Unexpected Discovery of the Food Additive Nonivamide as a Main Estrogenic Contributor in the Three Gorges Reservoir

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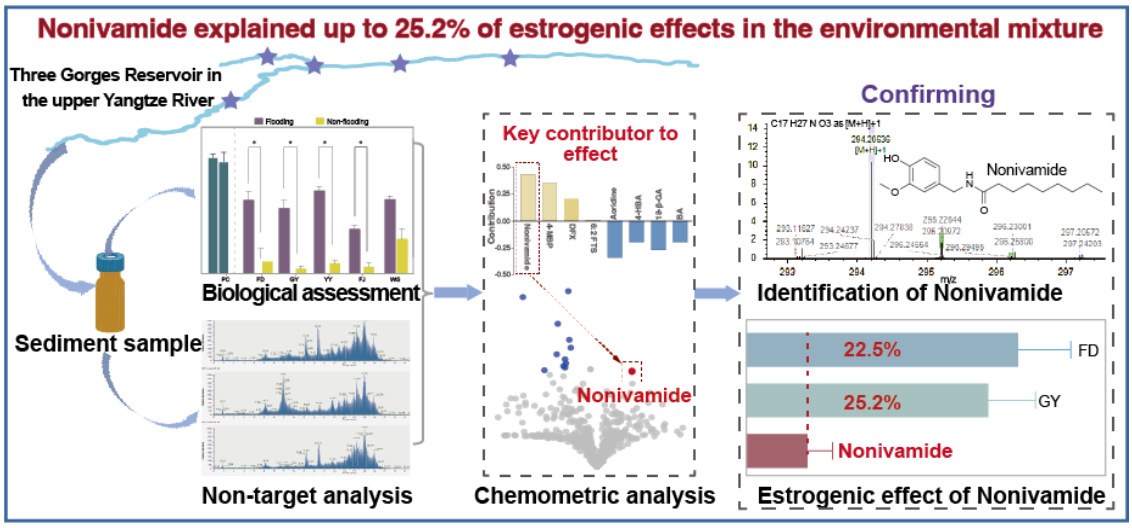
14 **TOC Artwork**



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Abstract

Thousands of natural and synthetic substances in the environment have been reported to exhibit estrogenic effects. However, targeted analysis of a limited number of typical estrogens or estrogenic candidates is insufficient for key estrogenic driver identification, since many estrogenic substances are poorly characterized or even unknown. In this study, chemometric methods were applied to link *in vitro* bioassays and non-target analysis to identify key drivers of estrogenic effects in complex environmental mixtures. The Three Gorges Reservoir (TGR), which is located in the middle and upper Yangtze River and provides drinking water to over 7 million people, was selected as the study area. Sediment samples were collected during flood and non-flood period to investigate toxic effects and chemical distribution. Bioassays, including cytotoxicity tests and yeast two-hybrid tests, revealed that samples collected during the flood period induced significantly higher estrogenic effects than those from the non-flood period. Non-target screening by high-resolution mass spectrometry (HRMS) detected 47,321 unique features, with 527 compounds being identified successfully (level 2a). Chemometric analysis by correlation and weighted contribution analysis indicated that *N*-[(4-hydroxy-3-methoxyphenyl)methyl]nonanamide (nonivamide) was significantly positively correlated with estrogenic activity ($p < 0.05$). Target analysis and the yeast two-hybrid assay confirmed that nonivamide induced up to 25.2% of the estrogenic effect of sediments at environmental concentrations. This study is the first report on nonivamide occurrence in environments and its unexpected estrogenic effects, highlighting its potential ecological and health implications.

Keywords

Non-target analysis; chemometric analysis; yeast estrogen screening; Nonivamide; Three Gorges Reservoir

Synopsis

By integrating *in vitro* bioassays with non-target analysis and chemometric approaches, nonivamide was unexpectedly identified as a key driver of estrogenic activity in sediment samples, highlighting its previously unrecognized environmental significance.

Introduction

Estrogenic substances, the first kind of defined EDCs (endocrine disrupting chemicals), have aroused people's concern since the early 1990s.[1] Over the past 30 years, accumulating evidence has indicated that estrogenic substances can affect the reproductive system, leading to reduced fertility, birth defects, sex ratio imbalance, abnormal development of sexual organs, and cancer of the breast, endometrium, and testes in organisms.[2-4] To manage and mitigate the environmental hazard posed by estrogenic substances, typical steroid estrogens, such as natural estrone (E1), 17β-estradiol (E2), and estriol (E3), synthetic estrogens, 17α-ethynylestradiol (EE2), and estrogen-like substances, including bisphenol A (BPA), nonylphenols (NPs), 4-tert-octylphenol (4-t-OP), and 4-nonylphenol (4-NP) have frequently been analyzed to assess estrogen-related risks in the environment.[5-7] In practice, however, approximately 1,000 natural and synthetic substances present in the environment are thought to have the potential to induce estrogenic effects.[1] This number is expected to continue rising with the ever-increasing diversity of chemical pollution. Many of these substances are poorly characterized or even unknown. Obviously, characterizing environmental estrogen pollution through targeted analysis of a few typical estrogens or estrogenic candidates is insufficient. Besides, estrogens or estrogenic candidates, occurring at pg g^{-1} or ng g^{-1} concentrations in sediment, are usually mixed with numerous other chemicals, forming a "cocktail" in the environment, which poses huge challenges for chemists and toxicologists in their endeavor to identify key estrogenic substances in complex environmental mixtures.[8-10]

Considering the low concentrations and vast amount of organic pollutants, high resolution mass spectrometry (HRMS), which can detect tens of thousands of "features", presents an opportunity for non-target screening (NTS) of environmental contaminants.[11] It further provides the possibility of identifying poorly characterized and unknown chemicals without prior knowledge of their presence or knowledge of their identity.[8] Recently, environmental chemists have utilized HRMS to analyze dozens to hundreds of contaminants in surface water, wastewater, food, sediments, indoor dust, and even organisms.[11-16] Determining whether a compound qualifies as a contaminant depends on understanding its hazard or potential hazard to humans and environments.

Bioanalytical techniques such as *in vivo* whole-organism bioassays and *in vitro* cellular bioassays are hence applied.[17] Bioassays are capable of capturing the effects of all chemicals in a mixture, even when chemicals are present below the instrumental detection limit or below the effect threshold of an individual chemical.[8] Strategies such as effect-directed analysis and component-based methods have been developed to combine chemical analysis and bioassays for key toxic driver identification from tens of thousands of chromatographic features.[18] Besides, statistics-assisted approaches provide opportunity to screen the toxic driver candidates by quantifying their individual contributions to the observed outcomes.[19] However, given the enormous complexity of environments and the diversity of chemicals, linking toxic effects with drivers of the mixture risk from tens of thousands of chromatographic features is still one of the major challenges that environmental scientists face.[8]

The aim of this work was to develop a feasible strategy to identify drivers of estrogenic effects from complex mixtures of pollutants in environmental samples by integrating *in vitro* cellular bioassays, HRMS equipped with ultra performance liquid chromatography (UPLC), and chemometrics. Sediment samples from the Three Gorges Reservoir (TGR) along the upper Yangtze River in China, which provides drinking water for more than 7 million people, were collected. The cytotoxicity assay, the yeast estrogen screening (YES) assay by two-hybrid yeast, and UPLC-HRMS were applied to investigate cytotoxicity, estrogenicity, and the chemical composition of the sediment samples, respectively. Chemometric and statistical analyses were subsequently conducted to identify potential drivers of estrogenicity in the environmental mixture.[20] Finally, target analysis by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS), and the YES assay were applied to confirm the effect contribution of the possible driver. This strategy can be applied for identifying the main toxic compounds in complex environmental mixtures and thus can be useful for risk assessment and river protection.

