A Female Reproductive System-on-a-chip to Identify Reproductive Toxicants

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Projects Contributors

a. Dr. Xiao and his team extracted organic pollutants from eight river water samples from six sites of three major rivers in New Jersey (NJ), including Delaware River, Passaic River, and Raritan River.

b. Dr. Xiao and his team collaborated with Drs. Hilly Yang and Brain Buckley from Rutgers Environmental and Occupational Health Sciences Institute (EOHSI) to perform targeted analytical chemistry and measurement of 72 major endocrine disrupting chemicals (EDCs) in the collected river water samples.

c. Dr. Xiao and his team tested the estrogenic and anti-estrogenic effects of river water organic extracts using the dual-luciferase reporter assay.

d. Dr. Xiao and his team used their established 3D hydrogel encapsulated *in vitro* follicle growth (eIVFG) system to test the potential endocrine disrupting effects and reproductive toxicity of river water organic extracts and investigate the toxic mechanisms involved.

Executive Summary

Rivers play a vital role in supporting diverse ecosystems and meeting various crucial human needs. There is a growing concern about various organic contaminants in the rivers, including dioxins, polychlorinated biphenyls (PCBs), per- and polyfluorinated substances (PFAS), polycyclic aromatic hydrocarbons (PAHs), and phthalates. Many of these contaminants are endocrine-disrupting chemicals (EDCs), posing potential risks to the reproductive health of both humans and wildlife animals.

The current study aims to develop a female reproductive tissue-on-chip, 3D *in vitro* microtissue culture, and other approaches to investigate the female reproductive effects of organic pollutants in major river waters in New Jersey (NJ). A total of eight surface water samples were collected from six sites in three NJ rivers, including the Delaware River (three sites, duplicates in one site), Passaic River (two sites, duplicates in one site), and Raritan River (one site). Organic pollutants were extracted using the solid-phase extraction approach, with double-distilled lab water as the control. High-resolution mass spectrometry (HRMS) was used to quantify 72 EDCs. The river water organic extract from the site of Elmwood Park of the Passaic River (PREP) had the highest concentrations of all measured EDCs, including alkylphenols, PFAS, phthalates, and pharmaceuticals.

A dual luciferase reporter assay was applied to determine the estrogenic and anti-estrogenic effects of river water organic extracts. The organic extracts from both sites of the Passaic and Delaware Rivers exhibited estrogenic effects, and the organic extract from one site of the Delaware River exhibited anti-estrogenic effects.

A 3D *in vitro* mouse ovarian follicle culture system was used to test the female reproductive toxicity of river water organic extracts. Organic extracts from both sites of the Passaic River and one site of the Delaware River significantly inhibited ovarian follicle growth, increased testosterone and progesterone secretion, and impaired ovulation. The organic extract that exhibited the most potent ovarian-disrupting effects from the PREP site (Passaic River site at Elmwood Park) was selected for *in vitro* concentration-response exposure and toxic mechanism assessments. The results showed that the PREP organic extract concentration-dependently inhibited ovarian follicle growth and suppressed the expression of genes essential for gonadotropin-dependent follicle development and hormone secretion. RNA sequencing (RNA-seq) analysis revealed that the PREP organic extract from the Passaic River altered the expression of genes related to Aryl hydrocarbon Receptor (AhR) signaling pathway, mitochondrial function, membrane disruption, and oxidative stress, which may contribute to the ovarian toxicities observed. Based on our own LC-HRMS results and publicly available data, we next mixed several major river water organic contaminants, including PFAS, phthalates, and several historical persistent organic pollutants (POPs) in the Passaic River (dioxin, PAHs, and PCBs). The mixture of POPs, but not PFAS or phthalate mixtures, exhibited similar ovarian toxicities to the organic extract from the PREP site of the Passaic River, which can be reversed by the co-treatment of a selective AhR inhibitor.

In summary, this study demonstrates that the female reproductive tissue organoids and organ-on-chip models, together with hormonal and molecular assays and analytical chemistry, are powerful models to assess the endocrine disrupting effects (e.g., estrogenic or anti-estrogenic effects) and female reproductive toxicity of real-world river water organic contaminant mixtures. The findings can be applied to support the development of prevention, mitigation, and remediation strategies to safeguard the health of humans and other species, with a particular focus on female reproductive health.

1 Introduction

Rivers play a vital role in supporting diverse ecosystems and meeting various crucial human needs. However, human activities such as urban development, agriculture, and industry have caused organic pollution in rivers, raising global concerns for both environmental and human health [1, 2]. In recent years, improved detection methods have revealed the presence of emerging pollutants (EPs) in aquatic environments. Previous studies have found pharmaceuticals, personal care products, and steroid hormones in river waters due to the discharge of treated wastewater [3]. Other persistent pollutants, such as polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), and per- and polyfluoroalkyl substances (PFASs) have also been detected and are mainly linked to industrial and agricultural waste [4-6]. One group of EPs is endocrine-disrupting chemicals (EDCs). Previous studies reveal that individual EDCs, such as bisphenols, PFAS, PCBs, PAHs, or dioxins, can bind to multiple nuclear receptors, including estrogen receptor (ER), androgen receptor (AR), aryl hydrocarbon receptor (AhR), and peroxisome proliferator-activated receptor (PPAR), which further interferes with associated signaling pathways and result in reproductive and endocrine disorders [7-9]. Despite these risks, current river monitoring programs still focus mainly on traditional and individual pollutants, due to a lack of guidelines and limited detection and treatment technologies for Eps [10].

Several methods are available for informing environmental risk assessment [11], but they each have their own advantages and disadvantages. Chemical analysis, which is widely employed in regulatory frameworks, is limited by the pre-selection of analytes and its inability to comprehensively assess mixture toxicity. In vivo analysis, involving the evaluation of whole animals within the receiving environment, is commonly used to detect toxicants and assess mixture toxicity of wastewater under real-world conditions. However, this method raises ethical concerns regarding animal use and exhibits greater variability due to complex interactions with environmental factors, such as seasonal variations. Bioanalytical tools offer unique advantages, such as lower detection limits and high-throughput screening capability. Current mixture studies, however, often rely on cell-based assays, such as the receptor binding assays, oxidative stress assays, or genotoxicity testing [12, 13], which do provide valuable insights into potential molecular targets but do not faithfully recapitulate real-life exposure of EDC mixtures to the organ/tissue as a whole. Furthermore, the EDC mixtures tested in previous studies often lack a well-defined characterization and quantification of various chemicals, which may overlook the vast number of unidentified compounds in real-world water samples and the potential interactions between chemicals.

To address these limitations, our study used a 3D *in vitro* reproductive microtissue culture model integrated with analytical chemistry and other hormonal and molecular approaches to investigate the endocrine disrupting effects and female reproductive toxicity of real-world organic extracts from three major rivers in New Jersey on female reproductive health.

2 Materials and Methods

2.1 Study sites

A total of eight surface water samples (six samples and two duplicates) were collected from six sites of three major New Jersey rivers in 2022. Selected sites included three Delaware River sites at Montague (DRMT), Phillipsburg above the Lehigh River (DRPB), and STP of Lambertville (DRLV), two Passaic River sites at Two Bridges (PRTB) and Elmwood Park (PREP), and one Raritan River site at the bridge access of Main Street in Millstone (RRMR). These sample locations are consistent with previous [14] and concurrent studies within the NJDEP evaluating intersex in smallmouth bass due to the prevalence of detectable estrogenicity as well as legacy concerns for endocrine-disrupting contaminants at these sites [15]. The specific site information, including latitude, longitude, sampling date, and ID, is summarized in Table 1. River water samples were collected following procedures described in the NJDEP Field Sampling Procedures Manual[16]. Briefly, grab samples were collected at each site in 1 L amber glass jars dipped approximately 6 inches below the water surface. Samples were immediately sealed with muffled aluminum foil and capped to prevent transfer of chemicals from the plastic lids (with a Teflon liner). Field samplers donned new gloves for each site to prevent cross-contamination and rinsed each sample jar with site water prior to collecting. The mouth of the jar was facing upstream to avoid any influence from the sampler. Glass jars with collected water samples were immediately placed on ice and transported to the Environmental and Occupational Health Sciences Institute (EOHSI) at Rutgers University. Laboratory-grade distilled water (ddwater, duplicates) was used for negative controls, and duplicate river samples from the sites of DRMT and PRTB were collected on each sampling day to assess quality control.

Table 1. Surface water collection information of six sites in three NJ rivers.

Waterbody	Site Name	Latitude	Longitude	Sample Date	Sample ID:
Delaware River	Delaware River at Montague*	41.308823	-74.797155	11-07-2022	**DRMT-1/2**
Delaware River	Delaware at Phillipsburg above the Lehigh River	40.69237	-75.2033	11-07-2022	**DRPB**
Delaware River	Delaware River at STP at Lambertville, NJ	40.360649	-74.945481	11-07-2022	**DRLV**
Passaic River	Passaic River at Two Bridges*	40.8978	-74.2799	11-10-2022	**PRTB-1/2**
Passaic River	Passaic River at Elmwood Park	40.909756	-74.131767	11-10-2022	**PREP**
Raritan River	Raritan River at Millstone; Bridge access under Main Street	40.553698	-74.580161	11-10-2022	**RRMR**
Blank	-	--	-	-	**Blank-1/2**

*Duplicate water samples were taken at sites with an asterisk following the site name.

2.2 Water Sample Extraction and Instrumental Analysis

2.2.1 Sample extraction

Organic chemical pollutants were extracted from river water samples (Figure 1) using the solid-phase extraction (SPE) approach. In brief, an Auto-SPE Manifold equipped with Oasis HLB 6 cc Vac Cartridge (500 mg Sorbent per Cartridge, 60 µm) was set up for simultaneous extraction of the river water samples. One liter of river water sample was loaded into the SPE Cartridges preconditioned with methanol and ultrapure water. The cartridges were dried and then eluted with 10 mL (8:2, v/v) methanol-dichloromethane. The eluent was evaporated under a stream of N_2 gas, and the residue was re-dissolved in dimethyl sulfoxide (DMSO). The relative enrichment factor (REF), the ratio of the initial water sample volume to the final volume after extraction and re-dissolution of the organic extracts, was 3,333.

Figure 1. Representative images of NJ river water collection and organic extracts.

2.2.2 Instrumental analysis

Organic contaminant concentrations in the surface water extracts of different study sites were determined using a Dionex UltiMate 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific) coupled with a Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer equipped with an electrospray ionization (ESI) source (UHPLC-high resolution mass spectrometry, or UHPLC-HRMS). Chromatographic separation was conducted on a Phenomenex Kinetex Coreshell C18 column (50 x 3 mm; 1.7 µm) at 30 °C. The injection volume of the samples was 5 µL. A mobile phase of H_2O (solvent A) and methanol with 5 mM ammonium acetate (solvent B) was used, following a 50-min linear gradient elution: starting composition of solvent B 25%; at 25 minutes solvent B 85%; at 35 minutes solvent B 100% and held for 5 minutes; from 40-43 minutes solvent B 25% and is then equilibrated for 7 minutes prior to the next analysis for a total run time of 50-minutes at a flow rate of 200 µL/min. Nitrogen was used for all gas flows. Data was collected in positive and negative ESI mode using Full MS and SIM mode with a resolution of 60000. Measured Precursor Ion, retention time, and ionization mode were listed in the Suppl. Table. Data acquisition and processing were carried out with Thermo Xcalibur (v.4.0.27.19) software. A solvent blank (HPLC grade ACN) was carried out after six samples to monitor for any background contamination.

