Development of Bioanalytical Techniques for Monitoring of Constituents/Chemicals of Emerging Concern (CECs) in Recycled Water Applications for the State of California

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Under Contract Agreement

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Final Report

Prepared by

Southern California Coastal Water Research Project

PROJECT TITLE: Development of bioanalytical techniques for monitoring of constituents/chemicals of emerging concern (CECs) in recycled water applications for the State of California

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EXECUTIVE SUMMARY

In 2009, the California Water Resources Control Board ("State Water Board") adopted the Recycled Water Policy that mandated monitoring of chemicals with the potential for human and ecological health effects that are not uniformly regulated (aka "chemicals of emerging concern" or CECs), in approved groundwater recharge applications statewide. To ensure that such monitoring requirements were based on the best available peer-reviewed science, the Water Board convened a panel of scientific experts ("Panel") to make recommendations that would ensure beneficial uses of recycled water were protected. In their final report[1], the Panel endorsed the development of bioanalytical techniques ("bioassays") to address both known and unknown CECs and to develop appropriate trigger levels for these techniques that correspond to responses posing a concern from a human health standpoint.

In 2011, the Southern California Coastal Water Research Project Authority (SCCWRP) was contracted by the Water Board to direct and manage a team of investigators to develop bioassays as envisioned by the Panel. Specifically, this team was tasked to (1) identify the most promising bioassay endpoints relevant to recycled water and the protection of human health; (2) develop extraction protocols for recycled water samples; (3) compare, evaluate and optimize the performance of commercially available bioassay technology; (4) deliver written bioassay protocols for successfully optimized endpoints; (5) provide interpretive guidance for bioassay results; (6) solicit stakeholder feedback and guidance on the application of bioassays; and (7) coordinate the team's activities with related efforts, including a similarly focused project (WateReuse Research Foundation WRF10-07) led by an Australian consortium faced with water supply and quality issues of their own. The all-compassing theme for this project was the practical application of bioassays whose performance and adaptability for monitoring of water quality was found promising. In response, SCCWRP assembled an investigative team consisting of experts in bioanalytical techniques from the University of California - Riverside, University of Arizona, University of Florida, and the University of South Florida.

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[1] Anderson et al. 2010. Monitoring Strategies for Chemicals of Emerging Concern (CECs) in Recycled Water: Final Report. Sacramento, CA.

To kickoff this effort, SCCWRP hosted the first of two Cross-Project Coordination meetings with WRF10-07 project researchers and advisors to identify commonality in project goals, exchange knowledge and information, and establish a working collaboration to promote leverage in achieving these goals. SCCWRP established a stakeholder advisory committee consisting of members representing the regulated, regulatory, research and commercial services communities to provide feedback on the likelihood of success and applicability of the bioassays developed by the SCCWRP team. The project teams reconvened at a second meeting in 2014 to discuss the findings of these concurrent projects, and to reach consensus on a path forward.

In collaboration with the WRF10-07 team, and supplemented by an extensive literature survey, the project team identified a list of eight (8) bioassay endpoints that were sufficiently mature in terms of performance potential and relevance for protecting human health. These endpoints target the activation of genetic processes at the cellular level that represent the initial biological response to exposure by foreign chemicals. Furthermore, each endpoint represents a specific mode of biological action (MOA) and thus responds to only those chemicals that act by the specified MOA. Many of the bioassays selected were initially developed and evaluated by federal initiatives to improve chemical screening and registration (e.g. Tox21[2] and USEPA's Endocrine Disruptor Screening Program or EDSP[3]). Targeting such responses are performed *in vitro*, i.e. by dosing engineered human, animal or bacterial cell lines with a small drop of concentrated sample extract in a high sample throughput format, e.g. using plates with an array of individual exposure wells. After allowing for chemicals in the sample extract to "activate" genes associated with the engineered test cells, each well is analyzed for light emitted resulting from the genetic activation. The intensity and wavelength of emitted light is proportional to the CEC concentration in the sample. Selected endpoints included those associated with endocrine/reproductive disruption (e.g. estrogenicity, androgenicity), genetic modification and cancer (genotoxicity) and general cell health (cytotoxicity). A survey of multiple vendors revealed that bioassays targeting each of these endpoints were commercially available, in contrast to the dozens of bioassays still in the research and development stage. Moreover, it was clear that the most technologically advanced and relevant endpoints for water quality screening purposes were *in vitro* receptor-based transactivation bioassays.

A standard procedure for water sample extraction was established by the SCCWRP team. The protocol utilized solid phase extraction (SPE) with two sorbents in series to maximize retention of CECs of interest, including hormones, pharmaceuticals and personal care products (PPCPs), pesticides and other consumer and commercial chemicals. The protocol was used to measure CECs in samples representative of recycled water feedstocks (e.g. domestic wastewater treatment plant effluent). Quality assurance/quality control (QA/QC) criteria to ensure robust bioassay results, as well as supporting chemical analyses, were documented in a project Quality Assurance Project Plan (QAPP).

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[2] http://www.epa.gov/ncct/Tox21/
[3] http://www.epa.gov/endo/

The first step in evaluating bioassay performance was to compare products from different vendors. The estrogen receptor transactivation endpoint (ER) was selected due its maturity (e.g. in Tox21 and EDSP) and widespread availability. Cell lines amenable for testing in multi-well plate format from three vendors were compared in dose response experiments using reference toxicants. Based on performance (sensitivity, reproducibility), ease of use and customer support potential, products from Life Technologies were selected for further optimization, in large part due to their availability in both culturable and cryo-preserved "thaw and use" (division arrested or DA) formats. It was noteworthy that the performance of cell lines from the other vendors was, in many cases, comparable to that observed for the Life Technologies product. Thus, future evaluations and/or applications of this technology should consider all possible commercially available products with similar performance characteristics.

Bioassay optimization focused on DA cell bioassay kits from Life Technologies in a 96-well plate format, which allow for intermittent application with little/no maintenance cost/effort and was compatible with basic detection instrumentation. Dose response experiments were conducted to identify (a) optimum seeding cell density and (b) endpoint-specific reference toxicants, which were critical in generating bioassay results that can be related to the responses of well-known CECs. Products targeting the 8 endpoints of interest, and in some cases, additional CEC-specific cell lines and multiple candidate reference toxicants were evaluated. Targets for minimum bioassay sensitivity were established based on the recommended reporting limits established by the Panel for estrogenic and genotoxic CECs. A seeding density range of 40,000-50,000 cells per well was established. Performance for 5 of the 8 targeted endpoints was found to be acceptable (**Table ES-1**); reference toxicants were identified and protocols for the DA bioassays were finalized based on feedback from potential end users. The 5 optimized bioassays were included in an interlaboratory calibration exercise, organized by the WRF10-07 investigators, that analyzed extracts from 10 water samples representing different levels of treatment (and thus water quality) using various bioanalytical techniques (103 endpoints in total). The study participants, including all 5 members of the SCCWRP led project team, represented 20 laboratories around the world. Among the most responsive and precise endpoints in this study included the ER and GR bioassays optimized by the project team.

Table ES-1. Receptor-based transactivation cell bioassays recommended for future evaluation.

ENDPOINT	MODE OF ACTION	POTENTIAL ADVERSE OUTCOMES
estrogen receptor (ER)	Estrogen signaling (estrogenicity)	Impaired reproduction; feminization of males,
androgen receptor (AR)	Male sexual phenotype (androgenicity)	Impaired reproduction; masculinization of females
glucocorticoid receptor (GR)	Cortisol binding, gene transcription regulation	Development, immune function, diabetes
progesterone receptor (PR)	Embryonic development, cell differentiation	Cancer, diabetes, hormone resistance syndrome
cytotoxicity	General cell toxicity	Tissue damage, death

Because the output from bioassays differs from conventional chemical analysis, guidelines for data analysis and interpretation are needed for decision-making applications. Working in concert with WRF10-07 researchers, the project team provided written guidance and a simple spreadsheet tool to convert bioassay results to concentrations that are equivalent to those of reference toxicants, so-called "bioassay equivalent concentrations" or simply BEQs. The second step in applying bioassay results considered health-based thresholds (e.g. no effect concentrations) as well as the difference in potency that CECs exhibit using *in vitro* bioassays compared to effects that may occur *in vivo* (i.e. on individual test organisms, humans or human populations) to derive appropriate bioassay-based action levels (ALs). The third and final component of the interpretive framework established ground rules for adaptive decision-making by managers based on the severity and persistence of exceedance of ALs.

At the second Cross-Project meeting held in January 2014, results from the SCCWRP led and WRF projects were presented before the combined research and stakeholder advisory teams. Consensus was reached collectively that the performance, applicability and relevance of the ER transactivation bioassay made it suitable as a screening level monitoring tool for recycled water. The participants further agreed that the next "pilot" stage of evaluation would focus on technology transfer and a real world simulation of how bioassay results could be used in a management context:

1. Could commercial services and water utility labs successfully perform the ER transactivation bioassay?
   Pilot study elements: (a) Provide training on bioassay performance, data analysis and interpretation. (b) Plan and conduct an inter-comparison exercise among commercial services and water industry laboratories.

2. Does the proposed bioassay interpretive framework provide managers with a robust decision making tool that better protects the beneficial uses of recycled water in California?
   Pilot study elements: (a) Collect and test recycled water samples across the State and analyze using the ER transactivation bioassay and by conventional chemical analysis. (b) Evaluate the bioassay results using the proposed interpretive framework and compare with decisions made based on chemical specific monitoring.

In addition, the project team identified a number of studies that would improve and/or better inform the application of bioassays for water quality screening purposes. These studies include identification and optimization of a broader suite of endpoints (e.g. aryl hydrocarbon receptor, genotoxicity, oxidative stress, neurotoxicity, immunotoxicity), assessment of the efficacy of water extraction protocols for a diverse set of bioassay endpoints, and a cost-comparison between chemical-specific and bioassay-based screening level monitoring.

PROJECT NARRATIVE

Task 1 – Project Management

It is estimated that 100% of this task has been completed.

Task 1 Deliverables

1.2 Subcontractor agreements (available upon request)

1.3. Project schedule with milestones deliverable due dates (below).

Task	Deliverable	Due Date
1.2	Signed subcontractor agreements with conflict of interest certifications	As Needed
1.3	Draft schedule with milestones for the team's activities	October 10, 2011
1.6	Quarterly Progress Reports	October 10, 2011 and quarterly thereafter
2.1	List of recommended bioassays selected for further development and evaluation	October 10, 2011
3.1	Written standardized water extraction protocols	January 10, 2012
3.2	Quality Assurance Project Plan (QAPP)	July 30, 2012
4.4	Written standardized bioassay protocols	July 10, 2013
4.5	Written results of inter-laboratory round-robin exercise	December 10, 2013
5.4	Written tiered framework for utilization of bioassays results	December 10, 2013
5.5	Agenda for stakeholder workshop, list of attendees, copies of presentations, and summary of stakeholder input	February 10, 2014
6.1	Project advisory committee meeting agendas and schedule, participant list of names and affiliations, and meeting minutes	As needed
6.2	Cross-project advisory committee meeting agendas, schedule, participant list of names and affiliations, and meeting minutes	As needed
7.2	Draft Project Report	April 1, 2014
7.3	Final Project Report	June 1, 2014

1.6 Quarterly Progress Reports (on file)

Task 2 – Literature Review

A comprehensive literature survey to compile relevant material to identify candidate bioassays for further consideration was completed by the project team. Peer-reviewed publications were compiled and contact with suppliers of relevant bioanalytical technology was initiated. Team members confirmed the interest of three commercial vendors of in vitro bioassay technology to participate in this study. **Table 2-1** lists the bioassay endpoints considered for development.