Material and Methods

Sample Collection and Preparation

Given the hydrophobicity of EDSs, coupled with the substantial surface area and elevated organic matter content characteristic of sediment,[21, 22] sediment was selected as the study matrix. In this study, sediment samples from the middle and upper reaches of the Yangtze River were collected in triplicates at each site and mixed thoroughly. A total of 10 sediment samples

were collected from five sampling sites, Fengdu (FD), Gaoyang (GY), Yunyang (YY), Fengjie (FJ) and Wushan (WS), using a stainless-steel grab sampler (Figure 1, Table S1). Five samples were collected during the non-flood period in August 2020, and an additional five samples were collected from the same sites during the flood period in April 2021 by boat. All the sediment samples were placed in aluminum foil, transported to the laboratory, and stored at -80 °C prior to use.

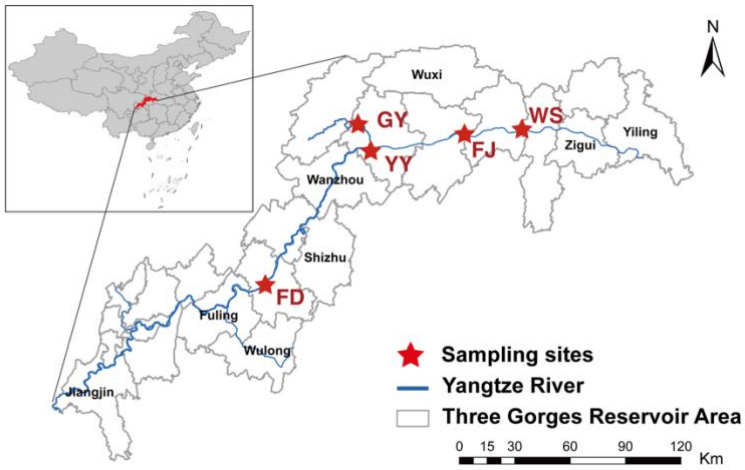


Figure 1. Study area and location of the sampling sites. The locally enlarged area is the Three Gorges Reservoir region. FD: Fengdu; GY: Gaoyang; YY: Yunyang; FJ: Fengjie; WS: Wushan.

Sediment samples were pretreated following previously established protocols with minor modifications.[23, 24] All sediment samples were freeze-dried, homogenized, and ground to pass through a 60 mesh (250 μm) sieve. Freeze-dried sediments (20 g) were extracted for 18 h with a hexane-acetone (1:1, v/v) solvent mixture using a Soxhlet extractor (JPSXT-06, Shanghai, China). Subsequently, the extracts were rotary evaporated to near dryness and re-dissolved in

1 mL of methanol. Thereafter, the extract was divided equally into two portions for chemical analysis and bioanalytical testing, respectively. The bioassay sample extracts were dried under the nitrogen gas and subsequently dissolved in 0.5 mL of dimethyl sulfoxide (DMSO). The final concentrations in these two aliquots were both 20 g sediment equivalents (SEQ) per mL solvent.

Bioassays

The H4IIE cell line is well-studied and proven to provide a diverse array of liver functions, and it has been extensively used for evaluating the toxicity of a wide variety of environmental contaminants.[25, 26] Thus, to avoid the interference of cytotoxicity on the estrogenic effects test, the MTT assay was performed using H4IIE rat hepatoma cells.[27] The MTT assay based on the H4IIE method was followed a previous study, with slight modifications.[28] The H4IIE rat hepatoma cells were kindly provided by the College of Environment and Resource Sciences of Zhejiang University. Cells were cultured at 37 °C with 5% CO_2, and 95% humidity in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (PS). Before exposure, a volume of 100 μL $well^{-1}$ of the cell suspension at a density of 2×10^4 cells mL^{-1} was seeded in 96-well plates for 24 h at 37 °C. After 24 h incubation, the cells were exposed to a 1:2 dilution series of each sample in triplicates, and the final DMSO concentration in each well was 0.10% (v:v).[25, 28] After 48 h exposure, 100 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.5 mg mL^{-1}) solution was added to each well and incubated for 30 min. Then the MTT media was exchanged with 200 μL $well^{-1}$ DMSO. Cell viability was estimated by

measuring light absorbance at 492 nm with a microplate reader (Synergy LX, BioTek, USA). The relative survival of exposed cells was compared to untreated control cells. Results were expressed as a percentage of the control results. Besides, to characterize the estrogenic effects of toxic driver candidates, the E-screen assay was applied. The E-screen assay measures the binding of estrogenic compounds to human estrogen receptors in MCF-7 cells (human breast cancer cells), resulting in increased cell proliferation.[29] Thus, it is widely used as a standard method for screening xenoestrogens.[30] Details of the E-screen assay method is provided in the Text S1. The estrogen receptor-mediated endocrine-disrupting effects of environmental samples were evaluated using the YES assay with a two-hybrid yeast screening system. Estrogen agonist activities of the samples were determined measuring β-galactosidase activity.[31] The recombinant yeast cells used in the current study were provided by the Research Center for Eco-Environmental Science, Chinese Academy of Sciences. The yeast assay was conducted using the previously described method.[32] In brief, yeast was incubated overnight at 30°C until the logarithmic growth phase. The final culture was adjusted to an optical density (OD_{600}) of 0.75 and then exposed to a 1:2 dilution series of samples in 96-well plates for 4 h. According to ISO 10993-5, the highest concentration of YES was defined as the concentration where cell viability exceeded 70% in the MTT assay.[33] The final DMSO concentration in each well was 0.50% (v:v). A dilution series of 17β-estradiol (E_2) (4, 8, 20, 40, 80, 200, 400, 800, 2000 pM) and a DMSO solvent control were included in each experiment. The yeast density was estimated by absorbance at 600 nm (OD600) after exposure to evaluate yeast cytotoxicity before measuring the estrogenic effect.[27] The enzymatic reaction was terminated by adding

Na_2CO_3 at a concentration of 1 mol L^{-1}. β-galactosidase activities were determined by the light absorbance at 420 nm and calculated according to the following equation:[34]

$$U = \frac{OD_{420} - OD'_{420}}{t \times V \times OD_{600}} \times D \quad (1)$$

Where U is the β-galactosidase activity. t, V, and D are enzyme reaction time, volume, and diluting factor. OD_{600} is the absorbance measured at 600 nm, OD_{420} and OD'_{420} are absorbances at 420 nm for the sample exposure group and negative control (yeast solution containing 0.50% DMSO (v:v)), respectively. Each sample was tested at nine 1:2 serial dilutions. All environmental samples were analyzed with three independent replicates. The details of compounds used in the bioassay are detailed in Text S2. The estrogenic equivalent (EEQ_{bio}) from the bioassay was calculated using the following equation:[35]

$$EEQ_{bio} = \frac{EC_{20,E2}}{EC_{20,Sample}} \quad (2)$$

Where $EC_{20,E2}$ is the concentration of the reference compound E2 required to elicit 20% estrogenic effect, while $EC_{20,Sample}$ denotes the concentration of sample produce the same 20% estrogenic effect as E2. The relative potency potency (REP) is used to quantify the estrogenic effect intensity of compound relative to the reference compound E2. The REP_i and EEQ_{chem} is calculated using the equation (3):[36]

$$REP_i = \frac{EC_{F,E2}}{EC_{F,i}} \quad (3)$$

Where $EC_{F,E2}$ is the concentration of the reference compound E2 required to elicit F% estrogenic effect, while $EC_{F,i}$ denotes the concentration of compound i produce the same F% estrogenic effect as E2.

