

Modulation of steroidogenesis by coastal waters and sewage effluents of Hong Kong, China, using the H295R assay

Tannia Gracia • Paul D. Jones • Eric B. Higley •
Klara Hilscherova • John L. Newsted •
Margaret B. Murphy • Alice K. Y. Chan •
Xiaowei Zhang • Markus Hecker • Paul K. S. Lam •
Rudolf S. S. Wu • John P. Giesy

Received: 7 February 2008 / Accepted: 16 April 2008 / Published online: 21 May 2008
© Springer-Verlag 2008

Abstract

Background, aim, and scope The presence of a variety of pollutants in the aquatic environment that can potentially

interfere with the production of sex steroid hormones in wildlife and humans has been of increasing concern. The aim of the present study was to investigate the effects of extracts from Hong Kong marine waters, and influents and effluents from wastewater treatment plants on steroidogenesis using the H295R cell bioassay. After exposing H295R cells to extracts of water, the expression of four steroidogenic genes and the production of three steroid hormones were measured. *Materials and methods* Water samples were collected during the summer of 2005 from 24 coastal marine areas and from the influents and effluents of two major waste water treatment plants (WWTPs) in Hong Kong, China. Samples were extracted by solid phase extraction (SPE). H295R cells were exposed for 48 h to dilutions of these extracts. Modulations of the expression of the steroidogenic genes *CYP19*, *CYP17*, *3 β HSD2*, and *CYP11 β 2* were determined by measuring mRNA concentrations by real-time polymerase chain reaction (Q-RT-PCR). Production of the hormones progesterone (P), estradiol (E2), and testosterone (T) was quantified using enzyme linked immunosorbent assays (ELISA). *Results* Extracts from samples collected in two fish culture areas inhibited growth and proliferation of H295R cells at concentrations greater or equal to 10⁵ L equivalents. The cells were exposed to the equivalent concentration of active substances in 10,000 L of water. Thus, to observe the same level of effect as observed in vitro on aquatic organisms would require a bioaccumulation factor of this same magnitude. None of the other 22 marine samples affected growth of the cells at any dilution tested. Twelve of the marine water samples completely inhibited the expression of *CYP19* without affecting E2 production; inhibition of *CYP17* expression was observed only in one of the samples while expression of *CYP11 β 2* was induced as much as five- and ninefold after exposure of cells to extracts from

Responsible editor: Henner Hollert

T. Gracia (✉) • P. D. Jones • E. B. Higley • X. Zhang • J. P. Giesy
Department of Zoology,
National Food Safety and Toxicology Center,
Center for Integrative Toxicology, Michigan State University,
East Lansing, MI 48824, USA
e-mail: tg3@sanger.ac.uk

K. Hilscherova
Research Centre for Environmental Chemistry and Ecotoxicology,
Masaryk University,
625 00 Brno, Czech Republic

J. L. Newsted
ENTRIX Inc.,
Okemos, MI 48864, USA

M. B. Murphy • A. K. Y. Chan • X. Zhang • P. K. S. Lam •
R. S. S. Wu • J. P. Giesy
City University of Hong Kong,
Tat Chee Ave,
Kowloon, Hong Kong, SAR China

M. Hecker
ENTRIX Inc.,
Saskatoon, SK S7N 5B3, Canada

M. Hecker • J. P. Giesy
Department of Veterinary,
Biomedical Sciences and Toxicology Centre,
University of Saskatchewan,
Saskatoon, SK S7N 5B3, Canada

J. P. Giesy
School of Environment, Nanjing University,
Nanjing, China

two locations. The expression of the progesterone gene 3β HSD2 was not affected by any of the samples; only one sample induced approximately fourfold the production of E2. Although more than twofold inductions were observed for P and T production, none of these values were statistically significant to conclude effects on the production of these two hormones. While influents from WWTPs did not affect gene expression, an approximately 30% inhibition in the production of E2 and a 40% increase in P occurred for the exposure with influents from the Sha Tin and Stonecutters WWTPs, respectively. Effluents from WWTPs did not affect the production of any of the studied hormones, but a decrement in the expression of the aldosterone gene CYP11 β 2 was observed for the Sha Tin WWTP exposure. No direct correlation could be established between gene expression and hormone production.

Discussion Observed cytotoxicity in the two samples from fish culture areas suggest the presence of toxic compounds; chemical analysis is required for their full identification. Although effluents from WWTPs did not affect hormone production, other types of endocrine activity such as receptor-mediated effects cannot be ruled out. Interactions due to the complexity of the samples and alternative steroidogenic pathways might explain the lack of correlation between gene expression and hormone production results.

Conclusions Changes observed in gene expression and hormone production suggest the presence in Hong Kong coastal waters of pollutants with endocrine disruption potential and others of significant toxic effects. The aromatase and aldosterone genes seem to be the most affected by the exposures, while E2 and P are the hormones with more significant changes observed. Results also suggest effectiveness in the removing of compounds with endocrine activity by the WWTPs studied, as effluent samples did not significantly affect hormone production. The H295R cell showed to be a valuable tool in the battery required for the analysis of endocrine disrupting activities of complex environmental samples.

Recommendations and perspectives Due to the intrinsic complexity of environmental samples, a combination of analytical tools is required to realistically assess environmental conditions, especially in aquatic systems. In the evaluation of endocrine disrupting activities, the H295R cell bioassay should be used in combination with other genomic, biological, chemical, and hydrological tests to establish viable modes for endocrine disruption and identify compounds responsible for the observed effects.

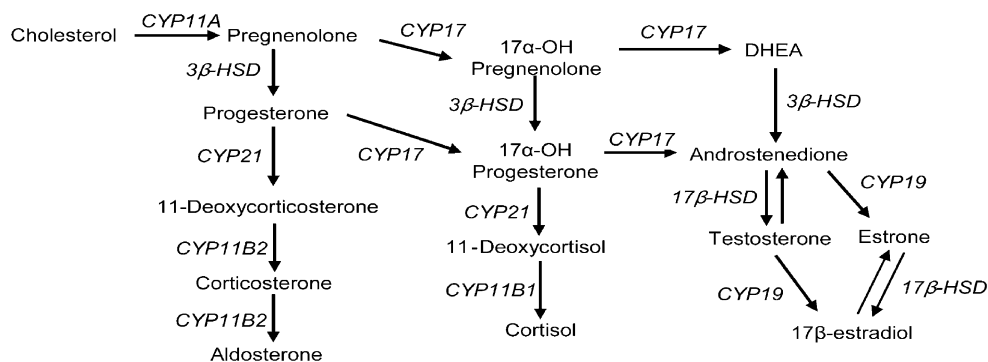
Keywords Aquatic environment · China · Coastal waters · Endocrine disruptors · Environmental samples · H295R cell bioassay · Hong Kong · Hormones · Influent · Marine waters · Sewage effluents · Sex steroid hormones · Steroidogenesis · Wastewater treatment plants

