SUPPORTING INFORMATION TO:

Using zebrafish embryo bioassays combined with high-resolution mass spectrometry screening to assess ecotoxicological water bodies quality status: a case study in Panama rivers

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Text S1. Details on the SPE followed by LC-HRMS screening approach

Water samples were shipped frozen to Santiago de Compostela, where they were subjected to solid-phase extraction (SPE) within 48 h. Eighteen isotopically labelled compounds were used as internal standards (IS) for semi-quantification purposes (see 2.4.2), amphetamine-d6, benzoylecgonine-d3, cocaine-d3, diazepam-d5, fluoxetine-d6, ritalinic acid-d10, sertraline-d3, temazepam-d5 and venlafaxine-d6 were supplied by Cerilliant (Round Rock, TX, USA) as 100 µg mL-1 solutions in MeOH; tri-n-butyl phosphate (TnBP)-d27, tris(2-chloroisopropyl) phosphate (TCPP)- d18 and 13C8-perfluorooctane sulfonic acid (13C8-PFOS) were supplied by Wellington Laboratories (Guelph, Ontario, Canada); Cyprodinil-d5 and 13C6 metalaxyl were supplied by Riedel-de Haën (Seelze, Germany); Methylparaben-d4 was supplied by CDN Isotopes (Quebec, Canada); Atenolol-d7 and irbesartan-d4 were supplied by Sigma-Aldrich (St. Louis, MO, USA); Metformin-d6 was supplied by TRC Canada (Ontario, Canada).

Before the SPE process, water samples were vacuum filtered through 0.45 µm PVDF filters (Merck Millipore, Darmstadt, Germany). Subsequently, 200 mL sample aliquots were spiked with 100 ng of a mixture of the 18 IS and extracted by SPE using Oasis HLB 200 mg cartridges (Waters, Milford, MA, USA). Samples were percolated through the SPE cartridges, previously conditioned with 5 mL of methanol (LC-MS grade, Scharlab, Barcelona, Spain) and 5 mL of ultrapure water. Then, the sorbents were washed with 10 mL of ultrapure water and dried for ca. 30 min by a nitrogen stream. Analytes were eluted with 10 mL of methanol by gravity and the eluates were evaporated to dryness using a Turbo-Vap II (Zymark, Hopkinton, MA USA) and a Mini-Vap concentrator (Sigma-Aldrich). Dried extracts were reconstituted with 0.5 mL of methanol, filtered with a GHP®

13 mm 0.2 µm Syringe filter membrane (Pall Corporation, Port Washington, NY, USA) and transferred into a glass micro insert prior ultra-high-performance liquid chromatography-high resolution mass spectrometric analysis (UHPLC-HRMS). Procedural blanks were run with the samples and chemicals detected in the blanks were excluded.

Two µL of sample or procedural blank extracts were injected into an Agilent Technologies (Wilmington, DE) 1290 Infinity II LC system. Chromatographic separation was carried out with a ZORBAX Extend-C18 1.8 µm (2.1 x 50 mm) column supplied by Agilent Technologies and connected to a Supelco ColumnSaver 0.5 µm Precolumn Filter (Supelco, Bellefonte, PA). As mobile phases, Milli-Q water (0.1% formic acid) (A) and methanol (0.1% formic acid) (B) were used at a flow rate of 0.4 mL min-1. The temperature of the column was fixed at 35°C. The gradient elution started with 98% A, increasing to 100% B in 22 min, held for 4 min. Subsequently, it returned to initial conditions (98% A), held for 4 min for column back-conditioning.

A quadrupole-time-of-flight (QTOF) mass spectrometer Agilent 6550 iFunnel Q-TOF LC/MS system equipped with a Dual Agilent Jet Stream electrospray (ESI) ion source was coupled to the UHPLC system. The ESI interface was operated in either in positive or negative modes and the voltage of the ESI needle was fixed at 3500 V. Nitrogen was used as nebulizing (30 psi) and drying gas (200°C, 12 L min^{-1}) in the ESI source, and also as collision gas in the MS/MS experiments.