2.3 Dual-Luciferase Reporter Assay for Estrogenic and Anti-Estrogenic Activity of River Water Extracts

Human 293T (h293T) cells were cultured *in vitro* and transfected with the plasmids pCMV-hERα, 3X ERE TATA luc, and pRL-SV40P using standard transfection procedures. To evaluate estrogenic activity, cells were exposed to river water organic extracts at a concentration of REF10x for 24 h, after which luciferase activity was measured, with 100 nM 17β-estradiol (E2) as a positive control. To assess potential anti-estrogenic effects, h293T cells were co-treated with each water organic extract and 100 nM E2 for 24 h. Following incubation, luciferase activities were determined using the dual luciferase reporter assay system, and relative activities were compared with those of blank controls (for estrogenic effects) or E2 alone (for anti-estrogenic effects).

2.4 3D Hydrogel-Based In Vitro Follicle Growth (eIVFG) Assay for Reproductive Toxicity Analysis

2.4.1 Animals

CD-1 mouse breeding colonies for ovarian follicle isolation and *in vitro* exposure were purchased from Envigo (Indianapolis, IN) and maintained at Rutgers University. Mice were housed in polypropylene cages in the Animal Care Facility of Research Tower at Rutgers University. Mice were kept in temperature- (72+/-2°F), humidity- (30%-70%), and light- (12/12 light/dark cycle) controlled facilities and were provided with food (PicoLab Mouse Diet 20 EXT 5R58, catalogue:3003269-712, LabDiet, Richmond, IN) and water ad libitum. All animals were maintained and treated according to the NIH Guide for the Care and Use of Laboratory Animals and the approved Institutional Animal Care and Use Committee (IACUC) protocol at Rutgers University.

2.4.2 Ovarian follicle isolation, encapsulation, and encapsulated *in vitro* follicle growth (eIVFG)

Postnatal day (PND) 16 old CD-1 female mice were euthanized using CO_2. Ovaries were removed and collected in Leibovitz's L-15 medium (catalog: 11415064, Gibco, Grand Island, NY) supplemented with penicillin-streptomycin and fetal bovine serum (catalog: FBS, Sigma-Aldrich, St. Louis, MO). Ovaries were cut into 6-8 pieces and enzymatically digested in L-15 media containing Liberase TM (catalog: 5401127001, Roche, Indianapolis, IN) and DNase I (catalog: 9003-98-9, Worthington Biochemicals, Freehold, NJ) for 25 minutes. Follicles were isolated using a pipette with a 200 µm diameter opening. Follicles with diameters between 130 and 160 µm were selected based on morphological similarity. 0.5% alginate hydrogel in PBS and calcium solution was used to encapsulate a single follicle in one alginate bead. Encapsulated follicles were maintained in the αMEM Glutamax media (catalog: 32561037, Gibco) with 1% FBS for 30 min. Follicles were then cultured in 96-well plates individually with 100 µL growth media (GM). The GM consisted of 50% αMEM Glutamax and 50% F-12 Glutamax (catalog: 31765-035, Thermo Fisher Scientific) supplemented with 3 mg/mL bovine serum albumin (catalog: BP9706100, BSA, Sigma-Aldrich), 1 mg/mL bovine fetuin (catalog: F3385, Sigma-Aldrich), 5 µg/mL insulin-transferrin-

selenium (ITS, Sigma-Aldric), and varying concentrations of recombinant rFSH (gifted by Organon, Jersey City, NJ, USA). Follicles were cultured in GM with FSH at 5 mIU/mL on day 0-4, then with FSH at 10 mIU/mL on day 4-6. Follicle images were captured using an Olympus inverted microscope every two days (Olympus Optical Co Ltd, Tokyo, Japan). Follicles were exposed to DMSO (less than 0.3%) or river water organic extracts from day 2 to day 6. Follicle size was calculated by averaging of two perpendicular diameters using Image J software (National Institutes of Health, Bethesda, MD). The exposure concentration of the water organic extract was approximately 10-fold higher than the original water organic extract concentration used for LC-MS, described below.

2.4.3 Ex vivo ovulation induction

Following six days of *in vitro* culture using eIVFG, grown antral follicles were freed from alginate encapsulation by digestion in an L-15-based lysis solution containing 1% FBS and 10 IU/mL alginate lyase (catalog: A1603, Sigma-Aldrich) for 20 minutes at 37°C. Follicles were then cultured in αMEM-based maturation media supplemented with 10% FBS and 1.5 IU/mL human chorionic gonadotropin (catalog: C1063, hCG, Sigma-Aldrich) in an incubator (37°C, 5% CO_2, Thermo Fisher Scientific) for 14 hours to induce in vitro ovulation. After hCG treatment for 14 hours, follicles were imaged to assess the follicle rupture rate. “Ruptured follicle” was defined by the broken follicular wall with an expanded cumulus-oocyte complex (COC), while “unruptured follicle” was defined by an intact follicular wall. Oocytes were collected from ruptured follicles and imaged to evaluate oocyte meiosis. Oocytes with the first polar body extrusion were defined as metaphase II (MII) oocytes, indicating the resumption of meiosis I and arrest at the MII stage of meiosis II. This stage is referred to as "Meiotic resumption". Most of the non-MII oocytes without the polar body extrusion were at either the germinal vesicle breakdown (GVBD) stage or the germinal vesicle (GV) stage, which were defined by the absence or presence of GV, respectively.

2.4.4 Measurements of hormones in the conditioned media

To examine the effects of river water organic extracts on ovarian hormone secretion, conditioned follicle culture medium was collected on day 6 before ovulation induction and at day 9 after ovulation. The concentrations of estradiol (E2), progesterone (P4), and testosterone (T) were measured using competitive enzyme-linked immunosorbent assay kits (ELISA, catalog: 501890, RRID: AB_2832924 for E2 ELISA kit; catalog: 582601, RRID: AB_2811273 for P4 ELISA kit; catalog: 582701, RRID: AB_2895148 for T ELISA kit; Cayman Chemical, MI, USA), based on the competition between the hormones and a hormone-acetylcholinesterase (AChE) conjugate. The dilution factors for E2, P4, and T on days 4 and 6 were 50-fold, 400-fold, and 5-fold, respectively, 400-fold for postovulatory P4 on day 9, to ensure the detection values within the standard curve range. The reported assay ranges of E2, P4, T, and cAMP are 0.61-10,000 pg/mL, 7.8-1,000 pg/mL, 3.9-500 pg/mL, and 0.078-10 pmol/mL, respectively. The lower limit of detection (LOD) of E2 and T are 6 and 5 pg/mL, respectively (no reported LOD for P4 and cAMP). The sensitivity of E2, P4, and T are 20 pg/mL, 10 pg/mL, and 6 pg/mL, respectively.

2.4.5 Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA of a single follicle was extracted using the Arcturus PicoPure RNA isolation kit (catalog: KIT0204, Applied Biosystems, Carlsbad, CA) following the manufacturer's instructions. cDNA was synthesized using the Superscript III First-Strand Synthesis SuperMix kit (catalog: 18080400, Invitrogen, Carlsbad, CA) and stored at -80°C to prevent degradation. qPCR was conducted in a 384-well plate, with reaction mixtures comprising Power SYBR Green PCR Master Mix (catalog: 4368708, Applied Biosystems), forward primer (0.25 μM), reverse primer (0.25 μM), and cDNA template. The qPCR was performed using the ABI ViiA 7 real-time PCR system (Applied Biosystems) with the following thermocycling program: 95°C for 10 min, 40 cycles at 95°C for 15 s and at 60°C for 1 min, followed by a melting stage at 63°C for 25 s. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an endogenous control for normalizing mRNA expression values of each gene, calculated using the 2(-ΔΔCT) method. The qPCR assay was performed in at least six biological replicates with 2-3 technical replicates. The specific primer sequences are listed in Supplemental Table 1.

2.4.6 Single-follicle RNA-seq and bioinformatic analysis

Because the river water organic extract from the PREP site (Elmwood Park at Passaic River) exhibited the most reproductive toxicity and the highest measured concentrations of EDCs, it was selected for subsequent mechanistic experiments. Follicles on day 6 treated with PREP organic extract at reference of 1, 5, 10, and 20-fold higher than the original river concentration were collected for single-follicle RNA-seq analysis. The cDNA library construction and low-input mRNA-Seq were performed on an Illumina NovaSeq PE150 platform by Novogene (Novogene Corporation, Sacramento, CA). High-quality paired sequencing reads were obtained with an effective rate of 96-99% and an error rate of 0.03%. The sequencing data were processed using the Partek Flow software for further analysis. The Bowtie 2 module was employed to filter out the rDNA and mtDNA contaminants. The filtered reads were aligned to the whole genome of Mus musculus-mm10 assembly via HISAT 2. The aligned reads were quantified based on the Ensembl Transcripts release 99 annotation model using the Partek EM algorithm. Gene counts were normalized using the log2-transformed Transcripts Per Million (TPM) plus 1. Gene symbols were mapped to the HUGO Gene Nomenclature Committee (HGNC) to filter out pseudogenes. Differential gene expression analysis was conducted via the DESeq2, with differentially expressed genes (DEGs) identified with a fold change of either > 2 or < 0.5 and a False Discovery Rate (FDR) value < 0.05. Principal component analysis (PCA) was performed using the R package. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were carried out using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (32,33) and Ingenuity Pathways Analysis (IPA).

2.4.7 Benchmark Dose Modeling (BMD)

The USEPA BMD Software (BMDS) tool (online version 2023.03.1)[17] was used to perform the frequentist BMD modeling and determine the point-of-departure (PoD) for endpoints examined, including follicle growth, ovulation, oocyte meiotic resumption, and hormonal secretion of E2, P4, and T. For dichotomous data, such as follicle rupture and ovulation, the

10% extra risk of these endpoints was set as the benchmark response (BMR10) level to derive the benchmark dose/concentration (BMC10) and the corresponding 95% lower confidence limit (BMCL10). For continuous data, such as hormone secretion and follicle diameter, a relative deviation of 10% from the background level was set as BMR, as this level of change has been regarded as a biologically significant response and provides comparability across endpoints. A relative deviation of 10 % is less affected by data variance than the more typical one standard deviation approach, and both the constant and non-constant variance cases were explored. The default model selection and restriction were used: for dichotomous data, the Dichotomous Hill, Gamma, Log-Logistic, Multistage and Weibull models were run restricted, and the Logistic, Log-Probit, Probit and Quantal Linear models were run unrestricted; for continuous data, the Exponential, Hill, Polynomial, and Power models were run restricted, and the Linear model was run unrestricted. Selection of the best models followed the EPA-recommended guideline to determine BMC10 and BMCL10[18].

