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In situ combined chemical and biological assessment of estrogenic pollution in a water recycling system

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ABSTRACT

Estrogenic pollution and its control in aquatic systems have drawn substantial attention around the world. The chemical and biological assessment approaches currently utilized in the laboratory or field cannot give an integrated assessment of the pollution when used separately. In this study, *in situ* chemical and biological methods were combined to detect pollution in a water recycling system. Data for the water quality index (WQI) demonstrated that the water treatment resulted in the decline of pollution from upstream to downstream. Wild male Nile tilapia, *Oreochromis niloticus*, was sampled in June and September. The concentrations of four common endocrine disrupting chemicals (EDCs) were determined in the tilapia liver by chromatographic analysis methods. The level of 17β-estradiol (E2) declined from upstream to downstream in both months. In contrast, the levels of bisphenol A (BPA), di-(2-ethylhcxyl) phthalate (DEHP), and perfluorooctane sulfonate (PFOS) did not display this declining tendency. The highest relative expression of vitellogenin 1 (VTG1) was observed in tilapia from upstream, then the level significantly decreased along the water system. The relative expression levels of CYP1A1 in the water system were also significantly higher than that of the control. However, no declining trend could be observed along the water system. The change of VTG1 expression corresponded well with that of E2 levels in the tilapia liver. Overall, our study assessed the pollution by endocrine disruptors using chemical and biological data with good correspondence. This study also demonstrated the effectiveness of the water recycling system in eliminating estrogen pollution in municipal sewage.

Introduction

Endocrine disrupting chemicals (EDCs) have received increasing attention because of their adverse influence on organisms. The aquatic environment is the ultimate destination for most environmental contaminants derived from industrial, agricultural and domestic wastewater. Studies have indicated that EDCs are prevalent in aquatic environments and induce endocrine dysfunction in aquatic organisms (Bertin et al., 2011; Oehlmann et al., 2000). EDCs are known to act by interfering with normal hormone biosynthesis (Patrick et al., 2014).

Vitellogenin 1 (VTG1) is synthesized in the liver and induced by estrogens. The induction of VTG1 synthesis in male fish by environmental estrogens has been proposed to be an effective and sensitive biomarker of estrogenicity (Goksøyr, 2006; Selcer and Verbanic, 2014). High levels of hepatic VTG1 were observed in Japanese medaka exposed to estrogenic EDCs (Yamaguchi et al., 2005). EDCs induced the increase of plasma VTG1 in a dose-related

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manner, and estrogenic chemicals shared the identical mechanism for inducing VTG1 synthesis (Chang et al., 2011). The cytochrome P450 (CYP450) gene signaling pathway plays an important role in the defense against environmental stress (Kim et al., 2015). CYP1A1, one of the CYP1 family isoforms, is the most relevant form described so far (Costa et al., 2012; Koenig et al., 2013; Solé et al., 2012). The level of CYP1A1 expression is inducible by exposure to certain EDCs (Zhu and Lee, 2005).

Oreochromis niloticus is a good biological model for toxicological studies. This fish has high growth rates, is efficient in acclimatizing to various diets and is resistant to diseases (Eshel et al., 2012; Fırat et al., 2011). *O. niloticus* is generally found in estuaries around the world and responds rapidly to environmental change. The biochemical parameters of *O. niloticus* are sensitive for detecting potential negative effects (Almeida et al., 2002).

Although laboratory studies have indicated negative influences on fish exposed to estrogen compounds, laboratory investigations of EDCs tend to use high chemical dose levels. The exposure cycle is relatively short. In general, chemical pollutants are present as a complicated environmental mixture and at relatively low concentrations in water ecosystems (Michel et al., 2013). Whether chronic exposures under environmentally relevant levels elicit adverse effects on wild fish populations (Kidd et al., 2007) in the natural environment has not been determined. Currently, measuring only the chemical characteristics does not allow one to judge the health of an aquatic ecosystem. Combined chemical and biological evaluations could provide a more systematic picture of water pollution (Oberholster et al., 2008). However, few previous studies combine chemical and biological data.

