Clustering of Sex Hormone Disruptors in Singapore's Marine Environment

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Abnormal sexual differentiation and other reproductive abnormalities in marine animals indicate the presence in seawater of endocrine-disrupting compounds (EDCs) that perturb the function of the sex hormone signaling pathways. However, most studies to date have reported on EDC effects in freshwater and sewage samples, and there is a paucity of bioassay data on the effects of EDCs in marine waters. Our aims in this study were to devise robust methodologies suitable for extracting potential EDCs and to measure their summated effects on activities of androgen receptors (ARs) and estrogen receptors (ER-α and ER-β) in marine samples from Singapore's coastal waters. In this study, we examined the ability of C18, hydrophilic and lipophilic balance, and diol cartridges to extract potential EDCs from seawater samples. Extracts from C18 cartridges exhibited the highest sex hormone bioactivities in reporter gene assays based on a human cell line expressing AR, ER-α, and ER-β. Examination of extracts from 20 coastal locations showed high androgenic and estrogenic agonist activities in confined clusters closest to the main island of Singapore. Sex hormone activity declined rapidly in clusters farther from the main coastline and in more open waters. Unexpectedly, surface and mid-depth samples from the confined high-activity clusters, in the presence of hormone, exhibited AR and ER-α activities that were 200–900% higher than those observed for the cognate hormone alone. This enhanced sex hormone activity suggests that analyses of complex seawater mixtures may uncover unusual bioactivities that may not be obvious by studying individual compounds. Our data present a "snapshot" of the sex hormone disruptor activity in Singapore's marine environment and indicate that C18 extraction for EDCs used in conjunction with reporter gene bioassays represents a robust and sensitive methodology for measuring summated androgenic and estrogenic activities in seawater. *Key words:* androgen, endocrine disruptor, estrogen, reporter gene bioassay, seawater, Singapore. *Environ Health Perspect* 111:1448–1453 (2003). doi:10.1289/ehp.6139 available via *http://dx.doi.org/* [Online 25 April 2003]

Hormone-disrupting compounds, although present in very low concentrations, can exhibit significant effects on the environment and human health (Barber et al. 2000; Melnick et al. 2002). A number of pollutants, including pesticides, certain polychlorinated biphenyls, dioxins, furans, alkylphenols, metal-containing organics, synthetic steroids, and excreted drugs, as well as naturally occurring phytoestrogens, cause disruption of normal hormonal signaling pathways in animals (De Guise et al. 2001; Keith et al. 2001; Tanabe 2002), and these have collectively been labeled endocrine-disrupting compounds (EDCs). Of particular concern are the organic pollutants that may interfere with sex hormone function in animals as well as humans. For example, EDCs have also been implicated in neurobehavioral deficits, breast cancer risk, and mortality in humans (De Guise et al. 2001). Androgens and estrogens are steroid hormones that mediate male and female sexual function, respectively. Excessive action of androgenic agonists or reduced action due to antagonists is known to have deleterious effects on the health of marine biota (Baatrup and Junge 2001; Hashimoto et al. 2000; Tillmann et al. 2001). Environmental androgens (Hotchkiss et al. 2002) and estrogens (Takao et al. 1999) can adversely affect reproductive behavior in many organisms. For example, fish caged in polluted waters have been shown to have abnormal levels of sex hormones and exhibit abnormal anatomical changes in their reproductive organs (Larsson and Forlin 2002).

Both androgens and estrogens exert their effects through specific intracellular receptors. In the presence of the cognate ligands, the androgen receptor (AR) and estrogen receptor (ER) bind to the specific androgen response element (ARE) and estrogen response element (ERE), respectively, in the responsive genes, resulting in gene activation and protein expression. All androgens act through a single AR to trigger male sexual development (Loy and Yong 2001). In contrast, two ERs, ER-α and ER-β, are known, and these receptors have significantly different properties and tissue distributions and may have opposing effects (Enmark and Gustafsson 1998). ER-α is expressed predominantly in reproductive tissues such as the uterus and breast, whereas ER-β is found in the cardiovascular, nervous, gastrointestinal, and urogenital systems (Omoto et al. 2001). Current studies on sex hormone disruptors focus on the effects of single compounds (Jobling 1998). This approach may be misleading because many different EDCs are present in the environment, and their combined effects may not be the sum of individual compounds (Carpenter et al. 2002; Safe et al. 1997). In addition, some compounds present may have

antagonistic activity, whereas others have agonistic activity, thereby limiting the interpretation of chemical data (Snyder et al. 1999) or assays dependent on receptor–ligand interactions alone (Takeyoshi et al. 2002). Therefore, there is a need for assays that can measure the combined effects of EDCs on sex hormone receptor function at the cellular level, in the presence and absence of the endogenous hormone.