For the RNA-sequencing data, dose-response analyses of log2(TPM + 1)-normalized counts were performed using BMD Express 2.2. Data were prefiltered using One-way ANOVA Test with an Adjusted P-value cut-off of <0.05, and fold change > |2| to remove genes for BMD analysis. Genes that passed the prefilter were fit to multiple continuous models (Hill, linear, exponential 2–5, polynomial of degree 2–3, and power). The best-fit model for each gene was selected based on the lowest AIC. The BMR was set to 1.349 × SD of replicate samples, corresponding to a 10% tail in a normal distribution. Hill models with a k parameter <1/3 the lowest positive dose were excluded from final model selection. BMDU and BMDL with 95% upper and lower bound values were calculated for each gene. Prior to functional analysis, genes that had global goodness-of-fit p-values of < 0.1 and BMDU/BMDL ratios (between upper and lower 95% confidence limits) of > 40 were removed. All genes that passed this selection criteria were used for Gene Ontology and Reactome enrichment analysis. GO processes or Reactome pathways with gene sets that contained more than three genes and had Fisher's Exact Two-Tailed < 0.05 (based on the total annotated gene number) were considered active. BMD was determined by calculating the median BMD for that gene set.

2.5 Quality assurance (QA) and Chemical quality control (QC) analysis

Compound concentrations were determined using the method established in Dr. Brian Buckley's laboratory from Rutgers EOHSI. Two procedure blanks were performed using Milli-Q water. The extraction method does not introduce a significant level of background contamination (for the targeted compounds) into the samples. Duplicate samples were collected from the sites of DRMT (Delaware River sites at Montague) and PRTB (Passaic River sites at Two Bridges) to assess the reproducibility of solid-phase extraction and bioassays. The relative differences of the total concentration values of 72 targeted chemicals between duplicates at the PRTB site were generally within acceptable analytical variation (<15%), indicating good reproducibility. Given the high degree of similarity, only one representative sample from each site is presented in the figures and tables to avoid redundancy. The limit of detection for 82% of compounds is < 30 ng/mL, with 44% of compounds < 1 ng/mL. Another quality assurance measure for the bioanalytical results involved processing MilliQ

water in the same manner as the samples. This procedural blank was evaluated as control group in all bioassays to assess potential effects from the extraction process or solvents.

2.6 Statistical analysis

Concentrations of pollutants below the detection limits are reported as 'not detected' (ND). ND values were excluded from total concentration average or mean concentration calculations to avoid introducing artificial bias into the dataset, since the proportion of non-detects was high for some analytes. All data on follicle growth, hormone secretion, follicle rupture rate, and the expression of related genes were shown as mean ± 1 standard deviation (SD). Normality of data distribution was verified using the Shapiro–Wilk test, confirming that all datasets met the assumption of normality required for ANOVA. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison using GraphPad Prism (SPSS Inc., Chicago, IL) to analyze the significance of different treatments on follicle growth, hormone secretion, follicle rupture rate, and the expression of related genes. A two-sided p-value less than 0.05 was considered statistically significant and denoted with asterisks as *p<0.05; **p<0.01; ***p<0.001; **** p < 0.0001.

3 Results and Discussion

3.1 Concentrations and composition of organic contaminants or EDCs in river waters

A total of 72 compounds in surface water extraction were detected using the Dionex UltiMate 3000 UHPLC system coupled with Q Exactive HF Hybrid Quadrupole-Orbitrap MS (High resolution). The detected EDCs were classified into eleven groups (Table 2), including Alkylphenols(n=16), bisphenol analogues (BPAs, n=3), hormones (n=3), pesticides (n=8), organophosphate flame retardants (OPFRs, n=3), parabens (n=2), pharmaceutical and personal care products (PPCPs, n=17), phthalates (n=3), quaternary ammonium compounds (QACs, n=7), zeranols (n=5), and PFAS (n=5). The percentages of each category of EDCs were summarized in Figure 3, and the specific concentrations of each EDC were listed in Table 2. Phthalates, PPCPs, alkylphenols, and PFASs exhibited high average concentrations of 94.1, 65.5, 61.8, and 25.9 ng/L, respectively. OPFRs, QACs, and pesticides showed moderate average concentrations of 10.8, 9.7, and 2.4 ng/L, respectively. BPAs, parabens, hormones, and zeranols were present at low levels, with average concentrations of 0.13, 0.13, 0.07, and 0.02 ng/L, respectively. The results also showed that the organic extract from the PREP site (Passaic River site at Elmwood Park) had the highest concentration of total 72 measured EDCs, followed by the sites of PRTB (Passaic River site at Two Bridges), DRMT (Delaware River site at Montague), DRPB (Delaware River at Phillipsburg), DRLV (Delaware River at Lambertville), and lastly, the site of RRMR (Raritan River just upstream of the Millstone River confluence) (Table 2 and Figure 2).

PFAS are widely used in consumer and industrial products such as food packaging, upholstery, and personal care items. As a result, PFAS contribute significantly to water and soil contamination. Epidemiological evidence revealed associations between PFAS and female reproductive disorders, including irregular menstrual cycles, infertility, and premature ovarian failure. In this study, PFAS demonstrated the highest concentration among eleven categories (Figure 2). The total concentration of 5 PFASs (∑PFASs) ranged from 2.9 to 64.5 ng/L, with a median concentration of 25.9 ng/L. The concentrations of PFOA were high at the two sites along the Passaic River, which may be caused by the river flowing through industrial areas, and the concentrations were even higher at the downstream sites.

Phthalates are used as plasticizers in various industrial and consumer products, including plastics, food packaging, and paints, which present potential health and environmental risk. The total concentration of 3 major phthalates (∑phthalates) in three major New Jersey rivers ranged from 0.02 to 651 ng/L. The level of di-n-butyl phthalate at the PREP site is the highest, contributing 97.3% of ∑phthalates.

Table 2. Concentration of 72 EDCs at study sites (ng/L)

	WATER COLLECTION SITE	**DRPB**	**PREP**	**RRMR**	**DRLV**	**PRTB**	**DRMT**
	Number of WATER SAMPLE	**S#3**	**S#4**	**S#5**	**S#6**	**S#7**	**S#9**
Chemical Class	**NAME**	**Concentration (ng/L)**					
Alkylphenols	4-hexylphenol	40.629	41.012	ND	ND	41.165	ND
	4-tert amylphenol	38.289	38.688	38.856	38.354	ND	38.462
	2,4,5-trichlorophenol	ND	0.023	ND	0.031	0.034	ND
	2,4,6-tribromophenol	3.399	0.061	1.634	2.795	1.054	2.180
	2,4,6-trichlorophenol	0.390	4.203	0.144	1.652	14.427	ND
	2-biphenylol	0.194	ND	1.193	0.353	0.208	0.339
	2-chlorophenol	12.611	ND	ND	ND	ND	ND
	4-chlorophenol	ND	ND	ND	ND	0.290	ND
	4-bromophenol	ND	0.000	0.002	0.002	0.001	ND
	4-chloro-2-methylphenol	1.765	0.011	1.418	ND	ND	1.413
	4-chloro-3-cresol	ND	ND	ND	ND	0.167	0.074
	3-chlorophenol	ND	0.071	0.125	ND	0.061	ND
	4-nitrophenol	1.467	7.354	3.549	3.770	2.431	0.602
	chlorothymol	0.059	ND	0.063	0.037	0.012	0.065
	hexachlorophene	ND	0.008	ND	ND	ND	0.082
	resorcinol	0.039	0.134	0.178	0.080	0.374	0.061
	Σ Alkylphenols	98.842	91.565	47.163	47.074	60.225	43.277
Bisphenol analogues (BPAs)	bisphenol A	ND	ND	ND	ND	ND	ND
	bisphenol F	ND	0.413	0.026	0.397	0.058	ND
	bisphenol S	ND	0.048	ND	ND	ND	ND
	Σ BPAs	ND	0.461	0.026	0.397	0.058	0.000
Hormones	diethylstilbestrol	ND	ND	ND	ND	ND	ND
	hexestrol	0.009	0.157	0.009	ND	0.044	0.006
	testosterone	ND	ND	ND	ND	ND	0.022
	Σ Hormones	0.009	0.157	0.009	0.000	0.044	0.028
Pesticides	metolachlor	0.340	0.199	1.050	1.005	0.185	0.094
	simazine	0.160	0.648	0.296	0.359	0.632	0.087
	atrazine	ND	0.009	2.431	1.995	ND	0.025
	prometon	0.312	2.204	1.044	1.922	1.499	0.145
	propazine	0.0030	0.0040	0.0513	0.0468	0.0135	ND
	ametryn	ND	0.0001	0.0022	0.0001	ND	0.0001
	prometryn	ND	0.0010	0.0001	ND	ND	0.0003
	terbutryn	0.0029	0.0333	0.0028	ND	0.0619	0.0002
	Σ Pesticides	0.8180	3.0985	4.8773	5.3281	2.3919	0.3522
Organophosphate flame retardants (OPFRs)	tris(2-chloroethyl) phosphate	0.183	4.648	0.911	0.469	4.480	0.077
	triethyl phosphate	0.321	6.877	1.305	0.638	9.509	0.182
	triphenyl phosphate	ND	ND	ND	ND	ND	ND
	Σ OPFRs	0.504	11.525	2.217	1.107	13.989	0.259
Parabens	ethyl paraben	0.002	0.001	0.013	0.013	0.019	0.004
	methyl paraben	ND	0.038	0.737	ND	0.163	ND
	Σ Parabens	0.002	0.039	0.750	0.013	0.182	0.004
Pharmaceutical and personal care products (PPCPs)	caffeine	0.663	26.294	0.152	9.706	12.112	0.041
	triclocarban	0.046	0.497	ND	0.475	2.036	0.331
	triclosan	0.058	2.068	0.119	ND	3.415	ND
	claritin	0.0002	0.0572	0.0041	0.0108	0.1607	ND

Table 2. Concentration of 72 EDCs at study sites (ng/L)