The EEQ_{chem} can be calculated based on the concentration addition (CA) model using the equation (4):[8, 37]

$$EEQ_{chem} = C_i \times REP_i \quad (4)$$

Where C_i represents the concentration of the target chemical, while relative estrogenic effects potencies (REP_i) denote the relative estrogenic potency of the target chemical in the YES assay.

Chemical analysis

In this study, 55 estrogenic compounds (Text S2), including 4 natural estrogens and 51 xenoestrogens,[38] were quantified in sediment samples using Acquity Ultra performance liquid chromatography coupled to a Xevo TQ-S triple quadrupole mass spectrometer (UPLC-MS/MS) and gas chromatography-mass spectrometry (GC-MS). Additional details on the elution procedure and the fragmentation patterns of the UPLC-MS/MS are described in Text S3 and Table S2. The temperature program, parameters, and the selected monitoring ions for GC-MS are detailed in Text S4 and Table S3.

To further investigate potential estrogenic compounds in the TGR, non-targeted analysis was performed using UPLC coupled with a quadrupole electrostatic field Orbitrap high-resolution mass spectrometer (UPLC-Exactive Orbitrap/HRMS, Thermo Fisher, USA).[39-41] A C_{18} column (Hypersil Gold C_{18}, 2.1 × 100 mm, 3.0 μm) was used for separation at a column temperature

of 40 °C, with an injection volume of 10 μL and a flow rate of 0.30 mL min^{-1}. Further details regarding the mobile phase gradient elution conditions and HRMS parameters are provided in Text S5.

The .raw data files from the UPLC-HRMS analysis, along with related blanks, were processed using Compound Discoverer (CD) 3.2 (Thermo Fisher Scientific, USA). The "Environmental w Stats Unknown ID w Online and Local Database Searches" analysis process in CD 3.2 software was used to screen for unknowns, with appropriate modifications. The data workflow and the parameters of CD are detailed in Figure S1 and Table S4. After data processing, the feature list was refined by applying filters and removing blank features. The screening criteria included a retention time deviation of less than 0.2 minutes, a precursor ion mass deviation of less than 5 parts per million (ppm), an isotope-matching threshold of 30%, a peak area greater than 1×10^6, and an mzCloud online database match score of at least 70.[42] The concentration of newly identified compounds in environmental samples was quantified *via* their standards using ultra-performance liquid chromatography coupled with a triple quadrupole mass spectrometer (AB SCIEX 5500, AB SCIEX, USA) operating in multiple reaction monitoring (MRM) mode. Details of the elution procedure and mass spectrometry parameters are shown in Text S3.

Quality assurance and quality control

To avoid introducing contamination during the experiment, all glassware and instruments used throughout the experiment were washed three times with ethanol and Milli-Q water, then dried at 105 °C for 24 hours. Quality assurance and control measures were implemented during the

analysis. For each batch of samples, procedural blanks were prepared using the extraction solvent and processed following the same procedures as samples.[43] These blanks were subtracted in subsequent non-targeted analyses. No detectable levels of the target analytes were found in the blank samples. The recovery rate and matrix effect of the extraction method were measured using spiked samples. The recovery rates of the target substances and nonivamide ranged from 88.6% to 104%, and the matrix effects ranged from 76.5% to 106%. The details of calibration curves for all chemicals, along with the method detection limit (MDL) and method quantification limit (MQL) of the target chemicals, are provided in supporting information Table S5.

Statistical analysis

Experimental data on cytotoxicity and estrogenic responses were plotted using GraphPad Prism 10 (GraphPad Software, San Diego, USA) and subsequently fitted with variable slope logistic regression and four-parameter logistic regression models. The Mann-Whitney *U* test was used to analyze differences in estrogenic activity and concentrations of nonivamide in samples. The correlation between estrogenic activity, determined by $1/EC_{50}$ values, and the concentrations of individual chemicals was assessed via pearson correlation. Pearson correlation analysis was also used to examine the relationship between compound peak areas and estrogenic effects in the non-target analysis.

Subsequently, the Quantile g-computation (Qgcomp) model was applied to assess the influence of mixed variable (established by the chemicals that significant correlations with estrogenic

effects) on estrogenic activity in sediment samples. A volcano plot was generated to compare the differential expression of chemicals in the non-target analysis between flood and non-flood periods at the same sampling site. Log_2 fold changes were calculated to represent the ratio of compound peak area between the two periods. Statistical significance was determined using a two-tailed t-test, and *p*-values were adjusted for multiple comparisons using the Benjamini-Hochberg method to control the false discovery rate (FDR). Compounds with a p-value < 0.05 and an absolute log_2 fold change greater than 1.5 were considered significant. The Qgcomp analyses were conducted in R (version 3.6.1; R Core Team) using the "Qgcomp" packages.

Results and Discussion

Bioassay-Derived Induction Equivalent Quantities

The results of H4IIE cells exposed to sediment sample extracts (0.08 - 20 g SEQ L^{-1}, 48 h) are shown in Figure S2. Cytotoxicity tests showed that, after 48 h of exposure, cell viability slightly decreased with increasing extract concentration. Nevertheless, under exposure to concentrations of 0.08 - 20 g SEQ L^{-1}, the cell viability of all samples still exceeded 70%.

The estrogenic activity of ten sediment sample extracts were determined by the YES test using the two-hybrid yeast system. All five flood period samples showed a concentration-dependent increase in β-galactosidase activity across a concentration range of 0.390 to 100 g SEQ L^{-1}, following an "S"-shaped dose-response relationship (Figure 2) with EC_{20} values ranging from 2.01 to 22.1 g SEQ L^{-1} (Table S6). The sediment extract from GY during the flood period exhibited the strongest estrogenic activity, with the lowest EC_{20} value of 2.01 g SEQ L^{-1},

followed by the extracts from WS, FD, FJ, and YY where EC_{20} values were 3.02, 3.27, 9.14, and 22.1 g SEQ L^{-1}, respectively.