1 Background, aim, and scope

During the past two decades, there has been increasing concern about chemicals that can interact with the endocrine systems of wildlife and humans, and that ultimately may cause reproductive disorders and population declines (Kavlock et al. 1996; EDSTAC Final Report 1998). A variety of chemicals are suspected to have the potential to act as so-called endocrine disrupting chemicals (EDCs) including certain polycyclic aromatic hydrocarbons (PAHs), pesticides, phthalate plasticizers, PBDEs (polybrominated diphenyl ethers), dioxins, alkyl-phenols, and natural and synthetic steroid hormones. Continuous exposure to such compounds can pose a threat to the development and reproductive success of aquatic organisms (Committee on Restoration of Aquatic Ecosystems 1992). EDCs can exert their disrupting effects by different mechanisms. However, much of the current research efforts have been focused on the development in vitro estrogen receptor (ER) and androgen receptor (AR) binding assays (Villeneuve et al. 1998) or on the development of transcriptional activation assays with ER and AR in stably transfected cell lines (Wilson et al. 2004; Wilson et al. 2002). The need to evaluate endocrine disruption exerted by pathways other than receptor-mediated mechanisms and the effects of EDC mixtures has resulted in development of new bioassays such as the H295R steroidogenesis assay (Hilscherova et al. 2004; Hecker et al. 2007). This in vitro system has been shown to be effective in characterizing the potential of individual chemicals, pharmaceuticals, pesticides, and their mixtures to modulate steroidogenesis (Sanderson et al. 2000; Zhang et al. 2005; Gracia et al. 2006; Blaha et al. 2006; Hecker et al. 2006; Xu et al. 2006). H295R cells express the entire steroidogenesis pathway required for the biosynthesis of mineralocorticoids, glucocorticoids, and steroid sex hormones (Fig. 1). Furthermore, the cells can be used to study steroidogenic pathways at multiple levels of organization including the expression of genes (Hilscherova et al. 2004), abundance and activity of enzymes (Sanderson et al. 2002), and the hormone products of these catalytic enzyme activities (Hecker et al. 2006). Therefore, the H295R cells offer an excellent in vitro model for studying effects and mechanisms of EDC interaction with steroidogenesis.

Some of the causes associated with the deterioration of the quality of coastal waters are inadequate sewage treatment, livestock waste, industrial effluents, and agricultural runoff. Relatively great levels of pollution have been identified in certain Asian waters (Connell et al. 1998; Monfils et al. 2006). For example, in Hong Kong, Special Administrative Region (SAR), China, decreasing water quality has been a concern over the past decades as a consequence of growing industrial activities and population increases in the nearby Pearl River Delta (Wong et al. 1995;

Fig. 1 Steroidogenic pathway in H295R cell. Enzymes are in *italics*, hormones are in **bold**, and arrows indicate the direction of synthesis

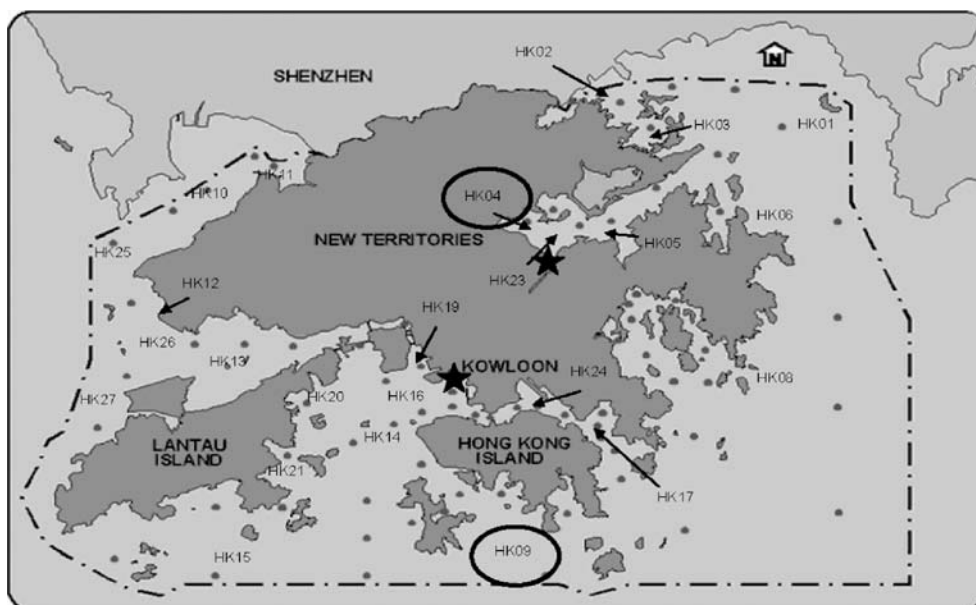


Yung et al. 1999; HKEPD 2002). Effects of bacteria, nutrients, and hypoxia have been identified in coastal areas of Hong Kong, including Victoria Harbor, around which 65% of the city's population lives. The lower sections of the East River (Dong Jiang), which provides approximately 80% of the drinking water for Hong Kong, is contaminated by metals such as cadmium, copper, and zinc (Ip et al. 2007; Ho et al. 2003; Hung et al. 2007) and persistent organic pollutants including polybrominated diphenyl ethers (PBDEs; Liu et al. 2005; Ramu et al. 2005), organochlorine pesticides (OCPs), and dioxin-like compounds (So et al. 2005). PBDE contamination has also been found in Hong Kong marine waters with concentrations ranging from 31 to 1.2×10^2 pg l^{-1} (Wurl et al. 2006), which probably arise from the disposal of electronics' industry wastes in southern China and the discharge of untreated wastewater of local origin. Moreover, residual levels of DDTs and polycyclic aromatic hydrocarbons (PAHs) have been found in freshwater and marine fish sold in Hong Kong markets (Cheung et al. 2007). Thus, there is a considerable body of evidence suggesting the presence of contaminants in Hong Kong waters that may have the potential to adversely affect

endocrine functions of marine organisms. Everyday, the approximately seven million people of Hong Kong produce on average of about 2.2 million m^3 of sewage that feeds into approximately 200 sewage treatment plants through 1,320 km of sewerage network (The Government of the Hong Kong Special Administrative Region 2006). Approximately 95% of Hong Kong's population is now served by the public sewerage system with over 98% of the sewage produced being collected and treated. All new towns in the New Territories have been designed and developed with modern secondary sewage treatment works.

The present study was conducted to evaluate the potential of coastal marine waters that were previously reported to be contaminated with a variety of EDCs and sewage effluents of a selection of WWTPs of Hong Kong to interact with steroidogenic functions using the H295R steroidogenesis assay (Fig. 2). Water samples were collected from WWTPs influents and effluents, fish culture zones, marine disposal, and WWTPs discharge areas. H295R cells were exposed to extracts from the collected samples, and the expression of the steroidogenic genes *3βHSD2*, *CYP11β2*, *CYP17*, and *CYP19* was measured using Q-RT-PCR

Fig. 2 Marine water monitoring stations in Hong Kong and sampling points for endocrine disruption evaluation. Sampling points in a *circle* are those that produced high toxicity in H295R cells. Stars: water treatment plants



methods. The production of the hormones progesterone (P), estradiol (E2), and testosterone (T) was quantified using ELISA methods. Correlations between gene expression and hormone production were evaluated statistically.