Instrument control and data acquisition were carried out with the MassHunter Workstation software B.10.00 from Agilent. Data acquisition was performed using two complementary methods, Auto MS/MS (i.e. data-dependent acquisition,

DDA) and All Ions pseudo-MS/MS (i.e. data independent acquisition, DIA). In all cases, a reference calibration solution, supplied by Agilent, was continuously sprayed in the source during the chromatographic run, providing the required accuracy of mass assignations.

The Auto MS/MS method consisted into three consecutive injections per sample and polarity operated in the iterative acquisition mode, where those precursors that were previously selected for MS/MS fragmentation in previous injections at a given retention time are automatically excluded by the software on a rolling basis in the following injections. Three collision energies (10, 20 and 40 V) were collected for each precursor ion, with a maximum of 2 precursor ions per cycle (absolute abundance threshold: 1000 counts). The acquisition frequency in the single-MS and the MS/MS were 3 and 6 spectra per second, respectively. During each chromatographic run, precursor ions previously selected for fragmentation were excluded after 3 spectra (1 per collision energy) and released after 0.5 min. The All Ions MS/MS method consisted into 1 injection per sample in each polarity, with two alternating collision energies of 0, where little fragmentation is expected, and 20 V where fragments are expected, but without selection of precursor ion. Hence, in this mode, assignations of potential MS/MS fragments are based on the coelution of such potential fragments in the 20 V channel with the precursor ion in the 0 V channel. The scanned range was 60-1100 m/z in MS and 30-1000 m/z in MS/MS modes.

Data files were processed using the Qualitative Workflows of Agilent MassHunter Workstation software B.10.00. Auto MS/MS data files were processed using the search algorithm Find by Auto MSMS, which automatically extract the MS and MS/MS information of those precursor ions which were selected by the software

for MS/MS acquisition, aligns the spectra, and sorts and display them as a single compound. Then, these chemicals are searched against an accurate mass MS/MS spectral library of ca. 3200 chemicals, including pharmaceuticals, pesticides and other emerging pollutants (full list presented as Table S1). Chemicals were considered as tentatively identified when there was a match in the MS/MS spectra of at least two product ions with a coherent precursor ion (score>80% and mass error lower than 5 ppm). All Ions data files were processed using the search algorithm Find by Formula, where the software searches for possible ions matching with the empirical formula considering $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ ions in positive mode and only $[M+H]^-$ in the negative mode (same score and ppm thresholds as in Auto MS/MS). When a match is found, up to 7 product ions from the MS/MS library spectrum are extracted as chromatograms from the 20V channel, generated a unique co-elution score, which indicates confidence of the correlation between the precursor and the product ions by abundance, peak shape, peak width and retention time. Analytes were deemed identified when there were at least 2 of these putative product ions coeluted with the precursor ion (coelution score>70%).

All the analytes detected through the screening process and IS were then integrated from the low collision energy MS chromatogram, using the $[M+H]^+$ or $[M-H]^-$ ion, depending on the source polarity employed using the Mass Hunter Quantitative Analysis B 08.00 software. Finally, the intensity of the detected substance was presented as area of such compound, divided by the area of the IS which ionized in the same polarity mode with the closest retention time, in order to correct for matrix effects in order to have a normalized signal.

For those chemicals (atenolol, benzoylecgonine, irbesartan, metformin, TCPP, TnBP and venlafaxine) detected where the analog isotopically labelled IS was available, a calibration curve from 1 ng mL^{-1} to 5,000 ng mL^{-1})