During the non-flood period, only a modest increase in β-galactosidase activity was observed with increasing sediment sample extracts concentration. A possible reason is the greater input by wash-out of estrogenically active substances from contaminated land to the aquatic environment during the flood period.[44] The EEQ_{bio} of flood period sediment sample extracts ranged from 0.638 to 6.99 ng EEQ g^{-1} (Table S6), which is comparable to China Liuxi River sediment sample extracts with an EEQ ranging from ND to 7.24 ng EEQ g^{-1}.[45] The highest EEQ was found in GY with the EEQ value of 6.99 ng EEQ g^{-1}. GY is located in the largest tributary's backwater zone of the TGR, where slow flow makes it susceptible to backflow from the main river during the flood period. Density currents may transport estrogenic compounds from the main river into the tributary, where they are likely to accumulate or deposit in sediments through sorption.[46] The EEQ values of the sediment sample extracts from WS, FD, FJ, and YY were 4.66, 4.31, 1.54 and, 0.638 ng EEQ g^{-1}, respectively, suggesting a high estrogenic activity of sediments during the flood period in TGR.[47] The estrogenic effect of sediment sample extracts downstream were higher than that upstream, which is consistent with previous studies.[48] A possible reason could be estrogenic contaminants that bind to particles followed by their sedimentation in the downstream region.[21, 49] In addition, the strong estrogenic effect detected from the FD site, which may be linked to pollutant migration slowed down by backflow of the dam.[50, 51]

To investigate the differences of estrogenic effects in the flood and non-flood periods, we analyzed estrogenic activity of sediment sample extracts from the same site across different periods, with the exposure concentration of 100 g SEQ L^{-1}. As shown in Figure 3, the sediment sample extracts during the flood period induced β-galactosidase activity ranging from 0.0921 U to 0.194 U, corresponding from 44.9% to 94.6% of the E2 max response (0.205 U). The β-galactosidase activities detected from the extracts during non-flood period were between 0.0110 U and 0.0717 U, which were about 5.37 - 35.0% of the E2 max response.

It is noticeable that except WS, all flood period sediment sample extracts exhibited significantly higher estrogenic activity than the sediment sample extracts in the non-flood period ($p < 0.05$) (Figure 3). The increased water level during the flood period may mobilize estrogenic compounds from the shoreline into the aquatic environment.[52] These compounds can easily bind to sediment and particulate matter, potentially enhancing the estrogenic effect in sediment sample extracts during the flood period.[21, 53]

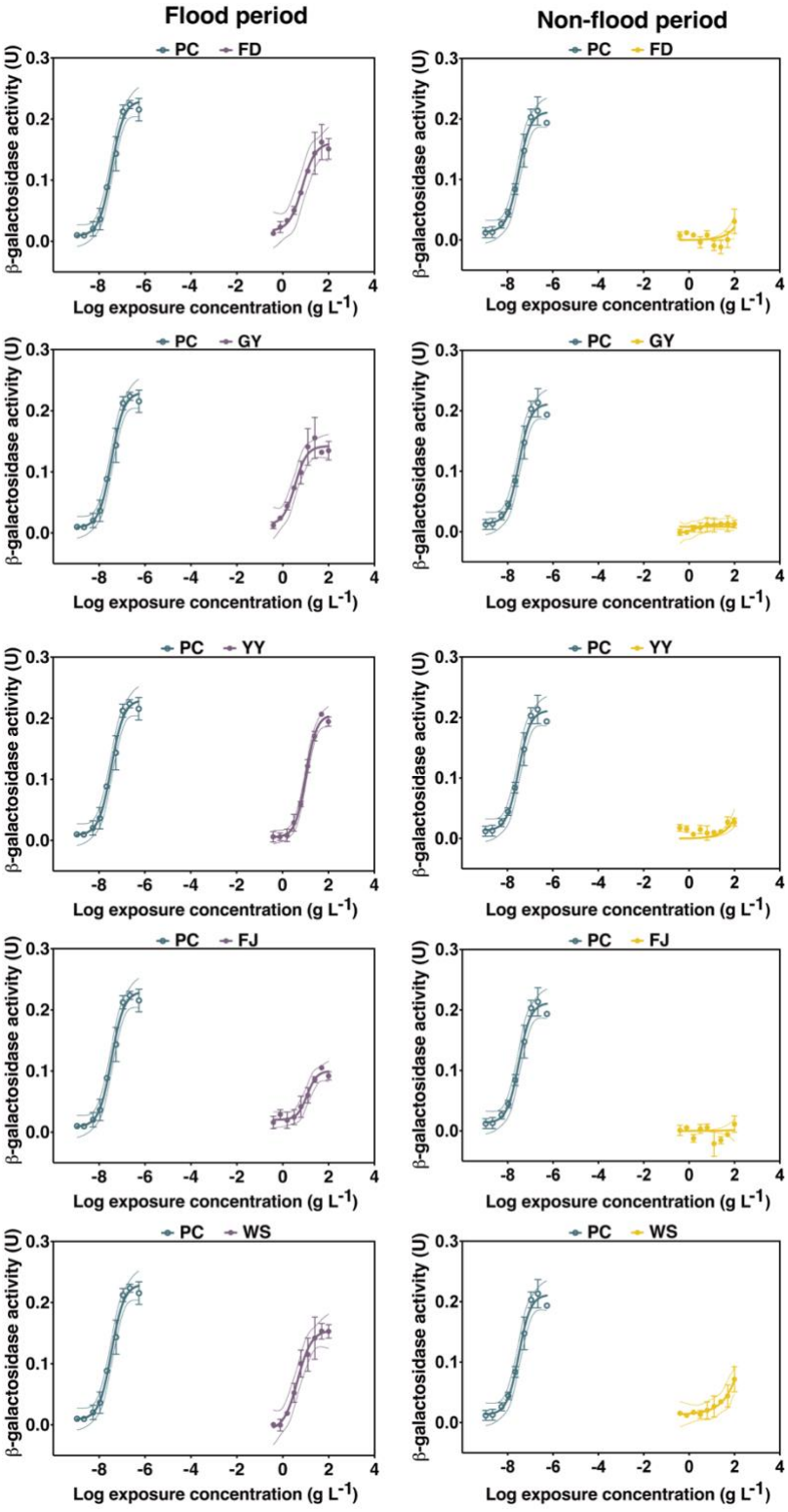


Figure 2. Concentration-response curves and corresponding 95% confidence bands in the YES assay for the TGR sediment sample extracts, and the E2 standard. PC: positive control (E2); FD: Fengdu; FJ: Fengjie; GY: Gaoyang; WS: Wushan; YY: Yunyang.

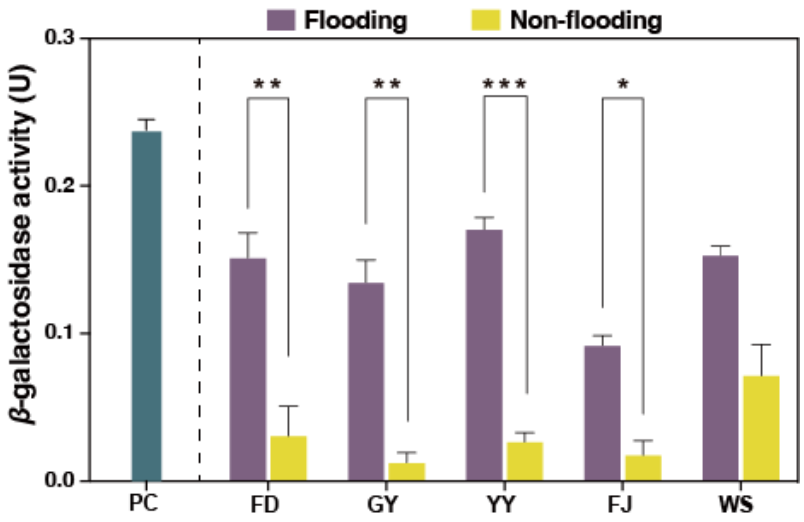


Figure 3. Compare the *β*-galactosidase activity induced by sediment sample extracts between the flood and non-flood periods at an exposure concentration of 100 g SEQ L^{-1}. The asterisks indicate *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. PC: positive control (E2 at a concentration of 5.44×10^{-7} g L^{-1}); FD: Fengdu; FJ: Fengjie; GY: Gaoyang; WS: Wushan; YY: Yunyang.