2 Materials and methods

2.1 Sampling locations and collection methods

Samples were collected in July, 2005 from 24 locations in coastal marine areas and from two major waste water treatment plants in the vicinity of the city of Hong Kong, China (Table 1, see Fig. 2). Among the different activities occurring in the sampling areas were intensive aquaculture, public waste filling, marine disposal, and sewage discharges. Tung Ping Chau was designated as a reference site because of its geographic location (upstream of most of the sampling locations). Water samples were collected using standard depth- and width-integrating techniques designed

to obtain a representative sample (Shelton 1994). Composite samples collected at each site were split into two pre-cleaned amber glass bottles. Samples were immediately chilled on ice and sent to the laboratory where they were stored at 4°C for a maximum of 24 h until they were filtered and prepared for extraction to keep analyte degradation to a minimum and to avoid the need for addition of preservatives.

2.2 Physical and chemical parameters

Water, temperature, pH, oxygen concentrations, salinity, and conductivity were measured with calibrated instruments at all sampling sites (pH meter HI8424, DO meter YSI-RS 232, Salinity YSI 33).

2.3 Extract preparation

Before extraction, each sample was vacuum-filtered through a 70-mm glass fiber GF/F filter (Whatman, Maidstone, UK)

Table 1 Sampling points for marine waters in Hong Kong, China

Sample	Name	Activity	Temp. (°C)	pH	DO ^a (mg/l)	Salinity (%)
HK01	Tung Ping Chau	Reference site	29.6	8.29	6.7	16.0
HK02	Ap Chau, Kat O	Fish culture	28.3	8.60	8.1	34.0
HK03	Sai Lau Kong	Fish culture	29.1	8.63	8.6	35.0
HK04	Yim Tin Tsai	Fish culture	27.8	8.63	8.0	30.8
HK05	Yung Shue Au	Fish culture	26.6	8.46	7.2	34.0
HK06	Tap Mun, Kau Lau	Fish culture	27.2	8.44	6.9	34.2
HK07	Ma Lam Wat	Fish culture	29.5	8.38	6.6	35.2
HK08	Leung Shuen Wan	Fish culture	26.5	8.39	6.6	33.0
HK09	Sok Kwu Wan	Fish culture	26.8	8.17	5.4	31.2
HK10	Oyster Site	Oyster production	29.4	8.04	5.4	16.0
HK11	Mai Po	Marine reserve	24.8	8.33	6.5	28.5
HK12	Tuen Mun	Public filling area	28.6	8.61	8.7	23.2
HK13	Sha Chau (East)	Marine disposal	26.2	8.43	6.0	28.0
HK14	Tsing Yi (South)	Marine disposal	24.4	8.40	5.5	30.2
HK15	Cheung Chau (South)	Marine disposal	26.0	8.56	8.0	31.8
HK16	Wan Chai	Disch. STW ^b	24.2	8.25	5.5	25.0
HK17	Tseung Kwan O	Reclamation site	26.8	8.37	5.3	36.0
HK19	Stonecutters Island	Disch. STW	24.7	8.36	6.4	28.2
HK20	Pennys Bay	Reclamation site	25.5	8.52	7.5	33.2
HK21	Mui Wo	Disch. STW	27.0	8.59	8.3	32.2
HK22	Aberdeen & Ap Lei Chau	STW & fish culture	26.6	8.21	4.9	30.0
HK23	Tolo Harbor	Disch. STW	26.7	8.37	5.7	34.0
HK24	North Point	Disch. STW	26.1	7.97	3.8	27.2
HK25	San Wai	Disch. STW	27.6	8.76	9.6	31.0
HK26	Pillar Point	Disch. STW	29.0	8.60	8.6	20.8
HK27	Cogeneration Plant	Power plant	29.6	8.29	6.7	16.0
HK28	Sha Tin—untreated	STW	29.3	8.34	0.2	13.2
HK29	Sha Tin—treated	STW	30.2	7.48	6.7	11.5
HK30	Stonecutters—untreated	STW	28.8	7.94	1.4	13.8
HK31	Stonecutters—treated	STW	28.8	7.56	0.8	13.8

^a DO Dissolved oxygen

^b STW Sewage treatment works, discharge, STW discharge area for a sewage treatment work

pore size of 0.7 μm , and the retained particulate material was washed with 0.5 mL of methanol that was added to the aqueous sample. After filtration, 1 mL of 200 mg mL⁻¹ Na₂EDTA was added to each liter of sample to prevent compounds such as the tetracycline antibiotics from forming complexes with divalent ions such as Ca²⁺ and Mg²⁺. The pH of water samples was then adjusted to 3 using glacial acetic acid. Solid phase extraction cartridges, 200 mg Strata X (Phenomenex, Torrance, CA, USA), were washed twice using 2 mL of methanol, followed by conditioning with three aliquots of 2 mL distilled water. One liter of the prepared samples was then pulled through each washed and conditioned cartridge at 10 mL/min using a vacuum pump. The cartridges were dried and the retained compounds were eluted three times with 2 mL of methanol. In a thermostatic bath, the extracts were then concentrated under a gentle nitrogen stream to achieve a final volume of 1 mL at 30°C. Extracts were stored at -20°C.

2.4 H295R cell bioassay

Protocols for culturing and exposure of H295R cells have been previously established and validated (Hilscherova et al. 2004; Zhang et al. 2005; Hecker et al. 2006). Briefly, H295R cells were cultivated in an incubator at 37°C in a 5% CO₂ atmosphere. Cells were cultured with supplemented medium containing DMEM with F-12 Ham's, Nu Serum, and ITS+ premium (BD Bioscience, San Jose, CA, USA) in 25 cm culture flasks. In preparation of exposure experiments, cells were seeded at a density of approximately 1 × 10⁶ cells/mL per well in a six-well cell culture plate. The culture medium was removed, and cells were exposed to 3 mL of dosing solutions. Before exposure, a live/dead cell viability kit (Molecular Probes, Eugene, OR, USA) was used to establish the range of extract concentrations that could be used without causing cytotoxicity to the cells. Extract dilutions in the range of 10³- to 10⁵-fold were evaluated. In instances, where exposure resulted in cell death or decreased viability (less than 85%), the data were not used to evaluate gene expression or hormone production. Dosing solutions of the extracts were prepared in medium by adding 10 μL extract to 10 mL medium to a final methanol concentrations of 0.1% to avoid significant effects on cell viability or any of the studied endpoints. A solvent control dose solution was 0.1% methanol in medium. Cells were examined under a microscope to assure good cell condition before dosing.

After a 48-h exposure period, but before nucleic acid isolation and hormone analysis, cell viability was determined by visual inspection under a microscope to evaluate viability and cell number. After inspection, the medium was removed from those treatments which exhibited no cytotoxicity and was stored at -80°C for later hormone analysis.

RNA was extracted from the cells using Absolutely RNA RT-PCR “miniprep” kits (Stratagene, La Jolla, CA, USA). Quantity and quality of the extracted mRNA was measured spectrophotometrically. After RNA extraction, reverse transcription was conducted with the Cloned AMV First-Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Dilutions of cDNA were prepared and Q-PCR conducted using previously published methods by Hilscherova et al. (2004) in a Smart Cycler System (Cepheid, Sunnyvale, CA, USA) in 25 μL sterile tubes using Sybr Green as quantification dye. Melting curve analyses were performed immediately after the final PCR cycle to differentiate between the desired amplicons and any primer-dimers or DNA contaminants. The steroidogenic genes analyzed by Q-PCR were *3 β HSD2*, *CYP11 β 2*, *CYP17* and *CYP19*. Specifics of the assay parameters such as primers, annealing temperatures, and efficiency of PCR for these genes have been published previously (Hilscherova et al. 2004). For quantification of gene expression, Ct values from the Q-RT-PCR reactions were used to express the results as fold difference with respect to the appropriate solvent control, and expression data was standardized to the expression of the β -actin gene. Gene expression was measured in triplicate for each control or exposed cell culture, and each exposure was repeated three times.