It is estimated that 100% of this task has been completed.

Task 2 Deliverables

2.1 List of recommended bioassays selected for development and evaluation (Table 2-1)

Table 2-1. List of recommended bioassays selected for development and evaluation.

ENDPOINT (MODE OF ACTION)	CANDIDATE BIOASSAY
Estrogencity[1]	Estrogen receptor (ER) reporter gene transactivation
Androgenicity[1]	Androgen receptor (AR) reporter gene transactivation
Progesterone activity[1]	Progesterone receptor (PR) reporter gene transactivation
Glucocorticoid activity[1]	Glucocorticoid receptor (GR) reporter gene transactivation
Genotoxicity[2]	P53 reporter; Ames II
Aryl hydrocarbon reactivity[2]	Aryl hydrocarbon receptor (AhR) reporter
CEC specific occurrence[2]	PPAR alpha, gamma
Cytotoxicity[1]	Presto Blue cell viability

*optimized (Task 4) and assessed in intercalibration exercise (Task 6)

**abandoned due to unacceptable performance during optimization (Task 4)

Task 3 – Water Extraction Protocol and Sample Analysis

Development of water extraction protocol. Water samples were tested for extraction efficiency using multiple solid phase sorbents and process sequences. In conjunction with the WRRF 10-07 Project Team, a final protocol that utilized Oasis HLB and coconut charcoal solid phase extraction (SPE) cartridges in series coupled with elution by methanol (MeOH) was adopted (see **Appendix 3.1**).

A quality assurance project plan (QAPP) was drafted to address quality assurance/quality control (QA/QC) guidelines for extraction and analysis of water samples for chemicals of interest using conventional instrumental and candidate bioanalytical techniques. Methods for chemical analysis with a list of target analytes and estimated analyte specific method detection limits (MDLs) are included in the QAPP (see **Appendix 3.2**).

Sample analysis. Wastewater treatment plant (WWTP) effluent samples characterized by bioassays (see Task 4) were analyzed by GC-MS/MS and LC-MS/MS at the University of Arizona. The sample was first filtered and then extracted using two sorbents in series. The method was validated by analyzing 26 target analytes, including ^{13}C labeled and perdeuterated analogs, spiked at 100 ng/L into deionized water. Recoveries of $\geq$ 75% of the spiked analytes exceeded 60%. The analysis of the water sample extract revealed that a number of target CECs were detectable, including PPCPs and commercial chemicals (e.g. TCPP. PFOS and bisphenol A). The hormones 17β-estradiol, 17α-ethinylestradiol and testosterone were not detected, however the corticosteroid prednisone was detected (**Table 3-1**).

It is estimated that 100% of this task has been completed.

Task 3 Deliverables

3.1	Written standardized water extraction protocol (Appendix 3.1)
3.2	Quality Assurance Project Plan (Appendix 3.2)

Table 3-1. Chemicals of emerging concern (CECs) analyzed in a wastewater treatment plant effluent sample from a facility in Tucson, AZ.

Compounds	Conc.(ng/L)	MRL(ng/L)	Compounds	Conc.(ng/L)	MRL(ng/L)
Atenolol	<MRL	2.0	Benzotriazole	<MRL	10.0
Caffeine	<MRL	0.5	PFBA	<MRL	1.0
Trimethoprim	<MRL	0.5	Naproxen	<MRL	5.0
Sucralose	2640	10.0	PFBS	27.7	0.2
Primidone	87.6	0.5	Diclofenac	5.6	1.0
Sulfamethoxazole	106	0.5	Ibuprofen	45.1	5.0
Meprobamate	494	0.5	PFOA	8.1	0.2
Diphenylhydramine	<MRL	0.5	Propylparaben	<MRL	2.0
Prednisone	22.7	2.0	Bisphenol A	<MRL	5.0
Ditiazem	<MRL	0.2	Gemfibrozil	1.5	1.0
Simazine	<MRL	0.5	PFDA	<MRL	5.0
Carbamazepine	<MRL	0.2	PFOS	4.2	2.0
Dexamethasone	<MRL	0.2	PFDoA	<MRL	10.0
Fluoxetine	<MRL	0.2	Triclocarban	1.2	1.0
TCEP	354	0.5	Triclosan	1.2	1.0
Atrazine	<MRL	0.5	BPA	52.1	0.1
DEET	356	0.5	Estrone	<MRL	0.02
Testosterone	<MRL	0.5	17β-estradiol	<MRL	0.1
TCPP	110000	2.0	Testosterone	<MRL	0.02
Norgestrel	<MRL	2.0	17α-ethinylestradiol	<MRL	0.15
Benzophenone	<MRL	10.0			

MRL – method reporting limit

Task 4 – Bioassay Performance Evaluation and Optimization

Comparison of commercially available cell biossays. Dose response curves for estrogen receptor (ER) test kits from three commercial vendors (**Table 4-1**) exposed to a series of dilutions of the reference toxicant (17β-estradiol or E2) revealed similar performance (sensitivity, dynamic range and precision) for kits from two of the three vendors (Companies A and B (**Figure 4-1**), with the kit from Company C exhibiting a higher threshold and lower precision compared with the others. It was determined that the division arrested (DA) cell test kit from Company A gave the best overall performance when graded by sensitivity, precision, ease of performance, cost and product support from the vendor. However, the team agreed that the test kit from Company B performed equally well and could represent a viable second source or alternative for entities soliciting this capability from a commercial services lab. The team negotiated with Company A to supply DA arrested cell kits for the remaining endpoints. Test cells from Company C appeared to be contaminated, therefore, the project team could not recommend the cell lines tested from this vendor at this time.

Table 4-1. Commercial suppliers for the estrogen receptor (ER) bioassay evaluated in this study.

Vendor	Location	Key Contact(s)
Life Technologies (Company "A")	Madison, WI	K. Bi
BioDetection Systems (Company "B")	Amsterdam, The Netherlands	P. Behnisch
Switchgear Genomics (Company "C")	Menlo Park, CA	S. Force-Aldred

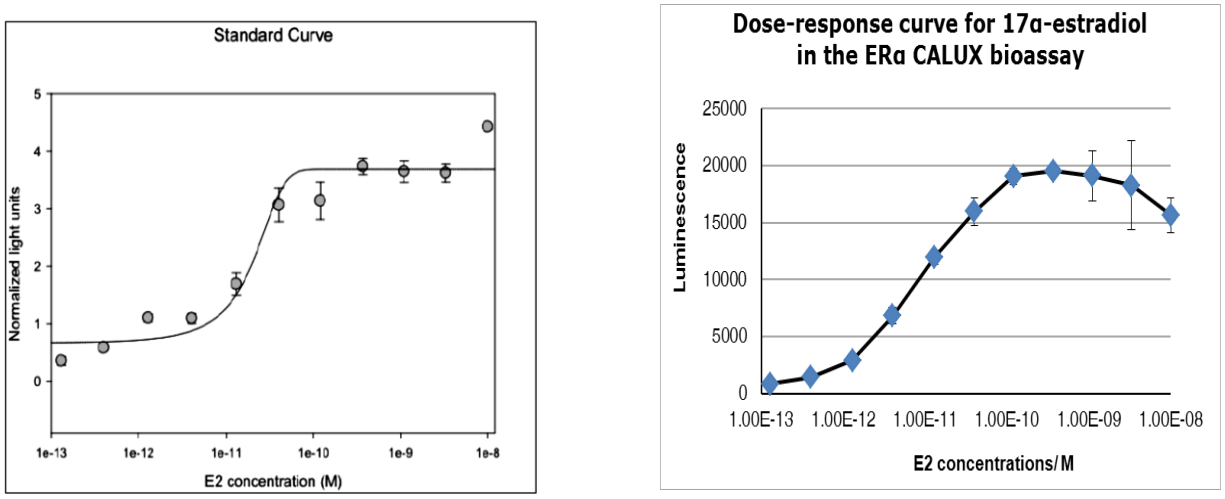

Fig. 4-1. Dose-response for the estrogen receptor (ER) test kits from Companies A (left) and B (right) illustrate a threshold of response at approximately 10^{-12} mol/L (< 0.5 ng/L) and a measurement range over two orders of magnitude for 17β-estradiol, a strong ER agonist.

Optimization of the GeneBLAzer ERα Assay. Work was performed to optimize the GeneBLAzer ERα Assay DA test kit resources provided by Life Technologies. In order to optimize the cell-density, 96 well plates were seeded with 5000, 10000, 30000 and 60000 cells/well and

stimulated with seven different concentrations of 17β–estradiol (E2) in the presence of 0.01% DMSO by the Universities of Florida and South Florida. A greater than two fold difference in fluorescence response was observed for the highest cell density (60000 cells/well) compared to the lowest cell density concentrations (**Figure 4-2**) when the blue/green ratio response was normalized to the vehicle control. Similar results were observed for the GeneBLAzer androgen receptor (AR), glucocorticoid receptor (GR) and progesterone receptor (PR) GripTite DA Cell-based assay kits (data not shown). Since a limited number of cells and sufficient reagents are provided in each DA kit, the number of assays possible per kit can be doubled (costs per sample cut in half) by decreasing the seeding cell density, assuming any decrease in dynamic (test response) range is acceptable. Thus, the team selected a seeding cell density range of 50 000 to 60 000 cells/well for the Life Technologies test kits. The team also concluded that thawed cells can be refrozen, stored at -80°C in a cryopreservation container, and then transferred to liquid nitrogen after 24 h for use at a later date.

