Bull Environ Contam Toxicol (2012) 89:990–994
DOI 10.1007/s00128-012-0821-6

Assessing Estrogenic Chemicals in Anchovy and Mussel Samples from Karachi, Pakistan with the Yeast Estrogen Screen Bioassay

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Received: 5 January 2012 / Accepted: 31 August 2012 / Published online: 16 September 2012

Abstract Endocrine disrupting chemicals (EDCs) are introduced into the aquatic environment through industrial and municipal effluents along with urban and agricultural runoffs. Exposure of aquatic organisms to EDCs may lead to hormonal disruption and adverse health effects. The goals of our study were: to collect anchovy and mussel samples from the coastal region of Karachi, to use the yeast estrogen screen (YES) bioassay in estimating xeno-estrogen content in these samples, and to investigate if the bioassay could be used to quantify known amounts of 17β-estradiol (E2) injected into cod and salmon fillets. Results of the studies showed that mussel estrogenic activity in Karachi decreased in the order of Buleji point 1 (8.91 $\pm$ 4.77, mean $\pm$ SD) > Paradise point 1 (1.72 $\pm$ 0.81) > Paradise point 2 (0.61 $\pm$ 0.84) ng E2 equivalents/g wet wt ($p < 0.05$). By comparison, anchovy estrogenic activity at Korangi/Phitti Creek was much higher than at Manora. Together, these results confirmed previous reports that both Buleji point 1 and Korangi/Phitti Creek were the most contaminated areas of Karachi. The YES bioassay was only a semi-quantitative method in determining the contents of xeno-estrogens in aquatic organisms; it consistently over-estimated the amounts of E2 injected into cod and salmon fillets due to additive and/or non-additive interactions between E2 and endogenous estrogens. Nevertheless, the YES bioassay was able to identify the contaminated sites in the coastal region of Karachi.

Keywords Estrogenic activity · Yeast estrogen screen · Pakistan · Aquatic species

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Endocrine disrupting chemicals (EDCs) are introduced into the aquatic environment through industrial and waste water treatment plants (WWTPs) effluents as well as urban and agricultural runoffs (Nelson et al. 2007; Lorenzen et al. 2004). The presence of EDCs in surface waters is of great concern because they may affect adversely the health of humans (Ejas et al. 2004) and aquatic organisms (Bjerregaard et al. 2006; Quinn et al. 2004). Even at very low concentrations, 17β-estradiol (E2) and other xeno-estrogens may adversely affect the estrogen receptors (ER), interfering with the normal functions of the female hormones in living organisms. Such disruption has been reported in egg-laying fish and embryo-producing snails at 0.1–10 and 25 ng E2/L water concentrations, respectively (Jobling et al. 2003).

The coastal region of Karachi, Pakistan (Fig. 1) is contaminated by organic and inorganic chemicals from industrial and farm effluents (WWF-Pakistan 2007). Untreated industrial effluents from Korangi/Phitti Creek and municipal wastewaters from the City of Karachi are released directly into the Arabian Sea. Because municipal wastewaters are not separated from industrial effluents, many trace metals have been detected in the Karachi harbour (WWF-Pakistan 2007). The environmental problems of the region are exacerbated by discharges from cattle farms and slaughterhouses which mix organic wastes with blood and release them into the Korangi Creek. Thus earlier studies (Shahzad et al. 2009; Saleem and Kazi 1998)

have reported high levels of contaminants along the coastal areas of Karachi.

The YES bioassay has been widely used to quantify xeno-estrogens in the influents, effluents and/or biosolids of WWTPs (Nelson et al. 2007; Johnson et al. 2007). However, very few studies have used the YES bioassays to assess estrogenic activity in aquatic organisms. An exception being the fish and mussels (Fu et al. 2006; Peck et al. 2007) which also have been studied with human cell-based bioassays (Bayen et al. 2004; Legler et al. 2003).

The goals of the present study were: to collect marine mussel (*Mytilus edulis*) and anchovy (*Engraulis purava*) samples from the coastal area of Karachi, to assess the contents of xeno-estrogens in these samples using the YES bioassay, and to examine the potential of using the YES bioassay to quantify known amounts of E2 injected into salmon and cod fillets. We also sought to study the stability of E2 in cod fillets after storage. This paper presents a novel application of the YES bioassay to identify EDC-contaminated sites in Pakistan.