2.5 Hormone quantification

Hormone extraction and quantification by ELISA were conducted as previously described (Hecker et al. 2006). Briefly, frozen media samples were thawed on ice, and the hormones were extracted twice with 5 mL of diethyl ether in glass tubes. To determine extraction efficiencies, a trace amount of ³H-testosterone was added to each sample before extraction. Concentrations of hormones in media were measured by competitive ELISA using Cayman Chemical® hormone EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA) for progesterone [Cat no. 582601], testosterone (Cat no. 582701), 17 β -estradiol [(Cat # 582251). Because the antibody to progesterone exhibits cross-reactivity with *pregnenolone* of approximately 61%, and the method does not allow for the separation of these two hormones, progesterone concentrations are expressed as the sum of progesterone and pregnenolone (P). Hormones in all media samples were measured in triplicate. The working ranges of these assays for the quantification of steroid hormones in H295R media were determined to be: progesterone (P): 7.8–1,000 pg mL⁻¹, testosterone (T): 3.9–500 pg mL⁻¹, 17 β -estradiol (E2): 7.8–1,000 pg mL⁻¹. Extracts were diluted 1:25 and 1:100 for T, while for P and E2, dilutions were 1:50 to 1:100 and 1:2 to 1:10, respectively. Hormone extraction is required especially for the determination of P and E2 because the antibodies used

in these ELISAs appear to cross-react with unknown compounds in the non-extracted medium. In fact, some of the antibodies used with the hormone ELISA systems utilized in this study cross-react with steroid metabolites and conjugates such as sulfates and glucuronates. Such compounds are likely to be produced by the cells and are removed during the extraction process. Cross-reactivity was checked quantifying hormones with extracted and non-extracted medium samples.

2.6 Statistical analysis

Statistical analyses of gene expression and hormone production were conducted using SYSTAT (SYSTAT Software, Point Richmond, CA, USA). Data are expressed as means and SDs. Before using parametric statistics, the normality of each sample set was assessed with the Kolmogorov–Smirnov one-sample test with Lillifor’s transformation. Variance homogeneity was determined with Levene’s test. For data sets determined not to be normal, the data were log transformed to approximate the normal probability distribution and reevaluated. Using either untransformed or transformed data when appropriate, differences in gene expression were evaluated by analysis of variance followed by Tukey’s test. Differences with $p < 0.05$ were considered significant. Principle component analysis (PCA) was performed with untransformed data using the covariance matrix with pair-wise deletion and “Varimax” rotation.

3 Results

3.1 Water quality parameters and cell viability

Values for the physical and chemical characteristics varied among locations (see Table 1). In general, the least pH values were measured in waters from the WWTPs where values were approximately 7.5. However, untreated waters from the Sha Tin treatment plant had a pH of 8.34. The greatest pH value (8.76) was measured in the vicinity of the effluents from San Wai (HK25) WWTP. Salinity values ranged from 11.5 to 36‰ among locations. The minimum and maximum salinities were observed in samples of treated effluents from the Sha Tin STP (HK29) and the public landfill Tseung Kwan O (HK17), respectively. Dissolved oxygen ranged from a minimum of 0.2 mg l^{-1} in untreated sewage from the Sha Tin STP (HK28) to 0.8 mg l^{-1} in treated effluents from the Stonecutters Island WWTP (HK31). Dissolved oxygen concentrations in coastal marine waters ranged from 3.8 to 9.6 mg l^{-1} . Only extracts from samples taken from two of the major fish culture zones, Yim Tin Tsai (HK04) and the Sok Kwu Wan

open-water fishing zone (HK09) were toxic to H295R cells to the point of inhibiting cell growth and proliferation with consequent death. As a result of this toxicity, gene expression and hormone production data were not measured for these samples. The other 26 samples did not affect growth and proliferation of the H295R cells at any dilution.

3.2 Effects on steroidogenic functions

Extracts from the marine water samples collected during this study primarily modulated expression of steroidogenic genes (Table 2). Of the 24 sample extracts evaluated in this group of samples, 15 significantly altered expression of at least one gene relative to that of the solvent control. Furthermore, of these 15 samples, the expression of the aromatase gene (*CYP19*) had the greatest frequency of statistically significant alterations (13 out of 24 samples) when compared to the other genes evaluated in this study. Other effects at the level of gene expression include, e.g., a significant increase in the expression of $3\beta\text{HSD2}$ and $\text{CYP11}\beta\text{2}$ (sample HK03). These changes were not associated with changes in expression of *CYP19*. In contrast to HK03, the extract from one sample (HK22) taken close to the Aberdeen and Ap Lei Chau WWTPs not only significantly decreased the expression of the *CYP19* but also the expression of the androgenic gene *CYP17*. Although extracts from this sample also increased the production of P and E2 hormones by more than two fold (Tables 2 and 3), these increases were not statistically significant when compared to the solvent controls. When extracts of waters from the central reclamation site (HK16) located between Kowloon and Hong Kong Island were evaluated, *CYP19* gene expression was significantly reduced from control levels, while there was approximately a tenfold up-regulation of expression of the $\text{CYP11}\beta\text{2}$, responsible for the production of aldosterone. In samples collected from a location that receives effluents from the old sewage treatment works (STW) from San Wai (HK27), there was a statistically significant decrease in *CYP19* expression that did not represent a change in E2 production. Furthermore, while *CYP19* expression was significantly decreased by several sample extracts taken from sites characterized by effluents of major public sewage treatment works, no consistent and statistically significant alterations in E2 production were observed in any of these samples.

Effects on gene expression only occurred in effluents from the Sha Tin WWTP. Here, a decrease of approximately 40% was observed in the expression of the aldosterone gene $\text{CYP11}\beta\text{2}$; the expression levels of the other genes were not affected (Fig. 3a). Several significant changes were observed in hormone production (Fig. 3b); the extract from the influents from the Sha Tin WWTP reduced E2 production by approximately 30% compared to solvent

Table 2 Gene expression results for methanolic water extracts of marine waters from Hong Kong, China