	WATER COLLECTION SITE	**DRPB**	**PREP**	**RRMR**	**DRLV**	**PRTB**	**DRMT**
	Number of WATER SAMPLE	**S#3**	**S#4**	**S#5**	**S#6**	**S#7**	**S#9**
Chemical Class	**NAME**	**Concentration (ng/L)**					
	dramamine	0.459	4.641	0.219	2.203	18.488	0.418
	dramamine1	0.424	ND	0.132	0.119	0.140	0.120
	glucophage	0.101	0.746	0.173	0.358	0.619	0.183
	keppra	ND	0.061	0.009	0.043	0.180	0.187
	lipitor	ND	ND	ND	ND	0.182	ND
	sensorcaine	0.0003	1.8965	0.0070	0.1028	1.7761	0.0018
	sudafed	0.085	0.097	ND	0.026	0.116	0.013
	tegretol	0.723	10.444	5.484	2.462	13.729	0.517
	topamaz	5.927	67.152	28.319	17.555	96.826	3.651
	zithromax	0.009	0.022	ND	0.056	0.009	ND
	zoloft	0.003	0.074	0.003	0.086	1.931	0.020
	zonegran	0.001	0.878	0.023	0.041	1.574	0.034
	zyrtec	0.490	2.543	0.561	0.376	4.527	0.468
	Σ PPCPs	8.988	117.471	35.207	33.619	157.821	5.985
Phthalates	di-n-butyl phthalate	ND	633.150	ND	34.133	ND	ND
	diethyl phthalate	ND	17.835	13.623	ND	8.006	ND
	dimethyl phthalate	0.016	ND	0.276	0.167	0.047	0.079
	Σ Phthalates	0.016	650.985	13.899	34.300	8.053	0.079
Quaternary ammonium compounds (QACs)	Dodecyl dimethyl ethylbenzyl ammonium chloride (AEB-12)	ND	ND	ND	ND	ND	0.2433
	Tetradecyl dimethyl ethylbenzyl ammonium chloride (AEB-14)	0.0432	0.6051	0.1688	0.1498	0.4051	0.1245
	Benzyl dimethyl decyl ammonium chloride (BAC-10)	ND	0.2775	0.0036	ND	0.4346	ND
	Benzyl dimethyl dodecyl ammonium chloride (BAC-12)	ND	0.727	1.378	0.529	ND	2.348
	Benzyl dimethyl tetradecyl ammonium chloride (BAC-14)	5.154	5.742	5.629	7.206	4.396	8.682
	Benzyl dimethyl hexadecyl ammonium chloride (BAC-16)	ND	0.940	ND	0.971	0.207	1.506
	Benzyl dimethyl octadecyl ammonium chloride (BAC-18)	1.230	3.288	ND	5.006	0.613	3.326
	Σ QACs	6.427	11.579	7.179	13.862	6.055	16.229
Zeranols	α-zearalanol (aZal)	ND	0.008	ND	ND	0.007	0.004
	β-zearalanol (bZal)	ND	0.006	ND	ND	ND	ND
	β-zearalenol (bZol)	ND	0.040	0.009	0.002	0.012	0.021
	zearalanone (Zan)	ND	ND	0.0029	0.0004	ND	ND
	zearalenone (Zen)	0.005	0.006	ND	0.007	ND	ND
	Σ Zeranols	0.005	0.059	0.012	0.009	0.020	0.025
Per- and polyfluoroalkyl substances (PFASs)	GenX (HFPO-DA)	ND	0.264	0.137	ND	0.544	ND
	perfluorodecanoic acid (PFDA)	0.160	1.918	0.340	0.516	1.485	0.157
	perfluorohexanoic acid (PFHxA)	0.280	4.701	1.487	0.679	4.494	0.151

Table 2. Concentration of 72 EDCs at study sites (ng/L)

	WATER COLLECTION SITE	DRPB	PREP	RRMR	DRLV	PRTB	DRMT
	Number of WATER SAMPLE	S#3	S#4	S#5	S#6	S#7	S#9
Chemical Class	**NAME**	**Concentration (ng/L)**					
	perfluorononanoic acid (PFNA)	2.005	11.319	3.650	3.923	9.111	1.283
	perfluorooctanoic acid (PFOA)	2.338	46.311	15.717	6.677	34.177	1.349
	Σ PFASs	4.78	64.51	21.33	11.80	49.81	2.94
	Σ 72 EDCs	120.898	962.977	134.885	148.613	312.637	69.4385

Note: Each value represents a single measurement. ND indicates 'not detected'. Pollutants marked as 'ND' were excluded from the calculation of total concentrations to avoid introducing artificial bias.

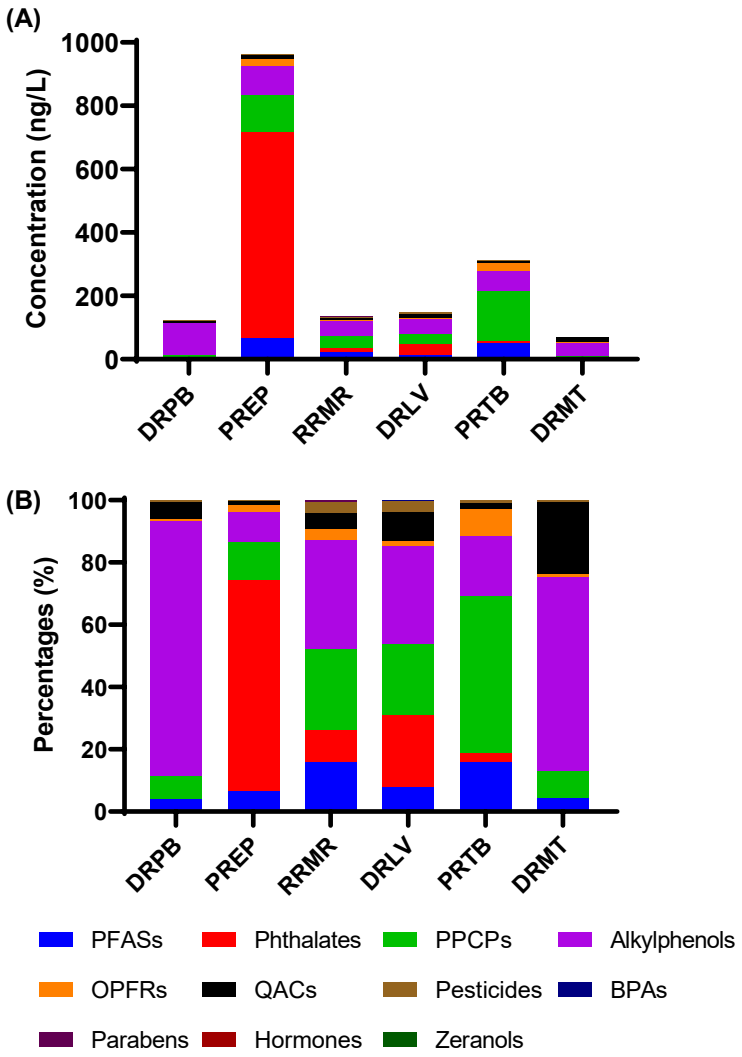

Figure 2. The concentrations of 72 measured EDCs in New Jersey river water samples. (A) The concentrations of the total 72 EDCs in surface water in different study sites. (B) The percentage of each chemical category in different water sample sites. The abbreviations used correspond to the following full terms: PFAS, Per- and polyfluoroalkyl substances; PPCPs, Pharmaceutical and personal care products; OPFRs, Organophosphate flame retardants; QACs, Quaternary ammonium compounds; BPAs, Bisphenol analogues.

3.2 Estrogenic and anti-estrogenic effects of river water organic extracts

We next determined the estrogenic and anti-estrogenic effects of river water organic extracts from six sites of three major New Jersey rivers using the dual luciferase reporter assay, based on the human 293T (h293T) cells transfected with the plasmids of pCMV-hERα, 3X ERE TATA luc, and pRL-SV40P. The results showed that the luciferase activity in h293T cells treated with 100 nM E2 was 4.94 ± 0.28-fold higher than that of cells treated with the blank (Figure 3A). Cells treated with the river water organic extracts from the sites of PREP (Passaic River site at Elmwood Park), PRTB (Passaic River site at Two Bridges), and DRMT (Delaware River site at Montague) exhibited significantly higher luciferase activities compared to the control (Figure 3A), suggesting estrogenic effects of these water organic extracts. To determine the potential anti-estrogenic effect of river water organic extracts, h293T cells were co-treated with each organic extract and 100 nM E2 for 24 hours. Compared to E2 alone, the co-treatment of organic extract from DRPB (Delaware River site at Phillipsburg) exhibited significantly reduced luciferase activity, suggesting an anti-estrogenic effect (Figure 3B).

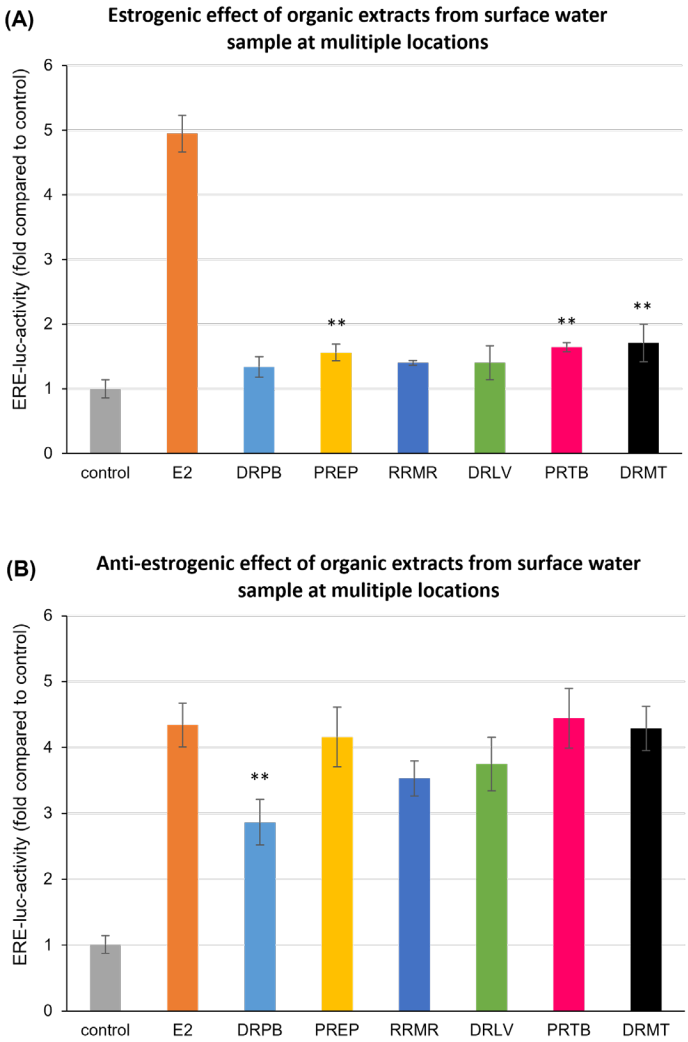

Figure 3. Estrogenic and anti-estrogenic effects of water organic extract tested in human 293T (h293T) cells transfected with plasmids of pCMV-hERα, 3X ERE TATA luc, and pRL-SV40P. E2 was used as a positive control. To measure anti-estrogenic effects, h293T cells were co-treated with each organic extract and 100 nM E2 for 24 hours. Data are presented as mean ± SD (n = 3–5) for the water sample at each site. Asterisks (**) indicate significant differences compared with the control group in panel A and with the E2-treated group in panel B (**p < 0.01). Location abbreviations are as follows: Delaware River sites at Montague (DRMT), Phillipsburg above the Lehigh River (DRPB), and STP of Lambertville (DRLV); Passaic River sites at Two Bridges (PRTB) and Elmwood Park (PREP); Raritan River site at the bridge access of the Main Street in Millstone (RRMR).

3.3 Female reproductive toxicity testing river water organic extracts

Figure 4 summarizes the experimental design of *in vitro* river water organic extract exposure and female reproductive toxicity testing using eIVFG. In Tier 1, cultured mouse ovarian follicles were exposed to the blank control, negative control, and eight river water organic extracts at a concentration of 10-fold higher than the original river water concentration (referred to as REF10x below). Key ovarian functions, including follicle growth, ovulation, oocyte meiosis, and hormone secretion of E2, T, and P4, were examined. In Tier 2, the organic extract with the most potent effects was selected for dose-response exposure and benchmark dose modeling. Single-follicle RNA-seq analysis was conducted to unbiasedly characterize the alternation of genes and signaling pathways at the whole transcriptomic level. In Tier 3, to further identify key organic chemicals or mixtures driving the observed ovarian toxicities, suspicious individual chemicals or their mixtures based on the results of Tiers 1 and 2 were used for *in vitro* exposure and mechanism examination.