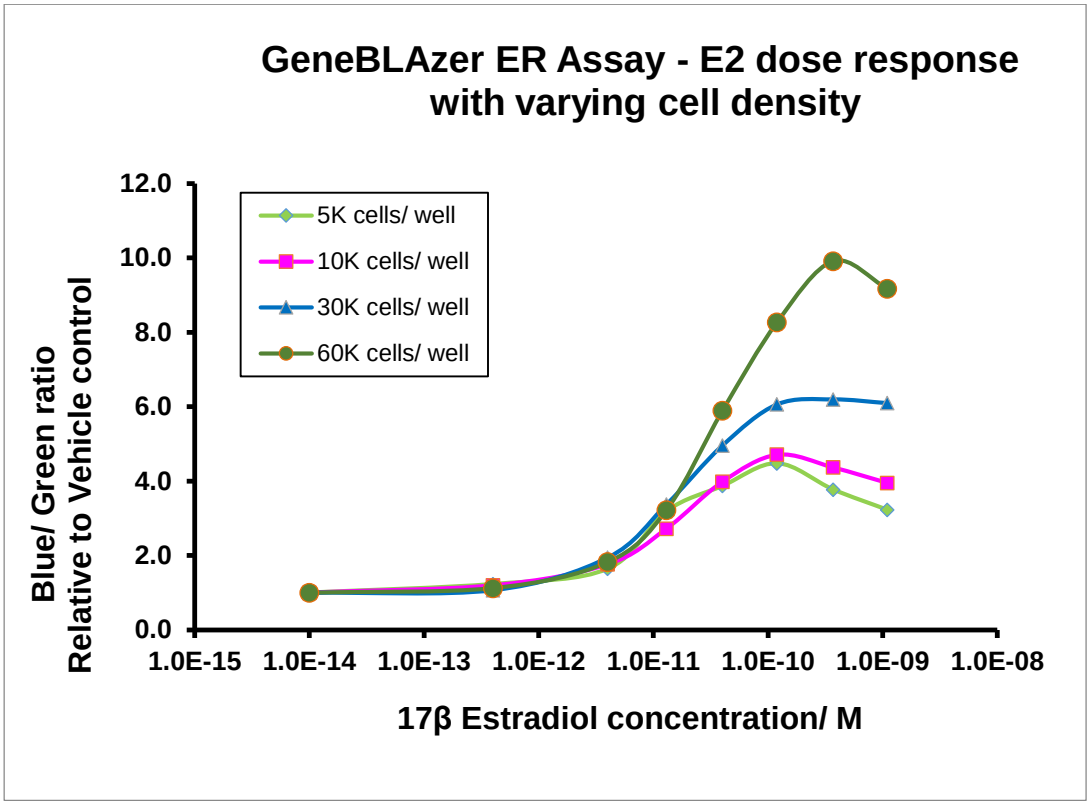

Fig. 4-2: Dose response (relative to vehicle control) of estrogen recepton (ERα) GripTite Division Arrested cells to 17β–estradiol (E2) at different seeding cell densities. Triplicate cells were plated in 96-well clear bottom plates and dosed with E2 for 24 h in the presence of 0.01% DMSO, loaded with LiveBLAzer™-FRET B/G substrate (2 h), and fluorescence emission was recorded at 460 and 530 nm. Data for 1.0E-14 M is for 0.01% DMSO.

Dose response experiments were also carried out on the GeneBLAzer ERα DA Assay with the weak ER agonists bisphenol A (BPA) and 4-octylphenol. The test was responsive to these chemicals, but as expected, at much higher concentrations (**Figure 4-3**).

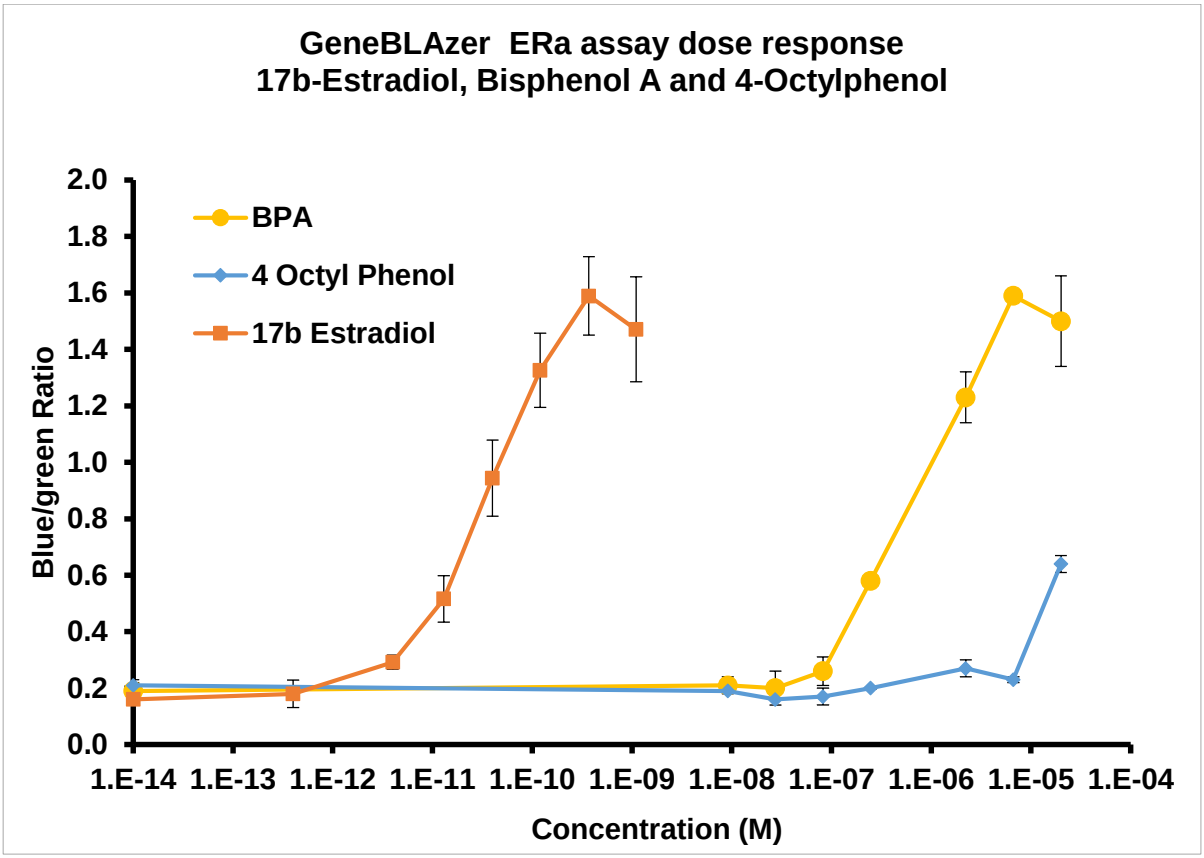

Fig. 4-3: Dose response (relative to solvent control) of ERα GripTite Division Arrested cells to the weak ER agonists bisphenol A (BPA) and 4-octylphenol. Cell plating density was 60,000 cells/well in the presence of 0.01% DMSO (n = 3 for each data point). The dose response data for 17β-estradiol was taken from earlier project results. Data at 1.0E-14 is for 0.01% DMSO.

Identification of reference toxicants for bioassays with adequate performance. Dose-response experiments were performed to determine the best reference toxicant for the Life Technologies progesterone receptor (PR) DA cell test kit (**Figure 4-4**). The results indicate that levonorgestrel, a component in human use pharmaceuticals, exhibited increased sensitivity compared to progesterone and trenbolone. A complete list of reference toxicants for the five successfully optimized bioassay endpoints is shown in **Table 4-2**.

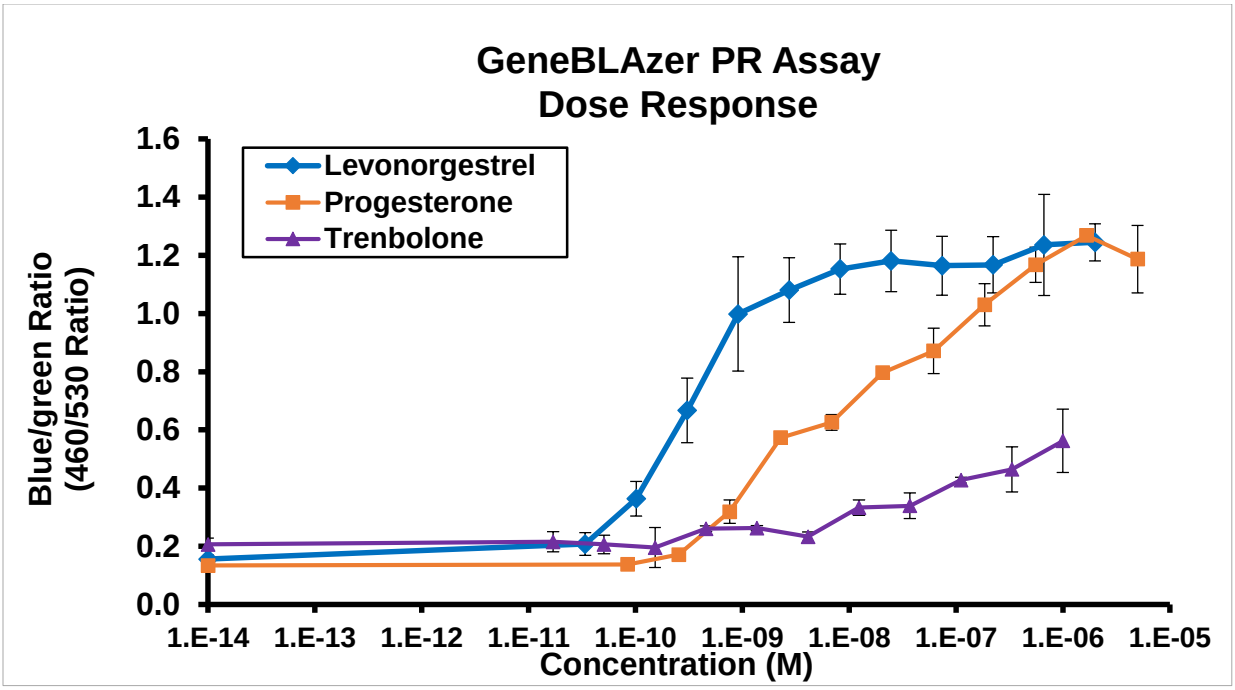

Figure 4-4. Dose response (relative to solvent control) of GeneBlazer progesterone receptor (PR) division arrested cells to candidate reference toxicants. Seeding cell density was 60 000 cells/well in the presence of 0.1% DMSO (n = 3 for each data point). Data for 1.0E-14 is for 0.1% DMSO.

Table 4-2. Reference toxicants for GeneBLAzer Division Arrested (DA) assays.

Assay	Reference Chemical	CAS #	Recommended Source	Part #
ERα	17β Estradiol (E2)	50-28-2	Sigma	E1024
AR	R1881 (Methyltrienolone)	965-93-5	Perkin-Elmer	NLP005005MG
GR	Dexamethasone	50-02-2	Sigma	D-4902
PR	Levonorgestrel	797-63-7	Sigma	N2260 Fluka
cytotoxicity	n/a	n/a	n/a	n/a

n/a – not applicable

Bioassays exhibiting inadequate performance. Dose-response experiments for the Life Technologies p53 and AhR CYP1A1 non-DA cell lines were performed using mitomycin, PCB-126 and benzo[a]pyrene as the reference toxicants. The p53 cell line showed a dose response to mitomycin and a similar response to seeding cell density observed with the ERα test kit (not shown). Although a dose-response was also observed for PCB-126 spiked with the AhR cell line, a corresponding response in control wells without DMSO was also observed. After consultation with technical representatives at Life Technologies did not resolve this issue, the

investigators abandoned the AhR CYP1A1 cell line from further consideration. The PPAR gamma 293H DA cells from Life Technologies were tested using gemfibrozil as the reference toxicant. The State's Science Advisory Panel for Recycled Water (SAP-RW) identified this compound as an indicator of treatment efficiency. Although this cell line performed well for the reference toxicant recommended by the vendor (Rosiglitazone) (data not shown), the assay was insensitive to gemfibrozil at concentrations expected to occur in recycled water (**Figure 4-5**). A second attempt to identify a bioassay that was sensitive to this indicator compound using the PPAR alpha cell test kit from Life Technologies was also unsuccessful. Thus, the investigators abandoned the PPAR cell line from further consideration.