Most studies to date have focused on the content of EDCs in freshwater and sewage samples. For example, EDCs have been detected in wastewater effluent (Zeng et al. 1999) and in the tissues of fish (Zeng and Tran 2002). There is a paucity of bioassay data on the levels of EDCs and their effects in marine waters (Atkinson et al. 2002; Dinan et al. 2001; Knap et al. 2002). Scattered evidence indicates that EDCs are present in the German Bight of the North Sea (Bester et al. 2001), and their accumulation in marine sediments has been correlated with concentrations in fish flesh in the coastal waters of southern California (Schiff et al. 2002). The discovery of feminized male fish in industrialized estuaries in Germany (Gercken and Sordyl 2002), the United Kingdom (Matthiessen et al. 2002), and the Mediterranean (Fossi et al. 2001) suggests that these contaminants may have potent effects on the sexual development of marine biota. It is possible that concentrations of EDCs in coastal waters may be elevated in industrialized and urbanized port cities such as Singapore, where industrial effluent, shipping, and outfall discharge are concentrated in a small area. This is especially important because Singapore is situated in the Strait of Malacca, which is one of the busiest shipping lanes (Mark and Goh 2000) in the world (Figure 1, inset). These coastal marine waters are also used for fish farming and recreational use, as well as proposed desalination plants for generation of potable fresh water.

Therefore, it is pertinent that potential effects of EDCs in these coastal waters are investigated. Key constraints on the application of bioassays to marine samples are, typically, the presence of high salt concentrations and the presence of complex mixtures of EDCs.

Our objectives in this study were to devise a robust method to extract potential EDCs from marine samples in a form suitable for cell-based bioassays that measure summated AR and ER-α and ER-β activities, and to determine the utility of these human cell-based bioassays to detect potential EDCs in Singapore's coastal marine waters.

Materials and Methods

All organic solvents were HPLC grade and obtained from Fisher Scientific (Fair Lawn, NJ, USA) and J.T. Baker (Phillipsburg, NJ, USA). Ultrapure water was obtained from Nanopure (Barnstead, Dubuque, IA, USA) treatment. Tap water was obtained directly from Singapore's potable supply without any further treatment. For solid-phase extraction, Oasis HLB (hydrophilic and lipophilic balance) cartridges were obtained from Waters (Milford, MA, USA), and Strata C18-E cartridges were purchased from Phenomenex (Torrance, CA, USA). Both HLB and C18 cartridges had 500 mg bonded silica in 6-mL barrels. Diol cartridges are self-packed with 500 mg bonded diol silica in a 6-mL barrel. Dihydrotestosterone (DHT) and 17β-estradiol (E_2) were purchased from Sigma (St. Louis, MO, USA).

Sample collection and solid phase extraction. Seawater samples (1.2 L) were collected at surface and mid-depth from four clusters of sites that contain a total of 21 sample locations (S0–S20) around Singapore's coastline (Figure 1). Cluster I contains five locations (S01–S05); cluster II contains seven locations (S06–S12); cluster III contains two locations (S13 and S14); and cluster IV contains seven locations (S15–S20 and S0). Sample locations were based on the presence of industrial activity on
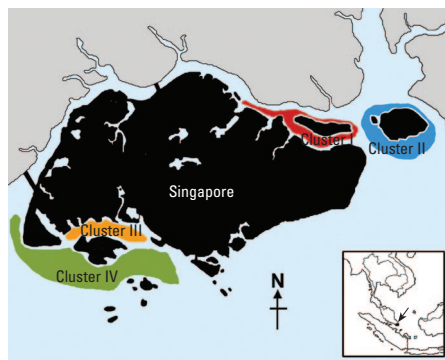
Figure 1. Map of Singapore indicating the seawater sampling locations, clusters I–IV. Inset: location of Singapore (arrow) at the tip of Peninsular Malaysia.

the northeast and southwest coastline of Singapore Island. Industrial sites include the presence of shipbuilding and maintenance yards, and petrochemical facilities, respectively.

Surface and mid-depth seawater samples were collected using a model 1015 rosette water sampler fitted with model 1010 Niskin bottles (General Oceanics Inc., Miami, FL, USA) and transferred to the polypropylene containers. After collection, the surface water was left to stand for 5 min, the top layer (5 cm) of water was removed, and the remaining seawater sample was transferred into labeled polypropylene containers that had been pre-cleaned with methanol and ultrapure water. The samples were kept chilled at 4°C in the dark and transferred to the laboratory, where they were passed through a 0.2 μm Nalgene filter (Nalge Nunc International, Rochester, NY, USA) and transferred into glass bottles. Solid-phase extraction (SPE) was carried out under negative pressure, and all SPE cartridges were sequentially conditioned with 3 × 5 mL methanol and 2 × 5 mL ultrapure water. The stored seawater was prewarmed to room temperature, and every 1 L water sample was then passed through a 6-mL 500 mg C18 cartridge, a 6-mL 200-mg HLB cartridge, and a 500-mg diol cartridge, respectively, at a flow rate of 10 mL/min. After all seawater was percolated, each cartridge was flushed with 3 × 5 mL ultrapure water to remove salts. Cartridges were then centrifuged at 3,000 rpm for 10 min, and then a gentle air stream was passed for 10 min to remove most of the water retained in the cartridges. This procedure resulted in optimal concentration without drying the seawater sample completely and has been demonstrated not to affect the behavior of polycyclic aromatic hydrocarbons, which are common EDCs (Li and Lee 2001). Chemical compounds retained in the cartridges were then eluted with 3 × 5 mL methanol. The elute was concentrated to a volume of approximately 0.5 mL at 40°C under a reduced pressure of 210 mBar. The concentrated elute was transferred to a 1.5 mL Eppendorf tube and further concentrated to 50 μL and stored at 4°C in the dark before exposure to the bioassay. Thus, the original seawater was concentrated 20,000× by reducing 1 L of seawater to 50 μL of SPE concentrate. This was the maximum concentration factor possible, because further concentration of seawater higher than 20,000× (e.g., 30,000×) resulted in supersaturation and precipitation of SPE elutes.