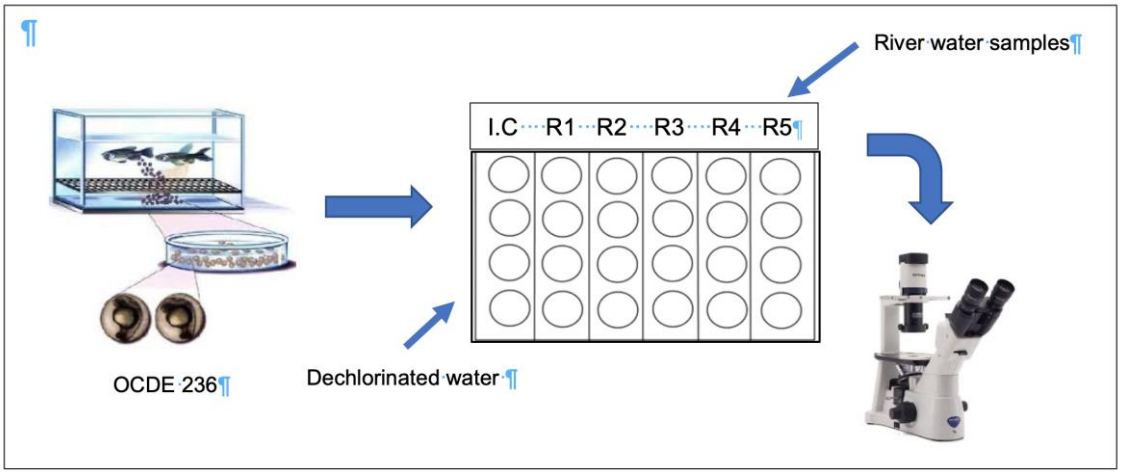


Figure S1. Experimental design of the zebrafish bioassay. 24 fertilized eggs were selected and transferred to 24-wells plates filled with 2mL of river water samples and dechlorinated water for the internal control per plate. Two plates per sample were performed. I.C (internal control); Rx (water sample).