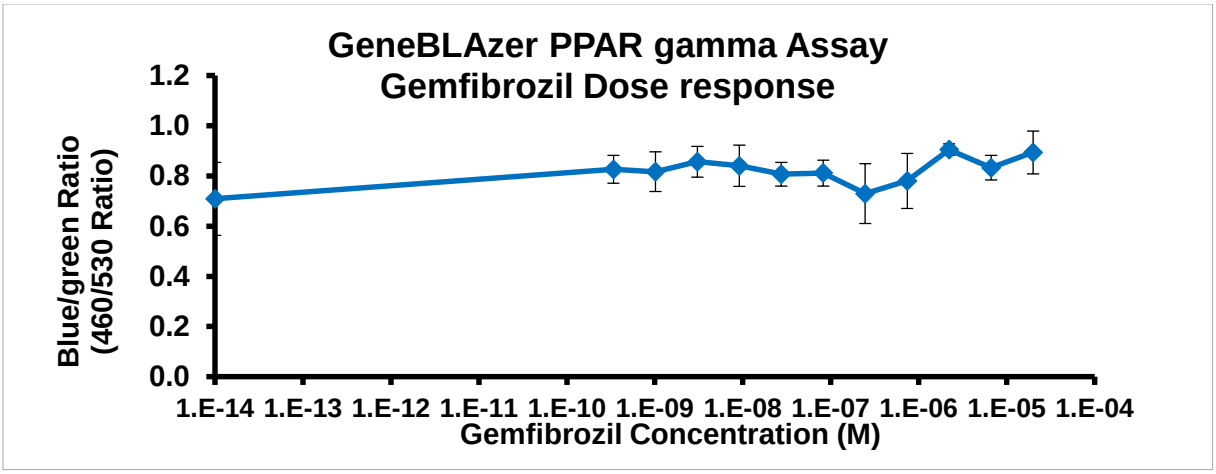

Figure 4-5. GeneBLAzer PPAR gamma 293H DA cells were stimulated with gemfibrozil for 16 h in 0.1% DMSO. Cells were incubated with LiveBLAzer™-FRET B/G Substrate (2 h) and fluorescence emission recorded at 460 and 530 nm. n = 4 per treatment.

A number of experiments were also conducted to identify a suitable assay for genotoxicity. Two suppliers were identified for the umuC-assay [Environmental Bio-detect Products, Inc. (www.biotoxicity.com) and Moltox, Inc. (www.moltox.com)], which is based on the use of a genetically modified *Salmonella typhimurium* strain TA 1535 that contains the plasmid pSK1002. The umuC gene (part of the SOS system) is fused in a reporter gene (lacZ) that encodes for ß-galactosidase. If genotoxicity induces the SOS function, the reporter gene is also activated and the formation of ß-galactosidase is quantified spectrophotometrically at 420 nm. The test is carried out with and without S9 rat liver cells, which metabolically activates mutagens that can damage DNA. Bacterial growth is measured as turbidity at 600 nm and biomass factors are considered in the test results. A reduction of cell growth by more than 50% is considered as a toxic effect and ß-galactosidase should not be evaluated for those wells. Reference genotoxicants were (−S9) 4-nitroquinoline -N-oxide (4NQO), (+S9) benzo[a]pyrene (BaP) and N-nitrosodimethylamine (NDMA). The latter compound was also identified by the SAP-RW as a chemical of interest for monitoring in recycled water applications. Using the Moltox product (**Figure 4-6**), the team found that concentrations of the selected reference toxicants that induced a measurable assay response were orders of magnitude higher than concentrations specified by the SAP-RW. As a result, the investigators abandoned umuC and other commercially available options (e.g. Ames test) from further consideration.

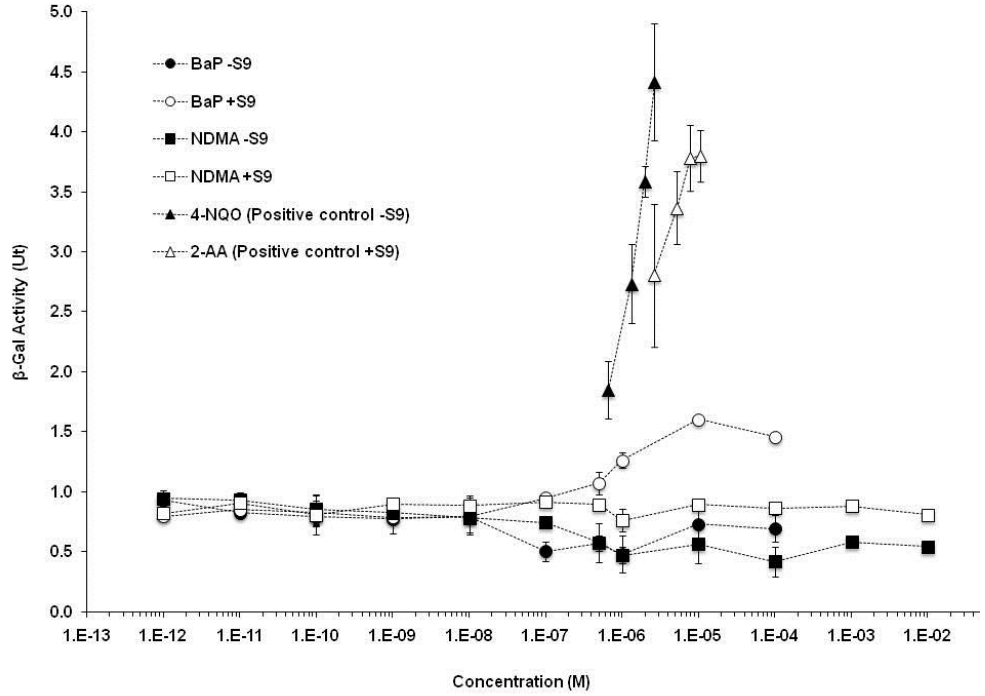

Fig. 4-6. Response of umuC assay supplied by Moltox, to benzo[a]pyrene (BaP), N-nitrosodimethylamine (NDMA) and 4-nitroquinoline -N-oxide (4NQO) with (+S9) and without (-S9) rat liver microsomes.

Standardized bioassay protocol. A draft bioassay protocol for 5 successfully optimized endpoints (ERα, AR, GR, PR, cytotoxicity) was submitted to SWB staff for comment. Based on feedback, the protocol was revised to include a list of materials and supplies. The final written protocol for the division arrested (DA) cell test kits obtained from Life Technologies Inc. is included as **Appendix 4.4**.

Results of the cross-project intercalibration exercise. The results of the interlaboratory bioassay comparison exercise, organized by Dr. Beate Escher (see also Task 6), is documented in the peer-reviewed article in *Environmental Science and Technology*. (**Appendix 4.5**). This publication describes in detail the results of the exercise where 20 academic, government and industry participants tested extracts of water samples representing a broad range of water quality using 100 individual bioassay endpoints. Excellent agreement among project team members for the ER and GR endpoints was achieved ($p<0.001$, $r2 = 0.81$ and 0.96, respectively; Table S4).

It is estimated that 100% of this task is completed.

Task 4 Deliverables

4.4. Written standardized bioassay protocols (Appendix 4.4)

4.5 Written results of laboratory intercalibration exercise (Appendix 4.5)

Task 5 – Data Interpretation Guidance

Data Analysis. The project team, in collaboration with Drs. Fred Leusch and Beate Escher of the WRRF10-07 project, created a spreadsheet data template to standardize data analysis and reporting for all five of the optimized bioassay endpoints (see **Appendix 5.1**). The project team identified two candidate approaches for quantifying assay output as bioassay equivalents (BEQs), a linear response and Hill ("S-curve") dose-response relationship. The team selected the Hill ("S-curve") dose-response relationship for quantifying bioassay output as BEQs, based on the data generated for the cross-project lab intercalibration exercise (see Deliverable 4.5 and Task 6).

Data Interpretation. The project team created a draft interpretive guidance document that addressed strategies and general guidelines for (1) analysis of in vitro bioassay results; (2) establishing science-based monitoring thresholds (considering uncertainty associated with bioassay and supporting toxicological data) and (3) making decisions based on the magnitude and persistence of bioassay results in exceedance of monitoring thresholds established in step (2) (**Appendix 5.4**). This framework was discussed at the second meeting of the Cross-Project Advisory Committee (CPAC), held at SCCWRP in January 2014. At that meeting, consensus was reached among the participants that the framework could and should be evaluated in the next phase of work on implementing bioassays for screening of recycled water quality, i.e. a pilot evaluation (**Appendix 6.2**).

Task 5 Deliverables

5.4 Written tiered framework for utilization of bioassay results (Appendix 5.4)

5.5 Written agenda for Stakeholder Workshop, held in conjunction with CPAC meeting #2 (Appendix 6.2)

Task 6 -- Project Coordination with WateReuse Research Foundation Project WRRF 10-07

Cross-project coordination. A project advisory committee (PAC) consisting of six (6) at large members was established to provide input on project directions (see **Appendix 6.1** for a list of names and affiliations). The purpose of the PAC was to provide guidance and perspective on the use of bioassays for screening of recycled water quality. Two face-to-face meetings of the PAC were planned, facilitated and hosted by the contractor in conjunction with the WateReuse Research Foundation (WRRF) Project 10-07, a multi-investigator project with overlapping and complementary objectives. The first Cross-project Advisory Committee (CPAC) Meeting was held at SCCWRP on Nov 10-11, 2011 (see **Appendix 6.2** for the final agenda). Critical agreements within the project and between the WRRF10-07 project were established, including an endorsement of the investigative team's approach by the PAC, and future collaboration on split water samples and common bioassay endpoints by advisors in attendance for both projects. Progress and agreements made at this meeting were memorialized on the project webpage.

The second Cross-project Advisory Committee (CPAC) meeting was also held at SCCWRP on Jan 23-24, 2014. Project summaries were presented by the CA and WRRF teams, sparking

discussion among the participants in attendance. Consensus was reached on a path forward, featuring a pilot evaluation of the estrogen receptor (ER) test by technology developers, industry labs/participants and commercial services representatives in a laboratory intercalibration exercise. The final agenda and presentations were posted on line at http://www.sccwrp.org/ResearchAreas/Contaminants/ContaminantsOfEmergingConcern/BioanalyticalScreeningToolsForCECs (see also Appendix 6.2).

Agreements were signed by team investigators to participate in a round robin organized by Dr. Beate Escher of the University of Queensland, managing principal investigator for the WRRF 10-07 project. Organic extracts of water and treated wastewater effluent samples were shipped to and received by members of the CA investigative team, for evaluation of the bioassay endpoints of interest. The results of this exercise were published in *Environmental Science and Technology* (Escher et al. 2014a; see Appendix 4.5). Release of the final WRRF10-07 project report is pending (Escher et al. 2014b).

It is estimated that 100% of this task has been completed.

Task 6 Deliverables

6.1 Project advisory committee (list of names and affiliations)(Appendix 6.1)

6.2 Final agendas for and powerpoint presentations from Cross-project advisory committee meetings #1 and #2 (Appendix 6.2)

Task 7 – Draft and Final Report

A draft final report generated based on the eleven previous progress reports was submitted for review by the SWB contract manager and stakeholder advisors of the PAC. Comments received within the allowable time were considered, and where warranted, revisions were made to the draft report. This submittal constitutes the final project report.