Materials and Methods

In May 2009, 12 marine mussels (*M. edulis*) and 9 anchovy (*E. purava*) were collected from each of the sampling sites 1–4 and 5–6, respectively (Fig. 1). An attempt was made to ensure the sizes of the mussels were similar and the lengths of the anchovies were about 15–20 cm. The mussel and fish samples were stored on ice and transported to the Wildlife Laboratory, Department of Zoology, University of Karachi where they were randomly divided into groups of mussels (n = 4) and fish (n = 3). The soft tissues were removed from the mussels and the fillets from the anchovies before they were cut into smaller, 3-g pieces and homogenized in methanol (see below). The methanolic extracts were couriered on dry ice to Simon Fraser University, Canada for analysis using the YES bioassay.

Cod (*Gadus macrocepalus*) and salmon (*Oncorhynchus nerka*) fillets were purchased from a supermarket in Burnaby BC, Canada. Cod and salmon fillets were cut into 1-g and 2-g pieces, respectively before being spiked with 0.6 (cod), 0.3, 0.15, or 0.075 (salmon) ng of E2/g fish tissue by injection. Marine mussels (*M. edulis*) also were obtained from an area near a WWTP operated by Metro Vancouver in October 2007. All samples were stored in a freezer at −40°C until use.

The fish fillets and mussel soft tissues were homogenized separately in 5 mL methanol using a Polytron Homogenizer (Brinkman Co., Rexdale, Ontario). The homogenates were centrifuged at 3,000 rpm for 20 min. The methanolic extract was removed and placed in a small test tube. The extraction procedure was repeated once. The remaining tissue pellets were extracted with 2 mL of chloroform. The methanolic and chloroform extracts were combined and the final volume measured before being evaporated to dryness in a rotary evaporator (Büchi Rotavapor Model No. RE 120, Flawil, Switzerland). The residues were reconstituted in 400 µl methanol and transferred to small vials.

The YES bioassay was conducted in a 96-well microplate using the yeast strain, *Saccharomyces cerevisae* which had been transfected with the human estrogen receptor (hER) and a plasmid carrying both the estrogen response element (ERE) and the reporter gene lac-Z (Nelson et al. 2007). Fish and mussel extracts were assayed in triplicate for estrogenic contents using the multiple point estimate approach of the YES bioassay. The bioassay was

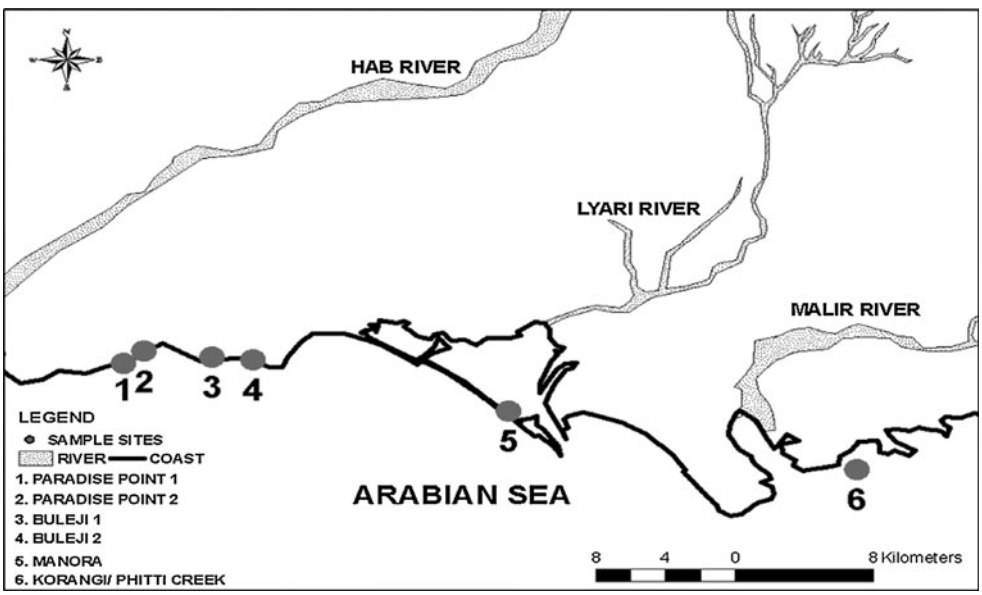

Fig. 1 Map of the coastal areas of Karachi with location of the six sampling sites: *1* Paradise point 1; *2* Paradise point 2; *3* Buleji point 1; *4* Buleji point 2; *5* Manora; *6* Koangi/Phitti Creek