Contribution of Substances to Estrogenic Effects

28 out of 55 substances were detected in sediment sample extracts (Figure S3, Table S7). Estrone (E1) was only detected in the YY samples during the flood period at a concentration of 0.136 ng g^{-1} dw, which is lower than the concentrations reported in the previous Taihu study (5.49 to 164 ng g^{-1} dw) and Catalonia, NE Spain study (1.00 to 11.9 ng g^{-1} dw).[54, 55] Ethinyl estradiol (EE2) was detected at concentrations ranging from 0.0245 to 2.67 ng g^{-1} dw , which were lower than the concentrations reported in the previous Taihu study (4.32 to 184 ng g^{-1} dw) and Tianjin study (0.930 to 7.67ng g^{-1} dw).[54, 56] Apart from EE2, 26 of the remaining 47 xenoestrogens were detected in concentrations between 0.0196 and 50.6 ng g^{-1} dw (Figure S3). The highest concentration was observed for endrin (50.6 ng g^{-1} dw) in FJ during the non-flood

period, which is much higher than that in the Buji River in Shenzhen and Huaihe in Anhui province, where the highest concentrations were 1.88 ng g^{-1} dw and 6.60 ng g^{-1} dw, respectively.[57, 58] Besides, hexachlorobenzene, methoxychlor and mirex were detected with 100% frequency across all sampling sites (Table S7).

To analyze the contribution of the detected target compounds to the estrogenic effects in the TGR, a pearson correlation analysis was conducted. Interestingly, no significant correlation was found in the tested samples (Figure S4). This result illustrated that the detected typical estrogens and estrogen-like chemicals may not be the major contributors to estrogenic activity in the sediments of the TGR. Similar results were observed for the Detroit River, where the estrogenic activity was not primarily induced by the detected 62 chemicals (16 polyaromatic hydrocarbons (PAHs), 38 polychlorinated biphenyls (PCBs), 5 chlorobenzenes, and 4 organochlorine pesticides)).[59]

To further explore how much of the environmental estrogenic effects can be explained by detected compounds, the REP_i values (Table S8) were firstly used to calculate the the EEQ_{chem} of the detected chemicals (Table S8). Then, the ratio of $\sum EEQ_{chem}$ to EEQ_{bio} was calculated. As can be seen in Figure 4, the $\sum EEQ_{chem}$ were much lower than the EEQ_{bio} in all samples. Except for FJ (29.5%), the EEQ_{chem} of other sampling sites explained less than 3.00% of the EEQ_{bio}, since the detected concentration of 17α-ethinyl estradiol at FJ (2.67 ng g^{-1} dw) was 1-2 orders of magnitude higher than at FD (0.499 ng g^{-1} dw) and WS (0.0245 ng g^{-1} dw). In TGR sediments, detected compounds accounted for a lower fraction of estrogenic effects than in the River Elbe, where they explained approximately 67.0% of the estrogenic effects.[60] These

results indicate that, compared with the correlation method, the REP approach provides a more accurate quantification of individual components' contributions to overall bioactivity, instead of being effective only for the predominant contaminants. Moreover, these results suggest that other chemicals contribute to the estrogenic activity and that the target compounds in this study are insufficient to explain the estrogenic effects in sediments.

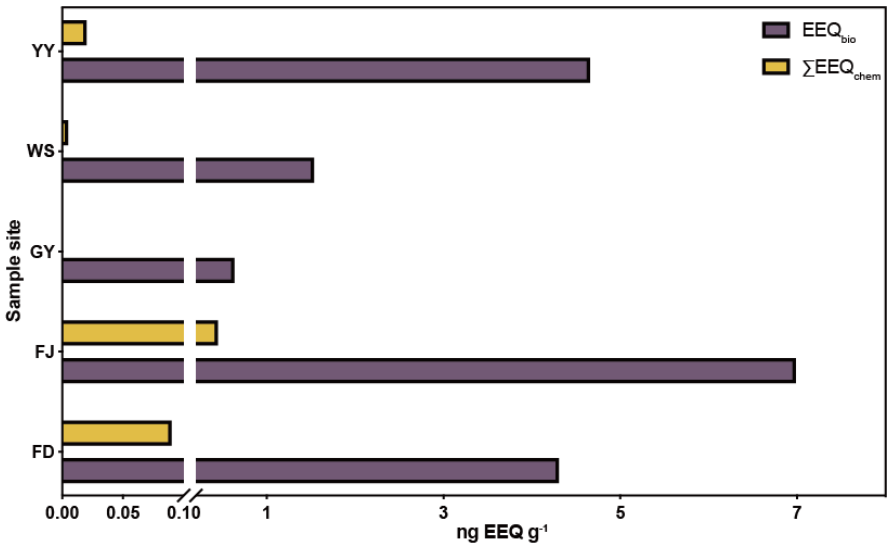


Figure 4. The EEQ_{bio} and the EEQ_{chem} of the detected chemicals during the flood period. $\sum EEQ_{chem}$ was calculated based on the concentration addition (CA) model as the sum of the EEQ_{chem} of the detected compounds in the flood period samples. EEQ_{bio} was derived from the YES assay, as the E2 equivalent concentration measured in the sediment sample extracts. FD: Fengdu; GY: Gaoyang; YY: Yunyang; FJ: Fengjie; WS: Wushan.

Non-target Analysis of the TGR Sediments by HRMS

To systematically screen key estrogenic chemicals in the TGR, the sediment sample extracts from the FD, GY, and YY sites in both flood and non-flood periods were selected, due to their

high estrogenic activity ($p < 0.01$).[61] The resulting set of non-redundant compounds detected across the full set of samples covered a wide range of molecular weights (100 to 1000 Da), hydrophobicity and polarity (chromatographic retention times from void volume elution up to 25 min) across more than 6 orders of magnitude of mass spectral abundance (i.e., chromatographic peak area). After subtracting the features found in blanks, non-target analysis extracted a total of 47,321 features in the samples from FD, GY, and YY (Figure S5), which are much higher than those detected in the sediments from the Taihu River with 1,064 - 2,780 features.[62] A total of 527 compounds were identified with the confidence level classified as 2a, which the structures matched by MS/MS spectra matching with databases (Table S9). The currently identified compounds are more diverse than the 448 compounds identified in sediments from the Taihu River in the Yangtze River Delta region.[62] As shown in Figure 5, 61.3% of the identified 527 compounds (n = 323) were natural compounds, including natural drugs, natural toxins, and endogenous metabolites. Previous studies have confirmed that endogenous estrogens, isoflavonoids, and lignans can induce significant estrogenic effects.[63] Our targeted analysis also detected E1, which contributed 1.09% of the estrogenic effect in YY. Industrial chemicals (n = 93) were followed by natural compounds, which account for 17.6% of the identified compounds. Earlier research has confirmed that industrial chemicals such as phthalates, BPA, and polybrominated diphenyl ethers (PBDEs) can induce considerable estrogenic effects.[64, 65]