The objective of this study was to assess estrogenic pollution through combined chemical and biological methods in a field survey of wild *O. niloticus* from upstream to downstream locations in a water system. The levels of four commonly used endocrine disruptors were measured in the tilapia liver. Meanwhile, the Messenger ribonucleic acid (mRNA) levels of both VTG1 and CYP1A1 were also measured. The correspondence of both data was analyzed to validate the effectiveness of the method. Through this study, we established an effective method to assess estrogenic pollution.

1. Materials and methods

1.1. Standards and reagents

All reagents were of high performance liquid chromatography (HPLC) grade. The standard chemicals were purchased as follows: di-(2-ethylhcxyl) phthalate (DEHP) (Supelco, Bellefonte, PA, USA), 17β-estradiol (E2) and perfluorooctane sulfonate (PFOS) (Sigma, St. Louis, MO, USA), and bisphenol A (BPA) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). $^{13}C_{12}$BPA (Cambridge Isotope Laboratories Ins, USA) and $^{13}C_4$PFOS (Wellington Laboratories Inc., Guelph, ON, Canada) were added as internal standards.

1.2. Sample collection in the water recycle system

Wild *O. niloticus* were collected using a fish net at eight sampling sites (S1–S3, upstream; S4–S6, midstream; S7–S8, downstream) in the water recycling system during June and September 2014 (Fig. 1). The water recycling system is located in Xiamen, China near the Xinglin Wan Reservoir. The sewage treatment unit is located upstream and includes an activated carbon filter, sand filters and plant absorption area. In the unit, effluent is first sent to a primary sedimentation tank and subsequently flows to the secondary sedimentation tank. Sewage treatment and recycling processes take place after the precipitation. Two small dams are present between the up- and middle-streams and middle- and down-streams. Each section has a plant adsorption area in the water reuse system. The tilapia were captured separately in each section. At each sampling point, dozens of fish were captured, and sex identification was subsequently performed according to the aquaculture industry standard of the People's Republic of China (SC/T 1105–2007). The male fish were used for the experiments. The weight and length of each fish were recorded, and the liver was then dissected and weighed. The hepatosomatic index (Huang et al., 2008) was calculated using the following equation: HSI = liver weight (g) / total fish weight (g) × 100%.

1.3. Measuring the water quality index (WQI)

The WQI was measured by a HACH Hydrolab multi-parameter probe (Hach Company, USA). The water was obtained from three sites (upstream, midstream and downstream). Each index was determined six times. The index included eight parameters: pH, temperature (T), oxidation reduction potential (ORP), turbidity (TUR), specific conductance (SPC), salinity (SAL), luminescent dissolved oxygen (LDO) and chlorophyll (CHL) content.

1.4. Determining DEHP and E2 by HPLC

To quantify DEHP and E2 in tilapia liver, the samples were extracted by solid phase extraction (SPE) with Oasis HLB cartridges (60 mg, 3 cc, Waters, Milford, MA, USA). The process was as follows: 3 mL of ethyl acetate was added to the liver samples, which were then immersed in an ultrasonic bath for 25 min. Next, 1 mL of Milli-Q water was added. The homogenates

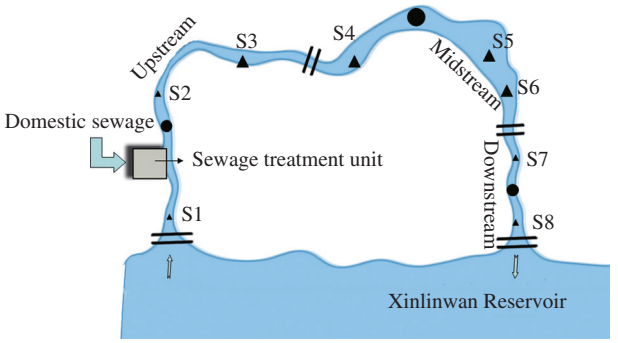

Fig. 1 – Sampling sites for wild *O. niloticus* in the water reuse system in Xiamen, China. S1–S8 (black triangle) were the sampling sites, S1–S3 was the upstream area, S4–S6 was the midstream area, S7–S8 was the downstream area, and the square was the sewage treatment unit, the black rounded areas represented plant purification areas.