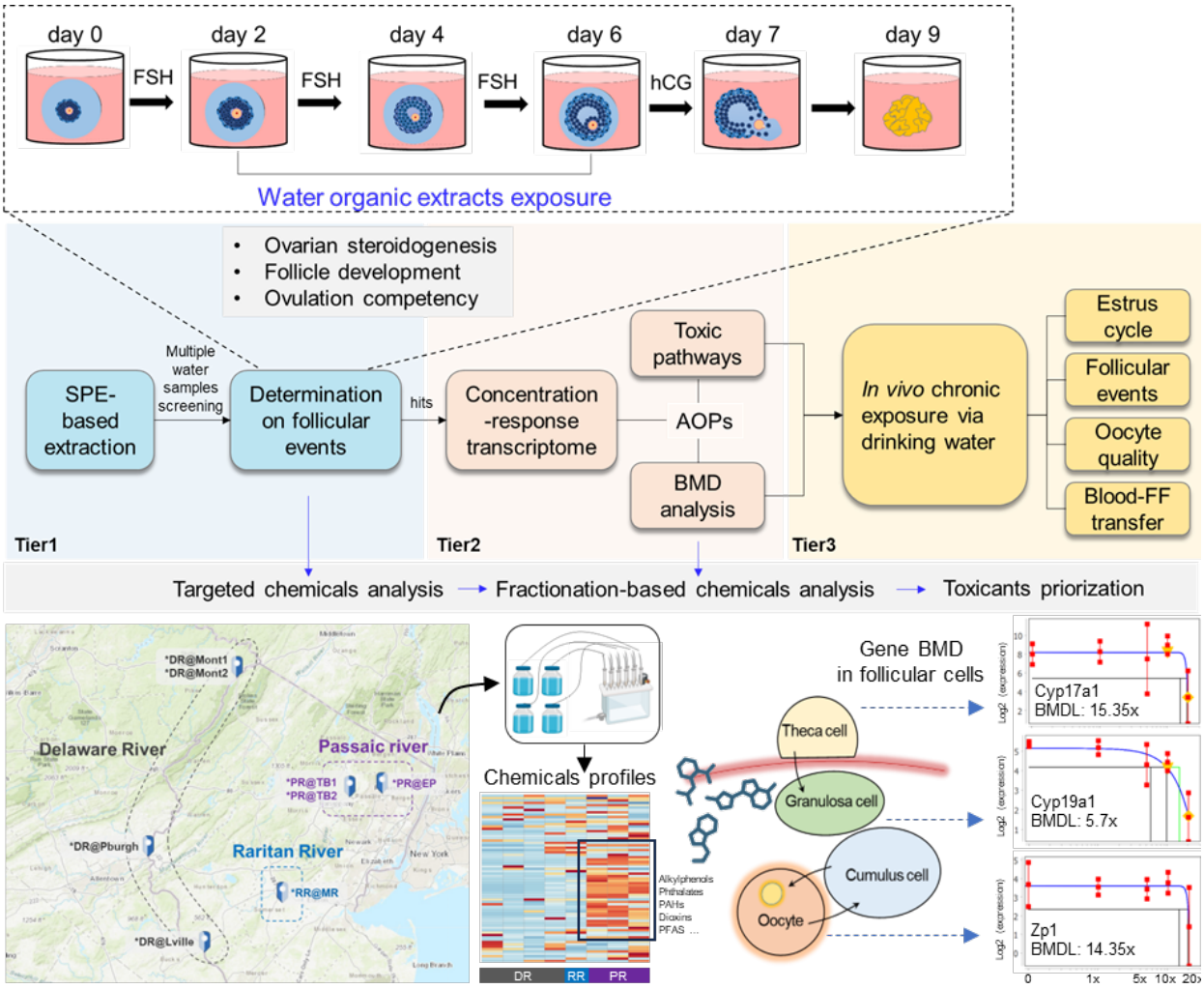

Figure 4. Workflow for the adverse effects of real-world NJ water samples on ovarian functions.

In Tier 1, mouse ovarian follicles cultured in eIVFG were treated with each organic extract at a concentration of REF10x from day 2 to 6 of eIVFG, which corresponds to the gonadotropin-dependent phase of ovarian follicle development. Key ovarian events or functions were examined, including follicle growth and survival, hormone secretion, ovulation, and oocyte meiotic resumption. The results showed that two ddwater blanks did not affect all endpoints. Organic extracts from the sites of PREP (Passaic River site at Elmwood Park), PRTB (Passaic River site at Two Bridges), and RRMR (Raritan River site at Millstone) significantly inhibited follicle growth, evidenced by the smaller follicle size on day 6 of eIVFG (Figure 5A).

Ovarian hormones of E2, T, and progesterone in the culture media on day 6 of eVIFG were measured using ELISA kits. There were no significant differences for the E2 concentrations between control and all organic extract/blank treatment groups (Figure 5B). The organic extracts from the sites of PREP (Passaic River site at Elmwood Park), PRTB (Passaic River site at Two Bridges), DRLV (Delaware River site at Lambertville), and DRMT (Delaware River site at Montague) significantly increased the secretion of T and P4 (Figure 5B).

Ovarian follicles from day 6 of eIVFG were next treated with hCG to induce *in vitro* ovulation and luteinization. The organic extracts from the sites of PREP (Passaic River site at Elmwood Park) and PRTB (Passaic River site at Two Bridges) significantly inhibited follicle rupture, reduced the percentages of oocytes with extruded polar bodies, and inhibited the secretion of post-ovulatory P4 (Figure 5C-5D). The other five river water organic extracts did not affect these follicular events.

3.4 River water organic extracts from the PREP site (Passaic River site at Elmwood Park) interfere with ovarian follicle growth, hormone secretion, and ovulation.

Because the river water organic extract from the site of PREP (Passaic River site at Elmwood Park, referred to as PREP below) exhibited the most potent endocrine disrupting effects and reproductive toxicity, it was selected for more comprehensive and in-depth assessments. Similar to the single concentration exposure described above, immature mouse follicles cultured in eIVFG were treated with various concentrations of organic extract from the PREP site, including REF1x, 5x, 10x, and 20x, from day 2 to day 6. The results showed that the PREP organic extract concentration-dependently inhibited ovarian follicle growth (Figure 6A). Although not statistically significant, PREP organic extract at REF10x exhibited a tendency to increase E2 secretion (Figure 6B), and it increased T and P4 secretion in a concentration-dependent manner (Figure 6C). The PREP organic extract at REF10x and 20x significantly inhibited follicle rupture, oocyte meiotic resumption, and post-ovulatory P4 secretion without inducing cytotoxicity (Figures 6E, 6F, and 6G). Together, these results indicate that the river water organic extract from the PREP site of the Passaic River at Elmwood Park exhibits endocrine disrupting effects and female reproductive toxicity in a concentration-dependent manner.

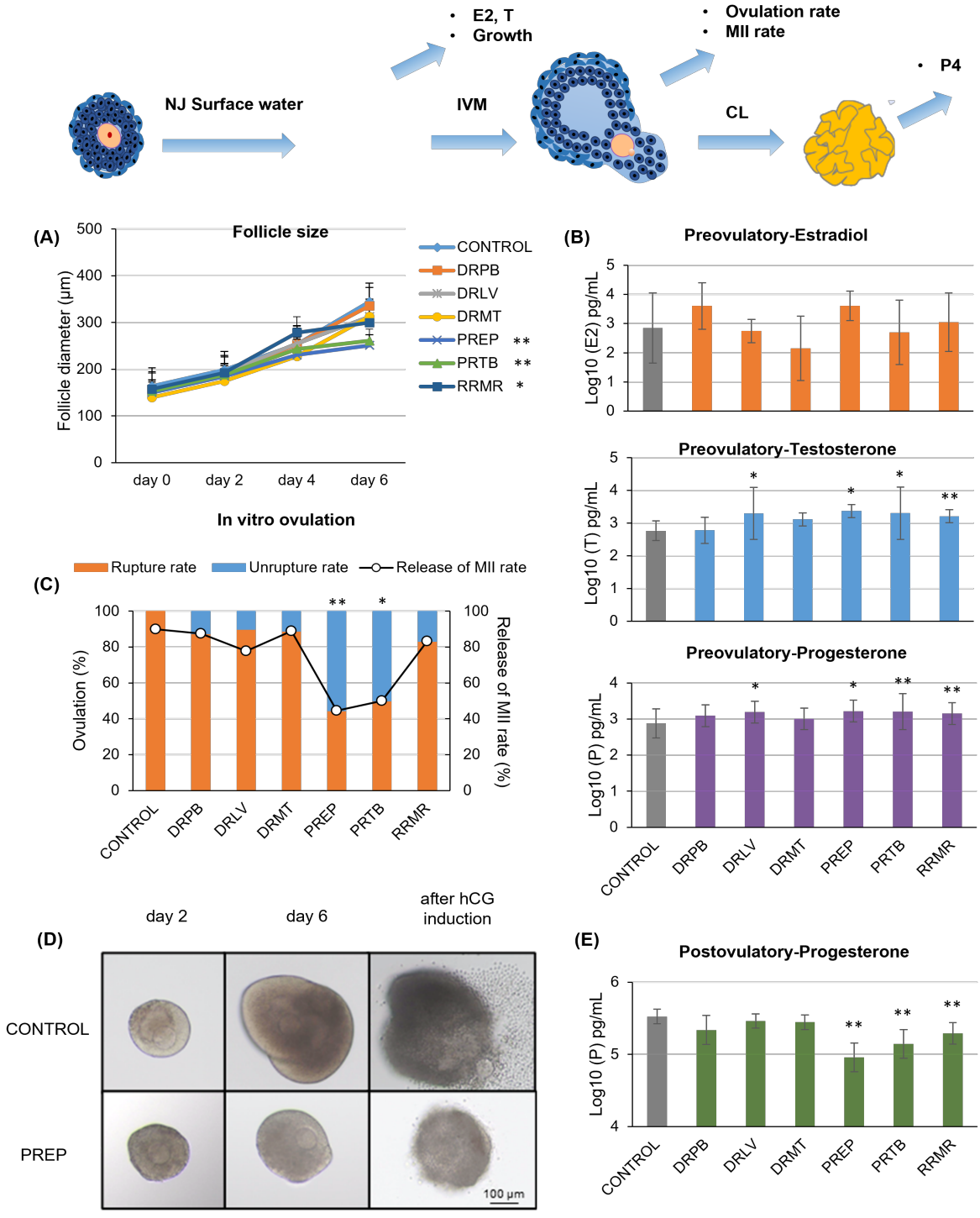

Figure 5. Ovarian disrupting effects of NJ river water organic extracts. (A) Follicle diameter from day 0 to day 6. (B) E2 , T and progesterone secretion in the conditioned follicle culture media on day 6. (C) Percentages of ruptured and unruptured follicles (D) and release of MII oocytes. (E) Post-ovulatory progesterone secretion in the conditioned follicle culture media two days after ex vivo ovulation. N=10-15 follicles per group and three replicates were performed.