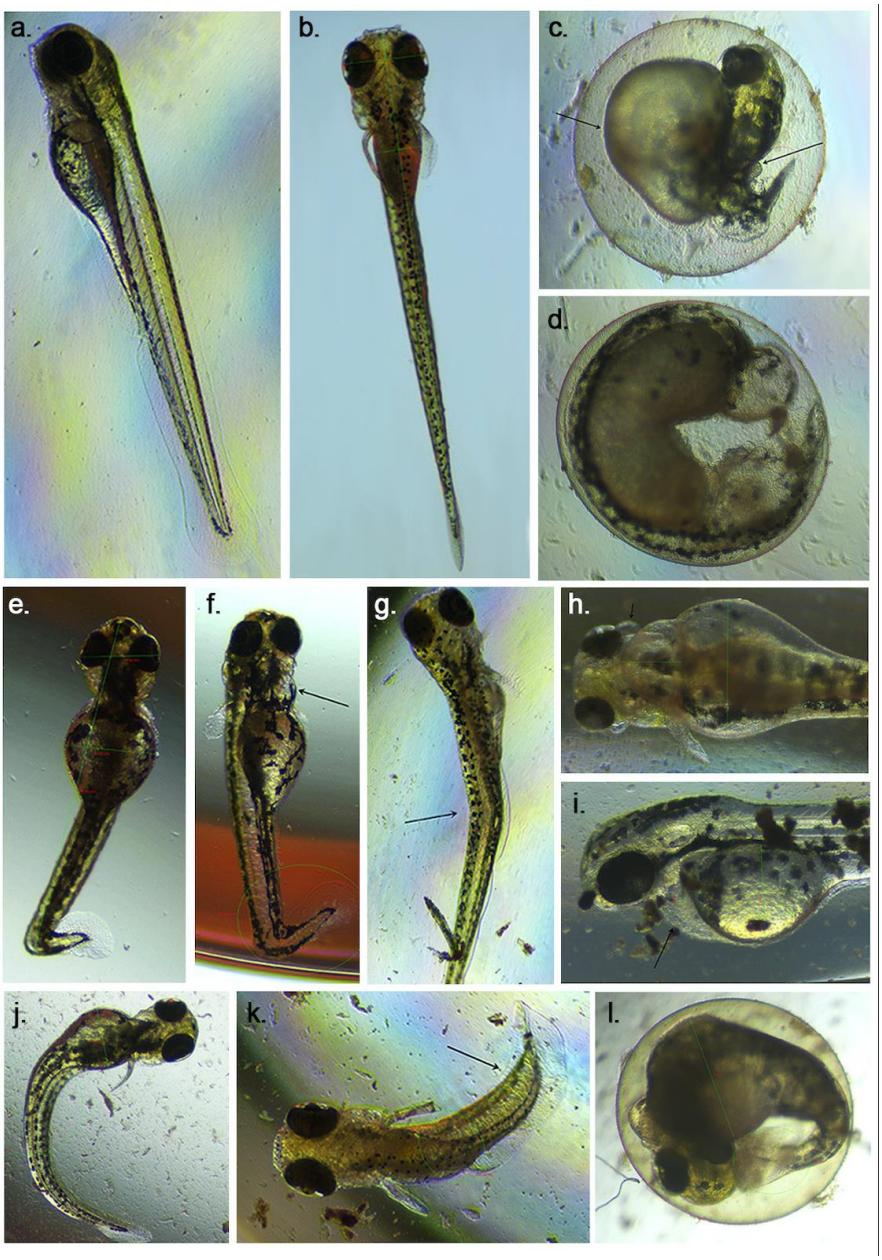


Figure S2 Examples of different abnormalities observed during *D.rerio* embryonic development exposed to river water samples in the present study. A. Normal larve *D.rerio* at 96hpf, lateral view; B. Normal larvae *D.rerio* at 96hpf front view. C. Non-development delay, enlarged yolk-sac and tail deformed; D. Head and tail deformed. E. Tail broken and enlarged yolk-sac. F. Pericardial edema and tail broken; G. Spine curvature. H. Yolk-sac and pericardial edema. I. Pericardial edema. J. Scoliosis; K. Short larvae; L. Yolk-sac enlarged/malformed, non-development delay. The arrows indicate the location of the abnormalities (→).