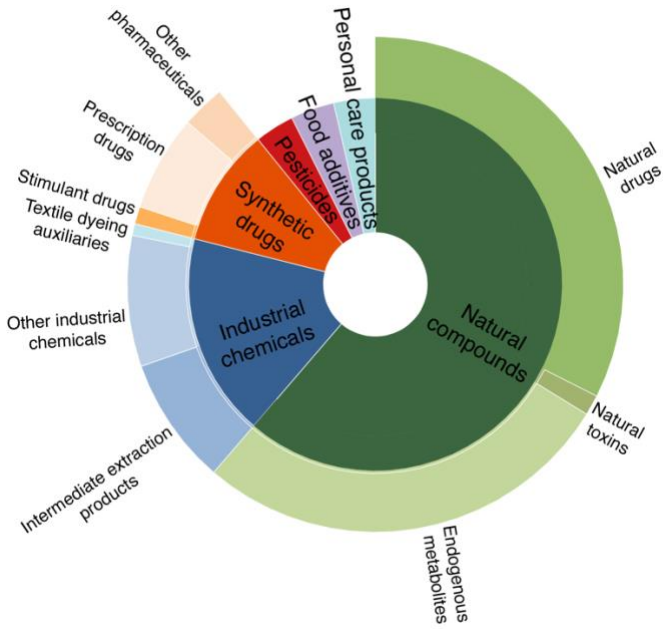
In addition, synthetic drugs (n = 54) accounted for 10.2% of the identified compounds. Oral contraceptives (OCs) and hormone replacement therapy (HRT) drugs contributed significantly

to environmental estrogenicity. OCs typically consist of combinations of estrogens and progestins, with EE2 being the most commonly used synthetic estrogen with an average daily dose of 30 - 35 μg per tablet.[66] It is estimated that the human body metabolizes 20 - 48% of the daily dose of EE2.[67] The remaining dose is excreted in its original form or as EE2 sulfate or glucuronide conjugates.[67] Approximately 60% of the ingested EE2 is excreted via urine or feces, primarily as conjugates, most of which dissociate back to EE2 in the environment.[68] In our targeted analysis, EE2 was detected in sediment samples from FD, YY, FJ, and WS with concentration ranging from 0.0245 - 2.67 ng g^{-1} dw. In FJ, 29.5% of the estrogenic effects in the flood period were attributed to EE2. HRT contain conjugated equine estrogens as the main active component.[69] A study from the UK found that equine estrogens and their metabolites can induce high estrogenic effects.[70]

Pesticides (n = 18) accounted for 3.42% of the identified compounds. Many pesticides exhibit estrogenic activity, for instance methoxychlor has been demonstrated to bind to the estrogen receptors (ERs) and modulate downstream signaling pathways,[71] such as the ER/ERK pathway, implicated in cell proliferation.[72] Dieldrin and endrin can agonize the ER mediated transcription in a dose-dependent manner.[73] Diazinon, malathion, chlorpyrifos, and prothiofos have been shown to exhibit estrogenic activity, as evidenced by previous research.[74-76] In our targeted analysis, the detection rates were 10/10, 7/10, 5/10 and 1/10 for methoxychlor, endrin, malathion and dieldrin, respectively. Furthermore, this result is consistent with previous studies on pesticides detected in the TGR.[77, 78]

Food additives (n = 20) accounted for 3.80% of the identified compounds. Some antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, have been confirmed to exhibit estrogenic activity in the MCF-7 cell,[79] as well as in fish exposure studies[80]. Furthermore, some food colorants such as tartrazine and sunset yellow have been found to bind to estrogen receptors, thereby inducing estrogenic activity.[81]

The remaining 19 substances are personal care products, accounting for 3.61%. Previous research indicated that cosmetics, specific parabens and ultraviolet (UV) filters, including butylparaben, propylparaben, and homosalate, effectively activate the ERα receptor, thereby inducing estrogenic effects.[82] However, the targeted analysis conducted in previous studies focused on only a subset of the above six types of substances, and many potential estrogenic substances are covered. This limitation may account for the limited explanatory power of the detected estrogenic substances in sediment sample extracts through targeted analysis for estrogenic effects. It further underscores the necessity of non-target analysis to identify potentially toxic substances in complex environmental samples.



399

400 **Figure 5.** Pie chart of the percentage of different types of non-target-identified compounds.

401 **Contribution of Non-Target Chemicals to Estrogenic Effects in the TGR**

402 As shown in Figure 6A, the result of the pearson correlation analysis showed that, among 527
403 identified compounds, only 4 chemicals (nonivamide, 4-methylbenzophenone, danofloxacin
404 and 6:2 fluorinated telomer sulfonate) were significantly positively correlated with estrogenic
405 effect ($p < 0.05$) and could hence be important contributors to the estrogenic activity of
406 sediment in the TGR. To evaluate the effect of the quadrivariate mixture (nonivamide, 4-
407 methylbenzophenone, danofloxacin, and 6:2 fluorinated telomer sulfonate) on estrogenic
408 activity, as well as the contribution of each compound, the Qgcomp model was implemented.
409 The Qgcomp model analysis showed that, for each quartile increase in these mixture's peak
410 area, the estrogenic effect increased by 11.1% (Table S10). It is noticed that nonivamide,
411 amongst them, account for the largest contribution (40.5%) to the increase in estrogenic effect,
412 followed by 4-methylbenzophenone (33.8%), danofloxacin (22.5%), and 6:2 fluorotelomer

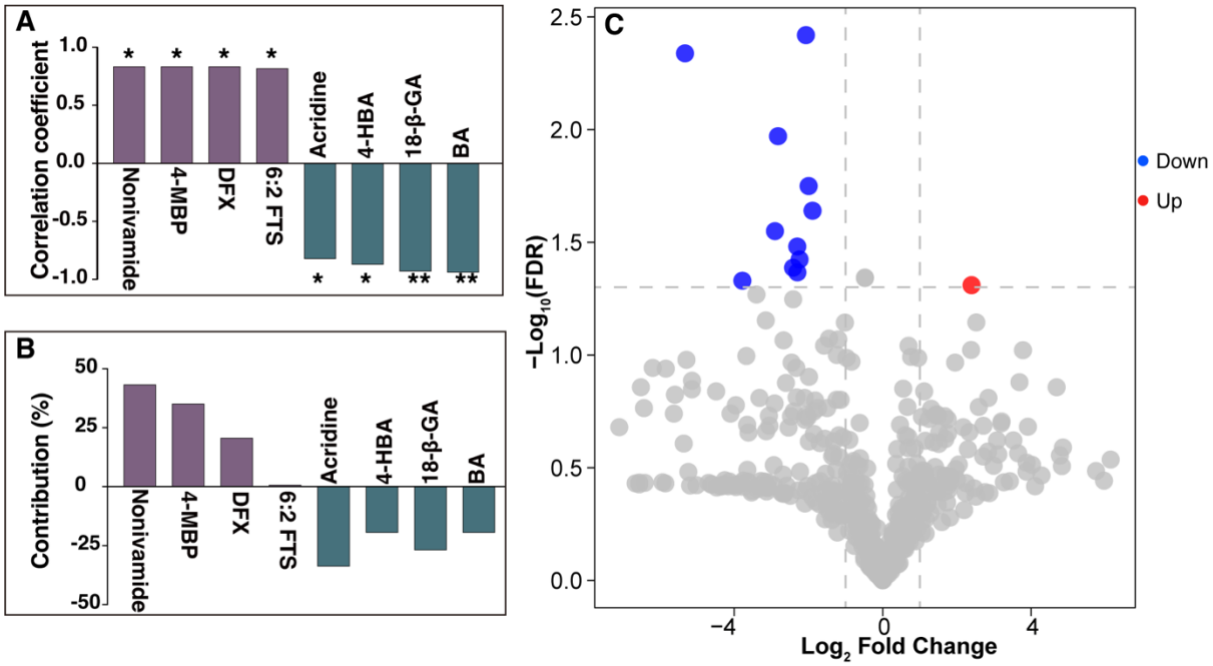
413 sulfonate (3.20%) (Figure 6B). 6:2 fluorotelomer sulfonate and 4-methylbenzophenone have
414 been proven to trigger estrogenic effect by binding to the estrogen receptor.[83, 84] However, to
415 the best of our knowledge, the environmental estrogenic effect of nonivamide and danofloxacin
416 have not been reported.