Human cell–based bioassay for activators of AR, ER-α, and ER-β. Plasmid constructs expressing AR, ER-α, or ER-β and the appropriate reporter constructs were cotransfected into HeLa cells on 24-well plates. The reporter vector for measuring AR activity was pARE-TATA-*Luc*, composed of two tandem copies of

an ARE from the aminotransferase gene driving a luciferase reporter gene (Wang et al. 2001). Estrogenic activity was measured by expressing ER-α or ER-β in the presence of the estrogen-responsive ERE-MMTV-*Luc* reporter gene, the promoter of which contains four ERE copies in the long terminal repeat of mouse mammary tumor virus (MMTV). HeLa cells were transiently transfected using the lipofection technique. A DNA mix containing expression vector (50 ng) and reporter vector (100 ng) was preincubated for 45 min at room temperature with 0.5 μL Lipofectamine (Gibco BRL, Invitrogen Corp., Carlsbad, CA, USA) in 200 μL serum-free phenol-red–free RPMI 1640 medium. The DNA–liposome complexes were overlaid onto 50–70% confluent HeLa cells in a total volume of 0.2 mL, and transfection continued for 5 hr before the addition of 0.6 mL growth medium containing 7% charcoal-stripped fetal calf serum, indicated amounts of steroid hormones, and the SPE extracts (1.2 μL). Thus, concentrated eluates were diluted 500× (1.2 μL extract in 0.6 mL media) for the bioassay. Higher amounts of the concentrated eluate led to reduced cell viability and adversely affected the bioassay. The original seawater sample was thus assayed at a 40× final concentration, on the assumption that the data can be extrapolated to reflect bioactivity at the original concentrations. After 36 hr of incubation, the cells were rinsed with phosphate-buffered saline and lysed with 100 μL reporter lysis buffer (Promega, Madison, WI, USA). Cells in the 24-well plates were sonicated in a water bath for 5 min, and the cell lysates were removed and cleared by centrifugation at 13,000 × *g* for 4 min. Cell lysates (10 μL) were added to 50 μL luciferase substrate, and luciferase activity [relative light units (RLU)] was measured with a luminometer. Sex hormone activities of the seawater extracts were expressed as percentages with respect to fixed concentrations of DHT (0.1 nM) and E_2 (10 nM). Control cells were exposed to vehicle (methanol) only. The data points were in triplicate. Error bars represent standard error of the mean (SE).

Statistical analyses. SPSS 11.0 (SPSS, Inc., Chicago, IL, USA) was used to perform the data analyses. The means, medians, and standard deviations of AR, ER-α, and ER-β bioassays for each cluster were computed. Differences in the median concentrations for surface and mid-depth locations for the various sites within clusters were assessed using the Kruskal-Wallis test. In the event that there were no significant differences among sites within clusters, differences in the median concentration among clusters were analyzed using Kruskal-Wallis with multiple Mann-Whitney *U*-tests performed to determine pairwise comparisons between two clusters, setting an adjusted *p*-value of < 0.005 for significance to control for type I error.

Results

Validation of bioassay and optimization of extraction methods for sex hormone disruptors. The sensitivity and specificity of the bioassay for androgens and estrogens were tested. As negative controls, ultrapure water and tap water concentrates, after C18 and HLB extraction procedures, were analyzed for AR, ER-α, and ER-β activity. These concentrates displayed activities that were no different from those of the methanol vehicle only. However, dose-dependent increases in reporter gene activity were observed with subnanomolar quantities of the cognate ligands in bioassays where AR, ER-α, and ER-β were coexpressed with their respective reporter genes (Figure 2). Conversely, no activity was detected on exposure to ligands from different, but related, steroid receptors (data not shown), indicating the high specificity of the bioassays. The lower limits of detection of our bioassay for DHT and E_2 are 0.001 nM and 0.01 nM, respectively (Figure 2). Test surface seawater was obtained 50 m offshore on the south coast of Singapore (S0 in cluster IV of Figure 1), and aliquots were subjected to SPE using C18, HLB, or diol cartridges, as described in "Materials and Methods." Basal sex hormone activity was detected in all samples, and activity was highest for the eluted extracts from C18 cartridges, and least with those from diol (Figure 2). In contrast, tap water and ultrapure water were subjected to C18 and HLB extractions, and the eluted extracts were found to have no sex hormone activity when tested with AR, ER-α, and ER-β. A dose–response study was conducted using various concentrations of C18 extract, and the cells on exposure to a 40-fold concentrate gave the most significant results (Figure 3). We found that the original SPE elutes of most of the seawater samples were supersaturated, and cell viability in the bioassay was affected if the final concentration of the extracts is more than 40-fold. Therefore, all subsequent seawater samples were extracted with solid-phase C18 columns, and HeLa cells in the bioassays were exposed to a final concentration of C18 elute at a concentration 40-fold higher than the original raw seawater sample.