Deliverables

7.2 Draft project report
7.3 Final project report

It is estimated that 100% of this task has been completed.

CONTRACTUAL

Table 1. Percentage of work completed by Grantee per contract agreement (attached).

Task	Description	Percent Complete
1	Project Adminstration & Management	100
2	Literature Review	100
3	Develop Water Extraction Protocols	100
4	Evaluate Bioassay Performance	100
5	Provide Data Interpretation Guidance	100
6	Coordination with Related Projects	100
7	Draft and Final Project Reports	100

LITERATURE CITED

Anderson P, Denslow N, Drewes JE, Olivieri A, Schlenk D, Snyder S. 2010. Monitoring Strategies for Chemicals of Emerging Concern (CECs) in Recycled Water: Final Report. Sacramento, CA.

Escher BI et al. 2014a. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. *Environ Sci Technol* 48:1940-1956.

Escher BI, Tang J, Poulsen A, Leusch F, Snyder S, Jia A. 2014b. Development of bio-analytical techniques to assess the potential human health impacts of recycled water. Final Report: WRRF-10-07, WateReuse Research Foundation, Alexandria, VA.

APPENDIX 3.1 – WATER EXTRACTION PROTOCOL FOR IN VITRO SCREENING

Extraction SOP for Bioassays

1. Cartridge Conditioning

   Condition cartridges separately. To condition, pass 2x 5mL 50:50 acetone:hexane, followed by 2x 5mL methanol, followed by 2x 5mL HPLC grade water. Let the solvent percolate by gravity; do not apply vacuum during the conditioning stage. It is important that the sorbent bed remain wet at all times once conditioning starts, so never allow solvent level to drop below the top frit.

Fig.1. Conditioning Oasis HLB cartridge (Oasis HLB 6cc 500mg, cat no. 186000115)

Fig.2. Conditioning Supelco coconut charcoal (Supelclean Coconut charcoal 6cc 2g, cat no. 57144-U)

2. Sample Loading

   Once conditioned, stack Oasis HLB cartridge (Oasis HLB 6cc 500mg cat no 186000115) on top of a Supelco coconut charcoal (Supelclean Coconut charcoal 6cc 2g cat no 57144-U). Ensure a tight seal. Fit tube from the top of the Oasis HLB to the sample bottle to be extracted. Once connected, apply gently vaccum to draw sample through the cartridges drop wise (aim for 10 mL/min). Vacuum may need to be increased over time to compensate for cartridge loading. Ensure that the sorbent bed remains wet at all time, ie ensure there is a small amount of water on top of the top frit of each cartridge. If not, stop that cartridge, refill reservoir with ultrapure water, and reconnect (ensuring a tight seal ... if the seal is tight from the start, you won't have this problem).

Fig.3. Sample extraction

3. Dry Cartridges

   Once all of sample has passed through both cartridges, disconnect tubing and apply vacuum for 2 hours to roughly dry sorbent bed

4. Elution

   Once dry, elute each cartridge with 2x 5mL methanol followed by 2x 5mL acetone:hexane. Collect the solvent with a large glass test tube (40ml vial). Allow the solvent to percolate with gravity first, then apply vacuum to draw the remainder into the test tube.

Fig.4. Coconut charcoal cartridge elution

Fig.4. HLB cartridge elution

5. Evaporation

   Blow down to dryness and reconstitute in 1ml of MeOH.

Fig.5. Evaporation

APPENDIX 3.2 – QUALITY ASSURANCE PROJECT PLAN (QAPP)

Quality Assurance Project Plan (QAPP)

for

Development of Bioanalytical Techniques for Monitoring of Constituents/Chemicals of Emerging Concern (CECs) in Recycled Water Applications for the State of California

AGREEMENT NO.: 10-096-250

Submitted to

California Water Resources Control Board
Division of Water Quality
1001 I Street, 15th Floor
Sacramento, CA 95819

Prepared by

Keith A. Maruya
Southern California Coastal Water Research Project
3535 Harbor Boulevard Suite 110
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June 2014

Agreement Signatures:

Name (print)	Role – Affiliation	Agreement Signature	Date
Keith Maruya	SCCWRP Project Director		7/5/12
Melenee Emanuel	State Water Board Contract Manager		7/30/12

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LIST OF ACRONYMS

CCC	Continuing calibration check
CDPH	State of California Department of Public Health
CEC	Constituent/chemical/contaminant of emerging concern
DCM	dichloromethane
DEET	N,N-Diethyl-meta-toluamide
EC50	half maximal effective concentration
ESI	electrospray ionization
GC-MS/MS	Gas chromatography – tandem mass spectrometry
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LFB	Laboratory fortified blank
MDL	Method detection limit
MeOH	methanol
MRM	Multiple reaction monitoring
MTBE	methyl-tert butyl ether
NaN3	Sodium azide
NDMA	N-Nitrosodimethylamine
QA/QC	Quality assurance/quality control
QAPP	Quality assurance project plan
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
RPD	Relative Percent Difference
RSD	Relative Standard Deviation
S/N	Signal to noise ratio
SAP	Science Advisory Panel
SCCWRP	Southern California Coastal Water Research Project
SOP	Standard operating procedure
SPE	Solid-phase extraction
SWAMP	Surface Water Ambient Monitoring Program
SWRCB	State of California Water Resources Control Board (“State Water Board”)
TBD	to be determined
TDCPP	tris(1,3-dichloro-2-propyl)phosphate
TCPP	tris (1-chloro-2-propyl) phosphate
TCEP	tris(2-carboxyethyl)phosphine
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
WRF	WateReuse Research Foundation

1.0 PROJECT DESCRIPTION AND OBJECTIVES

1.1 Background

On February 3, 2009, the State Water Board adopted the Recycled Water Policy (Policy). As regulatory requirements for recycled water must be based on the best available peer-reviewed science, it was deemed necessary to convene a Science Advisory Panel (SAP) to determine the current state of scientific knowledge regarding the risks of Constituents/Chemicals of Emerging Concern (CECs) to the public health and the environment and to make recommendations to ensure all uses of recycled water meet California Department of Public Health (CDPH) conditions. As part of their final recommendations released on June 25, 2010, the SAP endorsed the development of bioanalytical techniques (or "bioassays") to address both known and unknown CECs and to develop appropriate trigger levels for these techniques that correspond to a response posing a concern from a human health standpoint.

1.2 Objectives

The Southern California Coastal Water Research Project (SCCWRP) was selected to direct and manage a team of investigators to develop bioanalytical techniques for monitoring of CECs in recycled water applications in the State of California. SCCWRP will coordinate the team's activities with related parallel efforts, including an on-going WateReuse Research Foundation project (WRF 10-07). Successfully developed techniques will be used as part of an overall monitoring strategy to screen for CECs that may occur in recycled water and that are cause for concern in maintaining public health and confidence in this resource. The project will address the recommendations of the State's Science Advisory Panel (SAP) for CECs, established as a requirement of the Recycled Water Policy, regarding the development of appropriate trigger levels for bioanalytical screening techniques that correspond to a response posing a concern from a human health standpoint. SCCWRP has assembled an investigative team consisting of experts in bioanalytical techniques from the University of California - Riverside, University of Arizona, University of Florida, and the University of South Florida.

1.3 Scope

This Quality Assurance Project Plan (QAPP) outlines the quality assurance/quality control (QA/QC) program that will be followed by the project team during sample collection, transportation, testing, and data reporting to ensure that accurate, precise, and nonbiased data are produced using chemical and bioassays described herein. The QA/QC program includes the analysis of method blanks, calibration curves, replicates, laboratory-fortified samples, and the performance of method detection limit (MDL) studies. The study may require detection of analytes near the lower limits of what is analytically feasible. As concentrations approach these lower limits, analytical uncertainties increase. Detection within complex matrices, such as wastewater effluents is required. The project team will develop and/or improve

analytical methods capable of achieving MDLs appropriate to each analyte targeted in this study. The data obtained and screened via the QA/QC guidelines specified herein are intended to support the development and evaluation of the candidate bioassays, and are not intended for use to assess compliance to water quality standards or regulations.

2.0 PROJECT ORGANIZATION AND RESPONSIBILITIES

Dr. Keith Maruya of the Southern California Coastal Water Research Project (SCCWRP) ("Contractor") is the Project Director, and is responsible for the overall conduct of project tasks, including adherence in good faith to the guidelines and criteria set forth herein.

Drs. Nancy Denslow, Daniel Schlenk, Shane Snyder and Sandy Westerheide are co-Principal Investigators representing their home institutions ("Subcontractors") and are responsible for supporting the various project tasks dealing with developing and conducting chemical and bioanalytical analyses, in accordance with the guidelines and criteria set forth herein.

Ms. Melenee Emanuel is the project Contract Manager for the State Water Board.

Ms. Carolyn Brookshire is the project Contracts contact for the State Water Board.

3.0 METHODS

3.1 Water Sample Collection and Preservation

All samples will be collected in 1L amber glass bottles, which have previously been washed with MeOH, followed by DI water, and then heated in a 550^{o}C furnace overnight. Sodium azide at 1 g/L will be added to avoid microbial degradation immediately after the water samples are collected. Additionally, sodium thiosulfate (50 mg/L) will be added as well to quench residual chlorine if the samples are related to any chlorine/chloramine treatment. Alternatively, sodium azide and/or sodium thiosulfate may be added to sampling bottles in advance.

The samples will be placed in coolers during transport with ice packs to minimize light contact and high temperatures. Samples will be refrigerated at holding site (lab) at 4°C while awaiting shipping. After they arrive, samples will be filtered with a glass microfiber filter (0.45um), and the SPE procedure will be conducted within 48 hours.

3.2 Sample Preparation

HPLC grade solvents and Milli-Q water will be used for SPE. Field blank (replicates) will be conducted per sampling event using the same procedure as actual sample, substituting deionized (DI) water in place of the actual water sample. Absolute recovery experiment for target compounds in both DI water and real water samples

will be tested during method validation by spiking the analytical standards to water samples in triplicate. To ensure the method effectiveness, including bioassay, at least one positive control compound (aka reference toxicant) will be spiked to the sample matrix and the field blank to check both sample preparation and bioassay performance. Laboratory fortified reagent blanks will also be included in each extraction batch to evaluate performance of the entire analytical system, including all sample preparation and instrumental analysis steps.

3.2.1 Materials

All standards and reagents used will be of the highest purity commercially available. All pharmaceuticals used as reference or quantitation standards will be obtained from Sigma–Aldrich (St. Louis, MO, USA), Toronto Research Chemicals (Ontario, Canada) and from C/D/N Isotopes (Pointe-Claire, Canada). All solvents will be trace analysis grade from Burdick and Jackson (Muskegon, MI). Reagent water will be obtained using a Milli-Q Ultrapure Water Purification System (Millipore, Bedford, MA, USA). Concentrated stocks and standards will be prepared in MeOH and stored at −20 °C while spiking solutions will be stored at 4 °C.