Sample	Gene							
	<i>CYP19</i>		<i>CYP17</i>		<i>3βHSD2</i>		<i>CYP11B2</i>	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
Blank	1.08	0.57	1.37	1.38	1.20	0.18	1.03	0.03
MeOH	1.00	0.02	0.96	0.05	1.07	0.10	1.00	0.01
HK01	1.04	0.12	1.36	0.21	1.87	0.28	1.56	0.32
HK02	1.13	0.10	1.09	0.06	2.08	0.27	4.53 ^a	1.67
HK03	1.03	0.04	0.97	0.13	2.60 ^a	1.34	5.31 ^a	0.10
HK05	0.04 ^a	0.01	0.76	0.21	0.80	0.07	3.52	0.36
HK06	1.16	0.09	1.48	0.35	0.89	0.41	2.64	0.35
HK07	0.97	0.10	0.97	0.03	ND	ND	ND	ND
HK08	0.04 ^a	0.00	0.59	0.05	0.96	0.05	2.30	0.21
HK10	0.93	0.02	0.95	0.14	1.55	0.69	4.57	0.28
HK11	0.03 ^a	0.00	0.68	0.14	0.92	0.11	2.29	0.19
HK12	1.02	0.01	0.92	0.03	1.72	0.41	1.19	0.38
HK13	1.08	0.20	1.02	0.11	1.31	0.44	2.80	0.52
HK14	0.02 ^a	0.01	0.35	0.43	0.64	0.52	2.28	0.40
HK15	0.96	0.01	0.89	0.01	0.73	0.05	1.80	0.39
HK16	0.01 ^a	0.00	0.53	0.05	0.40	0.00	9.87 ^a	0.54
HK17	0.01 ^a	0.00	1.10	0.07	0.38	0.03	3.48	0.33
HK19	1.12	0.05	0.94	0.14	0.87	0.30	1.89	0.44
HK20	0.01 ^a	0.00	0.90	0.01	0.32	0.02	3.90	0.35
HK21	1.07	0.08	1.02	0.06	1.11	0.32	3.02	0.82
HK22	0.01 ^a	0.01	0.09 ^a	0.07	0.37	0.18	2.48	0.19
HK23	0.17 ^a	0.18	0.42	0.05	0.34	0.13	0.62 ^a	0.09
HK24	0.01 ^a	0.00	0.66	0.02	0.41	0.00	3.29	0.65
HK25	0.03 ^a	0.03	0.48	0.62	0.57	0.58	2.81	0.28
HK26	0.01 ^a	0.00	0.78	0.02	0.36	0.03	2.58	0.18
HK27	2.53 ^a	0.28	1.82	0.01	0.62	0.09	1.93	0.16

All exposures were conducted for 48 h under standard conditions. All gene expression values for fold change relative to MeOH the solvent control (=1), given as means and SDs.

^aIndicates statistically significant differences at $p < 0.05$.

control levels while P production was increased significantly by extracts of nontreated waters from the Stonecutters Island treatment plant. T production was not affected significantly by any of the samples collected from these treatment plants.

3.2.1 Correlation analysis

Pearson correlation analysis demonstrated several statistically significant associations between gene expression and hormone production data. For the gene expression data, statistically significant positive, correlations were found between *CYP19* and *CYP17* ($r=0.761$) as well as between *3βHSD2* and *CYP11B2* ($r=0.604$). For hormone production, statistically significant positive correlations were found between E2 production and P ($r=0.565$) and testosterone ($r=0.536$). The only significant correlation between gene expression and hormone production was a negative association between *CYP19* and E2 production ($r=-0.531$) indicating that, while there was only one

statistically significant alteration in E2 production observed in this study, there was still an association between these two variables.

3.2.2 PCA analysis

Results from the PCA analysis showed that approximately 82% of the variation in the HK sample set was accounted for by the first three factors. Of this variation, factor 1 accounted for approximately 42% of the variation, while factors 2 and 3 accounted for approximately 25% and 15% of the variation, respectively. Factor 1 was associated with hormone production (E2, T and P). In contrast, factor 2 was associated with the expression of the genes *CYP19*, *CYP17*, and *3βHSD2*. The only variable that was significantly segregated into factor 3 was the expression of *CYP11β2*. While a scatter plot of factors 1 2 (Fig. 4a) indicated a relatively diffuse grouping of samples that were not generally representative of activity or geographic location

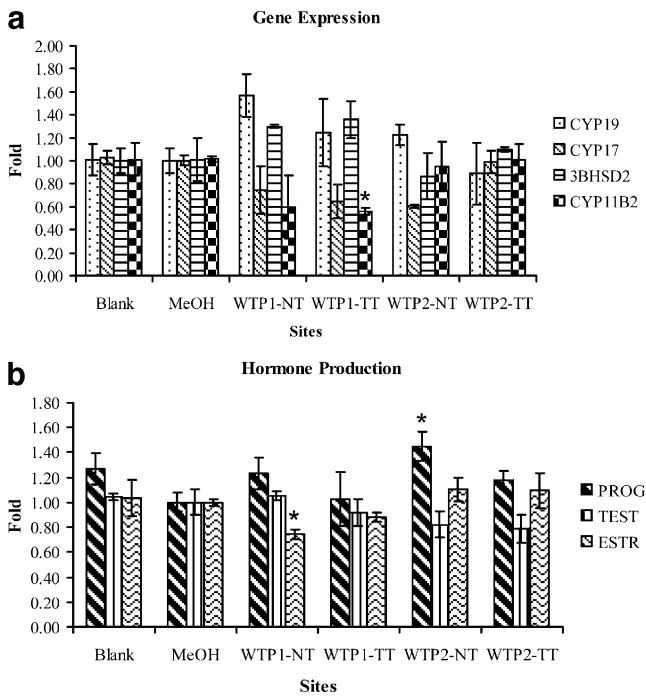


Fig. 3 Gene expression (a) and hormone production (b) for methanolic extracts from water samples from major waste water treatment plants—WWTP in Hong Kong, China. Nontreated water—NT. Treated water—T. WTP1—Sha Tin. WTP2—Stonecutters

within the study area, several clusters of samples were observed. The first group (HK1, HK2 and HK3) was segregated along factor 1 indicating the influence of hormone production on these samples. A second more diffuse group (HK5, HK8, HK14, HK22 and HK25) segregated along both factors 1 and 2 indicating that both hormone production and CYP19 and CYP17 gene expression influenced these samples. In a comparison of factors 1 to 3 (Fig. 4b), all samples were associated with hormone production except sample HK16 that was clearly influenced by the CYP11β2 factor. While we could separate the locations into distinct groups, the toxicological and ecological significance of these differences between the groups is unknown and would be difficult to assess without additional data.

4 Discussion

One of the advantages of the H295R cell bioassay is that it allows the study of simultaneous endpoints such as the expression of key steroidogenic genes, enzyme activities, and hormone production (molecular, protein, and functional levels), these endpoints can provide key information for establishing and/or understanding mechanism of actions and pathways in the study of potential endocrine disruption. Despite further characterization of autoregulatory and metabolic capabilities for this system is required, this

ability to integrate multiple effects at different biological levels (Hilscherova et al. 2004; Gracia et al. 2006) coupled with the observed low variability (Villeneuve et al. 2007) make the H295R assay a more realistic assay that—to some extent—can be more predictive of an in vivo effect than other in vitro assays such as H4IIE or MVLN cells that can only provide information about receptor-mediated effects. The H295R cell bioassay not only has shown to be responsive to changes of chemical concentrations and to chemical interactions that may occur in complex mixtures (Gracia et al. 2007), but also these responses have been consistent with the modes of action and observed effects of these chemicals in animal models (Hecker et al. 2006).