Survey of sex hormone disruptor activity in marine water samples. For statistical analyses, the 20 sampling points were grouped into four geographical clusters, I–IV. Clusters I and II were located in the northeast, and clusters III and IV in the southwest of Singapore Island (Figure 1). Because each sample was assayed twice, the frequencies (n) of sampling in clusters I, II, III, and IV were 10, 14, 4, and 12, respectively. No differences were detected between samples within each cluster. We therefore proceeded to analyze differences between clusters. There was a statistically significant gradation of EDC activities, being highest in cluster I and lowest in cluster IV. Surface and mid-depth samples from cluster I elicited significantly higher AR, ER-α, and ER-β bioactivities than those from clusters II, III, and IV, both in the presence and in the absence of the cognate ligands (Kruskal-Wallis, $p < 0.001$). There were no significant differences between clusters II and III. However, both AR (surface and mid-depth) and ER-α (surface) agonist activities were higher in clusters II and III than in cluster IV (Mann-Whitney U-test, $p < 0.005$). Detailed results for each bioassay are presented below.

AR activity. Basal androgenic activity was detected in almost all samples collected from both surface and mid-water depth samples. High levels of hormone activities were observed in seawater concentrates obtained from sample locations S01 to S05 in cluster I, which are in the confined coastal waters of the Johore Straits, adjacent to the northeast coast of Singapore (Figure 4A). Androgenic activity was particularly prominent in cluster I samples, where the AR activity ranged from 13 to 119% of 0.1 nM DHT. Both surface and mid-depth samples had a similar level of activity, indicating that EDC compounds are vertically mixed in the water column. In sharp contrast, seawater concentrates from the nearby locations in cluster II displayed much lower AR activity. A general trend can be observed in the data whereby the more enclosed areas where tidal flushing is weaker had a higher androgenic activity. Sex hormone activity tended to decline in sites farther from the coastline.

A lesser peak of activity, for both mid-depth and surface seawater samples, was observed in sites S13 and S14 (cluster III) taken from the opposite southwest end of Singapore Island, and were equivalent to 0.5–1.1% of 0.1 nM DHT (Tables 1 and 2). These locations are again confined with respect to marine hydrodynamic dispersion, because of the configuration of the coastline of the main and outlying islands of Singapore, and are adjacent to extensive petrochemical industries. In comparison, nearby samples S15–S20 in cluster IV, in less confined coastal waters, had significantly lower AR stimulatory activities, less than 0.34% of 0.1 nM DHT.

To determine the activity of these samples in the presence of androgens, the AR stimulatory activity was measured in the presence of 0.1 nM DHT. A large increase in activity was observed in seawater concentrates from samples in cluster I, whereby AR activity of concentrate plus DHT ranged from 200% to 900% higher than that observed for DHT alone (Figure 4B). Although variations
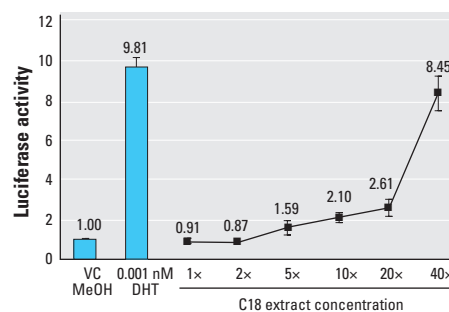
Figure 3. Optimization of extract concentration for bioassay. Abbreviations: MeOH, methanol; VC, vehicle. Increasing concentrations of C18 extracts of surface seawater from site S0 were added to HeLa cells expressing AR and androgenic activity measured with the reporter gene, pARE-TATA-*Luc*. The 40× concentrate induced a sharp increase in AR activity, and this concentration was used in all subsequent bioassays. Luciferase activities were expressed as fold increase in RLU over VC.
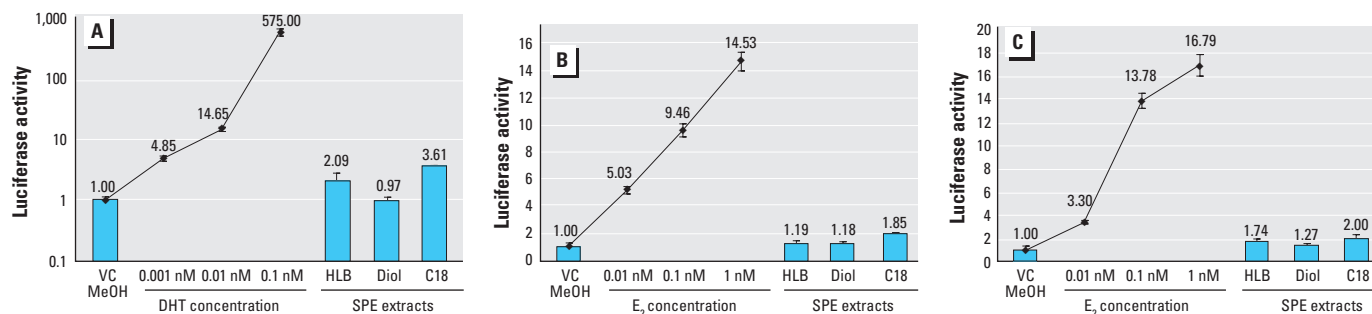
Figure 2. Validation of sex hormone receptor bioassays and optimization of SPE. Abbreviations: MeOH, methanol; VC, vehicle. (*A*) Increasing doses of the DHT were added to HeLa cells expressing AR, and androgenic activity measured with the reporter gene pARE-TATA-*Luc*. (*B* and *C*) Increasing doses of E_2 were added to cells expressing ER-α (*B*) and ER-β (*C*), and estrogenic activity was measured with the reporter gene ERE-MMTV-*Luc*. Seawater from site S0 in cluster IV was extracted with HLB, diol, and C18 cartridges, and their bioactivities compared in each bioassay. Luciferase activities were expressed as fold increase in RLU over VC.