3.2.2 Sample Extraction

For LC-MS/MS analytes, samples received in 1 L amber glass bottles will be spiked with isotopically labeled compounds (surrogates) prior to extraction with an Autotrace® system (Caliper Corporation, Hopkingon, MA) equipped with 5 mL, 200 mg HLB glass cartridges (Waters Oasis, Millford, MA). The SPE cartridges will be first conditioned sequentially with methyl-tert butyl ether (MTBE), methanol (MeOH), and LC/MS grade water. After conditioning, the samples will be loaded onto the SPE cartridges. After sample loading the SPE cartridges will be rinsed with reagent water and dried with nitrogen for 30 min. Analytes will be eluted with a combination of MTBE and MeOH into 15 mL calibrated centrifuge tubes. The extract will be concentrated with a gentle stream of nitrogen to a final volume of 1000 µL and then transferred to an autosampler vial for LC–MS/MS analysis (Vanderford and Snyder, 2006).

3.2.2.1. Sample Extraction for NDMA. Nitrosamines will be extracted from 500 ml of sample by passing a water sample through a solid phase extraction (SPE) cartridge containing 2 g of 80-120 mesh coconut charcoal per EPA 521. The cartridges will be conditioned with dichloromethane (DCM), MeOH and HPLC water prior to extraction. The analyte compounds will be eluted from the solid phase with DCM. The eluent will then be dried under gentle nitrogen stream for concentration followed by mass spectrometer analysis.

3.3 Instrumental Analysis

Identification and quantitation of the analytes listed in **Table 3-1** will be performed

by isotope dilution LC-MS/MS, GC-MS/MS and various other instrumental techniques described herein. HPLC fractionation may be further applied in order to isolate bioactive substances from the complex mixture. Recovery test for target compounds as well as field blanks and possible controls will be conducted using the appropriate detection method. Since the compounds of interest generally occur in the environment at concentrations below 100 ng/L, extensive care will be taken to prevent accidental contamination by sampling and laboratory personnel. Corrective action will be taken if laboratory fortified blank results do not meet established criteria. Principal component analysis, Venn diagram, as well as significance test will be conducted to compare different samples or samples and blanks.

Table 3-1. List of candidate analytes and estimated method detection limits (MDLs).

Analyte	Method	MDL (ng/L)
17a-ethinyl estradiol	LC-MS/MS (ESI+)	5.0
17b-estradiol	LC-MS/MS (ESI+)	0.5
Acetaminophen	LC-MS/MS (ESI+)	1.0
Atorvastatin	LC-MS/MS (ESI+)	1.0
Atrazine	LC-MS/MS (ESI+)	1.0
Bisphenol A	LC-MS/MS (ESI-)	20
Caffeine	LC-MS/MS (ESI+)	1.0
Carbamazepine	LC-MS/MS (ESI+)	1.0
DEET	LC-MS/MS (ESI+)	1.0
Dexamethasone	LC-MS/MS (ESI+)	1.0
Diazepam	LC-MS/MS (ESI+)	1.0
Diclofenac	LC-MS/MS (ESI-)	1.0
Dilantin (phenytoin)	LC-MS/MS (ESI+)	1.0
Dilitiazem	LC-MS/MS (ESI+)	1.0
Diphenhydramine	LC-MS/MS (ESI+)	1.0
Estrone	LC-MS/MS (ESI+)	1.0
Fluoxetine	LC-MS/MS (ESI+)	1.0
Galaxolide	GC-MS/MS	1000
Gemfibrozil	LC-MS/MS (ESI-)	1.0
Hydracortisone	LC-MS/MS (ESI+)	1.0
Iopromide	ICP-MS	1.0
Ibuprofen	LC-MS/MS (ESI-)	20
Meprobamate	LC-MS/MS (ESI+)	1.0
NDMA	GC-MS/MS	1.0
Naproxen	LC-MS/MS (ESI-)	1.0
Nonylphenol	LC-MS/MS (ESI-)	5.0
Norethistrone	LC-MS/MS (ESI+)	1.0
Norgestrel	LC-MS/MS (ESI+)	1.0

Table 3.1 (cont.)		
Octylphenol	LC-MS/MS (ESI-)	5.0
Prednisone	LC-MS/MS (ESI+)	20
Primidone	LC-MS/MS (ESI+)	5.0
Progesterone	LC-MS/MS (ESI+)	1.0
PFBA	LC-MS/MS (ESI-)	1.0
PFBS	LC-MS/MS (ESI-)	1.0
PFDA	LC-MS/MS (ESI-)	1.0
PFOA	LC-MS/MS (ESI-)	3.0
PFOS	LC-MS/MS (ESI-)	1.0
Simazine	LC-MS/MS (ESI+)	1.0
Sucralose	LC-MS/MS (ESI-)	5.0
Sulfamethoxazole	LC-MS/MS (ESI+)	1.0
TCDPP	LC-MS/MS (ESI+)	2.5
TCEP	LC-MS/MS (ESI+)	2.5
TCPP	LC-MS/MS (ESI+)	2.5
Testosterone	LC-MS/MS (ESI+)	1.0
Triclocarban	LC-MS/MS (ESI-)	1.0
Triclosan	LC-MS/MS (ESI-)	5.0
Trimethoprim	LC-MS/MS (ESI+)	1.0

3.3.1 LC-MS/MS. Analysis of CECs will be performed with an Agilent 6460 triple quadrupole LC-MS/MS (Agilent Technologies, Foster City, CA, USA) using electrospray ionization (ESI) in positive and negative multiple reaction monitoring (MRM) mode. Examples of mass spectrometer parameters and transitions are provided in Tables A3-1 and A3-2.

Liquid chromatographic separation will be performed with an Agilent ZORBAX Eclipse Column C-18 Rapid Resolution HD, 1200 bar set at 50°C (Agilent Technologies, Foster City, CA, USA). A binary solvent gradient consisting of water (A) and MeOH (B) (containing 0.1% acetic acid for negative mode) will be used for HPLC separation. The solvent gradient will be initialized at a flow rate of 0.4 mL/min with 30% of B and will be linearly increased to 100% with injection volume of 1 µL. Quality control samples at high, low and medium (random) concentrations will be included every 10 samples to ensure the integrity of mass spectrometric analysis. The data will be processed with MassHunter Quantitative Analysis B.04.00, with calibration curves ranging from 10 to 1000 µg/L and resulting correlation coefficients (R^2) shall be greater than 0.98. Instrument limits of detection will be determined by the lowest standard in calibration curve and had a signal to noise ratio of at least >3.

3.3.2 LC-FLD/DAD. The analysis of octylphenyl and nonylphenol will be performed on a Agilent 1290 Infinity LC/DAD/FLD by monitoring the elution of a 1µL injection of 4-nonylphenol and octylphenol at an excitation wavelength of 226 nm

and emission at 305 nm in a 10 min gradient run starting with 30% (A) water amended with 10% MeOH and (B) 70% MeOH. The MDL will be based on the lowest concentration of calibration standard exhibiting a signal to noise ratio (S/N) greater than 3 for both nonylphenol and octylphenol.

3.3.3. ICP-MS. An Agilent 7700x ICP-MS will be used for detection of iopromide under typical hot plasma conditions for elemental analysis. The eluent from the IC column and the 1.0 µg/L cesium standard will be mixed in a simple block mixer and introduced directly into the spray chamber of the ICP-MS. The cesium standard for signal correction will be added online via the Agilent peripump at an equivalent 1/20th total volume. The IC eluent program and the ICP-MS signal acquisition steps will be synchronized via unidirectional signal relays from the IC to the ICP-MS, and the time resolved signal will be converted into chromatographic data using Agilent's Plasmachrom software. The MDL for iopromide will be based on S/N of at least >3.

3.3.4 GC-MS/MS. The Agilent 7000 mass spectrometer in conjunction with the Agilent GC 7890 equipped with a 30m × 0.25 mm × 0.25 m DB-5MS capillary column will be used for analysis of galaxolide in MeOH sample extracts. Helium will be used as carrier at a constant flow rate of 1.0 mL/min and 1µL sample will be injected in splitless mode at 280 ◦C. After a holding time of 0.5 min, the GC oven will be programmed as follows: 70 ◦C to 180 ◦C at a rate of 20 ◦C/min; increase to 220 ◦C at 2 ◦C/min; increase to 280 ◦C at 30 ◦C/min, and a final hold time of 2 min. The MS/MS analysis will be carried out in electron impact (EI) in the MRM mode. The ion source will be operated at 200 ◦C with electron energy of 70 eV and a filament current of 200 µA. The MRM transitions will be 258 for the precursor and 243 as the quantifying transition and 243->143 as the qualifier. The temperature of the interface and source will be set at 200◦C and 250◦C, respectively. Nitrogen will be the collision gas and the pressure of the chamber maintained at 3.5 × 10−3 mbar. The electron multiplier will be set at 650 V. The MDL will be established using standards in MeOH with a S/N >3.

3.3.4.1 GC-MS/MS analysis for NDMA. The Agilent 7000 mass spectrometer in conjunction with the Agilent GC 7890 and equipped with a 30m × 0.25 mm × 0.25 m DB-WAXTER column will be employed for the analysis of nitrosodimethylamine (NDMA) in sample extracts reconstituted in DCM. Helium will be used as carrier at a constant flow rate of 1.7 mL/min, and 5 µL of sample extract will be injected in splitless mode at 200 °C. The Injection temperature will be 200 °C and the oven will be programmed as follows: 40 °C for 3 min, increase of 10 °C/min to 110 °C, increase to to 200 °C at 15 °C/min, and a final increase to 240 °C at 40 °C/min. The MS/MS analysis will be carried out in chemical ionization mode using ammonia (CI) using the MRM mode. The ion source will be operated at 200 ◦C with electron energy of 70 eV and a filament current of 200 µA. The MRM transitions are provided in Table A3-3. Nitrosodimethylamine-d6 (NDMA-d6) and N-Nitrosodi-n-propylamine-d14 (NDPA-d4) will be employed as the surrogate and internal standards for method and instrument

correction. The MDL will be based on S/N ratios of >3 for the lowest detectable calibration standard.

3.4 Bioassays

Five individual endpoints deemed relevant to CECs in recycled water and for their potential impacts to human health were evaluated and optimized in this project (**Table 3-2**). Detailed methods for each of the bioassays targeting each of the project endpoints are documented in the project final report.

4.0 QUALITY ASSURANCE/QUALITY CONTROL GUIDELINES

4.1 Instrumental Analysis

The data quality objectives listed in **Table 4-1** will be applied to the analysis of target CECs (see Table 3-1) in project water samples and QA/QC samples using the instrumental methods described in section 3.3.3, including but not limited to:

i. Reagent, laboratory procedural and field blanks

ii. laboratory fortified matrix spike samples that are representative of the matrix (recycled water) in question

iii. sample duplicates

4.1.1 Determination of method detection limit (MDL)

The determination of method detection limits (MDLs) shall be based on the statistical significance of 7 replicate standards whose concentration is roughly 3 times larger than the anticipated MDL (S/N ratio>3). The MDLs will be based on the peak-to-peak noise of the baseline near the analyte peak obtained by analyzing field samples and on a minimal value of signal-to-noise of 3. To fortify samples for MDL determination, analytes will be spiked at concentrations ranging between 20 to 200 ng/L. Analyses for this procedure should be done over at least three days. All reagents (including sample preservatives) must be added to the samples. The MDL shall be calculated as follows:

Table 3-2. Endpoints and candidate bioassays targeted for this project.

