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Fish Skin Mucus Vitellogenin as a Noninvasive, Sensitive Biomarker for Aquatic Xenoestrogens

Baili Sun, Chenyan Hu, and Lianguo Chen*

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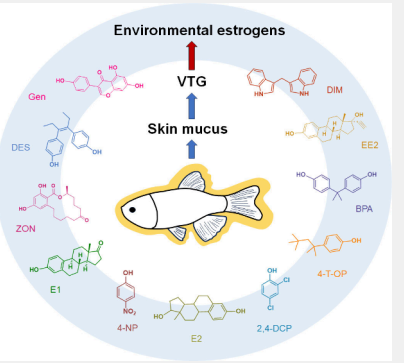
ABSTRACT: Environmental estrogens (EEs) can induce vitellogenin (VTG) in fish skin mucosa. However, the applicability of mucus VTG in aquatic xenoestrogen monitoring warrants comprehensive exploration. Here, we employed different estrogen exposure scenarios to compare the applicability of mucus VTG and other conventional biomarkers in zebrafish for EE monitoring. After acute exposure to 17α-ethynylestradiol (EE2) at various concentrations, mucus VTG demonstrated higher sensitivity in male zebrafish than in female zebrafish. Mucus VTG change patterns were similar to liver and blood VTG change patterns in males. Time-course exposure experiments revealed that male mucus VTG responded to EE2 much earlier than male liver and blood VTG, underlining the promise of mucus VTG as an early warning signal of aquatic estrogenicity. Exposure to multiple EEs further validated the high sensitivity of male mucus VTG. Proteomics analysis revealed that EE2 exposure potently shifted the proteome structure of male mucosa, and the VTG1 isoform was noted to be the most suitable biomarker. Overall, our results refine the roles of mucus VTG1 from male fish as a noninvasive, rapid, and sensitive biomarker of aquatic xenoestrogens, applicable to ecological risk assessment for animal welfare and ecosystem protection. Future ecological studies may only need to sample male fish mucus without sacrificing females.



KEYWORDS: *Environmental estrogens, Noninvasive biomarker, Mucus, Vitellogenin, Sensitivity, Animal welfare*

1. INTRODUCTION

Endocrine-disrupting chemicals (EDCs) refer to a large family of pollutants in the environment that can interfere with inherent hormone synthesis, release, transport, and function.[1] Because of the pivotal roles of endocrine homeostasis in development, growth, and reproduction, EDCs pose an emerging threat to the health and sustainability of impacted ecosystems. In recent decades, various EDCs with distinct chemical structures and physicochemical properties have been applied extensively in industrial, agricultural, and daily life activities.[2] However, EDC discharge, deliberate or unintentional, into water bodies leads to ubiquitous presence of various EDCs in different aquatic environment compartments.[3,4] Environmental estrogens (EEs), the most-studied EDCs, are widespread among aquatic environments. Moreover, by mimicking the modes of action of innate sex hormones, EEs compromise the normal physiology and endocrinology of teleosts significantly.[5−8] Considering the prevalence and adverse effects of EEs, the development of rapid waterborne EE detection methodology is warranted to protect aquatic ecosystems from endocrine-disrupting risks. Current methods based on analytical chemistry can be used to detect only EE compositions and concentrations in environmental samples without consideration of relevant endocrinological disturbances.[9] Thus, highly sensitive, robust biological measures that can assess the estrogenic activity of aquatic pollutants and consequently facilitate EE exposure monitoring and surveillance are needed.[10,11]

Numerous *in vivo* and *in vitro* assays available currently, such as yeast estrogen screening,[12] reporter gene assays,[13,14] gene editing,[15] and vitellogenin (VTG) biomarkers,[16,17] can assist in rapid estrogen mimic screening. However, data obtained *in vitro* cannot be completely extrapolated to the true endocrine events occurring *in vivo*.[18,19] *In vitro* evidence obtained thus far indicates the necessity of performing acute and chronic animal exposure experiments for estrogenic pollutants. However, animal use in research has long remained controversial, considering the potential harm caused to the animals.[20] The principles of the 3Rs (Replacement, Reduction, and Refinement) have been proposed and accepted worldwide as an ethical framework for animal welfare maintenance.[21,22] Conventional toxicological studies, including those related to endocrine disruptors, frequently require animal sacrifice for the collection of tissues of interest; some experiments can be injurious or even fatal to the study animals.[23−25] Moreover,

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large-scale *in situ* sampling of wild animals can impact the resilience of local populations. In alignment with the 3Rs, all toxicological studies are required to minimize the injuries caused to the experimental animals, reduce the number of animals used, and guarantee the maximum well-being of the animals. Hence, research on noninvasive alternative approaches is essential for the assessment of the estrogenic toxicity of aquatic pollutants *in vivo*.[26]

Various noninvasive ecotoxicological methods involving the use of feces,[27−29] environmental RNA,[30] skin mucus,[9,31,32] scale,[33] and behaviors[34−36] have been reported thus far. However, most of these noninvasive methods demonstrated a low EE specificity, hindering their application for direct estrogenic activity detection. VTG in the liver and blood is usually considered a reliable, sensitive biomarker for the identification of EE insults.[37,38] Notably, VTG is also abundantly expressed in fish skin mucus;[11,39,40] in other words, fish skin mucosa is a rich source of EE biomarkers, potentially applicable for noninvasive EE exposure monitoring. Maltais and Roy (2014) reported that EE exposure can increase VTG expression in fish surface mucus.[41] In addition, compared with traditional sampling procedures, mucus collection from fish skin costs less, demonstrates higher efficiency, and leads to no or minimal animal health damage.[42] The same test fish can be used repeatedly for parallel studies to reveal the actual time course of toxicity. In particular, mucus-based assays can facilitate field-intensive monitoring of EE exposures. Therefore, measurement of vitellogenesis in fish skin mucus may be an appealing noninvasive avenue for estrogenicity evaluations in aquatic environments.