In this study, the H295R bioassay was used for the analysis of marine water samples collected in the vicinity of Hong Kong, China, and results revealed a variety of endocrine effect patterns at the level of steroidogenic gene expression and hormone production indicating the presence of compounds with endocrine disrupting potential. At some locations, the endocrine disrupting potential could not be assessed due to the presence of toxic compounds. The cause of the cytotoxicity provoked by the two extracts from marine fish culture zones (HK04 and HK09) remains unknown, and further analysis, such as the utilization of fractionation techniques, are required to separate cytotoxic effects from potential endocrine disrupting activities. However, it may be speculated that high concentrations of different types of common environmental contaminants in Hong Kong waters, including chemicals commonly used by local fish farmers, may be responsible for the observed toxicity. Medical substances used in fish farming, such as antibiotics and pesticides, including tetracyclines and malachite green, will be directly placed in receiving waters because they are usually dosed as feed additives (Gulkowska et al. 2007; Halling-Sorensen et al. 1998; Richardson et al. 2005); however, these scenarios remain speculative, and until these samples are further characterized, the specific cause of this toxicity remains unknown. Our data showed that the most up-regulated gene in marine samples was the aldosterone gene CYP11β2 reaching between four- and tenfold increments in expression and at the same time down-regulation of the progesterone gene 3βHSD2 observed. In previous studies by Bláha et al. (2006) where the H295R was used to study the effects of organic contaminants associated with freshwater pond sediments, increments in the expression of CYP11β2 and decrements in 3βHSD2 were also observed at the same extent, and these observations were associated with the presence of polychlorinated biphenyls (PCBs) and OCPs which lead us to hypothesize the presence of similar compounds in our samples.

The CYP19 gene regulates the production of the aromatase enzyme responsible for converting T to E2 (see Fig. 1). Several of the sample extracts collected in the

Table 3 Hormone production in H295R cells exposed to methanolic extracts of marine waters from Hong Kong, China^a

Sample	Hormone					
	Progesterone		Testosterone		Estradiol	
	Mean	STD	Mean	STD	Mean	STD
Blank	1.88	1.13	0.95	0.37	1.24	0.51
MeOH	1.00	0.21	1.00	0.13	1.00	0.02
HK-01	2.57	0.66	0.96	0.46	2.29	1.05
HK-02	2.86	1.24	1.10	0.24	2.97	0.90
HK-03	1.58	0.33	1.01	0.23	2.43	0.04
HK-05	1.88	0.43	1.34	0.23	2.76	1.06
HK-06	1.13	0.42	0.73	0.10	0.92	0.05
HK-07	2.90	1.92	1.01	0.08	2.48	0.15
HK-08	1.31	0.34	0.91	0.09	2.67	0.12
HK-10	0.77	0.02	0.68	0.09	0.83	0.18
HK-12	1.66	0.88	0.69	0.14	2.22	0.81
HK-13	1.72	1.10	0.80	0.04	1.03	0.25
HK-14	3.01	0.72	1.04	0.07	2.32	0.13
HK-15	0.58	0.38	0.32	0.06	0.84	0.07
HK-19	1.96	0.83	0.77	0.15	1.15	0.16
HK-21	1.86	1.15	0.99	0.19	1.14	0.38
HK-22	2.26	1.29	0.99	0.20	3.04	0.57
HK-25	3.11	1.30	1.32	0.15	3.61 ^b	0.57
HK-27	1.14	0.05	0.95	0.08	0.89	0.10
HK-16	0.93	0.15	0.87	0.06	0.84	0.12

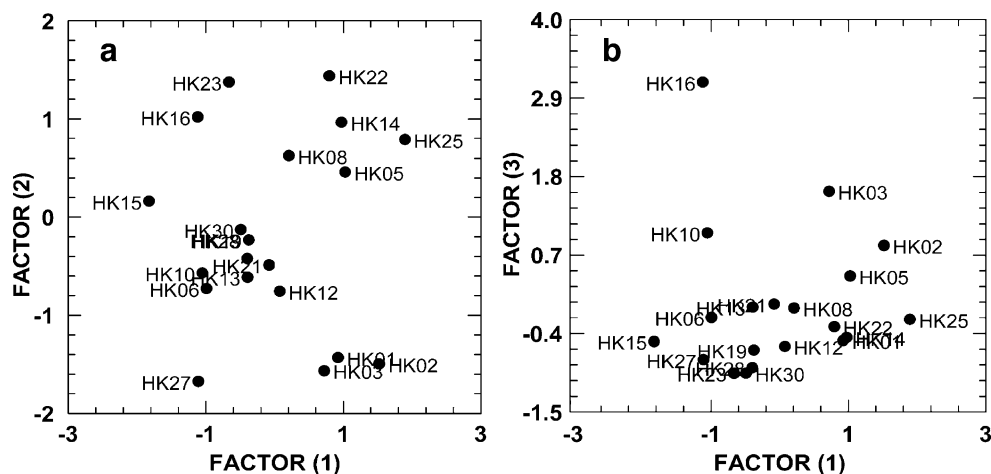
^a All exposures were conducted for 48 h under standard conditions. All hormone production values are expressed as fold change relative to control given as means and SDs.

^b Statistically significant differences at $p < 0.05$

marine environment around Hong Kong significantly decreased the expression of the *CYP19* gene, although no statistically significant changes were observed in E2 production, and in one instance, an increase in E2 hormone concentrations was observed. One potential reason for this phenomenon is that alternative steroidogenic pathways could be influencing the E2 production in the cells that are not directly dependent on *CYP19*. For instance, the conversion of estrone (E1) to E2 via 17 β -HSD (Poutanen

et al. 1995) could have contributed to the overall production of E2 such that even with significant changes in *CYP19* gene expression, the E1 pathways could still be maintained or even increased the overall E2 concentrations during the study. However, as 17 β -HSD gene expression was not evaluated in this study, this possible mechanism cannot be further evaluated. A second possible hypothesis would be the presence of mixtures of compounds that may affect aromatase gene expression but do not affect the activity of

Fig. 4 Plot principal component analysis (PCA). Factors of gene expression and hormone production for sample extracts collected from Hong Kong waters. **a** Factor (1) vs. factor (2). **b** Factor (1) vs. factor (3)



the aromatase enzyme. Some samples, particularly HK25, even showed an increase in E2 production suggesting a potential estrogen-stimulating activity caused by compounds that were not completely degraded by the San Wai sewage. Finally, it is possible that the decrease in CYP19 gene expression can be a compensatory mechanism in response to increased E2 production. Several studies have demonstrated that aromatase inhibitors such as fadrozole that decrease E2 production can result in an increase in gonadal CYP19 gene expression (Villeneuve et al. 2006; Tompsett et al. 2007). Similarly, increase in either endogenous or exogenous exposure to estrogens could trigger compensatory mechanisms in an attempt to maintain estrogen homeostasis. Studies conducted in the northwestern waters of Hong Kong have indicated the continued presence of PCBs and OCPs together with considerable bioaccumulation of metals in marine mammals native to the region (Tam and Yao 2002). Results of exposure of H295R cells to PCBs have previously demonstrated the down-regulation of the androgenic gene *CYP17* by these chemicals (Li and Wang 2005). Thus, the presence of these compounds in the samples collected in the Aberdeen and Ap Lei Chau area (HK22) may be responsible for the observed decreases in the expression of this gene. Several PCB congeners also have been found to significantly induce the expression of the *CYP11 β 2* gene (Xu et al. 2006), and therefore, the presence of these compounds in the sample from Sai Lau Kong (HK03) may be considered to be responsible, in part, for the fivefold up-regulation of this gene observed in our study. The significant estrogenic activity produced by sample HK25 corresponding to the extract of receiving effluents from the old sewage in San Wai, reflected by a nearly fourfold increase in E2 production, suggests that the waste water treatment used in this plant is not effectively removing compounds capable of inducing E2 production.