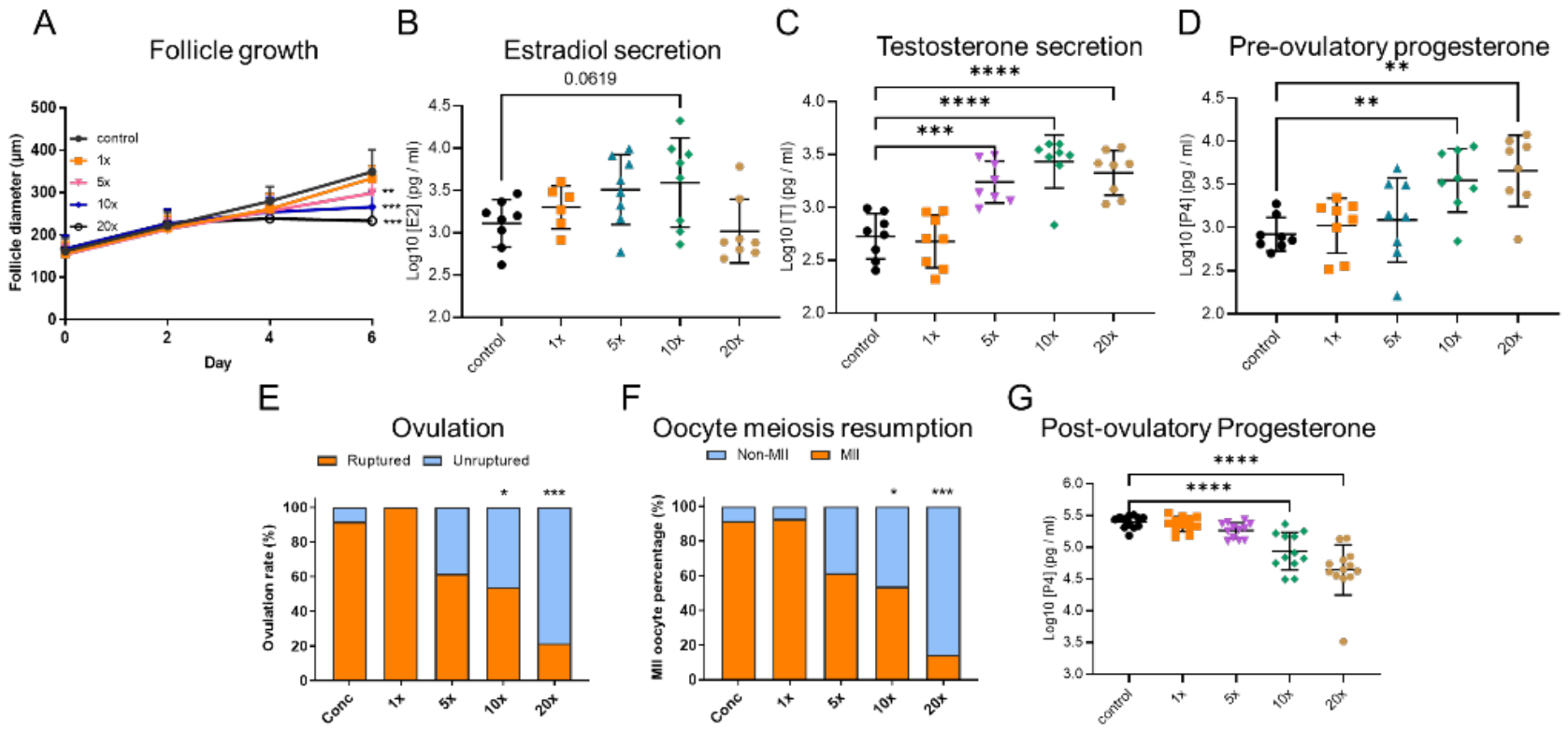

Figure 6. Effects of different concentration of PREP organic extract on ovarian follicle growth, hormone secretion, and ovulation. (A) Follicle diameter from day 0 to day 6. (B) E2, (C) T, and (D) pre-ovulatory P4 secretion in the conditioned follicle culture media on day 6. Percentages of ruptured and un-ruptured follicles (E) and released MII oocytes (F). (G) Post-ovulatory P4 in the conditioned follicle culture media on two days after in vitro ovulation. N=10-15 follicles per group with three replicates. Asterisks indicate statistical significance (*p<0.05; **p<0.01; ***p<0.001; ****P < 0.0001).

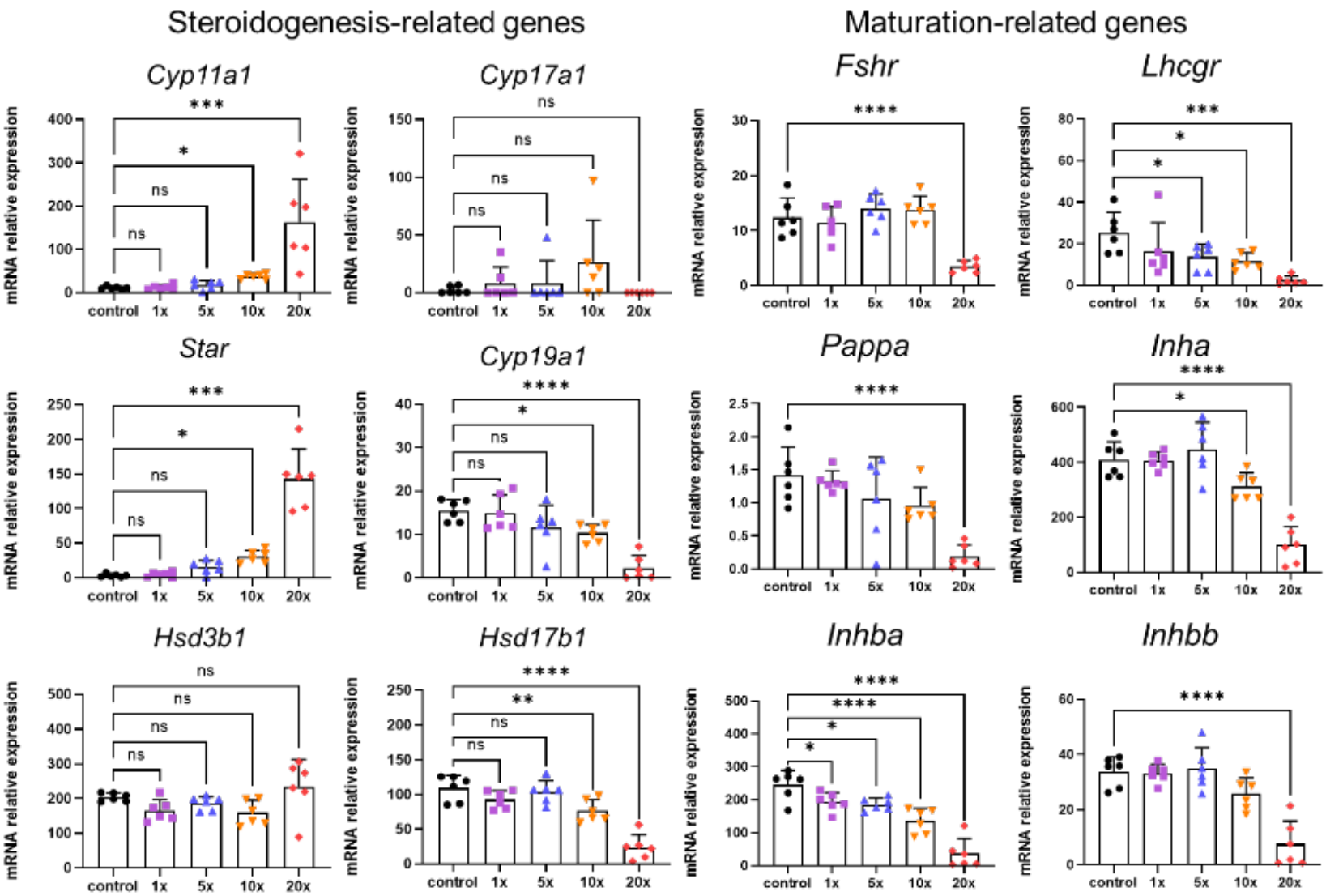

Figure 7. Effects of PREP organic extract on mRNA expression of genes related to ovarian steroidogenesis and gonadotropin-dependent follicle maturation. N=10-15 follicles per treatment group. Asterisks indicate statistical significance (*p<0.05; **p<0.01; ***p<0.001; ****P < 0.0001), and ns indicates no significant difference.

3.5 PREP organic extract alters the expression of genes related to ovarian steroidogenesis and follicle maturation.

To characterize the mechanisms of PREP organic extract-induced ovarian toxicity, we performed a similar *in vitro* dose-response exposure and collected follicles from day 6 of eIVFG for single-follicle RT-qPCR analysis. The results showed that the PREP organic extract significantly increased the gene expression of *Star* and *Cyp11a1* and reduced the expression of *Cyp19a1* (Figure 7). The PREP organic extract dose-dependently reduced the expression of several genes essential for regulating ovarian follicle maturation, including *Fshr, Lhcgr*, *Pappa*, *Inha*, *Inhba*, and *Inhbb* (Figure 7). The transcriptional changes of these genes were consistent with the morphological and hormonal changes shown in Figure 7. For example, *Star* and *Cyp11a1* critically contribute to androgen synthesis, and *Cyp19a1* encodes aromatase that converts androgen to estrogen. The induction of *Star* and *Cyp11a1* and reduction of *Cyp19a1* can result in hyper-androgen synthesis. The reduction of FHSR desensitizes ovarian follicles to the FSH-stimulated follicle maturation. The reduction of LHCGR desensitizes follicles to the LH surge-stimulated ovulation and luteinization. Collectively, these results suggest that the PREP organic extract from the Passaic River at Elmwood Park interferes with ovarian follicle maturation and hormone secretion.

3.6 PREP organic extract interferes with the follicular transcriptome and expression of genes related to AhR signaling.

Next, we conducted a single-follicle RNA-seq and bioinformatic analysis using total RNA from ovarian follicles treated with various concentrations of PREP organic extract. Principal component analysis (PCA) revealed that PREP organic extract concentration-dependently altered the follicular transcriptome, with follicles treated with REF10x and 20x separated from follicles from the control group (Figure 8A). The hierarchical clustering analysis further revealed the increasing/decreasing expression patterns of differentiall expressed genes (DEGs) (Figure 8B). The volcano plot (Figure 8C) highlighted that *Cyp1a1*, an established AhR target gene, was the most upregulated gene, together with the upregulation of other AhR target genes (e.g., *Cyp1a1*, *Cyp1b1*, and *Ahrr*). These results suggest the activation of AhR signaling pathway in response to PREP organic extract treatment. AhR is established to mediate the cellular response to environmental contaminants, particularly dioxins, PAHs, and PCBs, by inducing AhR target genes (Figure 8F). Specifically, crosstalk between the AHR and estrogen pathways has been well studied, and AhR activation in the ovary has been shown to disrupt several essential reproductive functions. For example, the model AhR agonist 2,3,7,8-TCDD has been shown to directly inhibit the estrogen-dependent vitellogenin pathway in zebrafish embryos via AHR activation [19, 20]. Mono(2-ethylhexyl) phthalate (MEHP) has been shown to act as an AhR agonist, leading to impaired follicle growth by reducing estrogen production and signaling in ovarian antral follicles [21].