were vortexed for 20 sec and centrifuged at 4°C for 15 min. The organic layer was obtained. After three repetitions, the organic layers were combined. The SPE column was activated with 10 mL of methanol according to the manufacturer's instructions, and 5 mL of Milli-Q water was later added to balance the column. The organic layer samples were then loaded, and 30 mL methanol–water (5:95, V/V) was added into the SPE column as the cleaning fluid. The eluate was collected in a flask. Subsequently, pure methanol (10 mL) was used to transfer the EDCs into the glassware. Next, the methanol was completely evaporated under a nitrogen stream. The sample was redissolved in 1 mL of methanol prior to HPLC analysis. The quantitative methods for DEHP and E2 employed an HPLC system. The HPLC analyses were performed using a Hitachi L-2000 system equipped with a diode array detector (DAD; L-2455), a pump (L-2130) and an auto-sampler (L-2200) operating with the Model D-2000 Elite Station software. The DEHP was detected using a previous method (Li et al., 2012). The HPLC method for DEHP involved an ODS-C_{18} column (250 mm ×4.6 mm, particle size 5 μm) at 30°C; a mixture of methanol–water (95:5, V/V) was used as mobile phase, and the detection wavelength was 275 nm. The linear regression equation was:

$$y = 12.766x + 86.899,\ \ R^2 = 0.9819.$$

Analyses of E2 were performed according to published methods (Abouzarzadeh et al., 2012; Wang et al., 2006). The linear regression equation was:

$$y = 18x + 17.92,\ \ R^2 = 0.9977.$$

The separation was performed at a flow rate of 1 mL/min with acetonitrile-water (60:40, V/V) as the mobile phase. The oven temperature was set at 25°C.

1.5. Quantification of BPA and PFOS by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The concentrations of BPA and PFOS were determined using LC (Shimadzu Prominence LC-20A, Japan) combined MS/MS (Applied Biosystems 3200Q TRAP, USA). The procedure for sample extraction was based on a previous study (Huang et al., 2010). A C_{18} 100 A column (100 mm × 4.60 mm, 2.5 μm particle size, Phenomenex, USA) was used for the BPA and PFOS analysis. The sample preparation and condition of the mobile phase were similar to those in the published literature (Liu et al., 2014). The mass transitions of monitored ions were 503 → 99 for $^{13}C_4$PFOS and 499 → 99/80 for PFOS. A BPA derivative reaction was performed on the basis of previous study (Fang et al., 2014). For BPA, the mass transition monitored ions were selected as follows: 707 → 171 for $^{13}C_{12}$BPA and 695 → 171.2 for BPA. The PFOS and BPA contents were calculated according to the standard curves:

$$y = 4.11x + 17.9,\ \ R^2 = 0.9752$$

$$y = 0.44x + 0.519,\ \ R^2 = 0.9936.$$

1.6. Precision and accuracy

To validate the accuracy and precision of the chromatography methods for the EDCs, recovery tests were performed using three known standard solutions (*i.e.*, 4, 8, and 20 ng/mL) for each EDC. Six repeated analyses of *O. niloticus* liver were carried out, and the results were expressed as a percentage. The recovery test samples were extracted and analyzed using the procedure for sample extraction and analysis. The spiked recoveries for EDCs were satisfactory. The average recovery test results for the four compounds at three spiked concentrations ranged from 82.5% to 106.0% (Table 1). The relative standard deviations (RSDs) ranged from 6.8% to 14.5%. These recoveries were acceptable.

1.7. RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

A total of 50 mg of liver samples were used for RNA extraction by Omega RNA extraction kits (Omega, USA) according to the manufacturer's instructions. Equal amounts of RNA were then reverse-transcribed by the PrimeScriptTM RT-PCR Kit (TaKaRa, China) following the manufacturer's instruction. qRT-PCR was performed using a SYBR Premix Ex Taq™ kit (TaKaRa) on a Roche 480 Light Cycler Real-Time PCR System (Roche Applied Science, Indianapolis, IN, USA). Primer sequences of the tested genes used in the qRT-PCR (Table 2) were designed by the Primer-BLAST tool in NCBI and then checked for validation by OLIGO 6.0 software. The PCR thermal profiles were as follows: 95°C for 30 sec, 40 cycles at 95°C for 5 sec, and 60°C for 34 sec, and finally, a dissociation curve analysis. The PCR procedure was performed three times for each sample. Gene expression levels were normalized to the 18s rRNA expression level. Tilapia in the Xinglin Wan Reservoir was used as the control fish. The fold change of the tested genes was analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

1.8. Data analysis

SPSS 19.0 for Windows (IBM, Armonk, NY, USA) was used for the statistical analysis. One-way analysis of variance followed by a Tukey *post hoc* test was used to test the significance of

Table 1 – Spiked recoveries of four EDCs at three different concentration levels (n = 6).