ENDPOINT	CANDIDATE BIOASSAY	REFERENCE TOXICANT(S)	MINIMUM THRESHOLD (ng/L)
estrogenicity	estrogen receptor reporter (ER)	17β-estradiol	0.5
androgenicity	androgen receptor reporter (AR)	Dihydrotestoterone (DHT)	20
progesterone activity	progesterone receptor reporter (PR)	levonorgestrel	50
glucocorticoid activity	glucocorticoid receptor reporter (GR)	dexamethasone	50
cytotoxicity	Presto Blue[#]	n/a[#]	n/a
n/a – not applicable			
[#] sample extracts of the highest concentration are analyzed			

Table 4-1. Data quality objectives (DQOs) for instrumental analysis of target CECs

MEASUREMENT	FREQUENCY	CONTROL LIMIT
Initial calibration		5 point minimum over the range of concentrations anticipated in project water samples. The correlation coefficient (R^2) for calibration shall be ≥ 0.98.
Sample batch		A maximum of 12 water samples, not including QA/QC samples
Calibration verification	1/batch	Performed every 12 sample injections or 12 hours of operation, whichever is shorter. Relative percent difference (RPD) compared to initial calibration < 25% for 80% of analytes
Reagent, laboratory and Field Blanks	1/batch	< MDL for all target analytes
Sample duplicate	1/batch	RPD < 50% for all target analytes >MDL
Lab Fortified Blank	1/batch	30-150% recovery of spike
Surrogate spikes	1/sample	30% < surrogate recovery < 150%

Calculate the DL using the following equation:

$$DL = s \times t_{(n-1,1-\alpha=0.99)}$$

where

$t_{(n-1,1-\alpha=0.99)}$ = Student's t value for the 99% confidence level with n-1 degrees of freedom (for seven replicate determinations, the Student's t value is 3.143 at a 99% confidence level),

n = number of replicates, and

s = standard deviation of replicate analyses.

Note that blank values are subtracted when performing DL calculations.

4.1.2 Initial and Continuing Calibration.

A minimum of 5 solutions across the expected range of analyte concentrations shall be prepared and analyzed for initial calibration of each instrument. The resulting calibration curve shall meet the guidelines specified in Table 4-1. A continuing calibration check (CCC) standard shall be analyzed periodically in accordance with the guidelines specified in Table 4-1.

4.1.3 Laboratory Fortified Blank (LFB)

An LFB is required with each extraction batch. The concentration of the LFB shall be rotated between low, medium, and high concentrations. The low concentration LFB shall be near to but no more than two times the MDL. Similarly, the high concentration LFB will be near the high end of the calibration range established during the initial calibration. Recoveries for samples fortified at all concentrations must be within the range specified in Table 4-1. If the LFB results do not meet these criteria, then all data for the analyte(s) in question shall be considered invalid for all samples in the batch, and investigative measures shall be initiatied.

4.1.4 Surrogate Recovery

Surrogate recovery compounds will be fortified into all field samples and blanks prior to extraction. Surrogates will also be added to standard solutions prepared for instrument calibration. The percent recovery for each surrogate shall be as specified in Table 4-1. If a surrogate fails to meet this criterion, the following checks will be conducted to rectify the out-of-spec condition:

1. errors in calculation
2. evidence of degradation or contamination in standard solutions
3. stability and performance of instrument

If none of the above are found to be the cause for the out-of-spec condition, the extract(s) in question will be reanalyzed. If the repeat analysis meets the surrogate recovery criterion, the report will contain only data for the reanalyzed extract.
If the extract reanalysis fails once again, a CCC standard will be analyzed to verify temporal stability of instrument performance. If this check fails the specified criteria, the instrument shall be recalibrated. If the CCC is acceptable, extraction of the sample should be repeated provided a sample is available and still within the holding time. If the re-extracted sample also fails the recovery criterion, all data associated with the sample in question shall be reported as “suspect/surrogate recovery.”

4.2 Bioanalytical Testing

4.2.1 Sensitivity

Each of the candidate bioassays shall exhibit a minimum threshold response to a

bioassay specific reference toxicant as specified in **Table 4-2**.

Table 4-2.
Table 4-2. Data quality objectives (DQOs) for performance of in vitro bioassays.

MEASUREMENT	FREQUENCY	CONTROL LIMIT
Sensitivity	1/plate	minimum threshold concentration[#]
Dose Response	1/plate	demonstration of dose response; EC50 for reference toxicant within 20% of historic value
Replication & Precision	1/plate	20%RSD among triplicate well determinations for all samples, including QA/QC samples.
Method, Field Blank	1/plate	< minimum threshold concentration[#]
Cell Viability	1 test/plate	90% cell viability

[#] specified in Table 3-2.

4.2.2 Dose Response

Each candidate bioassay shall exhibit a dose-effect response upon testing with a minimum of 5 dilutions of the bioassay specific reference toxicant at a frequency specified in Table 4-2. The evaluation of dose response may include concentrations of the reference toxicant that are lower than the specified minimum threshold (see also 4.2.1) and shall be sufficient to estimate the half maximal effective concentration (EC50). Each dose-response curve for the reference toxicant shall be recorded in control charts maintained locally for each assay type for comparison across time and performing entities.

4.2.3 Replication and Precision

Extracts representing individual water or QA/QC samples (e.g. method blanks, matrix spikes) shall be run in replicate wells per bioassay (plate) as specified in Table 4-2. For example, each plate (e.g. in 96 well format) must have the specified number of replicate wells dedicated to each sample, solvent blank, negative control, and positive control (or reference toxicant). The relative standard deviation (RSD) among replicate measurements shall not exceed that specified in Table 4-2.

4.2.4 Method and Field Blanks

The average contribution of method and field blanks shall not exceed the percentage of the corresponding minimum threshold response specified in Table 4-2. Method blanks shall include those that isolate test solvents, media/reagents, media/reagents and test cells. Field blanks shall include sample collection equipment and travel blanks. Care shall be undertaken by personnel performing sample collection and bioassays to avoid contamination of samples, laboratory and field equipment and supplies.

4.2.5 Cell Viability and Toxicity

The cells used in each bioassay shall be tested for health/activity ("viability") by visual inspection followed by a test of cytotoxicity prior to or in parallel with the conduct of testing of field and/or QA/QC samples to ensure that a lack of response is not due to low cell viability. The minimum viability for cells shall be as specified in Table 4-2.

4.2.6 Test Conditions

The participating labs performing bioassays shall possess capital equipment and facilities necessary to control the ambient environment to promote cell growth and minimize external biological contamination of test cells, media, reagents and water and QA/QC sample extracts. As a minimum, labs shall possess a laminar flow, humidified environment with controlled temperature (37 $\pm$ 2^{o}C) and UV-sterilized 5% CO2/95% air.

4.2.7 Equipment Checkout, Calibration and Maintenance.

All equipment used in the performance of bioassays, including electronic pipettes, balances for weighing reagents/standards, and plate readers shall be tested for proper performance prior to each day. Instruments such as micro-balances that require periodic calibration shall be re-calibrated once a year.

5.0 DOCUMENTATION AND REPORTING

5.1 Documentation

Chemical and bioanalytical results will be compiled in electronic format, complying with State environmental data reporting formats, including the Surface Water Ambient Monitoring Program (SWAMP) , where applicable.

5.2 Reporting

The results of all chemical and bioanalytical analyses will be made available in interim and final project reports as specified under Agreement No. 10-096-250. Standard operating procedures (SOPs) for bioassays who performance is deemed acceptable by the project investigators will be submitted as part of the project final report.

References

Vanderford, B.J., Snyder, S.A., 2006. Analysis of pharmaceuticals in water by isotope dilution liquid chromatography/tandem mass spectrometry. Environ. Sci. Technol. 40, 7312-7320.

APPENDICES

Table A3-1. Transitions for Multiple Reaction Monitoring (MRM) experiments in Electrospray Positive Mode.

Compound Name	Precursor Ion	Product Ion	Fragmentor	Collision Energy	Cell Accelerator (V)	IDL (µg/L)
Acetaminophen	152	110	100	15	7	12
Atorvastatin	559.2	466	145	15	7	16
Atrazine	218	176	140	15	7	13
Caffeine	195.1	110.1	104	24	7	12
Carbamezapine	237	194	120	15	7	12
DEET	192	119	110	15	7	16
Fluoxetine	310	148	90	5	7	14
Simazine	202.1	132	72	16	7	12
Sulfamethoxazole	254	156	80	10	7	17
Testosterone	289	109	115	25	7	12
Trimethoprim	291	261	75	25	7	13

Table A3-2. Transitions for Multiple Reaction Monitoring (MRM) experiments in Electrospray Negative Mode

Compound Name	Precursor Ion	Product Ion	Fragmentor	Collision Energy	Cell Accelerator (V)	IDL (µg/L)
Diclofenac	294	250	75	4	7	11
Gemfibrozil	249.2	121	75	6	7	11
Ibuprofen	205	161	50	0	7	59
Naproxene	229	170	55	4	7	65
PFBA	213	169	60	0	7	11
PFBS	298.8	98.9	133	29	7	11
PFOA	412.9	368.9	86	5	7	11
PFOS	498.9	99	210	50	7	59

Table A3-3. NDMA Target and Qualifier ions by GC-MS/MS.

Compound	Abbreviation	Exact Molecular Mass (g/mol)	Monitoring ions (m/z)	
			Target [M+18]+	Qualifier [M+1]+
N-Nitrosodimethylamine	NDMA	74.048	92	75
N-Nitrosodimethylamine-d6	NDMA-d6	80.086	98	81
N-Nitrosodi-n-propylamine-d14	NDPA-d14	144.198	162	145

Note: Ammonia CI gas supplied the following ions for reaction with target analytes $[NH_4]^+$, $[NH_4\text{-}NH_3]^{+,}$ and $[NH_4\text{-}(NH_3)_2]^+$ ions with mass over charge (m/z) values of 18, 35 and 52, respectively.

APPENDIX 4.4 – STANDARDIZED BIOASSAY PROTOCOL

GENEBLAZER DIVISION ARRESTED CELL ASSAY AND PRESTOBLUE CYTOTOXICITY ASSAY (*INVITROGEN*)

Cytotoxicity general description: PrestoBlue™ Cell Viability Reagent is a cell permeable resazurin-based solution used to quantitatively measure cell viability and cytotoxicity. Metabolically active cells reduce the reagent and turn red, becoming highly fluorecent.

Protocol overview: This assay uses ERα and PR division-arrested cells. On day one, the cells are thawed, plated, and compounds are added (same as GeneBLAzer assay protocol). On day two, PrestoBlue is added, the plate is incubated and then read on a fluorescent plate reader.

- - - - - - - - - - - - - - -

GeneBLAzer general description: Mammalian one-hybrid system consisting of mammalian (HEK293) cells stably transfected with 5X UAS β-lactamase and a chimera consisting of the GAL4 DNA binding domain fused to the ligand-binding domains of the human soluble receptors. The reporter gene product is β-lactamase (bla). Cells are loaded with an engineered fluorescent substrate containing two fluoroprobes, coumarin and fluorescein. In the absence of bla expression, the substrate molecule remains intact. In this state, excitation of the coumarin results in fluorescence resonance energy transfer to the fluorescein moiety and emission of green light. In the presence of bla expression, the substrate is cleaved, separating the fluorophores, and disrupting energy transfer. Excitation of the coumarin in the presence of enzyme bla activity results in a blue fluorescence signal. The resulting blue:green ratio provides a normalized reporter response.