Results from our study strongly suggest that treatment used in both the Sha Tin and Stonecutters Island WWTPs can be considered effective in removing most of the compounds and metabolites with the potential to interact with steroidogenesis despite some slight effects observed at the level of steroidogenic gene expression from the effluents' exposures. However, only influents affected hormone production, while effluents did not affect this endpoint significantly. Hormone production was more affected by influents, effluents did not affect. In this context, it is important to note that the H295R bioassay evaluates the potential of extracts to alter the steroidogenic pathways associated with the biosynthesis of androgens, estrogens, progestagens, and corticoids, thus the presence of compounds that express their endocrine activity through receptor-mediated responses or other nonsteroidogenic pathways may not be ruled out, although H295R results has been consistent with results from receptor-mediated

assays such as H4IIE or MVLN cells (Bláha et al. 2006). Several studies have been conducted to identify effects of WWTPs effluents in vivo. The variability of exposure to mixtures of EDCs in WWTP effluents and the inherent biological responses make it difficult to identify causative factors; however, some WWTP effluents have been found to induce feminization, modulate immune function, and cause genotoxic damage in exposed animals, and these detrimental effects have been linked to the presence of EDCs (Filby et al. 2007; Liney et al. 2006). Furthermore, reproductive responses have indicated both beneficial and detrimental effects from exposure to WWTP effluents (Barber et al. 2007). To our knowledge, no studies with undefined environmental mixtures have been tested with both H295R and in vivo models, but it will be beneficial to have a comparative study of such kind.

5 Conclusions

Changes observed in gene expression and hormone production suggest the presence of pollutants in Hong Kong coastal waters that can cause minor disruptions of steroidogenic processes. Considerable efforts have been made to upgrade the infrastructure of the newly constructed WWTPs in Hong Kong and to couple these upgrades with new strategies to established more effective sewage disposal processes on a regional basis. While these efforts may result in the elimination and/or reduction of EDCs from waste waters, as is suggested from the results here obtained when comparing treated and nontreated samples, there is an indication that the presence of pollutants in Hong Kong coastal waters will persist due to past practices and other current activities that will act as sources of these contaminant to this system. Additional studies are needed to further characterize the endocrine-disrupting potential of these sources to Hong Kong coastal waters. However, the inclusion of bio-analytical approaches such as the H295R steroidogenesis assay, along with other in vitro receptor-based bioassays, can be used to help direct future analytical chemical efforts to identify and quantify these chemicals in a cost-effective manner. Furthermore, the H295R assay can be used in a bioassay-directed fractionation scheme to allow the isolation and or identification of the causative agents.

6 Recommendations and perspectives

Due to the intrinsic complexity of environmental samples, a combination of analytical tools is required to realistically assess environmental conditions, especially in aquatic systems. In the evaluation of endocrine disrupting activities,

the H295R cell bioassay should be used in combination with other biological, chemical, and hydrological tests to establish reliable mechanisms of endocrine disruption and identify compounds responsible for the observed effects.

Acknowledgement The work described in this paper was supported by the Area of Excellence Scheme under the University Grants Committee of the Hong Kong Special Administration Region, China (Project No. AoE/P-04/2004) and supported by U.S.EPA, ORD Service Center/NHEERL, contract GS-10F-0041 L. PKSL was supported by a strategic research grant of City University of Hong Kong (Grant number 7001818). The authors gratefully acknowledge the invaluable help of Mr. and Mrs. Kwok on the journeys of sample collection. Many thanks to Dr. Eric Ching and Dr. James Lam from City University of Hong Kong for their excellent job on the planning and execution of the field sampling strategies required for this study.