Although the use of skin mucus systems for toxicological research is attracting increasing attention, the applicability of mucus VTG as a noninvasive, sensitive EE biomarker requires systemic exploration. In the current study, we concurrently employed various typical EE exposure scenarios with adult zebrafish to compare the responsive VTG patterns in their livers, blood, and mucus. First, we conducted dose-dependent experiments of 17α-ethynylestradiol (EE2) exposure in male and female zebrafish to elucidate the responsiveness and sensitivity of mucosal vitellogenesis as a function of sex. Then, because they were more sensitive than females, only male fish were used in the subsequent exposure experiments. Time-course EE2 exposure experiments were performed to track temporal trends of VTG expression changes in the mucosal layer. Male zebrafish were further exposed to 10 typical EEs to validate the sensitivity of mucus VTG. Finally, proteomic mechanisms underlying mucosal vitellogenesis were examined. In general, the objective of this study was to develop mucus VTG as a rapid, noninvasive, and sensitive estrogenic pollutant biomarker applicable in toxicological research and environmental surveillance.

2. METHODS

2.1. Chemical Reagents

EE2 (CAS 57-63-6; purity >99.9%), zearalenone (ZON; CAS 5975-78-0; purity >99.9%), estrone (E1; CAS 53-16-7; purity >99.8%), 3,3′-diindolylmethane (DIM; CAS 1968-05-4; purity >99.9%), bisphenol A (BPA; CAS 80-05-7; purity >99.8%), and 4-tert-octylphenol (4-T-OP; CAS 140-66-9; purity >98.9%) were purchased from Med Chem Express (Monmouth Junction, NJ, USA). Diethylstilbestrol (DES; CAS 56-53-1; purity >99.5%), 17-estradiol (E2; CAS 50-28-2; purity >99.5%), 2,4-dichlorophenol (2,4-DCP; CAS 120-83-2; purity >99.7%), genistein (GEN; CAS 446-72-0; purity >98.0%), and 4-nitrophenol (4-NP; CAS 100-02-7; purity >99.5%) were procured from Macklin Biochemical Technology (Shanghai, China). All EDC and estrogen mimic stock solutions were prepared in and diluted using dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA).

2.2. Zebrafish Culture and Exposure Scenarios

Wild-type AB-strain adult zebrafish (*Danio rerio*), aged 5 months, were maintained in a semistatic system with charcoal-filtered, fully aerated tap water (dissolved oxygen: 6.9 mg/L; pH: 7.2). The temperature was constantly controlled at 28 ± 0.5 °C with a photoperiod of 14:10 h (light:dark). To minimize interference and highlight sex-specific responses, male and female zebrafish were cultured separately. Thirty zebrafish were randomly allocated to each glass tank containing 25 L of water. Zebrafish were fed with brine shrimp and commercial feeds twice daily. Water was refreshed daily to remove the feces and debris in a timely manner. After acclimation for 2 weeks, zebrafish adults were used for subsequent exposure experiments. The primary purpose of this study is to test the sensitivity of mucus VTG to environmental xenoestrogen pollution. If there is an immediate response of mucus VTG to estrogen exposure, it would be preferred in the future to employ mucus VTG in the early warning system of environmental estrogen surveillance. Hence, merely acute exposures were performed herein. All the animal operations have been approved by the Animal Research and Ethics Committee of the Institute of Hydrobiology, Chinese Academy of Sciences.

2.2.1. Dose-Dependent EE2 Exposure. Adult female and male zebrafish were exposed to different concentrations of EE2 (0, 1, 10, and 100 ng/L) for 7 days (n = 3 replicate tanks per group for each sex). The selection of EE2 concentrations in this study is environmentally relevant and is well representative of typical concentrations in aquatic environments. An equivalent amount of DMSO (<0.001%, v/v) was added per tank. On exposure termination, the zebrafish were anesthetized on ice, and their body weights and lengths were recorded, followed by dissection of the brain, gonad, and liver tissues. The blood samples were collected from caudal veins and centrifuged at 6000 × g at 4 °C for 15 min to obtain the plasma supernatants. Moreover, mucus was collected from zebrafish skin in 400 μL of phosphate-buffered saline containing protease inhibitor cocktail (cOmplete; Roche, Manheim, Germany) by using a cell scraper, followed by centrifugation at 10000 × g at 4 °C for 15 min and collection of the mucus supernatant. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.2.2. Time-Dependent EE2 Exposure. To determine the time course of EE2 toxicity, adult male zebrafish were exposed to 100 ng/L EE2 for 1, 2, 4, or 7 days (n = 3 replicate tanks per group). An equivalent amount of DMSO (<0.001%, v/v) was added to each tank. At each exposure time point, zebrafish were selected randomly and anesthetized to obtain liver tissue, blood, and mucus samples, which were immediately frozen in liquid nitrogen and then stored at −80 °C until VTG measurement.

2.2.3. Typical Estrogen Mimic Exposure. To reveal the differential responsiveness and sensitivities of liver, blood, and mucus VTG to chemicals with estrogenic activity, adult male zebrafish were exposed to one of 10 typical EEs [DES (100 ng/L), E2 (200 ng/L), E1 (2.7 μg/L), ZON (3.2 μg/L), DIM (10 μg/L), 4-NP (14 μg/L), BPA (100 μg/L), 2,4-DCP (300 μg/L), 4-T-OP (500 μg/L), and GEN (5 mg/L)] for 7 days (n = 3 replicate tanks per group). The exposure concentrations for these EEs were based on the effective thresholds that have been verified to cause estrogenic activity potently.[43−53] An equivalent amount of DMSO (<0.001%, v/v) was added to each tank. After exposure, zebrafish were selected randomly and anesthetized to obtain liver tissue, blood, and mucus samples, which were immediately frozen in liquid nitrogen and then stored at −80 °C until VTG measurement.

2.3. EE2 Concentration Measurement in Exposure Media

True EE2 concentrations in exposure media were determined, as described previously.[54,55] In brief, an aliquot of 500 mL exposure solution was extracted using C18 Cartridges (Waters, Milford, MA, USA) and eluted in 3 mL of methanol. After concentration under a

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nitrogen stream, the extract was redissolved in 100 μL of methanol and analyzed at 204 nm on an Agilent 1260 Infinity HPLC system (Agilent, Palo Alto, CA, USA) equipped with an Aligent Zorbax SB-AQ C18 column (5 μm, 4.6 × 250 mm). The mobile phase comprised methanol and ultrapure water at 75:25 (v/v). The flow rate was 1.0 mL/min, and the injection volume was 20 μL. The peak of EE2 appeared at 4 min. EE2 concentrations were quantified on the basis of the peak area against the standard curve. The detection limit of EE2 was 0.4 mg/L; in the procedural blanks, EE2 concentrations were below the detection limit.