Chemicals	Concentration (ng/mL)	Recovery (%)
DEHP	4	90.2 ± 7.2
	8	98.7 ± 11.2
	20	86.1 ± 10.8
E2	4	92.4 ± 9.2
	8	103.6 ± 14.3
	20	93.4 ± 9.7
PFOS	4	82.5 ± 12.5
	8	86.3 ± 14.5
	20	96.5 ± 6.8
BPA	4	95.4 ± 8.6
	8	96.3 ± 8.7
	20	106.0 ± 11.4

EDCs: endocrine disrupting chemicals; DEHP: di-(2-ethylhcxyl) phthalate; E2: 17β-estradiol; PFOS: perfluorooctane sulfonate; BPA: bisphenol A.

Table 2 – Primers used in the quantitative RT-PCR analysis.

Gene name	Nucleotide accession number	Primer sequences used for qRT-PCR (5′ to 3′)
VTG1	XM_003452574	F: CTTGGTGGCTGGATGGAAAGGA
		R: ATCAGTGCAACAAGTGCCAACG
CYP1A1	FJ664151	F: CAGAGACAACGACGTAGCGGAA
		R: CAACTTAGAGGGCACAACCCCA
18S	U67340	F: TGCTCAATCTCGTGTGGCTGAA
		R: TGGCCGTTCTTAGTTGGTGGAG

qRT-PCR: quantitative reverse transcription polymerase chain reaction.

data differences. The significant difference was set at $p < 0.05$. Values are expressed as the mean ± standard deviation (SD).

2. Results

2.1. Water quality data and HSI of tilapia

A HACH Hydrolab multi-parameter probe was employed to measure the WQI data (Table 3). Results showed that most indexes (TUR, SPC, SAL, CHL and LDO) declined from upstream to downstream. The water quality improved across the water system. Wild tilapia in the water system were used to assess the pollution. The basic parameters of the fish are listed in Table 4. The hepatosomatic index (HSI) of the fish was not significantly different among the three sites.

2.2. EDC concentrations in water system

Concentration levels of BPA, E2, DEHP and PFOS in tilapia liver are presented in Table 5. No significant difference in EDC concentration levels was noted between June and September. However, a declining trend was noted in the concentration of E2 from upstream to downstream in a single month. The levels of E2 ranged from 15.79 ± 0.33 to 9.77 ± 0.68 ng/g in June and ranged from 15.91 ± 1.09 to 10.65 ± 0.42 ng/g in September. By contrast, no significant difference in the levels of BPA, DEHP and PFOS was observed among the three sites.

2.3. Biomarker analysis

The mRNA levels of VTG1 and CYP1A1 genes in *O. niloticus* liver were compared among the fish from different sites (Fig. 2). Tilapia from the Xinglin Wan Reservoir were used as the control fish. The relatively highest VTG1 mRNA levels were observed in the upstream samples both in June and September. The level was significantly higher than any other sites. The VTG1 level in fish from the midstream was also significantly higher than that of the control. No significant difference was observed between fish in the downstream when compared to the control. A significant decrease from the upstream to downstream was observed. Similar relative expression tendencies appeared between June and September. The mRNA level of CYP1A1 reached maximum expression in the midstream and was significantly different when compared with the control group ($p < 0.05$). No significant difference was observed downstream ($p > 0.05$) both in June and September.

2.4. Relationship between the chemical data and biological data

Good positive correlations were observed between the E2 level and the VTG1 mRNA level (Fig. 3). The relationship between E2 and VTG1 was measured by linear equations, and statistical correlations were determined in the SPSS software. A bivariate correlation analysis was performed using the Pearson's correlation coefficient (r). The results showed a strong correlation between E2 and VTG1. The regression equations were:

in June: $y = 576.91x - 5786.2$, $R^2 = 0.9669$ $r = 0.9833$.

in September: $y = 1.703x - 17.881$, $R^2 = 0.9967$ $r = 0.9983$.