were noted, both surface and mid-depth water samples from cluster I displayed significantly higher degrees of AR additive activity. Because the agonist activity of the samples alone ranged from 13 to 119% of DHT, the 200–900% increase in AR activity observed with seawater concentrates in cluster I, in the presence of DHT, represented an exaggerated additive effect (Figure 4B). In contrast, no additive activity was detected in samples from clusters II–IV, wherein no obvious differences in DHT-stimulated AR activity in the presence or absence of seawater concentrate was apparent.

ER-α activity. A similar clustering of ER-α activity in samples from cluster I was observed. Tables 1 and 2 show the percentage induction of total bioactivities of surface and mid-depth seawater samples with and without hormone. Seawater samples from cluster I elicited an ER-α activity ranging from 13 to 288% of that observed with 10 nM E_2. The ER-α agonist activities of both surface and mid-depth samples from cluster I were significantly higher than in clusters II–IV. Similar to AR activity, ER-α activity tended to be lower in less confined sample locations, and a minor peak of ER-α activity was also detected in the enclosed sites S13 and S14 in cluster III. Samples obtained from other locations had a lower baseline ER-α activity that was equivalent to 1–7% of 10 nM E_2. In the presence of E_2, the seawater concentrates from cluster I also increased estrogenic activity by up to 9-fold that observed with E_2 alone. This increased activity was observed for both surface and mid-depth samples obtained from cluster I but was not observed for samples from clusters II–IV.

ER-β activity. The clustering of sex hormone activity was less obvious when the samples were examined for ER-β activity. Although increased ER-β agonist activities were detected in some samples from cluster I, these differences were not significant compared with other sample locations. This was in contrast to the highly

significant changes noted above for AR and ER-α. No significant augmentative activity was observed (Tables 1 and 2).

Discussion

There was a concordance of data with respect to the patterns of androgenic and estrogenic activity detected in marine water samples collected from Singapore's coastal environment. Measured androgenic and estrogenic variables were highest in the most confined waters of cluster I and declined rapidly in locations farther from the main coastline and in more open waters (clusters II–IV). Levels of EDC activity were lowest in the open waters of cluster IV, where tidal flushing and marine hydrodynamics are strongest. The effect of confinement can still be observed in cluster III, which displayed significantly higher levels of AR (surface and mid-depth) and ER-α (surface) agonist activity compared with the adjacent cluster IV. In contrast, no differences were observed with the ER-β bioassay.

Thus, there were marked differences in the distribution of EDC effects in Singapore's coastal marine waters. Enclosed areas have weaker tide flushing and generally higher levels of EDC activity. Confinement of marine waters, limited hydrodynamic dispersion of pollutants, proximity to adjacent land masses, and exposure to industrial effluents may all influence the concentration of EDCs in this coastal marine environment. Interestingly, mid-depth samples from sites S10 and S11 in cluster II had lower AR and ER-α agonist activities compared with corresponding surface water samples, possibly reflecting the concentration of hydrophobic contaminants on thin hydrophobic surface layers prevalent in semi-enclosed bodies of water adjacent to industrial activity. The surface microlayer has been described as a potential site of enrichment of organic pollutants, including organochlorines and other pesticides (Booij and VanDrooge 2001; Kucklick and Bidleman