based on β-galactosidase production as a result of estrogenic chemical(s) binding to hER. Galactosidase activity was determined using a UV/Vis spectrophotometer at 415 nm (colorimetric change) and 595 nm (turbidity caused by the yeast cells). A serial dilution of the E2 standard solution (25.9, 7.78, 2.59, 1.56, 0.78, 0.52, 0.31, 0.16, 0.10, 0.016, and 0.010 ng/mL) was used to construct the concentration–response curve of E2 standard.

EC_{20}, EC_{30} and EC_{50} were determined from each concentration–response curve using Prism 5 software (GraphPad Software Inc, San Diego, CA, USA). When the results merited, E2 equivalents (EEQs) were calculated for the samples using the equation reported by Lorenzen et al. (2004):

$$\text{E2 equivalents (ng/g)} = (\text{E2 EC}_{\text{ave}}/\text{extract EC}_{\text{ave}}) \times (\text{VAM/VET}) \times (\text{VRD/DWR})$$

where E2 EC_{ave} is the average of EC_{20}, EC_{30} and EC_{50} for the E2 standard (ng/mL); extract EC_{ave} is the average of EC_{20}, EC_{30} and EC_{50} for the sample extract (dilution volume, unitless); VAM is the volume of the assay medium (mL); VET is the volume of extract tested (mL); VRD is the volume of ethanol used to re-dissolve the RR residues (mL); DWR is the wet weight of the tissue used to prepare the ethanolic extract (g). The EEQs at each site are expressed as mean ± standard deviation (SD).

Statistical analysis was conducted using the Prism 4 program (GraphPad Software, La Jolla, CA, USA). For multiple group comparisons, we used the two-way ANOVA followed by the Fishers least significant difference test. For a two-group comparison, the two-sided, non-paired Student's t test was employed. Differences were considered significant when $p < 0.05$.

Results and Discussion

The YES bioassay was used to determine the estrogenic activity in anchovy fillets and mussel soft tissues obtained from the coastal region of Karachi (Fig. 1), an identified pollution area of Pakistan (Shahzad et al. 2009; Saleem and Kazi 1998). The mussel population is quite dense in certain areas of the region as the rocky bottom provides an ideal habitat and ecosystem for the mussels. Table 1 shows the mussels from Buleji point 1 have the highest estrogenic activity in the region since mussel EEQs decrease in the order of Buleji point 1 (8.91 ± 4.77, mean ± SD) > Paradise point 1 (1.72 ± 0.81) > Paradise point 2 (0.61 ± 0.84) ≥ Buleji point 2 (about 0.41) ng E2 equivalents/g wet wt. In comparison, the mussels collected near a WWTP in British Columbia, Canada showed an average estrogenic activity of 1.66 ± 1.87 ng E2 equivalents/g wet wt (unpublished observation) which was very close to that found in Paradise point 1, Karachi. The estrogenic activities in the mussels of Pakistan and BC are quite variable and are of concern because even a minute amount of E2 may affect adversely the reproductive capabilities of fish and snails (Jobling et al. 2003). Peck et al. (2007) also have reported that the estrogenic activity of zebra mussels from a non-contaminated area is <1 ng E2 equivalents/g wet wt. Since all the mussel samples except those from Buleji point 2 and Paradise point 2 have EEQs >1 ng E2 equivalents/g wet wt, we conclude that sampling sites 1 and 3 are contaminated by xeno-estrogens. Results of the anchovy study also identify Korangi/Phitti Creek as an EDC-contaminated area of Karachi (Table 1). It is important to study the estrogenic activity of anchovy in Korangi/Phitti Creek and Manora because this species usually are found in these sites and migrates to other places only during the monsoon season. Anchovy also is regularly consumed by humans residing in the coastal region of Karachi. However, no mussels have been collected from Korangi/Phitti Creek and Manora in the present study (Table 1) because mussels do not flourish in these areas. Legler et al. (2003) have reported the EEQs in flounder and bream from the Netherlands are 0.46 and 0.06 ng EEQs/g lipids, respectively. Since the data are from other fish species and are expressed in ng EEQs/g lipid, it would be difficult, if not impossible, to compare results with our studies. However, our results confirm the finding that fish are able to accumulate xeno-estrogens from the water (Legler et al. 2003).

