# Juxtaposition of Intensive Agriculture, Vulnerable Aquifers, and Mixed Chemical/Microbial Exposures in Private-Well Tapwater in Northeast Iowa – Supplementary Information

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**1. Methods**

*1.1. Analytical methods*

TW samples were analyzed by the 1) USGS using 7 target-organic (437 unique analytes), 3 inorganic (35 analytes), 3 field parameter, and 11 microbial methods (Table S2), as described ([Bradley et al., 2021a](#_ENREF_7); [Romanok et al., 2018](#_ENREF_29)), 2) Center for Health Effects of Environmental Contamination and Iowa State Hygienic Laboratory at the University of Iowa for 6 neonicotinoid insecticides (acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, thiamethoxam), as described ([Evelsizer and Skopec, 2018](#_ENREF_16); [Thompson et al., 2021](#_ENREF_34)), 3) EPA using 3 *in vitro* bioassays targeting 3 (androgen [AR], estrogen [ER], and glucocorticoid [GR]) receptor classes ([Medlock Kakaley et al., 2020](#_ENREF_24); [Medlock Kakaley et al., 2021](#_ENREF_25)), and 4) National Cancer Institute (NCI) using 6 *in vitro* bioassays targeting 5 (AR, ER, GR, aryl hydrocarbon [AhR], thyroid hormone [TR]) receptor classes ([Jones et al., 2020](#_ENREF_21); [Stavreva et al., 2012b](#_ENREF_32); [Stavreva et al., 2016](#_ENREF_33)) (see SI for details/citations). Per/poly-fluoroalkyl substances (PFAS) method details are provided in Kolpin et al. ([2021](#_ENREF_22)) Pharmaceutical and pesticide samples were syringe filtered (0.7 µm nominal pore size, glass fiber) in the field. A subset of 10 replicate TW samples was sent to the USGS Organic Chemical Research Laboratory for comparative analysis of the 6 neonicotinoid analytes, as described ([Hladik and Calhoun, 2012](#_ENREF_20)). All results are in Tables S3-S6 and in Meppelink et al ([Meppelink et al., 2022](#_ENREF_26)).

In vitro estrogen (ER), androgen (AR), and glucocorticoid (GR) bioactivities were assessed by EPA using the T47D-KBluc cell line (American Type Cell Culture, Manassas, Virginia; ATCC CRL-2865; human estrogen receptor α/β) ([Wilson et al., 2004](#_ENREF_43); [Wilson et al., 2002](#_ENREF_44)) and the CV1 cell line (ATCC CCL-70) transduced (adenovirus) with the chimpanzee androgen receptor ([Hartig et al., 2002](#_ENREF_18); [Hartig et al., 2007](#_ENREF_19)) or the human glucocorticoid receptor ([Conley et al., 2017a](#_ENREF_10)), as described previously ([Conley et al., 2017a](#_ENREF_10); [Conley et al., 2017b](#_ENREF_11); [Medlock Kakaley et al., 2020](#_ENREF_24)). Cells for bioassays were plated in 96-well luminometer plates (T47D-KBluc: Greiner, Bio-One North America, Monroe, NC, CV1‑hGR/chAR: Costar 3610, Corning Inc., Corning, NY) and standards, controls, and samples were run in quadruplicate, and each sample screen was at least duplicated. After 24 h *in vitro* exposure, cells were visually scored for cytotoxicity and any wells with cells exhibiting cytotoxic effects were excluded from subsequent analysis ([Bhatia and Yetter, 2008](#_ENREF_2); [Conley et al., 2017c](#_ENREF_12)). Luminescence was quantified using a CLARIOstar luminometer (BMG Labtech, Cary, NC) ([Medlock Kakaley et al., 2020](#_ENREF_24)). Endocrine-active samples were identified using a tiered screening process for tapwater ([Medlock Kakaley et al., 2021](#_ENREF_25)). Biological equivalency values (BioEq) were calculated using an enrichment factor (EF) of 10,000 ([Escher and Leusch, 2011](#_ENREF_15)). BioEq above the respective assay minimum detectable concentration (T47KBluc: 0.068 ng 17β-Estradiol equivalents (Eq)/L; CV1-chAR: 0.9 ng 4,5α-Dihydrotestosterone Eq/L; and CV1-hGR: 5.41 ng Dexamethasone Eq/L) were considered positive for endocrine activity ([Medlock Kakaley et al., 2020](#_ENREF_24); [Medlock Kakaley et al., 2021](#_ENREF_25)).