2.4. Reproductive End Point Recording

For dose-dependent exposure to EE2 (0, 1, 10, and 100 ng/L), adult male and female zebrafish were randomly paired and transferred to breeding tanks every morning to monitor their fecundity under light stimuli (n = 6). After exposure, 100 newly spawned eggs were collected from each tank and cultured in a Petri dish containing 200 mL medium (n = 6). Until 96 h postfertilization (hpf), the following offspring developmental end points were monitored daily: egg weight (n = 6), egg protein content (n = 5), egg lipid content (n = 5), fertilization rate (n = 6), hatching rate (n = 6), mortality percentage (n = 6), malformation (e.g., spinal curvature, yolk edema, and pericardial edema) percentage (n = 6), larval body weight (n = 6), and larval body length (n = 15).[56,57]

2.5. Blood Sex Hormone Measurement

After acute exposure to 0, 1, 10, and 100 ng/L EE2 for 7 days, blood collected from five fish of the same sex in the same tank was pooled as a replicate sample (n = 5). The plasma concentrations of sex hormones, namely estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT), were measured using commercially available immunoassay kits (Cayman Chemical Company, Ann Arbor, MI, USA; Catalog number: 501890, 582701, and 582751, respectively; sensitivity: 19 pg/mL, 6 pg/mL, and 1.3 pg/mL, respectively), according to the manufacturer's instructions. The hormone concentrations were normalized to protein concentrations in plasma.

2.6. Liver, Blood, and Mucus VTG Concentration Measurement

Varying VTG expressions after exposure to EE2 at different concentrations, to EE2 for different durations, and to different EEs were measured. Three livers from zebrafish of the same sex were pooled as a replicate (n = 5). Blood from five fish and mucus from eight fish of the same sex were pooled as replicate samples (n = 5). Liver samples were homogenized in 0.9% saline on ice and centrifuged at 12,000 × g at 4 °C for 10 min. Finally, VTG concentrations in the liver, plasma, and mucus supernatants were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (Meimian, Jiangsu, China; Catalog number: MM-3367501 and sensitivity: 30 μg/L), according to the manufacturer's instructions.

2.7. Gonad Pathohistological Analysis

Concentration-dependent effects of EE2 on gametogenesis were determined in zebrafish gonads (n = 5). Ovary and testis tissues were treated with 10% buffered formalin, embedded in paraffin, and serially sectioned at 5-μm thickness. The sections were then subjected to hematoxylin–eosin staining. For females, we counted the numbers of oocytes at different developmental stages. For males, we analyzed the areas of different spermatogenic stages in the testes by using ImageJ (NIH, Bethesda, MD, USA).

2.8. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

After exposure, changes in the transcriptional levels of key genes along the hypothalamus–pituitary–gonad (HPG) axis were determined in zebrafish brains, livers, and gonads. Three tissues from zebrafish of the same sex were dissected and pooled as a replicate sample (n = 3). mRNA abundance was estimated using our previously established protocol of quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).[58] The primers used in the present study are listed in Table S1 of Supporting Information (SI). Ribosomal protein L8 (*rpl8*) was employed as the reference gene.

2.9. Mucus High-Throughput Proteomic and Bioinformatic Analyses

After 7 days of exposure to 100 ng/L EE2, perturbations in mucosal proteome structure were profiled using a shotgun proteomic workflow.[59] Mucus samples from eight fish of the same sex in each tank were collected and pooled as replicates (n = 3). After protein extraction, purification, and quantification, a 100-μg aliquot of mucus protein was separated through 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then subjected to in-gel reduction in 10 mmol/L dithiothreitol for 45 min at 56 °C, alkylation in 55 mmol/L iodoacetamide for 30 min at room temperature in the dark, and digestion by sequencing grade trypsin at 37 °C overnight. The peptide fragments were extracted from the gel and identified on a U3000-CapLC chromatography system coupled with the Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Rockford, IL, USA). After annotation according to the *D. rerio* protein library, we filtered differentially expressed proteins (DEPs) by using the following criteria: detection in at least two replicates; average spectral count of >10; fold change of >1.5 or <0.667; p value of <0.05.

DEPs were hierarchically clustered using Gene Cluster (version 3.0) by using the Pearson correlation distance metric and average linkage clustering algorithm. Principal component analysis (PCA) was performed using PAST based on the variance–covariance matrix of

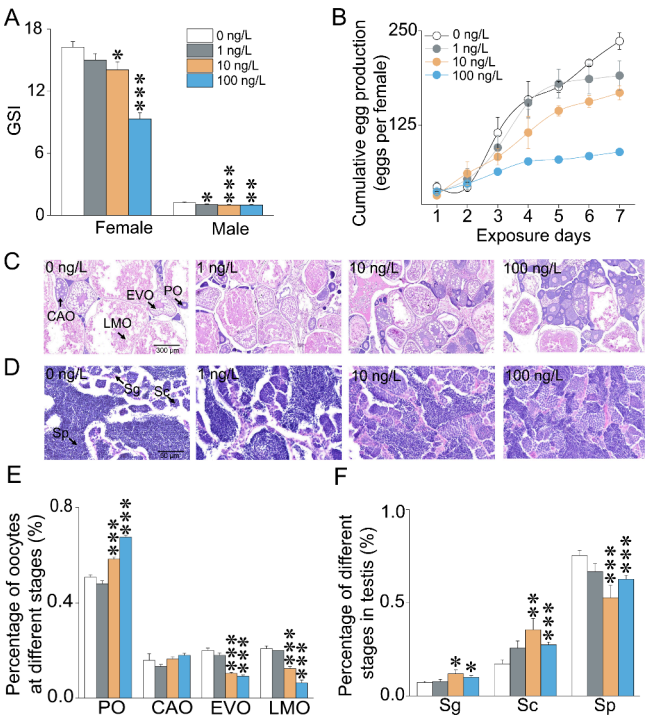

Figure 1. Reproductive deficits in adult zebrafish after acute exposure to 0, 1, 10, and 100 ng/L EE2. (A) GSI values (n = 75). (B) Cumulative fecundity during the entire exposure period (n = 6). (C) Representative images of ovarian histology from different groups. (D) Representative images of testicular histology from different groups. (E) Aberrant oogenesis in EE2-exposed females (n = 5). (F) Aberrant spermatogenesis in EE2-exposed males (n = 5). PO: primary oocytes; CAO: cortical alveolar oocytes; EVO: early vitellogenic oocytes; LMO: late/mature oocytes; Sg: spermatogonia; Sc: spermatocytes; Sp: spermatids and spermatozoa. Data are presented as means ± SEMs. *p < 0.05, **p < 0.01, and ***p < 0.001 indicate statistically significant differences compared with the control group.