3. Discussion

Water quality criteria have been proposed for water quality management to protect aquatic organisms (Colt, 2006). In the water system, our WQI data showed that the water quality improved gradually along the flowing water. Most indexes (TUR, SPC, SAL, CHL and LDO) declined from upstream to downstream. The water quality improvement from upstream to downstream showed the favorable treatment effect of the water system.

EDCs have been frequently detected in domestic sewage discharge. In this study, estrogenic compounds (including BPA, E2, PFOS and DEHP) were detected in the water system. The maximum concentrations of BPA were found in the midstream. The concentrations in our study were close to those in the water systems of the Netherlands (Belfroid et al., 2002), indicating that BPA ranged from 2 to 75 ng/g in the fish liver. These levels were lower than those in supermarket

Table 3 – The WQI data from upstream to downstream in June and September.

Time	Sampling sites	ORP(mV)	TUR(NTU)	SPC(μS/cm)	SAL(ppt)	CHL(μg/L)	LDO(mg/L)
Jun	Upstream	355.25 ± 16.76	18.15 ± 3.38	6662.25 ± 115.26	3.76 ± 0.14	85.37 ± 2.49	171.95 ± 5.29
	Midstream	324.51 ± 8.50	12.76 ± 5.03	3240.25 ± 280.69	2.04 ± 0.20	29.49 ± 1.52	142.475 ± 5.79
	Downstream	377.23 ± 16.63	9.17 ± 2.79	2499.34 ± 201.40	1.187 ± 0.45	16.70 ± 1.94	128.26 ± 12.99
Sep	Upstream	405.52 ± 33.71	6.25 ± 1.29	1003.12 ± 45.25	0.52 ± 0.03	27.33 ± 5.76	52.25 ± 27.43
	Midstream	323.66 ± 1.15	9.07 ± 0.94	427.45 ± 37.48	0.21 ± 0.02	18.27 ± 0.25	123.15 ± 11.61
	Downstream	333.33 ± 10.02	5.75 ± 0.39	266.20 ± 6.44	0.13 ± 0.01	0.25 ± 0.49	37.55 ± 3.29

WOI: water quality index.
OPR: oxidation reduction potential; TUR: turbidity; SPC: specific conductance; SAL: salinity; LDO: luminescent dissolved oxygen; CHL: chlorophyll.

Table 4 – Basic parameters of wild *O. niloticus* from the water reuse system in June and September.

Sites	Time	Fish length (cm)	Fish weight (g)	HIS (%)	Number
Upstream	Jun	6.41 ± 0.75	9.72 ± 1.78	2.58 ± 0.45	63
	Sep	6.32 ± 0.63	8.58 ± 0.69	2.22 ± 0.42	59
Midstream	Jun	14.08 ± 2.67	56.68 ± 17.33	2.88 ± 0.56	77
	Sep	7.38 ± 1.56	18.75 ± 4.54	2.34 ± 0.38	60
Downstream	Jun	12.09 ± 1.87	14.37 ± 2.76	2.63 ± 0.65	67
	Sep	7.93 ± 0.83	11.43 ± 1.95	2.17 ± 0.43	47

seafood species in Singapore (13–213 ng/g) (Song et al., 2010). The concentrations of BPA in carp samples ranged from 70 to 1020 ng/g (Yang et al., 2014). E2 has frequently been selected as a model hormone (Cespi et al., 2014). In the present study, the maximum values of E2 concentrations appeared in the upstream and gradually dropped from upstream to downstream. Previous studies demonstrated that fish containing 10 μg E2/g body weight show a chronic increase in the circulating levels of VTG (Guerreiro et al., 2002; Mosconi et al., 2002). Similar to BPA, the occurrence of PFOS displayed the highest concentrations in the midstream. However, these concentrations did not indicate remarkable temporal variations within the sample locations. The average liver concentration of PFOS was 18.8 ng/g for 30 liver samples (Olsen et al., 2003). Our results were similar to these values. A mean DEHP level of 5 ng/g was detected in marine fish (Stales et al., 1997). Phthalate compounds were tested in fish in 17 Taiwanese rivers, and the maximum levels of DEHP were detected in *Liza subviridis* at 253.9 mg/kg and *O. niloticus* at 129.5 mg/kg (Kalaitzidis and Gilmore, 2005). In an overall study of DEHP in fish in Austrian rivers, DEHP was measured at concentrations of up to 1 mg/kg in many species (Zheng et al., 2013). The DEHP concentrations in our study are also comparable to these levels. These results showed the estrogenic pollution and its effects on fish in the water system.