TW samples were also analyzed by NCI using 5 mammalian-cell based steroid receptor bioassays targeting 5 endocrine disrupting compound receptor (aryl hydrocarbon [AhR], androgen [AR], estrogen [ER], glucocorticoid [GR] receptor, and thyroid [TR]) classes), as described previously ([Stavreva et al., 2012b](#_ENREF_32); [Stavreva et al., 2016](#_ENREF_33)). Briefly, for each assay batch, samples were divided into four replicates and concentrated at 200x. Concentrated replicates were added to treated cells for 30 min when screening for glucocorticoids and androgens, and for 3 h when screening for aryl hydrocarbon, estrogenic and thyroid activity. Each batch included blanks, solvent controls, and blind duplicates. Automated imaging and a customized image analysis pipeline (Perkin Elmer) were employed to calculate the mean green fluorescent protein (GFP) intensity across the four replicates per sample and was further normalized with the control sample on the same plate. Results were analyzed using SigmaPlot v. 11 (SPSS Inc., Chicago, IL) as previously described ([Stavreva et al., 2012a](#_ENREF_31); [Stavreva et al., 2016](#_ENREF_33)) and with RStudio version 4.0.2 (Boston, MA, USA). The normalized average GFP ratio for each sample was compared to negative controls using one-way ANOVAs followed by Holm-Sidak correction for multiple comparisons. Sample results with a p-value ≤ 0.05 were considered to have statistically significant bioactivity.

*1.2. Risk assessments*

A screening-level assessment ([Goumenou and Tsatsakis, 2019](#_ENREF_17); [U.S. Environmental Protection Agency, 2011](#_ENREF_35)) of potential cumulative biological activity of mixed-organic contaminants in each TW sample was conducted as described ([Blackwell et al., 2017](#_ENREF_3); [Bradley et al., 2019](#_ENREF_5); [Bradley et al., 2018](#_ENREF_6)). The toxEval version 1.2.0 package ([De Cicco et al., 2018](#_ENREF_14)) of the open source statistical software R ([R Development Core Team, 2019](#_ENREF_28)) was used to sum (non‑interactive concentration addition model ([Altenburger et al., 2018](#_ENREF_1); [Cedergreen et al., 2008](#_ENREF_9); [Stalter et al., 2020](#_ENREF_30)) individual EAR (ratio of the detected concentration to the activity concentration at cutoff (ACC) from the Toxicity ForeCaster ([ToxCast,U.S. Environmental Protection Agency, 2020](#_ENREF_38)) high-throughput screening data ([U.S. Environmental Protection Agency National Center for Computational Toxicology, 2019](#_ENREF_41); [U.S. Environmental Protection Agency National Center for Computational Toxicology, 2020](#_ENREF_42))) to estimate sample-specific cumulative EAR (∑EAR) ([Blackwell et al., 2017](#_ENREF_3); [Bradley et al., 2020](#_ENREF_4); [Bradley et al., 2018](#_ENREF_6)). ACC estimates the point of departure concentration at which a defined threshold of response (cutoff) is achieved for a given biological activity and is less prone to violations of relative potency assumptions (for discussion see Blackwell et al. ([2017](#_ENREF_3))). ACC data in the toxEval v1.2.0 employed in the present study were from the August 2020 invitroDBv3.2 release of the ToxCast database ([U.S. Environmental Protection Agency National Center for Computational Toxicology, 2020](#_ENREF_42)). Non-specific-endpoint, baseline, and unreliable response-curve assays were excluded ([Blackwell et al., 2017](#_ENREF_3); [Bradley et al., 2021a](#_ENREF_7); [Bradley et al., 2021b](#_ENREF_8)). ∑EAR results and exclusions are summarized in Tables S7a-c.

An analogous human-health-benchmark HI assessment ([Goumenou and Tsatsakis, 2019](#_ENREF_17); [U.S. Environmental Protection Agency, 2011](#_ENREF_35); [U.S. Environmental Protection Agency, 2012](#_ENREF_36)) of the combined inorganic and organic contaminant risk also was conducted using toxEval v1.2.0 ([De Cicco et al., 2018](#_ENREF_14)) to sum the TQ (ratio of detected concentration to corresponding health‑based benchmark) of individual detections to estimate sample-specific cumulative TQ (∑TQ) ([Corsi et al., 2019](#_ENREF_13)). A precautionary screening‑level approach was employed based on the most protective human-health benchmark (i.e., lowest benchmark concentration) among MCLG ([U.S. Environmental Protection Agency, 2017](#_ENREF_37); [U.S. Environmental Protection Agency, 2021b](#_ENREF_40)), WHO Guideline Values (GV) and provisional GV (pGV) ([World Health Organization (WHO), 2011](#_ENREF_45)), USGS Health-Based Screening Level (HBSL) ([Norman et al., 2018](#_ENREF_27)), and state drinking-water MCL or health advisories (DWHA). For the ∑TQ assessment, MCLG values of zero (i.e., no identified safe‑exposure level for sensitive sub-populations, including infants, children, the elderly, and those with compromised immune systems and chronic diseases) ([U.S. Environmental Protection Agency, 2021a](#_ENREF_39); [U.S. Environmental Protection Agency, 2021b](#_ENREF_40)) were set to the respective method reporting limit, except for Pb, which was set to 1 µg L‑1 as suggested by the American Academy of Pediatrics ([Lanphear et al., 2016](#_ENREF_23)). ∑TQ results and respective health‑based benchmarks are summarized in Tables S8a-b.

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