Our results show that the YES bioassay successfully identifies some of the EDC-contaminated areas along the coastal region of Karachi, although the EDCs in these sites have yet to be characterized chemically. The measured EEQs in the mussel and anchovy samples are quite variable (Table 1), due perhaps to the different background EEQs in individual aquatic organisms and/or potentiating/inhibitory interactions among the xeno-estrogens in the EDC mixtures (see below).

Figure 2 shows that the background EEQs in untreated cod fillets is about 2.0 ± 0.6 ng E2 equivalents/g wet wt. After spiking cod fillets with 0.6, 0.3, and 0.15 ng of E2/g fish tissue, the measured EEQs in the fillets were found to be (mean ± SD) 4.2 ± 0.8, 3.4 ± 0.4, and 2.5 ± 1.0 ng E2 equivalents/g wet wt, respectively. The measured EEQs were much higher than the amounts of E2 injected into the cod fillets. An explanation for the difference in measured and actual EEQs is not readily available but may be related to the potentiating/synergistic interaction between the injected E2 and the endogenous estrogens in the fish fillets. In contrast, the background EEQs in untreated salmon were found to be below the detection limit of the YES bioassay (data not shown). Thus, after injecting salmon fillets with 0.3, 0.15, and 0.075 ng of E2/g fish tissue, the measured

Table 1 Estrogenic activity in anchovy and mussel samples collected from the coastal areas of Karachi, Paskistan

No. on map	Location	Estrogenic activity (E_2 equivalents ng/g, wet wt)	
		Anchovy	Mussel
1	Paradise point 1	NS	1.72 ± 0.81 (n = 3)**
2	Paradise point 2	NS	0.61 ± 0.84 (n = 3)**
3	Buleji point 1	NS	8.91 ± 4.77 (n = 3)**
4	Buleji point 2	NS	0.41 (n = 2)
5	Manora	0.79 ± 0.27 (n = 3)	NS
6	Korangi/ Phitti Creek	112.9 (n = 2)	NS

NS not sampled

** Significantly different from the other sampling sites ($p < 0.05$) based on the two-way ANOVA and the Fishers least significant difference test. Buleji point 2 data was not included in the statistical analysis

EEQs (mean ± SD) were 7.7 ± 4.2, 3.5 ± 0.7 and 1.5 ± 0.5 ng E2 equivalents/g wet wt, respectively (Fig. 3). These EEQs also were much higher than the amounts of E2 injected into the fillets. Thus, the YES bioassay appeared to be a useful tool for determining the total contents of estrogens in salmon and cod fillets. Indeed, the measured EEQs of salmon and cod fillets were not found to differ significantly ($p > 0.05$) from each other when they were spiked with 0.3 or 0.15 ng of E2/g fish tissue (Figs. 2 and 3) although the measured EEQs of salmon fillets appeared to be higher than those of cod and the salmon background EEQs were below the detection limit of the YES bioassay. An explanation for the discrepancy in results between salmon and cod fillets is not readily available but may be related to the variable EEQ background in the aquatic species and potentiating/inhibitory interactions among the EDCs. Despite this, the YES bioassay is able to show an increase in EEQ values for cod and salmon fillets that have been spiked with an increasing amount of E2 (Figs. 2 and 3). However, the YES bioassay can only be used as a semi-quantitative method to assess xeno-estrogen content in the aquatic species. As the cod and salmon fillets presumably are from clean or uncontaminated fish, the background EEQs of fish fillets (Figs. 2 and 3) should be very similar to the EEQs of anchovy in the pristine areas of Karachi. Indeed, a comparison of results shows that the background EEQs of salmon and cod fillets (Figs. 2 and 3) are well below the measured EEQs of anchovy from Korrangi/Phitti Creek, Pakistan (Table 1). Our results also are in agreement with the findings of Shahzad et al. (2009) that this area is highly contaminated by organic and inorganic chemicals.