Our results showed that the change in the trend of the VTG1 mRNA level agreed with the level of E2 in the male tilapia liver. Both levels decreased significantly from upstream to downstream. By contrast, other EDCs did not correspond well with the trend of VTG1 expression. E2 was the main contributor to the estrogenic effects in fish (Zheng et al., 2015).

EDCs including BPA, DEHP and PFOS may induce estrogenic effects in organisms. BPA is one of the most widely produced compounds worldwide, with determinate estrogenicity measured by VTG1 mRNA induction (Muncke et al., 2007). As observed by Segner, zebrafish exposed to BPA showed damage to reproduction, as well as an induction of VTG levels (Segner et al., 2003). BPA could obviously induce VTG plasma concentrations in male zebrafish at 1000 μg/L after 3 weeks of exposure (Van den Belt et al., 2003). In Japanese medaka, exposure to DEHP caused an apparent reduction in VTG, suggesting an anti-estrogenic mode of action. Also, in zebrafish, DEHP was found to influence signals involved in VTG (Maradonna et al., 2013). VTG gene expression was distinctly up-regulated in zebrafish after long-term exposure to low concentrations of PFOS. The results further verify the estrogenic activities of PFOS (Du et al., 2009). However, in our study, *in situ* exposure to BPA, DEHP and PFOS did not express a significant induction of VTG mRNA in a dose-dependent manner. Possible explanations are differences in studied species and life-stage-dependent sensitivity (Rose et al., 2002).

The assessment of environmental pollution risk is important for water quality monitoring. Currently, *in vitro* and *in vivo* laboratory studies are widely used to determine pollution risk. However, laboratory exposure cannot fully simulate a realistic field environment. Both physical and chemical factors could affect the toxicity of target chemicals. Thus, direct field exposure has received increasing attention (Burton and Nordstrom, 2004; Gust et al., 2014). Chemical and biological methods are two major ways to detect water pollution. Chemical detection has been widely used to measure the levels of contaminants. This process could be a useful tool to keep track of their existence in the environment. The pollution risk could be assessed based on reference data. However, chemical evaluations do not adequately cover byproducts and metabolites that may generate effects at low levels. Moreover, pollutant mixtures could behave additively, in a subtractive manner, or synergistically with each other. Thus, biological methods are required to provide a full picture of the contaminant risk. Chemical and biological analyses should be combined to evaluate the potential risk of pollutants (Blasco and Picó, 2009). In our study, the wild tilapia was captured and used as a model to assess the pollution in the water recycling system. This fish has been used for many toxicological studies (Govindasamy and Rahuman, 2012; Omar

Table 5 – EDCs concentration in *O. niloticus* liver samples from different locations around the water reuse system (ng/g).

Time	Sampling site	BPA	E2	DEHP	PFOS
Jun	Upstream	1.89 ± 0.66	15.79 ± 0.33	17.43 ± 1.58	19.18 ± 3.57
	Midstream	2.27 ± 0.80	12.53 ± 0.58	17.08 ± 4.59	22.96 ± 3.41
	Downstream	2.05 ± 0.28	9.77 ± 0.68	14.35 ± 2.49	19.25 ± 2.91
Sep	Upstream	1.97 ± 0.76	15.91 ± 1.09	15.95 ± 0.81	21.04 ± 1.47
	Midstream	2.42 ± 0.51	13.94 ± 0.49	22.91 ± 2.18	25.41 ± 1.86
	Downstream	1.92 ± 0.25	10.65 ± 0.42	20.81 ± 0.88	19.65 ± 0.78

EDCs: endocrine disrupting chemicals; BPA: bisphenol A; E2: 17β-estradiol; DEHP: di-(2-ethylhcxyl) phthalate; PFOS: perfluorooctane sulfonate.