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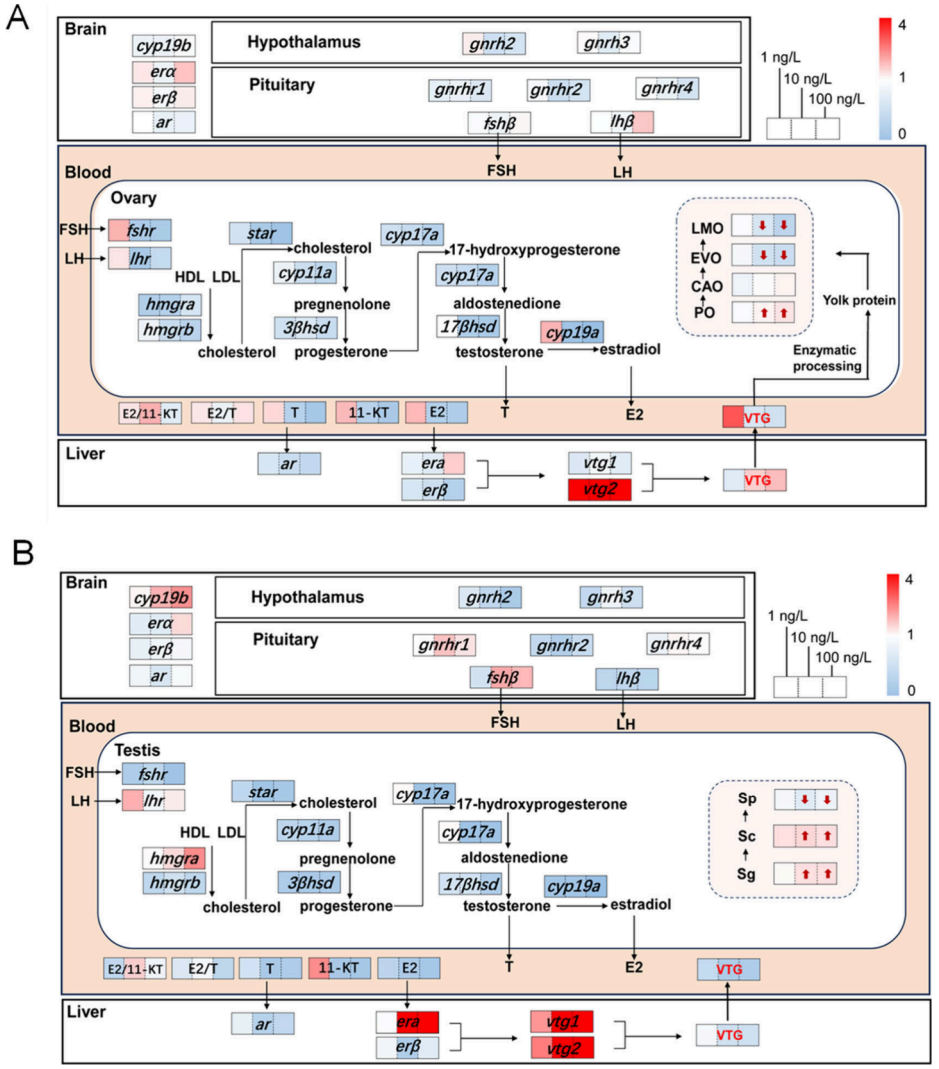

Figure 2. Holistic changes along the HPG axis after acute exposure to 0, 1, 10, and 100 ng/L EE2. (A) Females. (B) Males. Red and blue represent upregulation and downregulation, respectively, with color intensity proportional to the changing magnitude. Gene transcription is denoted in italics. The arrows in the oocytes and sperms section indicate the changing trend of significant difference at each developmental stage.

DEPs. Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEPs were performed using the online server KOBAS (version 3.0). Protein−protein interaction (PPI) networks of DEPs were constructed using the STRING database and visualized using Cytoscape (version 3.9.0).

2.10. Statistical Analyses

The Shapiro−Wilk and Levene tests were used to evaluate the normality and variance homogeneity of the data, respectively. Next, one-way analysis of variance (ANOVA) with the post hoc least significant difference test or Kruskal−Wallis ANOVA with the post hoc Dunn−Bonferroni test was used to determine the significant differences between the control and exposure groups. If there were only two groups, independent samples *t*-test was used to determine the significant differences between the control and exposure groups.

Statistical analyses were performed using SPSS (version 26.0; IBM, Armonk, NY, USA). Moreover, Pearson correlation analysis was performed using Origin 2024 (OriginLab, Northampton, MA). All data are presented as means ± standard errors of the means (SEMs). Differences with $p < 0.05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. True EE2 Exposure Concentrations

The actual concentrations of EE2 in exposure solutions were measured to be 0.86 ± 0.02, 8.28 ± 0.12, and 87.53 ± 2.29 ng/L in nominal 1, 10, and 100 ng/L exposure groups, respectively. No EE2 was detected in the control group.

3.2. Reproductive Toxicity of EE2

To assess the effects of estrogenic pollutants on conventionally used biomarkers of reproductive endocrine disruption, we first determined holistic changes along the HPG axis after acutely exposing male and female zebrafish to EE2. By incorporating the data regarding reproductive adverse outcomes, gonadal gametogenesis, sex hormone concentration, and critical gene transcription, we profiled the responsiveness and sensitivity of conventional estrogen biomarkers.