The BMD modeling of RNA-seq data pinpointed sensitive molecular events associated with mitochondrial dysregulation, p53 signaling, and DNA damage, with mitochondria-related pathways exhibiting the lowest BMD at REF2.23x (Figure 8D-8E). Mitochondria play a crucial role in cellular energy production and apoptosis in ovarian cells. Disruption of mitochondrial

functions can lead to inadequate ATP production and impaired follicular growth and steroidogenesis. AhR activation [20, 22] has also been associated with upregulation of p53 signaling and DNA damage pathways, which can cause cell cycle arrest or trigger apoptosis, potentially impairing ovarian and reproductive health. The concentration-dependent upregulation of *Cyp1a1* and other AhR-target genes suggests that AhR activation is a potential mechanism by which PREP organic extract exerts its ovarian toxicity.

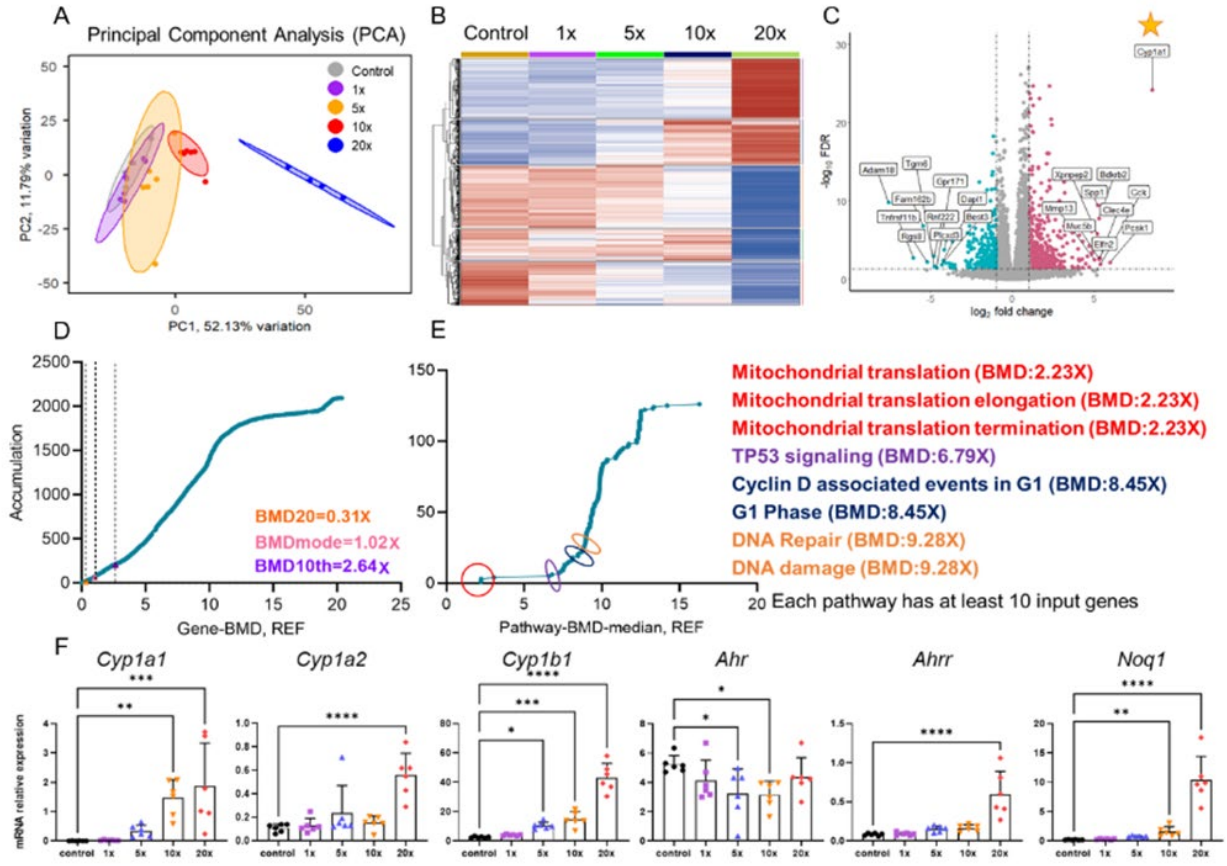

Figure 8. Dose-response transcriptomic analysis of PREP organic extract from Passaic river. (A) PCA of follicles treated with different concentrations of water sample #4. (B) Heatmap of DEGs altered by S#4. (C) Top 10 genes regulated by S#4 at 10x. BMD analysis at the gene level (D) and pathway level (E). (F) mRNA expression of AhR target genes.

3.7 The lab-made mixture of key organic contaminants detected from the PREP exhibited similar ovarian toxicity to the PREP organic extract

Passaic River water samples collected from the PREP site (Passaic River site at Elmwood Park) primarily contain high concentrations of phthalates and PFAS (Figure 2). Existing historical data reported that the Passaic River has been subjected to a long-term contamination of POPs, including dioxins, high molecular weight (HMW) PAHs, low molecular weight (LMW) PAHs, and PCBs [23]. To determine which class of EDCs

contributes to endocrine disrupting effects caused by the PREP organic extract we observed, we made mixtures of each class of EDCs and used a similar *in vitro* exposure regimen to test their effects on follicle ovulation, a sensitive endpoint affected by the PREP organic extract (Figure 9A). The concentrations of each EDC were similar to REF20x based on the targeted analytical data. The mixture of PFAS includes PFOA, PFOS, PFNA, PFHxA, PFDA, and GenX. The phthalate mixture includes dibutyl phthalate (DBP) and diethyl phthalate (DEP). The mixture of POPs includes 2,3,7,8-TCDD, benzo(a)pyrene (BaP), naphthalene (NaP), and PCB153. The results showed that the mixtures of PFAS and phthalates did not affect follicle ovulation. However, the POP mixture significantly inhibited follicle ovulation as the PREP organic extract did (Figure 9B). Furthermore, the POP mixture also elicited similar ovarian disrupting effects from molecular perspectives, including altering follicle maturation-related genes and steroidogenic genes to the real-world PREP organic extract (Figure 10). The co-treatment of 1 or 5 μM α-Naphthoflavone, a selective AhR / CYP1A1 inhibitor, was able to reverse failed follicle ovulation induced by the POP mixture (Figure 9C-9E). The co-treatment of α-Naphthoflavone also prevented the reduction of follicle maturation genes caused by the POP mixture, including *Lhcgr, Pappa*, *Cyp19a1*, and *Fshr*; as well as the reduced expression of ovarian steroidogenic genes, such as *Star* and *Cyp11a1* (Figure 10).

To further identify specific chemical(s) of the POP mixture that primarily contributed to the failed follicle ovulation and molecular changes, we examined the ovarian toxicity of each individual POPs. The results showed that BaP alone, but not TCDD, PCB153, nor NaP, was able to cause similar ovarian defects such as inhibited follicle maturation and failed follicle rupture, suggesting that HMW PAHs such as BaP play a key role in ovarian toxicities induced by the PREP organic extract (Figure 11). Together, these results demonstrate that exposure to the real-world river water organic extracts, particularly HMW PAHs, disrupts ovarian functions through activating AhR signaling in follicular cells.

AhR activation has been shown to disrupt estrogen receptor (ER) signaling through competing for transcriptional machinery, downregulating ER expression, and inducing enzymes that metabolize estrogen, such as CYP1A1 and CYP1B1 [24]. Both ERα (primarily in theca cells) and ERβ (primarily in granulosa cells) are essential for gonadotropin-dependent follicle development. The crosstalk between AhR and ER may indicate that the activation of AhR caused by PAHs interferes with the ER signaling, which further disrupts normal follicle maturation, ovulation, and hormone secretion [25]. For instance, our results showed that the PREP organic extract markedly increased the expression of AhR target genes such as *Cyp1a1* and *Cyp1b1* and reduced the expression of ER-responsive genes such as *Cyp19a1*, a gene encoding aromatase that metabolizes androgens to estrogens. Moreover, the co-treatment with α-Naphthoflavone, a selective AhR inhibitor, partially rescued follicle rupture and restored the expression of genes critical for follicle maturation, including *Lhcgr* and *Cyp19a1*, highlighting AhR's role in the observed ovarian toxicity.

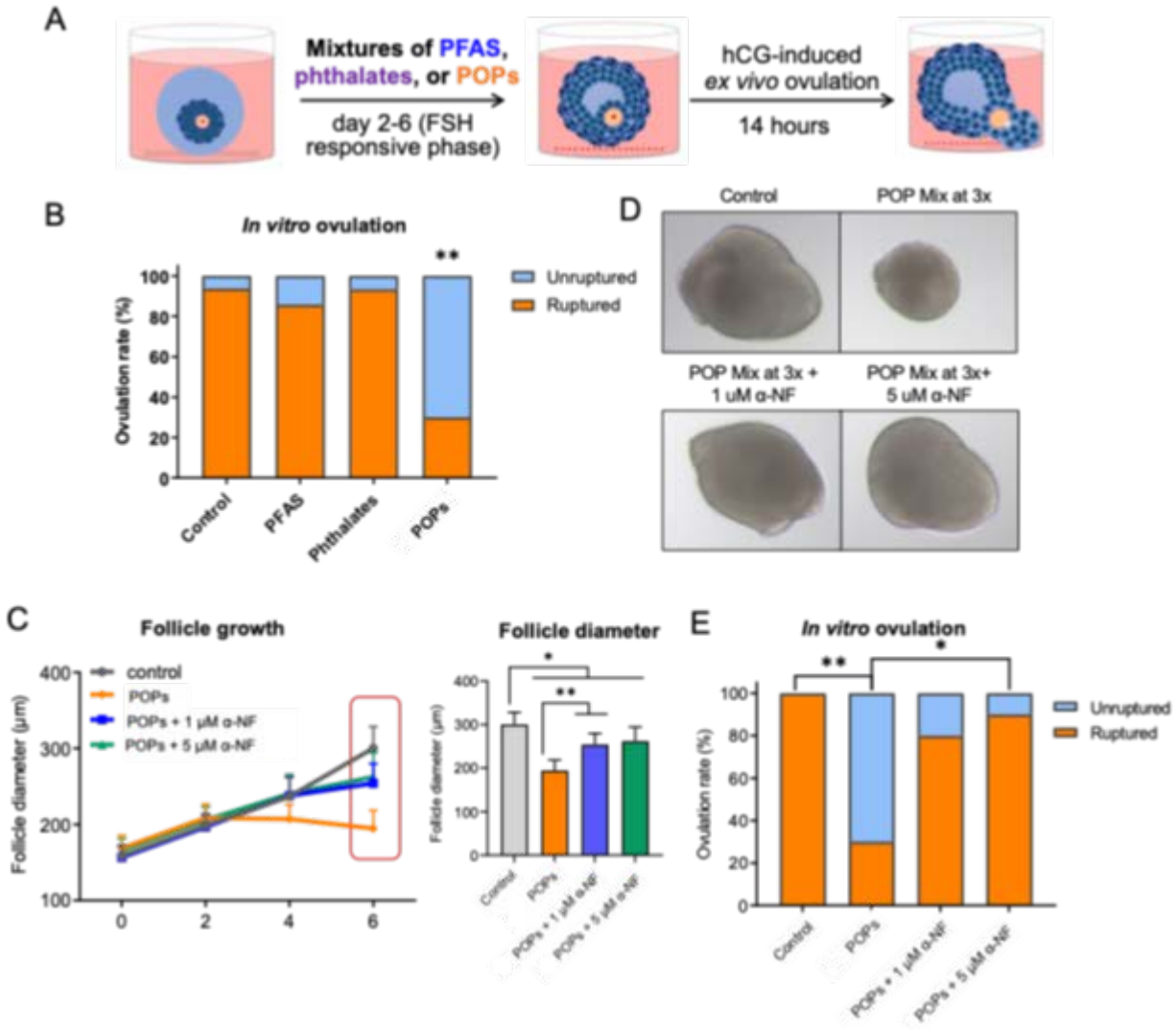

Figure 9. Ovarian impact of mixtures of PFAS, phthalates, and POP. (A) Experimental design based on eIVFG model. (B) Effects of mixtures of PFAS, phthalates, or POP on follicle ovulation. (C-E) POP mixture (TCDD, PCB153, BaP, and NaP) induced ovarian disrupting effects by activating AhR signaling. Follicles were treated with individual dioxins or their mixture, or co-treatment with the AhR antagonist. (C-D) Follicle diameter and morphology from day 0 to day 6. (E) Percentages of ruptured follicles. N=15 follicles in each group.