1994). Contaminant concentrations in the surface microlayer also decreased with distance from an industrialized coastline in Los Angeles, California, USA (Cross et al. 1987). Such accumulation may be accentuated by water stratification resulting in local thermoclines and haloclines (Gomez-Gutierrez et al. 2001). Although the existence of such hydrophobic layers remains to be determined in Singapore, their likely presence is important because their rich hydrophobic nutrients contents may attract zooplankton, possibly amplifying the effects of hydrophobic contaminants in the food chain. Assuming a 100% recovery rate of steroid-active compounds and the 40-fold concentration of samples used in our assays, the androgenic activity of the raw seawater from the high activity sites (cluster I) would be equivalent up to 3% of 0.1 nM DHT. Such levels of androgenic activity may be physiologically significant for marine biota, especially after prolonged exposure. Similarly ER-α activity in raw seawater in cluster I was within the range observed in the sera of normal women, because the physiologic level of E_2 in women at mid-cycle is known to be approximately 1 nM (Scheffer et al. 2003). Bioaccumulation in the marine food chain may also be expected to produce higher concentrations of bioactive compounds in marine organisms than in the surrounding seawater.

A noticeable effect was observed when samples from location cluster I, in the northeastern coastal area of Singapore, were examined for AR and ER-α activities in the presence of the cognate endogenous hormone. Both surface and mid-depth samples from cluster I displayed AR and ER-α activities of between 200 and 900% higher than those observed for the cognate ligand (DHT or E_2) alone. Because the agonist effects of samples from cluster I on AR activity and ER-α activities are 13–119% of DHT and 13–288% of E_2, respectively, the activity of the seawater sample plus endogenous hormone represents an exaggerated
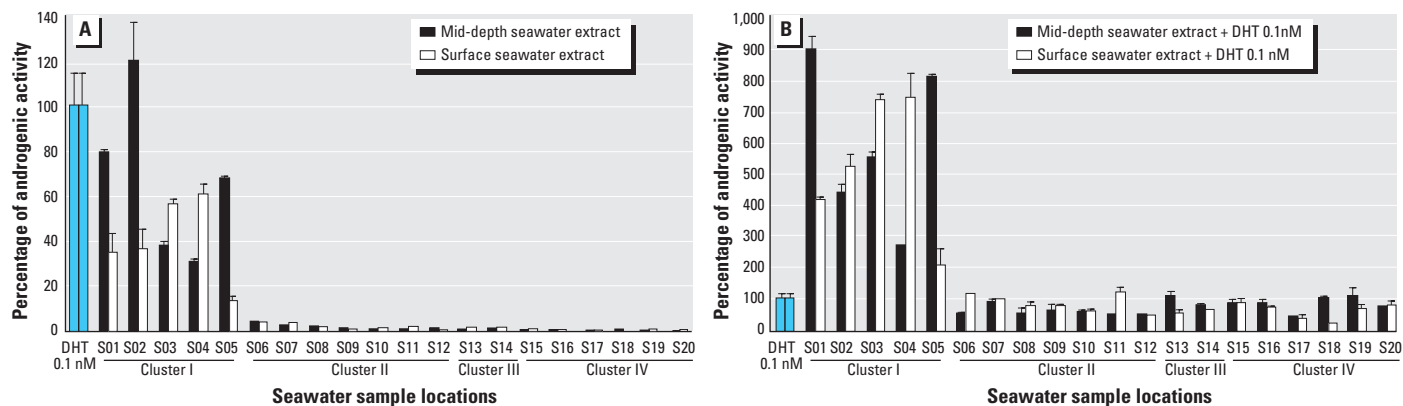
Figure 4. Androgenic activity of seawater extracts. C18 extracts of mid-depth and surface seawater samples were added to HeLa cells expressing AR, and androgenic activity was measured with the reporter gene pARE-TATA-*Luc*. Androgenic activity of extracts was expressed as a percentage of the reference androgen (0.1 nM DHT). Extracts were concentrated 40× for bioassays. (*A*) Androgenic activity of seawater extracts alone. (*B*) Androgenic activity of seawater extracts in the presence of 0.1 nM DHT.

additive activity. This finding illustrates the value of our receptor-based approach for detecting combinatorial effects that might not be obvious from studies of single chemical entities. Other evidence indicates that the combined effects of xenoestrogens may lead to a dramatic enhancement of hormone action, although each single agent was present below its no-observed-effect level (Rajapakse et al. 2002). Our data support the hypothesis that complex mixtures of compounds can have effects that are strikingly different from what can be predicted based on the actions of their individual constituents alone. If replicated *in vivo*, this augmentative effect of seawater concentrate on endogenous sex hormone activity could feasibly result in endocrine disruptor activity in excess of that predicted by its agonist effects alone. Indeed, the levels of androgenic and estrogenic activity detected in Singapore's coastal waters may be physiologically significant for some marine organisms, especially after chronic exposure. Furthermore, androgenic compounds may accumulate in the tissues of organisms, because the partition coefficients between the nonaqueous phases in fish liver and muscle and contaminated water, for example, are known to favor the bioaccumulation of organic contaminants (Zeng and Tran 2002). Because Singapore's marine

Table 1. Sex hormone activities of mid-depth seawater samples.