3.2.1. Changes in Reproductive End Points. EE2 acute exposure for 7 days led to significant increases in the hepatosomatic index (HSI) but significant decreases in the gonadosomatic index (GSI) in both male and female zebrafish (SI Table S2 and Figure 1A). Similar findings have been noted in previous studies on EE2.[60−62] In line with ovary shrinkage,

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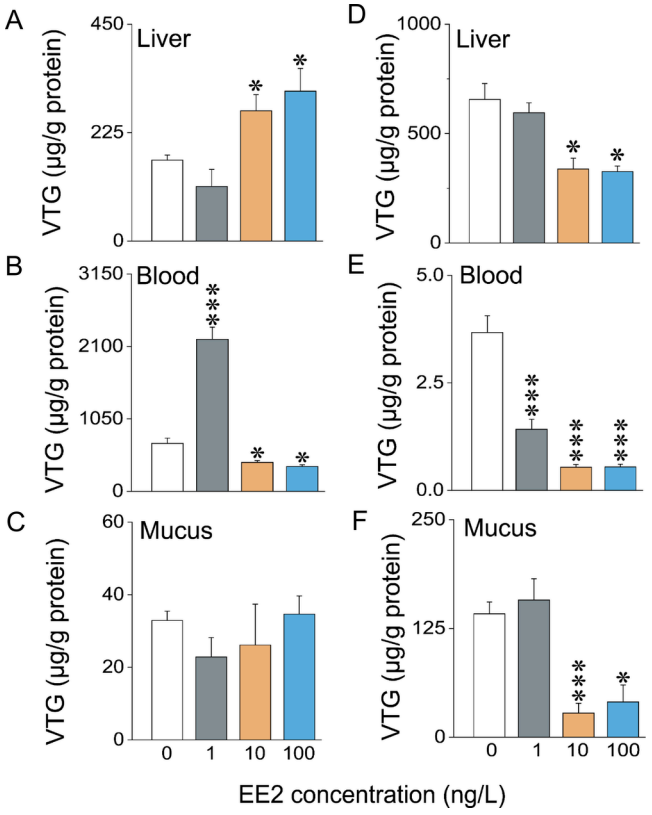

Figure 3. Concentration-dependent alterations in VTG concentrations in the livers, blood, and mucus of female zebrafish (A–C, respectively) and male zebrafish (D–F, respectively) exposed to 0, 1, 10, and 100 ng/L EE2. Data are presented as means ± SEMs from five replicates (n = 5). *p < 0.05 and ***p < 0.001 indicate statistically significant differences compared with the control group.

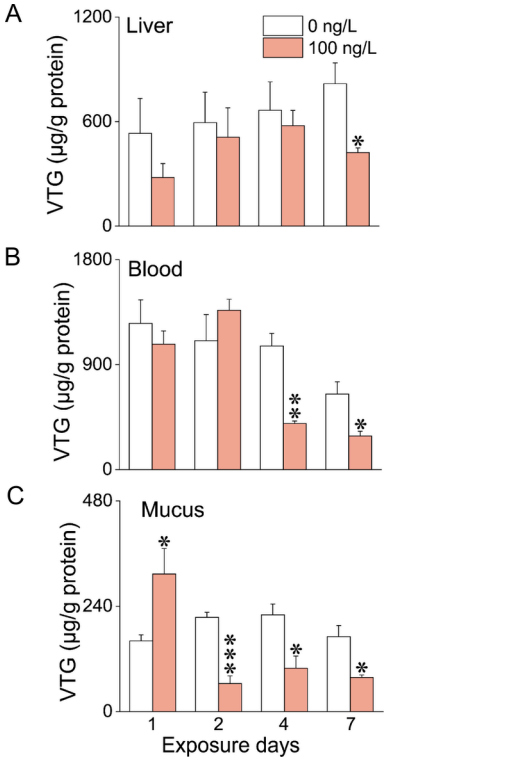

Figure 4. Temporal alterations in VTG concentrations in the liver (A), blood (B), and mucus (C) of male zebrafish after exposure to 100 ng/L EE2 for 1, 2, 4, or 7 days. Data are presented as means ± SEMs from five replicates (n = 5). *p < 0.05, **p < 0.01, and ***p < 0.001 indicate statistically significant differences compared with the control group.

the fecundity in the females decreased in a concentration-dependent manner (Figure 1B). Moreover, reduced protein deposition compromised the egg quality (SI Table S2). By examining EE2-exposed gonad histology (Figure 1C,D), we noted that EE2 simultaneously blocked oogenesis and spermatogenesis, as characterized by higher percentages of primary gametes and lower percentages of mature gametes (Figure 1E,F). Similarly, inhibition of both gonadal morphogenesis and gamete maturation is a representative adverse outcome of EE exposure.[63−65] In the exposed group offspring, EE2 significantly impaired early embryonic development by inducing multiple deficits, such as a decline in the fertilization rate, a delay in hatching success, an increase in mortality percentage, and a reduction in the body length (SI Table S2).[66]

3.2.2. Reproductive Endocrine System Disturbances. In fish, EE2-induced sex hormone imbalance generally occurs because of changes in circulatory E2 and T levels.[67] Acute exposure to 10 and 100 ng/L EE2 significantly reduced the blood levels of sex hormones (E2, T, and 11-KT) in our females, whereas that to 1 ng/L EE2 increased them (SI Table S3 and Figure 2A). Moreover, all our EE2-exposed male zebrafish demonstrated decreases in blood E2 and T levels (SI Table S3 and Figure 2B).

In fish, sex hormone homeostasis and reproductive performance are closely regulated by the HPG axis.[57,68] In the current study, the most pronounced effect of EE2 along the HPG axis exhibited in the ovaries (SI Table S4 and Figure 2A) and testes (SI Table S5 and Figure 2B), where the key genes involved in steroidogenesis were considerably downregulated at the transcriptional level. Notably, 0.08- and 0.01-fold decreases in the levels of the aromatase transcript (*cyp19a*) were noted in the ovaries of 10 and 100 ng/L EE2-exposed females, respectively; in contrast, exposure to 1 ng/L EE2 led to a 1.92-fold increase in ovarian *cyp19a* transcription (Figure 2A). Because aromatase is responsible for the conversion of T to E2,[69] the changing patterns of *cyp19a* transcription aligned with those of blood E2 levels in females. In contrast, a consistent upregulation was noted in *vtg* transcription in both female and male livers from all EE2 exposure groups, regardless of the changes in circulatory sex hormone levels. Studies have indicated this inverse correlation between sex hormone and VTG biomarker levels,[70−72] implying dynamic fluctuations occurring across the entire HPG axis.