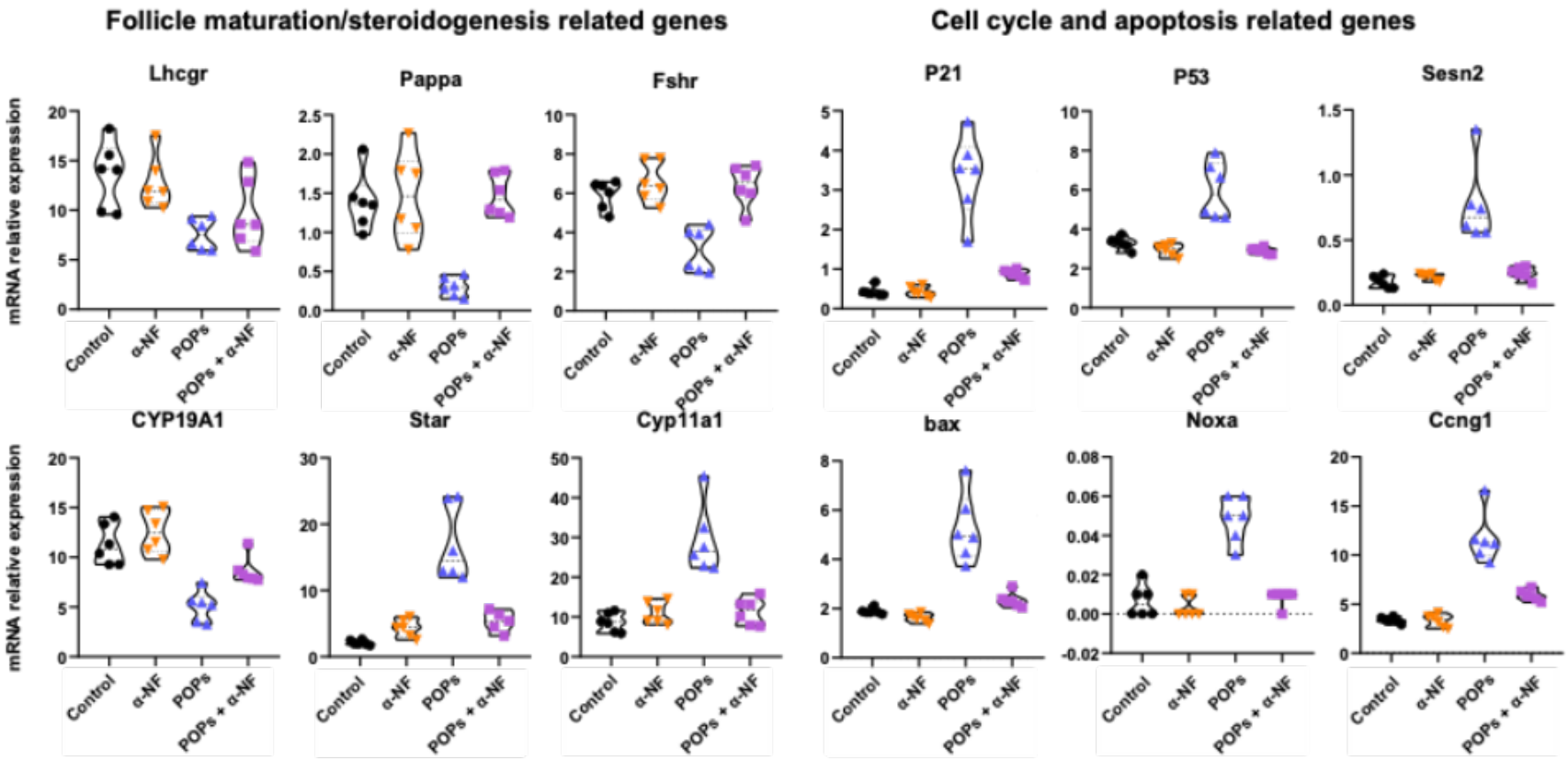

Figure 10. mRNA expression of follicle maturation and steroidogenesis related genes examined by qRT-PCR. N=8-10 follicles in each group.

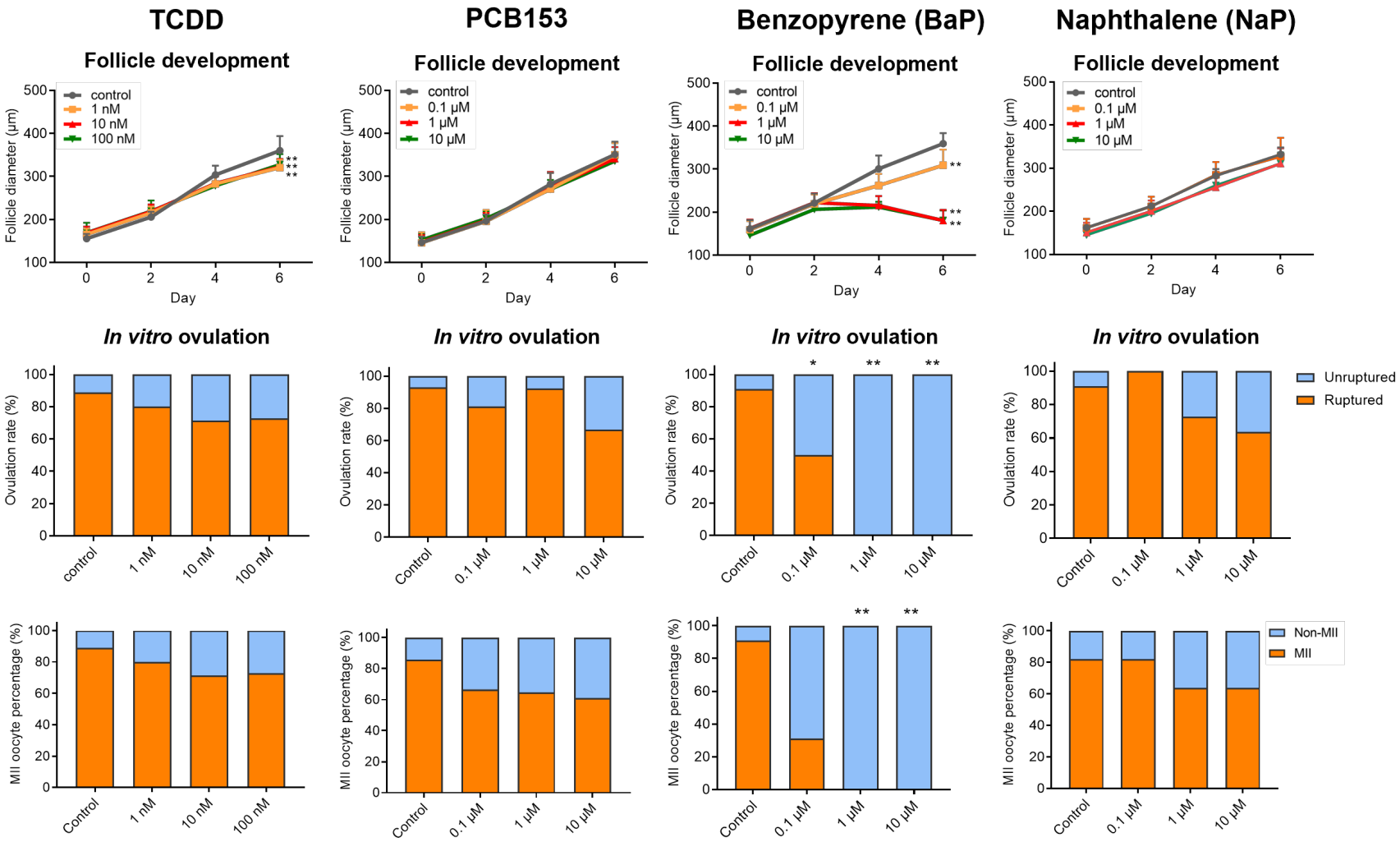

Figure 11. Follicle growth and ovulation in response to in vitro exposure of TCDD, P153, BaP, or NaP. N=15 follicles in each group.

4 Summary and Recommendations

The results generated from the current NJDEP research project revealed that New Jersey river waters from the sample collection sites contain various organic contaminants, some of which have been established as EDCs. Using our 3D *in vitro* ovarian follicle culture models, together with analytical, molecular, and computational approaches, our results showed that the mixture of organic pollutants found in river water from several sample collection sites, particularly the Passaic River site at Elmwood Park, exhibit endocrine disrupting effects and interferes with ovarian follicle development, hormone secretion, and ovulation. Mechanistically, HMW PAHs such as BaP activate AhR signaling pathway in follicular cells to alter the ER signaling and related reproductive functions. Collectively, the studies presented here represent a small piece of a much larger puzzle that can provide a new avenue for identifying/prioritizing waters with higher contamination and develop new approaches to handling complex mixtures of contaminants in our state.

In conclusion, the results of our study demonstrate that:

(1) The organ-on-chip models, hormonal and molecular assays, and bioinformatic assays developed and applied in the current study are sensitive and effective for testing the endocrine disrupting effects and specific female reproductive toxicity of river water organic contaminants and their mixtures, as well as elucidating the toxic mechanisms involved.
(2) The generated results can help develop prevention, mitigation, and remediation methods to protect the health of humans and other species, particularly for endpoints related to female reproductive and endocrine health.
(3) These established approaches can also be applied to prioritize river sites with high endocrine disruption and reproductive toxicity concerns and restrict access to river sites with elevated endocrine disrupting and reproductive toxicity risks to protect vulnerable populations, including prepubertal girls and young adult women.

Based on the experience and expertise gained from this NJDEP project, future studies are recommended to:

(1) Include more biomarkers of oxidative stress, genotoxicity endpoints, and a wider array of hormonal effects.
(2) Develop a more complex *in vitro* model, such as a tissue chip incorporating other reproductive microtissues (fallopian tube, uterus, and cervix), neuroendocrine components (hypothalamus and pituitary microtissue), and hepatic metabolism (liver microtissue), which will more accurately recapitulate the endocrine and reproductive functions in vivo.
(3) Include GC-MS-based analytical methods to complement the LC-MS results. Moreover, calculating ToxPi scores and risk indices can help assess the exposure risks associated with the detected pollutants.

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