417 Anti-estrogen effects result from substances that inhibit or block the normal actions of
418 estrogen.[85] These anti-estrogens primarily exert their effects by competing with estrogen for
419 binding to estrogen receptors.[86] In the current study, the four compounds (acridine, 4-
420 hydroxybenzaldehyde, benzoic acid, 18-β-glycyrrhetinic acid) were significantly negatively
421 correlated with estrogenic effect, implying that these compounds may exhibit anti-estrogenic
422 activity. These compounds maintained these negative correlations in the Qgcomp analysis.
423 Acridine made the largest contribution to the inhibition of estrogenic activity (35.9%), followed
424 by 4-hydroxybenzaldehyde (23.7%), benzoic acid (20.2%), and 18-β-glycyrrhetinic acid
425 (20.2%) (Figure 6B). Benzoic acid and 18-β-glycyrrhetinic acid have been confirmed without
426 any estrogenic activity in the yeast test and have strong anti-estrogenic effects, consistent with
427 the results of our current study.[87, 88] 4-hydroxybenzaldehyde is an important intermediate
428 metabolite of the estrogen active substance bisphenol A (BPA), which could indicate the
429 transformation and decomposition of BPA, resulting in a negative correlation with estrogenic
430 activity, which are consistent with the results of this study.[89]

431 In consideration of the significant difference of estrogenic activity of sediment sample extracts
432 in flood and non-flood periods in FD, GY and YY ($p < 0.05$), a differential analysis was
433 performed for the 527 substances identified in the non-target screening. Only 12 compounds

(nonivamide, 2,11,11-trimethyl-3-oxo-undecane-9-carboxylic acid, 4-(3-hydroxybutyl)-5-methyl-10-methylidene-decan-9-one, 1,5,9-trimethyl-tetraoxatetracyclo-hexadecan-10-one, 16-hydroxyhexadecanoic acid, 18-*β*-glycyrrhetinic acid, 4-hydroxybenzaldehyde, acridine, benzoic acid, isophorone, norharman), differed significantly in peak areas between flood and non-flood periods (Figure 6C). Notably, only nonivamide exhibited a significantly higher peak area during the flood period compared to the non-flood period (Figure 6C). This result further supports that nonivamide is one of the most important toxic drivers to estrogenic activity in the TGR sediment.

Nonivamide is a capsaicin formed by the condensation of the amino group of 4-hydroxy-3-methoxybenzylamine with the carboxy group of nonanoic acid. Owing to its superior heat stability and lower production costs, it is frequently employed as a food additive to satisfy the population's demand for spiciness, thus replacing natural capsaicin.[90] Furthermore, nonivamide exhibits antifouling activity and, as a safer biocide, has replaced organotin compounds in widely used antifouling paints for ships.[91] However, to the best of our knowledge, there has been no report on the occurrence of nonivamide in the environment.



449

450 **Figure 6.** Association analysis of substances identified by non-target screening in samples and
451 estrogenic effects. (A) Correlation coefficient of estrogenic effects and substances identified
452 by non-target screening from FD, GY and YY from the flood and non-flood periods. * $p < 0.05$,
453 ** $p < 0.01$. (B) The contribution of substances significantly correlated with estrogenic effects
454 assessed by the Qgcomp model. 4-MBP: 4-methylbenzophenone, DFX: danofloxacin, 6:2 FTS:
455 6:2 Fluorotelomer sulfonate, 4-HBA: 4-hydroxybenzaldehyde, 18β-GA: 18-β-glycyrrhetinic
456 acid, BA: benzoic. (C) Volcano plot of the differences in substances identified using non-target
457 screening of sediment sample extracts from FD, GY, and YY during the flood and non-flood
458 periods. FD: Fengdu; GY: Gaoyang; YY: Yunyang; FJ: Fengjie; WS: Wushan.

459 **Confirming the Contribution of Nonivamide to Estrogenic Activity**

460 The MS spectrum of nonivamide is shown in Figure 7A. As can be seen from Figure 7B,
461 nonivamide was only detected in FD and GY with concentrations ranging from 1.02 ± 0.07 ng

462 g^{-1} dw and 0.948 ± 0.043 ng g^{-1} dw in flood period. It is noticed that the concentrations in flood
463 samples were significantly higher than those in non-flood samples ($p < 0.05$) (Figure 7B),
464 which is in accordance with the differential analysis and the observed estrogenic activity. To
465 our knowledge, this is the first report on the occurrence of nonivamide in real environments.
466 To verify the contribution of nonivamide to the estrogenic activity in the TGR, YES test was
467 implemented for nonivamide. The results showed that nonivamide induced a concentration-
468 dependent increase in β-galactosidase activity, with concentrations ranging from 2.00×10^{-5} to
469 7.80×10^{-8} g L^{-1}, displaying an "S"-shaped dose-response relationship (Figure 7C). The REP
470 of nonivamide was determined to be 3.53×10^{-2}, which is 2.35 times that of typical estrogen-
471 like chemicals such as BPA (1.50×10^{-2}) and 35.3 times that of NPs (1.00×10^{-3}), placing it in
472 the same order of magnitude as E1 (5.30×10^{-2}).[92] According to the "S"-shaped dose-response
473 relationship, nonivamide can induce 0.0340 ± 0.0140 U of β-galactosidase in the YES assay at
474 the detected concentration, which accounted for 22.5% and 25.2% of the estrogenic effects in
475 the flood period at FD and GY, respectively (Figure 7D).
476 To further characterize the estrogenic activity of nonivamide, the E-screen assay was
477 conducted using ER-positive MCF-7 cells. As shown in Figure 7E, nonivamide induced a
478 concentration-dependent increase in the MCF-7 cell proliferation rate across a concentration
479 range of 1.00×10^{-4} g L^{-1} to 3.09×10^{-7} g L^{-1}. The REP of nonivamide was calculated to be
480 5.89×10^{-2}, which is 1.64 times higher than that obtained from the YES assay (3.59×10^{-2}),
481 indicating a stronger estrogenic response in the mammalian system. The elevated estrogenic
482 potency observed in this mammalian model underscores the need for further toxicological

evaluation of nonivamide, especially considering its possible health risks associated with estrogenic effects.