3.3. Development of Mucus VTG as a Noninvasive, Sensitive Biomarker of EEs

By using conventional markers of reproductive endocrine-disrupting effects (e.g., aromatase gene transcription in gonads, sex hormone levels in the blood, and VTG expression in the liver), studies have indicated that xenoestrogens such as EE2 can potently overturn the reproductive endocrinology and functions. However, to obtain the required tissues and determine these conventional biomarkers, test animals must be dissected; this causes major damage to the organismal fitness and viability. Moreover, the use of these conventional biomarkers for field EE exposure surveillance tends to defy the 3Rs principles for animal welfare protection and worldwide

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awareness of ecosystem conservation.[9] Therefore, the development of novel biomarkers that not only sensitively diagnose the estrogenic toxicity of pollutants but also minimize the interference with aquatic ecosystems is warranted.[11]

The skin of animals is constantly and directly exposed to environmental stressors.[73] Compared with endogenous tissues (e.g., the brain, blood, liver, and gonads), the skin has a higher risk of EE exposure, leading to endocrine-disrupting toxicity. Mucosa is a chemical barrier mediating the interplay between environmental factors and fish skin.[74,75] When the unintended accessibility to EEs occurs, the cutaneous mucosal microenvironment also responds immediately. In aquatic environments, EEs can induce VTG expression in teleost skin, resulting in estrogenic activities.[31,41,76] The skin is postulated to serve as an excretory pathway of VTG into mucosal layer.[31] Compared to traditional noninvasive methods (e.g., environmental RNA and behaviors), the main advantage of skin mucus VTG is the high specificity to estrogen exposure. To develop and validate mucus VTG as a rapid, noninvasive, and sensitive diagnostic biomarker of estrogenic pollutants further, we integrated various paradigms of exposures, including dose–effect exposure, time-course exposure, and typical EE validation.

3.3.1. Vitellogenic Response in Zebrafish Exposed to EE2 at Different Concentrations. We performed dose-dependent EE2 exposure experiments to compare VTG changes as the functions of tissue and sex. Although *vtg* expression was significantly upregulated in the livers of females (*vtg2*) and males (*vtg1* and *vtg2*; Figure 2), EE2 exposure increased liver VTG levels in females (Figure 3A) but reduced them in males (Figure 3D). This inconsistency may be attributable to the latency from gene transcription to protein translation and modification.[39,77] In addition, there are various VTG isoforms that respond to EEs differently, which provides another potential mechanism underlying the inconsistency between isoform gene transcription and total protein abundance of VTG;[78,79] thus, future studies should focus on assessing the unique responsiveness and sensitivity of each VTG isoform. The sex-specific response is assumed to result from the inherent distinction between males and females in endocrine regulatory mechanisms.[56,57]

In females, exposure to 1 ng/L EE2 increased blood VTG levels, but that to 10 and 100 ng/L EE2 reduced them (Figure 3B); these results were in contrast to those noted in the liver (Figure 3A). This suggests the complexity of VTG expression regulation in females as a response to EE exposure.[80] Mucus VTG expression in females demonstrated no changes even after exposure to EE2 at high concentrations, further ensuring the unsuitability of using female fish for estrogenic risk assessment. In contrast to those in females, liver, blood, and mucus VTG in males demonstrated concordant reductions in protein abundance caused by EE2 exposure (Figure 3D–F). VTG is normally absent in males, but xenoestrogens can easily stimulate its biosynthesis.[81,82]

3.3.2. Temporal VTG Responses in Male Zebrafish Exposed to EE2. To differentiate temporal changes in VTG in male zebrafish further, the zebrafish were exposed to EE2 for different durations. The use of biomarkers demonstrating temporal responses, such as VTG, may provide credible interpretations.[83] The results showed that only 100 ng/L EE2 suppressed liver VTG expression by 0.64-fold after 7 days of exposure (Figure 4A), whereas blood VTG concentration started to decrease at exposure day 4 and 7 by 0.39- and 0.40-fold, respectively (Figure 4B). In contrast, mucus VTG in male zebrafish significantly increased in abundance by 1.99-fold within 1 day of EE2 exposure, but it began to decrease at 2, 4, and 7 days of EE2 exposure by 0.45-, 0.71-, and 0.55-fold, respectively (Figure 4C). This further highlighted that mucus VTG is much more sensitive at detecting estrogenic toxicity than liver and blood VTG. Employing liver and blood VTG alone may lead to the underestimation of EE pollution severity. Therefore, skin mucus VTG may provide early warning signals in xenoestrogen exposure and effect monitoring. Considering the applicable value of mucus VTG, future targeted studies should be conducted to unveil why mucus VTG is changed by environmental estrogens by dissecting the skin and measuring the gene transcriptions involved in vitellogenesis and steroidogenesis.

3.3.3. Validation of Vitellogenic Response in Male Zebrafish Exposed to Typical EEs. To validate the applicability of mucus VTG in estrogenic detection further, male zebrafish were exposed to 10 typical EEs, and their potential to interfere with reproductive endocrinology was verified. The results showed that in males, liver VTG levels decreased at varying magnitudes after exposure to DES, E2, E1, ZON, DIM, and 4-T-OP (Figure 5A). Moreover, male blood VTG levels decreased after exposure to all EEs except 4-NP and GEN (Figure 5B). Compared with the liver and blood, all