Sample	Extract alone			Extract with hormone added		
	AR (% of DHT)	ER-α (% of E$_2$)	ER-β (% of E$_2$)	AR (% of DHT)	ER-α (% of E$_2$)	ER-β (% of E$_2$)
Cluster I						
S01	79.11 ± 0.74	288.77 ± 36.31	556.00 ± 20.60	896.04 ± 44.52	609.58 ± 0.74	79.84 ± 3.98
S02	119.71 ± 17.01	239.93 ± 17.89	32.50 ± 9.91	438.43 ± 26.26	639.8 ± 77.30	131.58 ± 1.54
S03	37.39 ± 1.89	34.87 ± 1.89	80.00 ± 29.10	550.86 ± 16.02	735.26 ± 17.78	118.41 ± 0.34
S04	30.22 ± 1.55	13.36 ± 1.01	13.80 ± 0.15	267.31 ± 1.33	322.19 ± 20.09	153.72 ± 0.14
S05	67.79 ± 0.07	105.91 ± 4.52	32.70 ± 5.80	808.80 ± 7.37	412.13 ± 20.60	214.41 ± 4.23
Cluster II						
S06	4.07 ± 0.07	8.92 ± 1.43	8.60 ± 5.80	53.07 ± 3.46	111.69 ± 1.69	58.18 ± 0.12
S07	1.95 ± 0.29	7.35 ± 2.07	8.80 ± 1.36	89.85 ± 7.81	244.32 ± 0.74	58.05 ± 1.33
S08	1.14 ± 0.24	7.39 ± 2.77	4.80 ± 0.57	51.48 ± 17.26	96.57 ± 0.33	61.03 ± 1.03
S09	0.80 ± 0.08	8.61 ± 1.83	2.50 ± 0.57	63.23 ± 16.50	152.03 ± 5.61	60.78 ± 6.78
S10	0.32 ± 0.01	0.56 ± 0.26	11.38 ± 2.13	58.85 ± 3.43	68.08 ± 3.34	46.30 ± 22.70
S11	0.32 ± 0.01	0.48 ± 0.32	16.70 ± 4.45	48.51 ± 2.87	71.13 ± 3.20	34.38 ± 0.47
S12	0.86 ± 0.01	16.53 ± 5.6	9.20 ± 2.98	50.86 ± 3.25	60.32 ± 2.31	68.42 ± 4.13
Cluster III						
S13	0.50 ± 0.01	5.90 ± 0.96	6.70 ± 1.21	105.46 ± 13.41	50.96 ± 2.12	64.43 ± 2.58
S14	0.68 ± 0.01	4.47 ± 0.05	11.08 ± 0.81	74.36 ± 6.21	36.57 ± 1.43	73.27 ± 5.45
Cluster IV						
S15	0.17 ± 0.01	1.62 ± 0.02	2.05 ± 0.12	85.04 ± 12.52	64.49 ± 1.92	69.30 ± 11.80
S16	0.29 ± 0.06	2.16 ± 0.06	2.07 ± 0.79	84.63 ± 12.93	76.51 ± 7.37	65.58 ± 4.13
S17	0.16 ± 0.04	1.34 ± 0.20	12.50 ± 1.20	44.63 ± 0.92	55.58 ± 11.64	47.06 ± 2.36
S18	0.34 ± 0.10	0.62 ± 0.40	83.50 ± 2.38	101.19 ± 5.12	75.23 ± 0.56	54.36 ± 7.97
S19	0.26 ± 0.03	3.99 ± 0.05	4.99 ± 0.39	109.74 ± 24.76	33.79 ± 2.14	98.54 ± 5.23
S20	0.19 ± 0.04	2.26 ± 0.11	7.54 ± 1.44	74.76 ± 4.37	46.82 ± 4.73	51.65 ± 2.70

All data are expressed as percentages (± SE) of bioactivity observed with the cognate reference hormone (10 nM E$_2$, 0.1 nM DHT). SPE extracts were 40× concentrated for bioassay.

Table 2. Sex hormone activities of surface seawater samples.