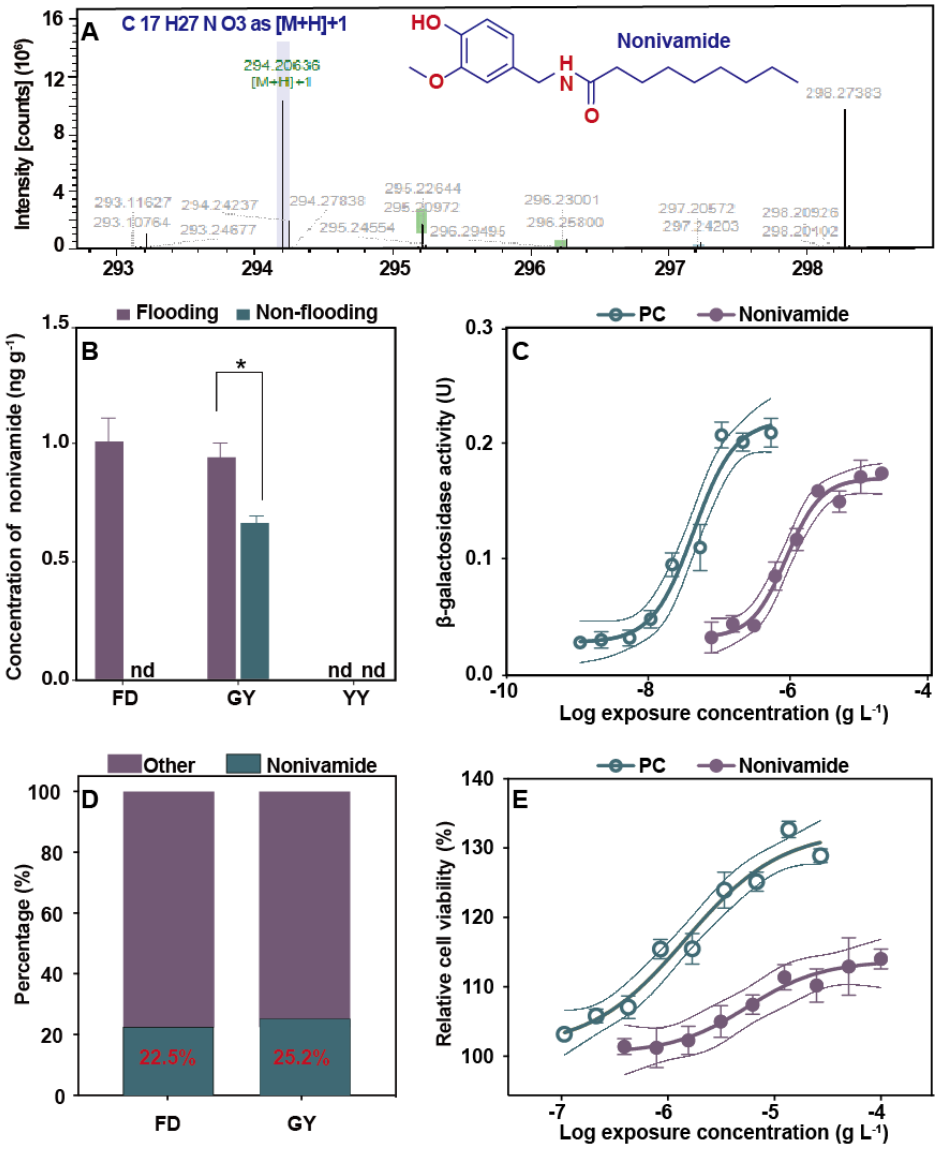


Figure 7. Environmental distribution and estrogenic effect assessment of nonivamide. (A) The MS spectrum of nonivamide. (B) The distribution of nonivamide in sediments. (C) Effect-dose curves of nonivamide and E2 standards in the YES test. (D) The expression level of nonivamide in the estrogenic effects from flood periods in FD and GY. (E) Effect-dose curves of nonivamide and E2 standards in the E-screen test. FD: Fengdu; GY: Gaoyang; YY: Yunyang; FJ: Fengjie; WS: Wushan; nd refers to concentrations below the method quantification limit

(MQL).

In the current study, we confirmed that compared to traditional EDCs such as BPA and NPs, nonivamide is more potent and induces higher estrogenic effects in the real environment. To the best of our knowledge, there is no literature reporting the environmental persistence or bioaccumulation potential of nonivamide. Data from the OU Chemical Safety Data exhibits that nonivamide is chemically stable and not readily oxidized.[93] According to ACD/Labs predictions, the predicted bioconcentration factor (BCF) of nonivamide is 480 L/kg,[93] indicating its ability to bioaccumulate in aquatic organisms. A previous study reported that nonivamide exerts a significant growth inhibitory effect on *Phaeodactylum tricornutum* ($p < 0.01$), with a 4-d EC_{50} of 5.10 mg L^{-1},[94] which may pose a considerable environmental risk. Therefore, our findings underscore the importance of incorporating nonivamide into future environmental monitoring and risk assessments. Nonivamide is widely used as a food additive and in industrial applications. In the current study, our findings highlight its contribution to estrogenic effects in sediments, emphasizing the need to consider it in future environmental monitoring and risk assessments.

Furthermore, our study highlights the critical role of chemometrics to provide links between *in vitro* bioassays and non-target analysis to identify potential key toxicants in complex environmental samples. Non-targeted analysis facilitates the identification of potential toxic compounds that may elude detection by targeted analysis, due to the lack of awareness and appropriate reference standards. Based on the non-target data, chemometrics effectively extracts valuable information. Simultaneously, integrated with bioassays, this methodology

facilitates the identification of compounds with significant biological activity or toxic effects. Our analytical strategy effectively detected the main and novel contributor to the estrogenic activity in TGR sediments, which provides a valuable reference for future key toxicants in complex environmental mixtures.

Supporting Information

Text S1. Detailed protocol of the E-Screen assay. Text S2. The details of chemical and standards. Text S3. The gradient elution process of chromatography and mass spectrometry parameters for UPLC-MS/MS. Text S4. The temperature program and mass spectrometry parameters for GC-MS. Text S5. The mobile phase gradient elution conditions and HRMS parameters. Figure S1. Compound Discoverer data workflow. Figure S2. The toxic effects of sediment extracts on the cell viability of rat hepatocellular carcinoma cells (H4IIE). Figure S3. Distribution of 4 natural estrogens and 51 xenoestrogens in sediment samples. Figure S4. Correlations between estrogenic effects and 4 natural estrogens along with 51 xenoestrogens. Figure S5. Ion flow spectrum and distribution of non-targeted detection compounds. Table S1. The details of sampling sites. Table S2. MRM parameters for 4 natural estrogens and 4 xenoestrogens. Table S3. Quantifying and qualifying ions for 47 xenoestrogens in GC-MS analysis. Table S4. Parameters set in the Compound Discoverer workflow for the non-target analysis. Table S5. The calibration curves and the method detection limit (MDL) and method quantification limit (MQL) of the target chemical. Table S6. The EC_{20} and EEQ_{bio} in different samples during the flood period. Table S7. Concentrations of 55 estrogenic compounds in the samples. Table S8. The REP values and EEQ_{chem} of detected chemicals in each sample. Table S10. Effect estimates of increasing chemical abundance on estrogenic effects based on Qgcomp analysis.

Table S9

The details of non-target-identified compounds for samples from FD, GY, and YY during the flood and non-flood periods.

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Author Contributions

Y.S. and Z.C. designed the study and supervised all parts of the project. M.Z. performed experiments. M.Z., Z.Q. performed data analyses. Y.S. and Z.Q. drafted the first versions of the paper. Y.S. provided funding support. A.J. and A.S. contributed on experimental design and manuscript reviewing and editing.

Notes

The authors declare no competing financial interest.

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