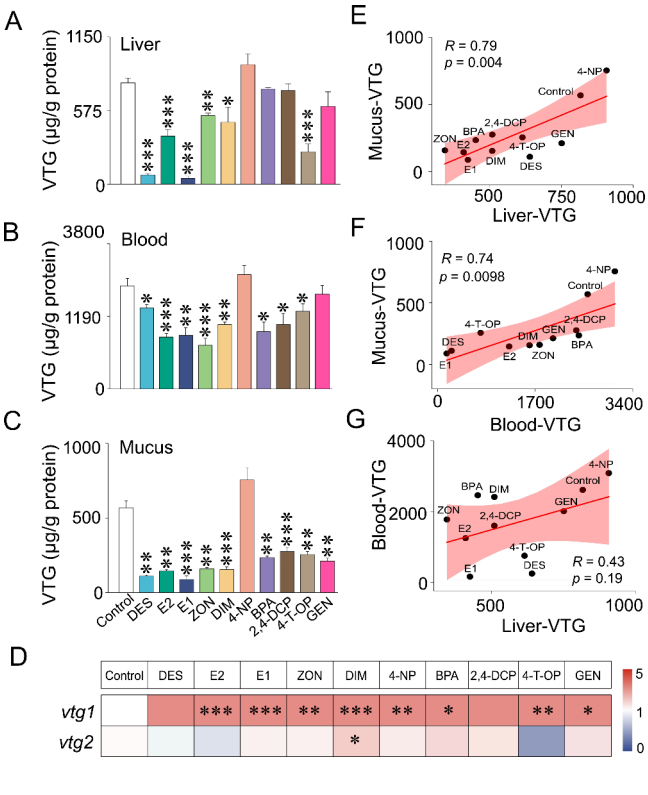

Figure 5. Alterations in VTG concentrations in the livers (A), blood (B), and mucus (C) of male zebrafish after acute exposure to typical estrogenic pollutants ($n = 5$). (D) Alterations in liver transcription of *vtg1* and *vtg2* in male zebrafish from different exposure groups ($n = 3$). Correlation pattern of VTG expression between mucus and liver (E), between mucus and blood (F), and between blood and liver (G) in male zebrafish exposed to multiple estrogenic pollutants. Data are presented as means ± SEMs. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistically significant differences compared with the control group.

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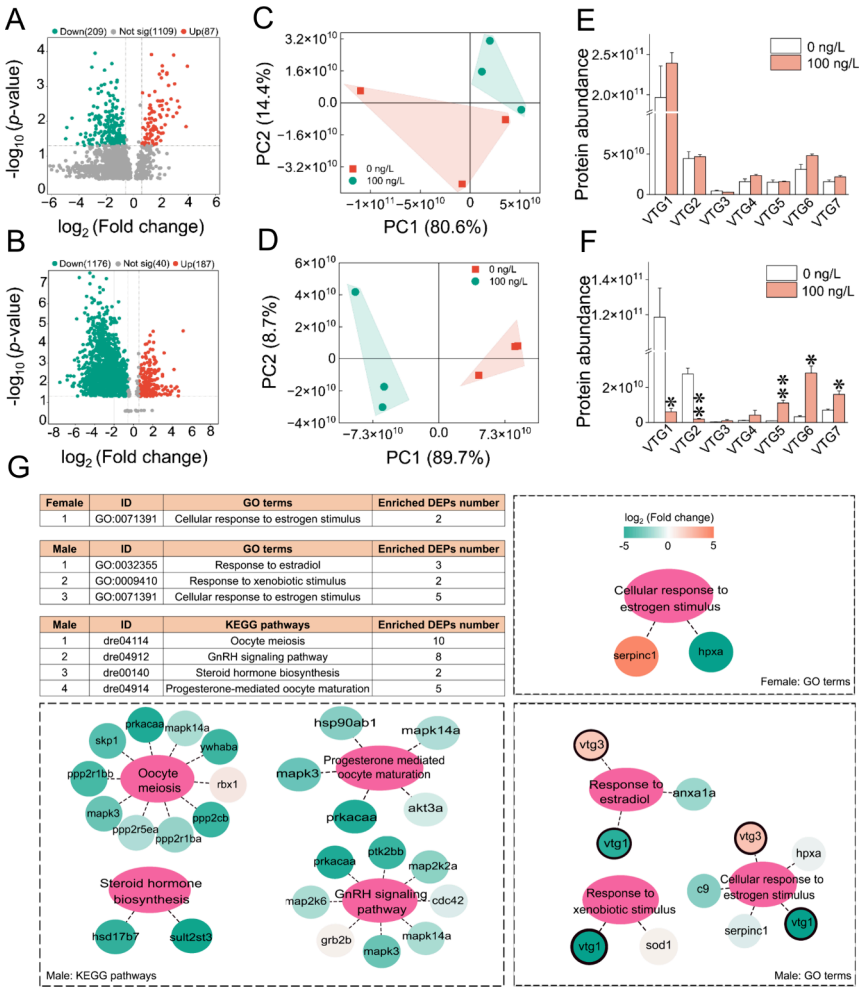

Figure 6. Proteomic insights into mucosal vitellogenesis after acute exposure of zebrafish to 100 ng/L EE2. Volcano plot of mucus DEPs in females (A) and males (B). Red and green dots represent significantly upregulated and downregulated DEPs, respectively. PCA plots based on the differential mucosal proteins in females (C) and males (D). Changes in the abundance of various VTG isoforms in the mucus of female (E) and male (F) zebrafish after EE2 exposure. (G) PPI network of GO terms and KEGG pathways in the mucus of female and male zebrafish. Color intensity is proportional to the $\log_2$(fold change). Data are presented as means ± SEMs of three replicates (n = 3). *p < 0.05 and **p < 0.01 indicate statistically significant differences compared with the control group.

EEs except 4-NP significantly reduced VTG expression in male skin mucosa (Figure 5C). Hence, mucus VTG showed more positive detection frequency of estrogenic chemicals only after an acute exposure for 7 days. Besides, the estrogen mimics detected by mucus VTG had a good overage of those detected by liver and blood VTG, confirming the higher sensitivity of skin mucosal vitellogenesis to estrogenic toxicity. An inverse trend of changes between *vtg* transcription and protein abundance was also noted in the livers of males exposed to typical EEs (Figure 5D and SI Table S6). In addition, different VTG isoforms genes (*vtg1* and *vtg2*) demonstrated differential responses to EEs. Mucus VTG levels had a strong correlation with liver VTG levels (Figure 5E; R = 0.79, p = 0.004) and blood VTG levels (Figure 5F; R = 0.74, p = 0.0098). In general, these validation results confirmed that mucus VTG in male zebrafish is extremely sensitive to EE activity and thus is a rapid, simple modality for noninvasive surveillance of xenoestrogen pollution in aquatic environments. In other fish species including Atlantic salmon and copper redhorse, previous studies also provided supportive evidence about the rapid responsiveness of skin mucus VTG to EEs,[31,41] highlighting the universal sensitivity of skin mucus VTG across different fish species.