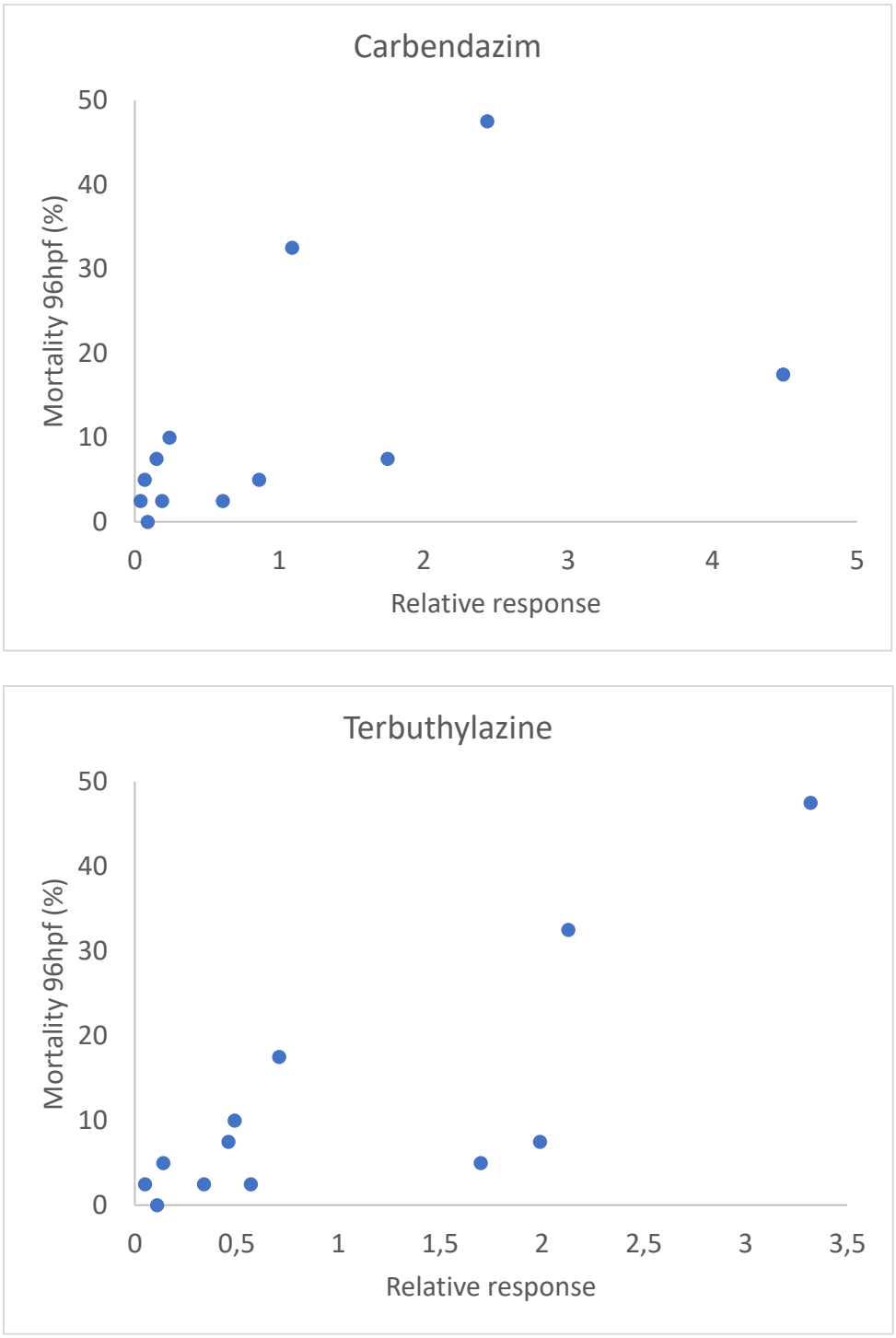
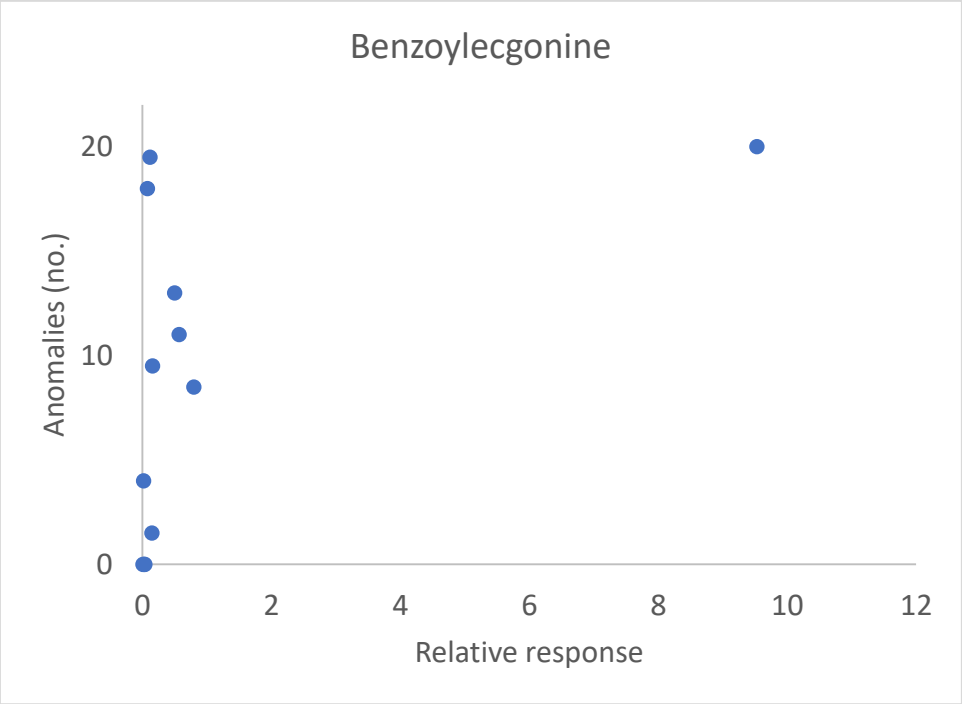


Figure S3. Scatter plots for chemicals (showing the relative response: area/area of internal standard in the sample) associated with ecotoxicological endpoints (either mortality at 96 hpf or number of anomalies) where a statistically significant p-value (<0.05) in the Spearman's rank test was obtained.