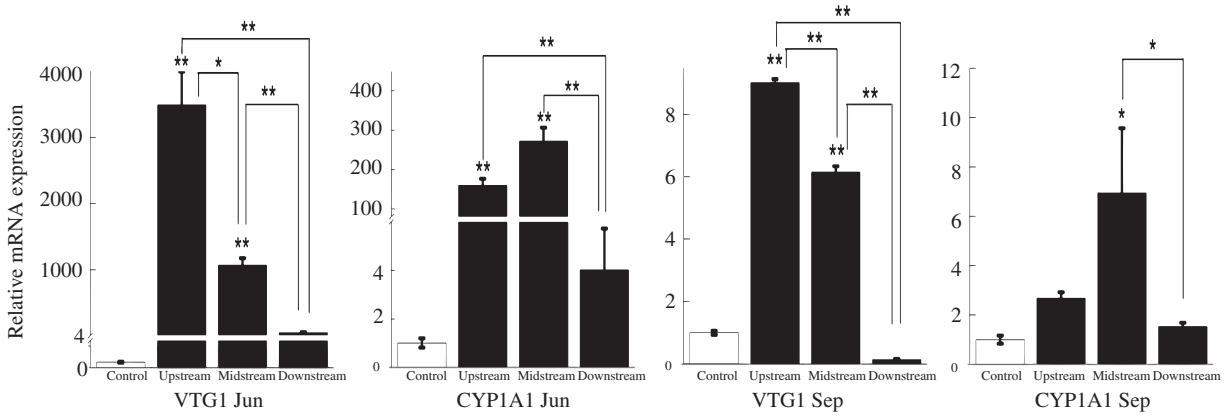

Fig. 2 – Gene expression analyses of VTG1 and CYP1A1 by relative quantitative real-time PCR. *O. niloticus* liver sampled from different locations in June and September. At least three duplicate samples were adopted. One-way analysis of variance followed by a Tukey *post hoc* test was used to test the significance of data differences. The level of $p < 0.05$ was considered to be significant.

et al., 2013; Villarreal et al., 2014). Chemical levels and biological responses were both detected in the liver of male tilapia. The chemical concentrations of E2 resulted in changes in the expression of VTG1 from upstream to downstream. This finding validated the effectiveness of methods combining chemical and biological procedures to assess pollution.

4. Conclusions

Overall, estrogenic pollution in a water reuse system was evaluated by combining chemical and biological analyses based on the *in situ* sampling of wild *O. niloticus*. The relative mRNA levels of VTG1 significantly decreased from upstream to downstream, corresponding well with the change in the E2 concentration in tilapia liver. This field survey method with combined chemical and biological analyses could be a useful tool for assessing water pollution in the watershed. E2 induced the VTG1 mRNA expression in a dose-related manner. E2 could be recommended as a suitable biomarker for the estrogen effect evaluation of *O. niloticus* in aquatic environments. Among the four target EDCs, E2 was responsible for the majority of the endocrine effects in the wild *O. niloticus*. Furthermore, all of the selected EDCs were detected at different levels, demonstrating that fish in the environment may be at a greater risk of EDC contamination. Additional appropriate measures should be taken to reduce the emission of effluents containing EDCs into the water recycling system to protect the aquatic ecosystem.

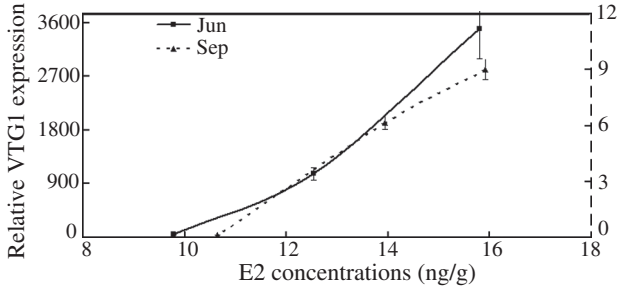

Fig. 3 – Positive correlations between E2 and VTG1 expression level. From left to right, the order is upstream to downstream. The regression equations were:
$y = 576.91x - 5786.2$, $R^2 = 0.9669$, $r = 0.9833$ in June.
$y = 1.703x - 17.881$, $R^2 = 0.9967$, $r = 0.9983$ in September.

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