3.4. Proteome Mechanisms in Mucosal Vitellogenesis after EE2 Exposure

Mucosal composition fluctuates along with environmental changes,[84] whereas an intact mucosa barrier plays major roles in the defense against pathogen colonization and contaminant penetration.[85,86] Fingerprinting skin mucus DEP profiles can provide a further understanding of the mechanisms of action of EEs.[87] After exposing female and male zebrafish to 0 and 100 ng/L EE2 for 7 days, we performed high-throughput proteomic analysis of their mucus samples. After acute exposure to EE2, 296 and 1,363 proteins were noted to be differentially expressed in female (Figure 6A and Figure S1A) and male (Figure 6B and Figure S1B) mucus, respectively. Most of these DEPs were downregulated in EE2-exposed mucus regardless of sex. PCA and heatmap clustering demonstrated that EE2 exposure drove the formation of a distinct proteome landscape in female and male mucosa compared with the controls (Figure 6C,D and Figure S1C,D). Functional annotation revealed that the DEPs were enriched in various GO terms associated with peptide synthesis, transport,

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and metabolism in the cytoplasm (Figure S2A,B). Moreover, KEGG pathway enrichment analysis revealed that EE2 led to disturbances in protein, lipid, and carbohydrate metabolism as well as immunity and endocrine system (Figure S3A,B). Skin mucosa acts as the first line of immune defense, and exposure to environmental pollutants can compromise innate immunity and promote inflammation in the skin mucosal layers of animals.[88−91] In the current study, the top 20 enriched pathways were closely related to the modulation of protein transcription and translation processes (Figure S4A,B). In the PPI networks, the key protein nodes were involved in protein expression and inflammation (Figure S5,B).

Next, we screened for changes in the protein abundance of various VTG isoforms. In both male and female zebrafish mucus, VTG1 was the most abundantly expressed isoform (Figure 6E,F). Acute exposure to 100 ng/L EE2 exerted no obvious alterations in the expression of any VTG isoform in female mucus (Figure 6E). However, in male mucus, EE2 exposure significantly reduced VTG1 and VTG2 abundance but increased that of VTG5, VTG6, and VTG7 (Figure 6F), indicating the isoform specificity of VTG responsiveness to estrogenic pollutants.[79] Moreover, in female mucus, DEPs were significantly enriched in a GO term related to cellular response to estrogen stimulus (Figure 6G). In male mucus, EE2 exposure significantly affected three GO terms related to endocrine homeostasis processes involving VTG1: response to estradiol, response to xenobiotic stimulus, and cellular response to estrogen stimulus. Considering the high abundance, EE sensitivity, and functional implications of mucus VTG1, future studies should focus on targeting VTG1 to develop specific ELISA kits and detect subtle changes in VTG1 abundance rather than a sum of all mucus VTG isoforms. By focusing on the VTG1 isoform, the sensitivity and reproducibility of EEs exposure monitoring can be enhanced further; consequently, this may reduce the sampling volume of mucus and alleviate any discomfort borne by the animals. Finally, after EE2 exposure, male zebrafish mucus demonstrated enrichment of four KEGG pathways associated with reproductive endocrine functionality: oocyte meiosis, the GnRH pathway, steroid hormone biosynthesis, and progesterone-mediated oocyte maturation.

4. CONCLUSIONS

In summary, the present study compared the sensitivity and response mode of mucus VTG with those of conventional biomarkers in zebrafish exposed to EEs at different doses, to EEs for different durations, and to different EDCs. Mucus VTG expression in male zebrafish demonstrated higher sensitivity to estrogen than that in females, indicating that our method can demonstrate desired results only by sampling male zebrafish mucus. In male fish, vitellogenesis was much stronger in mucus than in the liver or blood, supporting that mucus VTG can be an early warning of EE aquatic pollution. Different EEs also affected mucus VTG abundance significantly. Proteomic analysis of male mucus further revealed that the VTG1 isoform demonstrates the highest sensitivity for estrogenic activity, thereby refining the scope of further research and improving relevant diagnostic representation. In general, our results provided a strong rationale for the use of male mucus VTG1 as a noninvasive, sensitive, and rapid biomarker for the risk evaluation of estrogenic pollutants. Changes in VTG1 abundance on male fish skin mucus may indicate the impacts of various xenoestrogens. This approach can facilitate ecological monitoring projects because of its low labor and instrument costs. During risk assessments, the nondestructive nature of mucus sampling can also prevent damage to animals, especially those endangered and protected. In the future, the responsive pattern of mucus VTG to a broad concentration range of estrogen pollutants will be established.

■ ASSOCIATED CONTENT

Data Availability Statement

All relevant data supporting this study are available within the paper and Supporting Information. The raw MS data have been deposited to the public iProX proteome database (https://www.iprox.cn/page/SSV024.html;url=1734598511719HZpQ).

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.4c00235.

> Numbers and heatmap clustering of mucus DEPs, GO term and KEGG pathway enrichment analysis of DEPs in mucus, Top 20 KEGG pathways with significant enrichment in mucus, PPI network for mucus, primer sequences for qRT-PCR assays, F0 adult health and F1 offspring development end points, sex hormone concentrations in blood, gene transcriptions along the HPG axis in female and male zebrafish, and transcriptions of *vtg1* and *vtg2* genes in the liver of male zebrafish after exposure to typical estrogen mimics (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Lianguo Chen − *Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China;* orcid.org/0000-0003-3730-7842; Phone: +86 27 68780042; Email: lchenam@ihb.ac.cn; Fax: +86 27 68780123

Authors

Baili Sun − *Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China; University of Chinese Academy of Sciences, Beijing 100049, China*

Chenyan Hu − *School of Chemistry and Environmental Engineering, Wuhan Institute of Technology, Wuhan 430072, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/envhealth.4c00235

Notes

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