Sample	Extract alone			Extract with hormone added		
	AR (% of DHT)	ER-α (% of E$_2$)	ER-β (% of E$_2$)	AR (% of DHT)	ER-α (% of E$_2$)	ER-β (% of E$_2$)
Cluster I						
S01	34.22 ± 8.87	101.40 ± 3.77	57.11 ± 6.60	414.68 ± 9.75	687.32 ± 2.59	169.78 ± 10.80
S02	35.97 ± 8.76	73.64 ± 10.40	34.97 ± 2.70	521.56 ± 40.86	948.41 ± 9.82	159.80 ± 7.10
S03	55.67 ± 2.41	90.67 ± 0.88	143.70 ± 2.59	731.21 ± 20.13	349.25 ± 34.9	167.13 ± 20.30
S04	60.59 ± 3.67	100.20 ± 1.33	41.27 ± 0.71	741.17 ± 78.54	524.23 ± 50.00	269.66 ± 3.37
S05	12.92 ± 2.01	12.67 ± 4.82	42.25 ± 2.19	203.66 ± 55.67	115.27 ± 10.01	100.70 ± 5.50
Cluster II						
S06	2.69 ± 0.07	13.01 ± 1.60	14.24 ± 6.40	114.11 ± 2.32	52.61 ± 3.09	52.11 ± 27.55
S07	3.16 ± 0.44	11.19 ± 0.27	19.72 ± 0.19	97.35 ± 3.99	66.11 ± 1.51	26.98 ± 12.58
S08	1.35 ± 0.21	6.12 ± 0.43	7.90 ± 6.65	77.64 ± 10.81	66.14 ± 10.7	58.68 ± 4.28
S09	0.52 ± 0.12	5.73 ± 0.22	6.70 ± 3.39	75.20 ± 5.73	67.17 ± 3.68	49.34 ± 23.40
S10	0.95 ± 0.21	4.16 ± 0.46	21.40 ± 6.80	59.83 ± 5.51	73.43 ± 4.71	113.40 ± 14.33
S11	1.67 ± 0.35	4.46 ± 0.13	26.70 ± 5.42	118.04 ± 18.74	54.8 ± 6.40	157.90 ± 26.30
S12	0.06 ± 0.01	4.25 ± 0.17	43.80 ± 7.43	46.43 ± 2.6	51.42 ± 3.31	159.70 ± 7.70
Cluster III						
S13	1.10 ± 0.16	6.80 ± 0.08	18.91 ± 0.52	51.74 ± 8.39	50.96 ± 2.12	49.80 ± 10.53
S14	1.09 ± 2.70	6.31 ± 0.11	18.64 ± 6.06	59.85 ± 1.15	36.57 ± 1.43	38.52 ± 8.26
Cluster IV						
S15	0.21 ± 0.09	2.04 ± 0.13	8.04 ± 2.01	87.04 ± 12.68	64.49 ± 1.92	59.88 ± 2.67
S16	0.29 ± 0.01	2.35 ± 0.28	4.26 ± 1.01	73.33 ± 0.89	76.51 ± 7.37	48.61 ± 3.30
S17	0.03 ± 0.01	2.40 ± 0.07	20.50 ± 7.17	35.96 ± 9.26	59.40 ± 4.92	64.80 ± 10.40
S18	0.07 ± 0.04	3.02 ± 0.07	30.58 ± 8.19	18.17 ± 0.13	80.72 ± 2.22	82.80 ± 2.60
S19	0.66 ± 0.03	3.99 ± 0.05	11.65 ± 0.65	67.25 ± 14.03	33.79 ± 2.14	62.96 ± 3.99
S20	0.33 ± 0.04	2.26 ± 0.11	7.41 ± 1.71	80.02 ± 12.31	46.82 ± 4.73	65.76 ± 0.59

All data are expressed as percentages (± SE) of bioactivity observed with the cognate reference hormone (10 nM E$_2$, 0.1 nM DHT). SPE extracts were 40× concentrated for bioassay.

waters are used for leisure, fishing, aquaculture, and desalination activities, significant effects on human health are possible.

In this study we have devised and validated an SPE method to extract EDCs from seawater samples. The optimal conditions for efficient extraction of sex hormone disruptors have been empirically established. A C18 matrix produced the highest yield for the detection of EDCs, where a 40-fold concentrate gave the greatest sensitivity in the human cell–based reporter gene assay. We have also demonstrated that reporter gene assays based on human cell lines can be used to detect the summated sex hormone effects of EDCs in seawater. Although reporter gene assays based on yeast have been used to measure summated endocrine activities (Garcia-Reyero et al. 2001; Murk et al. 2002), bioassays based on human cell lines more accurately reflect potential toxic effects in humans (Simons 1996). There are few data on the application of the human cell–based sex hormone receptor bioassays on marine environmental samples. This study is the first, to our knowledge, to test surface and mid-depth seawater at defined locations in a coastal area with heavy shipping traffic, to extract potential EDCs using optimized solid phase matrices, and to examine these extracts for sex hormone disruptor activity using sensitive reporter genes in a human cell line. Although our data represent only a "snapshot" of the prevailing EDC activity in Singapore's coastal marine waters, they indicate that our method of SPE extraction for EDCs used in conjunction with reporter gene analysis is a robust and sensitive assay that can provide reproducible bioassay results for androgenic and estrogenic activities in seawater. The existence of potential EDCs such as organotin arising from shipping activities in Singapore's marine environment has been documented (Basheer et al. 2002), and there is evidence that pollutants are disrupting sexual differentiation in local mollusks (Tan 1999). It is likely that hundreds or more as yet unidentified xenobiotics may be present in the coastal waters of a busy industrialized port city such as Singapore. Our extraction techniques and receptor bioassays provide a reliable methodology for monitoring the summated effects of the all potential EDCs in seawater samples, thereby supplementing studies based on measurement of single compounds. This approach is likely to provide a more holistic measure of the environmental impact of complex multichemical mixtures. A site-specific coastal water monitoring program over the longer term is now being undertaken in Singapore, and work is continuing in order to identify the specific EDC compounds with sex hormone–disrupting properties.

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