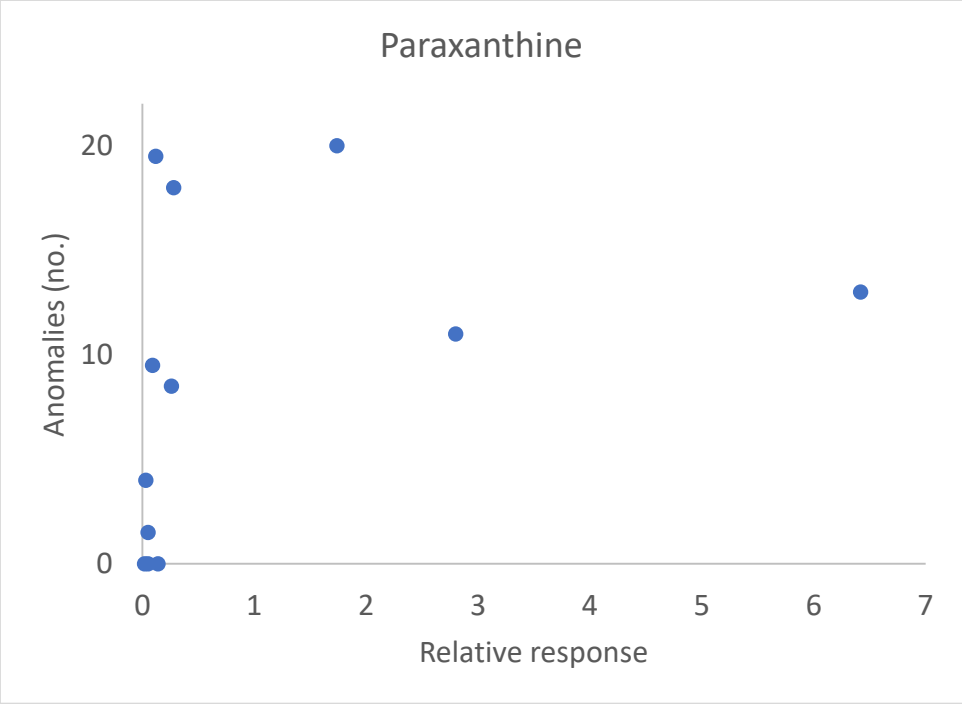


Figure S3 Cont.

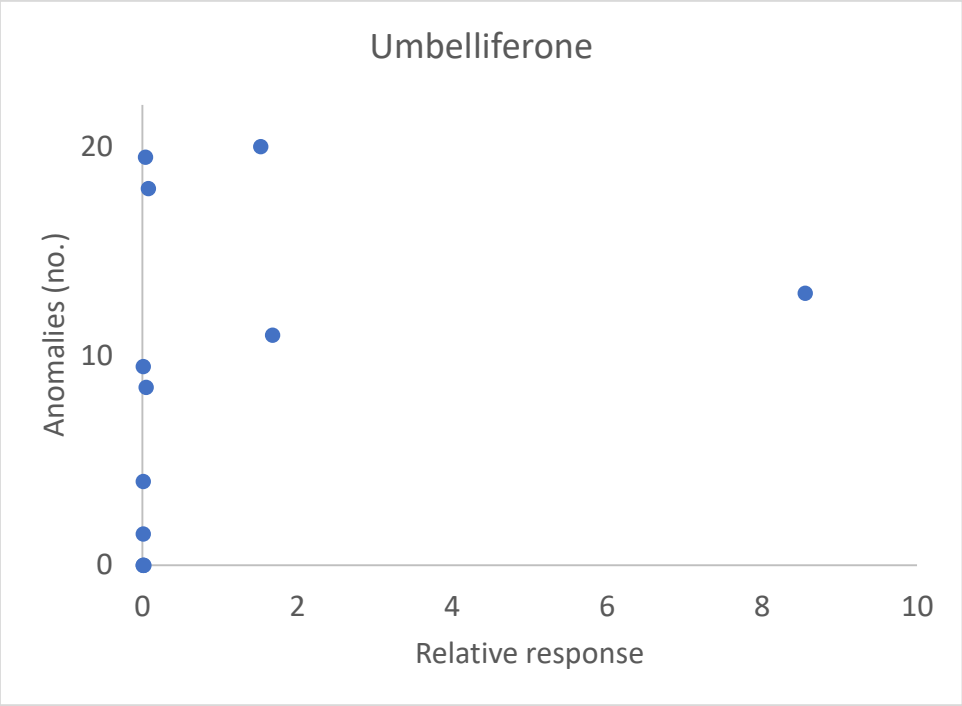


Figure S3 Cont.