References

- Barber LB, Lee KE, Swackhamer DL, Schoenfuss HL (2007) Reproductive responses of male fathead minnows exposed to wastewater treatment plant effluent, effluent treated with XAD8 resin, and an environmentally relevant mixture of alkylphenol compounds. *Aquat Toxicol* 82:36–46
- Blaha L, Hilscherova K, Mazurova E, Hecker M, Jones P, Newsted J, Bradley P, Gracia T, Duris Z, Horka I, Holoubek I, Giesy JP (2006) Alteration of steroidogenesis in H295R cells by organic sediment contaminants and relationships to other endocrine disrupting effects. *Environ Int* 32:749–757
- Connell DW, Wu RSS, Richardson BJ, Leung K, Lam PSK, Connell PA (1998) Occurrence of persistent organic contaminants and related substances in Hong Kong marine areas—an overview. *Mar Pollut Bull* 36:376–384
- Cheung KC, Leung HM, Kong KY, Wong MH (2007) Residual levels of DDTs and PAHs in freshwater and marine fish from Hong Kong markets and their health risk assessment. *Chemosphere* 66(3):460–468
- Committee on Restoration of Aquatic Ecosystems: Science, Technology, and Public Policy. Commission on Geosciences, Environment, and Resources. Water Science and Technology Board (1992) Restoration of Aquatic Ecosystems. National Academy Press. Washington, DC
- EDSTAC (1998) Endocrine Disruptor Screening and Testing Advisory Committee Final Report. USEPA, <http://www.epa.gov/opptint/oppntendo/finalrpt.htm>
- Filby AL, Neuparth T, Thorpe KL, Owen R, Galloway TS, Tyler CR (2007) Health impacts of estrogens in the environment, considering complex mixture effects. *Environ Health Perspect* 115:1704–1710
- Gulkowska A, He MK, So L, Yeung WY, Leung HW, Giesy JP, Lam PKS, Martin M, Richardson BJ (2007) The occurrence of selected antibiotics in Hong Kong coastal waters. *Mar Pollut Bull* 54:1278–1293
- Gracia T, Hilscherova K, Jones P, Newsted J, Zhang X, Hecker M, Higley E, Sanderson J, Yu RMK, Wu RSS, Giesy JP (2006) The H295R system for evaluation of endocrine-disrupting effects. *Ecotoxicol Environ Saf* 65:293–305
- Gracia T, Hilscherova K, Jones P, Newsted J, Zhang X, Hecker M, Higley E, Yu RMK, Wu RSS, Giesy JP (2007) Modulation of steroidogenic gene expression and hormone production of H295R cells by pharmaceuticals and other environmentally active compounds. *Toxicol Appl Pharmacol* 225:142–153
- Halling-Sorensen B, Nors S, Lanzky PF, Ingerslev F (1998) Occurrence, fate and effects of pharmaceutical substances in the environment—a review. *Chemosphere* 36:357–393
- Hecker M, Newsted JL, Murphy MB, Higley EB, Jones PD, Wu R, Giesy JP (2006) Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis—hormone production. *Toxicol Appl Pharmacol* 217:114–124
- Hecker M, Hollert H, Cooper R, Vinggaard A-M, Akahori Y, Murphy M, Nellesmann C, Higley E, Newsted J, Wu R, Lam P, Laskey J, Buckalew A, Grund S, Nakai M, Timm G, Giesy JP (2007) *Environ Sci Pollut Res* 14(Special Issue 1):23–30
- Hilscherova K, Jones PD, Gracia T, Newsted JL, Zhang X, Sanderson JT, Yu RMK, Wu RSS, Giesy JP (2004) Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. *Toxicol Sci* 81:78–89
- Ho KC, Chow YL, Yau JTS (2003) Chemical and microbiological qualities of the East River (Dong Jiang) water, with particular reference to drinking water supply in Hong Kong. *Chemosphere* 52:1441–1450
- Hong Kong Environmental Protection Department (HKEPD) (2002) Marine water quality in Hong Kong in 2001. Hong Kong SAR Government, Hong Kong, PR China, pp. 130–145
- Hung CLH, Lau RKF, Lam JCW, Jefferson TA, Hung SK, Lam MHW, Lam PKS (2007) Risk assessment of trace elements in the stomach contents of Indo-Pacific humpback dolphins and finless porpoises in Hong Kong waters. *Chemosphere* 66:1175–1182
- Ip CCM, Li XD, Zhang G, Wai OWH and Li YS (2007) Trace metal distribution in sediments of the Pearl River estuary and the surrounding coastal area, South China. *Environ Pollut* 147:311–323
- Kavlock RT, Daston GP, De Rosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C, Miller R, Moore J, Rolland R, Scott G, Sheehan DM, Sinks T, Tilson HA (1996) Research needs for the risk assessment of health and environmental effects of endocrine disruptors—a report of the USEPA sponsored workshop. *Environ Health Perspect* 104:715–740
- Li LA, Wang PW (2005) PCB126 induces differential changes in androgen, cortisol, and aldosterone biosynthesis in human adrenocortical H295R cells. *Toxicol Sci* 85:530–540
- Liney KE, Hagger JA, Tyler CR, Depledge MH, Galloway TS, Jobling S (2006) Health effects in fish of long-term exposure to effluents from wastewater treatment works. *Environ Health Perspect Suppl* 1:82–89
- Liu Y, Zheng G, Yu H, Martin M, Richardson BJ, Lam HW, Lam PKS (2005) Polybrominated diphenyl ethers (PBDEs) in sediments and mussel tissues from Hong Kong marine waters. *Mar Pollut Bull* 50:1173–1184
- Monfils R, Gilbert T and Nawadra S (2006) Sunken WWII shipwrecks of the Pacific and East Asia: The need for regional collaboration to address the potential marine pollution threat. *Ocean Coastal Manage* 49:779–788
- Poutanen M, Isomaa V, Peltoketo H, Vihko R (1995) Role of 17 beta-hydroxysteroid dehydrogenase type 1 in endocrine and intracrine estradiol biosynthesis. *J Steroid Biochem Mol Biol* 55:525–532
- Ramu K, Kajiwara N, Tanabe S, Lam PKS and Jefferson T (2005) Polybrominated diphenyl ethers (PBDEs) and organochlorines in small cetaceans from Hong Kong waters: Levels, profiles and distribution. *Mar Pollut Bull* 51:669–676
- Richardson BJ, Lam PKS and Martin M (2005) Emerging chemicals of concern—pharmaceuticals and personal care products (PPCPs) in Asia, with particular reference to southern China. *Mar Pollut Bull* 50:913–920
- Sanderson JT, Seinen W, Giesy JP, Van den Berg M (2000) 2-Chloro-5-triazine herbicides induce aromatase (CYP19) Activity in H295R human adrenocortical carcinoma cells—a novel mechanism for estrogenicity. *Toxicol Sci* 54:121–127
- Sanderson JT, Boerma J, Lansbergen WA, Van der Berg M (2002) Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. *Toxicol Appl Pharmacol* 182:44–54

- Shelton LR (1994) Open-File Report. US Geological Survey, No 94-455
- So MK, Zhang X, Giesy JP, fung CN, Fong HW, Zheng J, Yoo KH, Lam PKS (2005) Organochlorines and dioxin-like compounds in green-lipped mussels *Perna viridis* from Hong Kong mariculture zones. *Mar Pollut Bull* 51:677–687
- Tam NFY and Yao MWY (2002) Concentrations of PCBs in coastal mangrove sediments of Hong Kong. *Mar Pollut Bull* 44:642–651
- Tompsett A, Park J, Hecker M, Jones P, Newsted J, Giesy J (2007) Gene expression and histological structure as biomarkers of chemical exposure in *Japanese medaka*. 34th Annual Aquatic Toxicity Workshop, 30th September to 3rd October, Halifax, Nova Scotia
- The Government of the Hong Kong Special Administrative Region—drainage services department (2006) Sewerage strategy, http://www.dsd.gov.hk/sewerage/sewerage_strategy/index.htm
- Villeneuve DL, Blankenship AL, Giesy JP (1998) Estrogen receptors—environmental xenobiotics. In: Denison MS, Helferich WG (eds) *Toxicant–receptor interactions and modulation of gene expression*. Lippincott-Raven Publishers, Philadelphia, pp 69–99
- Villeneuve DL, Knoebl I, Kahl M, Jensen K, Hammermeister D, Greene K, Blake L, Ankely G (2006) Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (*P. promelas*). *Aquat Toxicol* 76:353–368
- Villeneuve DL, Ankley GT, Makynen EA, Blake LS, Greene KJ, Higley EB, Newsted JL, Giesy JP, Hecker M (2007) Comparison of fathead minnow ovary explant and H295R cell-based steroidogenesis assays for identifying endocrine-active chemicals. *Ecotoxicol Environ Saf* 68:20–32
- Wilson VS, Bobseine K, Gray LE (2004) Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol Sci* 81:69–77
- Wilson VS, Bobseine K, Lambright CR, Gray LE (2002) A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicol Sci* 66:69–81
- Wong YS, Tam N, Lau PS, Xue XZ (1995) The toxicity of marine sediments in Victoria Harbour, Hong Kong. *Mar Pollut Bull* 31:464–470
- Wurl O, Lam PKS, Obbard J (2006) Occurrence and distribution of polybrominated diphenyl ethers (PBDEs) in the dissolved and suspended phases of the sea-surface microlayer and seawater in Hong Kong, China. *Chemosphere* 65:1660–1666
- Xu Y, Yu RMK, Zhang X, Murphy MB, Giesy JP, Lam MHW, Lam PKS, Wu RSS, Yu H (2006) Effects of PCBs and MeSO₂-PCBs on adrenocortical steroidogenesis in H295R human adrenocortical carcinoma cells. *Chemosphere* 63:772–784
- Yung YK, Yau K, Wong CK, Chan KK, Yeung I, Kueh CSW, Broom MJ (1999) Some observations on the changes on physico-chemical and biological factors in Victoria Harbour and vicinity, Hong Kong, 1988–1996. *Mar Pollut Bull* 39:315–325
- Zhang X, Yu RMK, Jones PD, Lam GKW, Newsted JL, Gracia T, Hecker M, Hilscherova K, Sanderson JT, Wu RSS, Giesy JP (2005) Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. *Environ Sci Technol* 39:2777–2785