As the field samples from Karachi had to be stored for a certain time interval before analyses, the concern was that

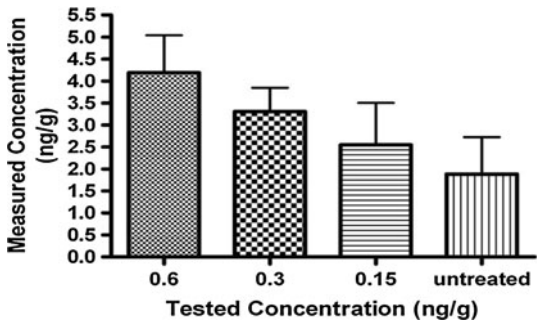

Fig. 2 EEQs of E2-spiked cod fillets. One-g pieces of cod fillets were spiked with 0.6, 0.3, or 0.15 ng of E2 before homogenization and extraction as described in "Materials and Methods". The *bars* represented the mean ± SD of measured EEQs from three independent assays

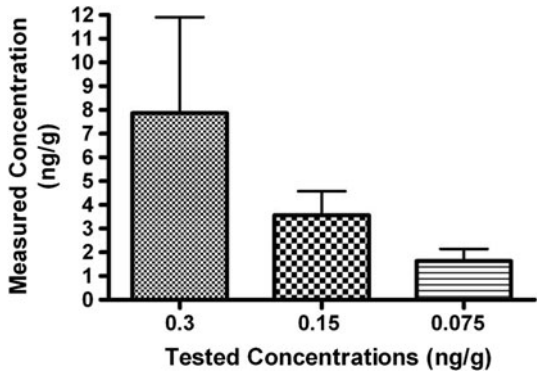

Fig. 3 EEQs of E2-spiked salmon fillets. Two-g pieces of salmon fillets were spiked with 0.6, 0.3, or 0.15 ng of E2 before homogenization and extraction as described in "Materials and Methods". The bars represented the mean ± SD of measured EEQs from three independent assays. The EEQs of untreated salmon fillets were below the detection limit of the YES bioassay

the EDCs would degrade over time, yielding inaccurate EEQs. Therefore, E2-spiked cod fillets were stored in a freezer and the EEQs of these samples were measured periodically over a 2-month period. Because the samples were kept at −40°C in sterile containers and hidden from light, the EEQs of the stored samples are not found to be significantly different to those of the controls (samples without storage) for at least 2 months (Fig. 4).

The YES bioassay is an inexpensive and rapid screening procedure for determining E2-like chemicals in aquatic animals. Results of the salmon and cod fillets show that the YES bioassay is reproducible, but the bioassay is confounded by the endogenous estrogens in the fillets. As a result, the YES bioassay can only be used as a semi-quantitative tool to assess the contents of xeno-estrogens in the aquatic organisms. It is important to assess the estrogenic activities in the aquatic species along the coastal region of Pakistan in light of the report that endocrine

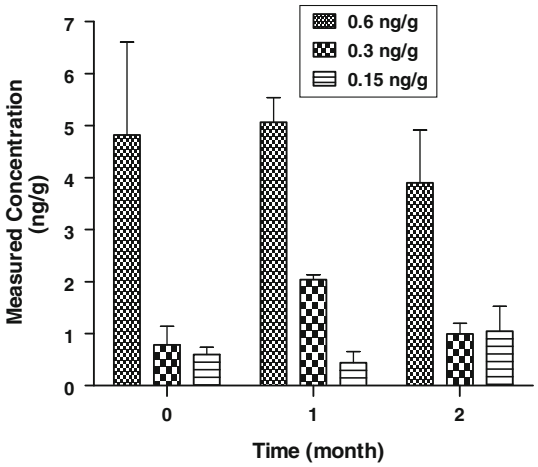

Fig. 4 Storage of E2-spiked cod fillets. One-g pieces of cod fillets were spiked with 0.6, 0.3, or 0.15 ng of E2 before homogenization and extraction as described in "Materials and Methods". The bars represented the mean ± SD of measured EEQs from three independent assays. The data were analyzed using the two-sided, non-paired Student's *t* test. No significant difference ($p > 0.05$) was found between the EEQs of stored and non-stored fillets after a 2-month storage period

disrupting pesticides have caused cancer in the rural Pakistan population (Ejas et al. 2004).

Acknowledgments The authors would like to thank the Pakistan Higher Education Commission (HEC) for the Sabbatical Fellowship Program grant in supporting this research project.

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