Protocol overview: This assay comes as a kit and uses division-arrested cells. On day one, the cells are thawed, plated, and compounds are added. On day two, the substrate (CCF4-AM) is added and the assay is read on a fluorescent plate reader.

For instruction manuals from Invitrogen for specific assays:

ERα : http://tools.invitrogen.com/content/sfs/manuals/geneblazer_ERalphaUASblaGripTite_man.pdf

AR: http://tools.invitrogen.com/content/sfs/manuals/geneblazer_ARGripTite_man.pdf

GR: http://tools.invitrogen.com/content/sfs/manuals/geneblazer_GRblaHEK293T_man.pdf

PR: http://tools.invitrogen.com/content/sfs/manuals/geneblazer_PRUASblaHEK293T_man.pdf

LIST OF REAGENTS AND EQUIPMENT

Table 1. Materials and equipment required.

Reagents	**Recommended Source/ Cat#**
LiveBLAzer FRET B/G loading kit with CCF4-AM (includes DMSO for solution A, solution B, solution C, cell line)	Invitrogen (GeneBLAzer assay ONLY)
PrestoBlue cell viability reagent (cytotoxicity assay ONLY)	Invitrogen #A-13261
DMSO	Fluka #41647
70% ethanol solution for decontamination	Various
Isopropanol	Various
Reference compounds (see Table C1 p12)	Various
Trypan Blue, 0.4% in PBS	e.g. Sigma Aldrich T8154
Laboratory consumables	
Aluminum adhesive film	E&K Scientific #T592100
Black-wall, clear bottom, 96-well plates (with low fluorescence background)	Corning #3603
'Mr. Frosty' freezing container (for 1 - 2mL vials)	Thermo Scientific #5100-0001
Pipetting reservoirs, sterile (25 mL capacity)	e.g. Sigma Aldrich or VWR
Serological pipettes, sterile disposable (5, 10, 25 mL)	
PCR strips (350 µL capacity) and PCR plates 15 mL conical tubes, 1.5-2 mL tubes with screw cap lids	Various
Laboratory equipments	
Class II biological safety cabinet	Various
Cryogenic Freezer	Various
Fluorescence plate reader, bottom read capabilities with correct filters (see page 5)	Various
Humidified cell culture incubator (5% CO_2 and 37°C)	Various
Cell counting chambers (hemocytometer)	Various
Centrifuges for 96-well plates and 15 mL tubes	Various
Microscope (10X objective)	Various
Pipettes, multichannel pipettors (and tips)	Various
Water bath (set at 37°C)	Various

Table 2. Reagents for assay media (vary depending on the assay).

Component	**Recommended Source**	**Cat #**
Phenol red-free DMEM	Invitrogen	21063-029
Opti-MEM	Invitrogen	11058-021
Charcoal-stripped fetal bovine serum (FBS)	Invitrogen	12676-011
Dialyzed fetal bovine serum (FBS) **(Do not substitute)**	Invitrogen	26400-036
Non-essential amino acids (NEAA)	Invitrogen	11140-050
Penicillin/Streptomycin (antibiotics)	Invitrogen	15140-122
Sodium Pyruvate	Invitrogen	11360-070
HEPES,1 M, pH 7.3	Invitrogen	15630-080

PREPARATION OF ASSAY MEDIA

Table 3: Composition of assay media for each assay.

Component	**Assay**			
	ERα	**AR**	**GR**	**PR**
Phenol red free DMEM	**500 mL**	-	**500 mL**	**500 mL**
Opti-MEM	-	**500 mL**	-	-
Charcoal-stripped FBS	**10 mL**	-	**10 mL**	**10 mL**
Dialyzed FBS	-	**50 mL**	-	-
Sodium pyruvate	**5 mL** (1 mM)	**5 mL** (1 mM)	**5 mL** (1 mM)	-
Non-essential amino acids (NEAA)	**5 mL** (0.1 mM)	**5 mL** (0.1 mM)	**5 mL** (0.1 mM)	-
Penicillin/Streptomycin	**5 mL** (100 U/mL and 100 µg/mL)	**5 mL** (100 U/mL and 100 µg/mL)	**5 mL** (100 U/mL and 100 µg/mL)	**5 mL** (100 U/mL and 100 µg/mL)
HEPES	-	-	**12.5 mL** (25 mM)	

Notes: All media components can be added directly to the 500 mL bottle of base medium.

- If only preparing reagents for 1 plate, you can divide the volumes listed above by 10.
- Assay media can be stored at 4°C and the mixture is stable ~ 1 month.

SECOND INTERCALIBRATION

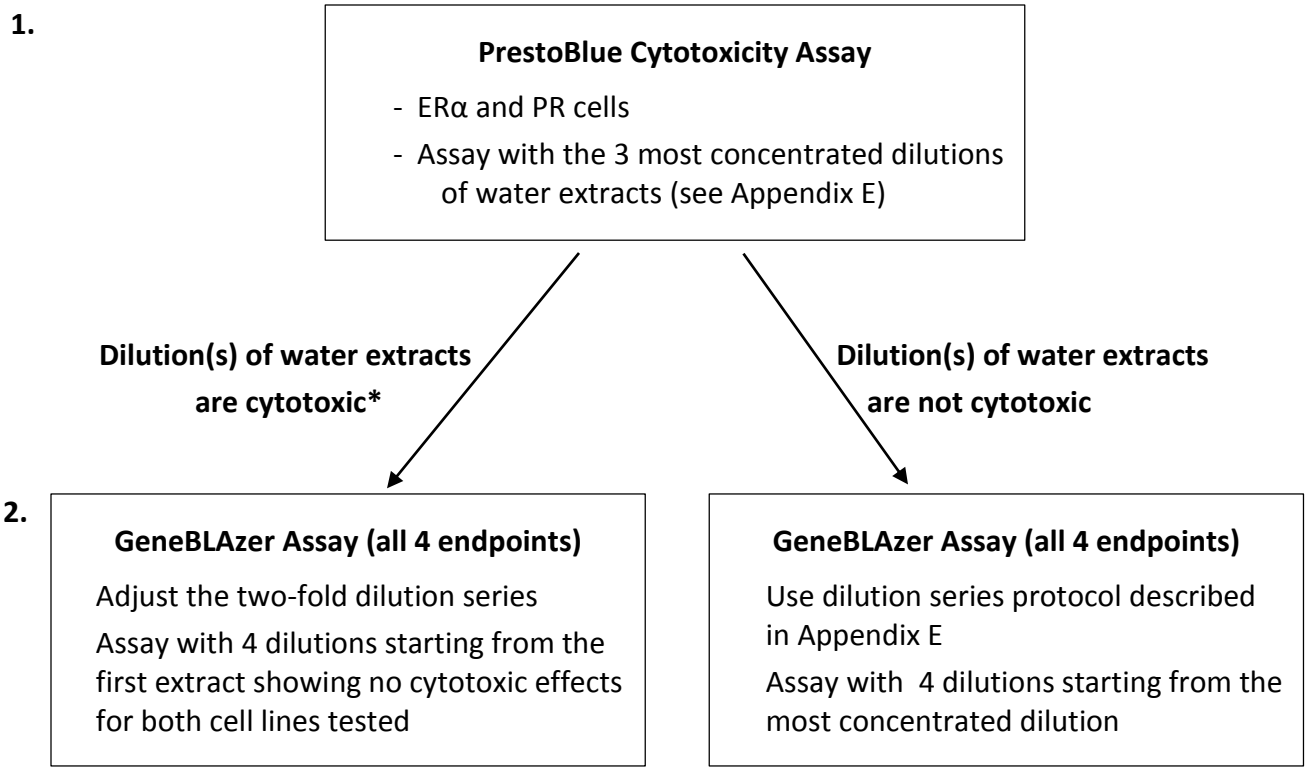
Participating laboratories: UAz, UCR, UF, USF and SCCWRP

Endpoints: AR, ERα, GR, PR, cytotoxicity

Samples to test (provided by UAz):

- 12 water extracts from Tucson and West Basin (1000X in DMSO)
- 1 DMSO sample for DMSO control (sample #13)
- 1 spiked sample – RR effluent LEV (vial dated 10-8-13 from 2nd shipment)

Work strategy



* If ANY cytotoxicity is measured (for one or both cell lines), the dilution series should be adjusted for ALL 4 endpoints.

PRESTO BLUE ASSAY PROTOCOL

DAY ONE: Plate cells, add positive control and water extracts. Incubate overnight

1. Thaw DA cells by placing at 37°C in a water bath with gentle agitation for 1 - 2 minutes. Do not submerge vial in water.
2. Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet using sterile technique.
3. Calculate the volume of cell suspension needed for the assay (50,000 cells/well for ER and 40,000 cells/well for PR). Add an extra ~ 50 µL of cell suspension to the amount calculated.
4. Transfer the cells drop-wise into 10 mL of Assay Medium in a sterile 15-mL conical tube.
5. Refreeze the leftover cells following the instructions given in **Box 1**.
6. Centrifuge cells at 200 × *g* for 5 minutes.
7. Aspirate supernatant and resuspend the cell pellet in 6 mL fresh Assay Medium.
8. Count the cells using a hemocytometer (see **Appendix A**).
9. Dilute cells in assay medium to a density of 5.5 x 10^5 ERα cells/mL or 4.4 × 10^5 PR cells/mL.
10. Plate cells in black, clear bottom 96-well plate (Corning 3603). See plate layout **Appendix B**. Do not touch bottom or use dusty plate.
11. Add 90 µL of Assay Medium to the cell-free control wells only.
12. Add 90 µL of cell suspension to the other wells.
13. Add 10 µL of DMSO to the positive control well (10% DMSO).
14. Prepare dilutions for the water extracts and 0.5% DMSO control (see **Appendix E**; dilutions 1, 2 and 3 are needed for this assay).
15. Add 10 µL of the appropriate solution to each well.
16. Incubate overnight (~ 16 hours) at 37°C with 5% CO_2 .

Box 1. Freezing instructions for leftover cells

- Immediately place the vial with the leftover cells in a "Mr. Frosty" freezing container (filled with 250 mL of isopropanol) and refreeze at -80°C.
- The following day store, the vial in Liquid N_2 (in a Cryo freezer). Limit number of freeze/thaws.

DAY TWO: Add PrestoBlue and read the cytotoxicity assay

1. Add 11 µL of the PrestoBlue to each well.
2. Cover the plate with an aluminum adhesive film to protect it from light and evaporation.
3. Incubate at 37°C /5% CO_2 for 15 minutes.
4. During incubation
    - Set up the plate reader.
    - Set the fluorescence plate reader to bottom-read mode with optimal gain and 5 reads.
    - Allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes before making measurements.
    - Before reading the plate make sure it's free of dust (use compressed air) and fingerprints.

Format	Excitation	Emission
General	540-570 nm	580-610 nm
Fluorescence (Monochrometer)	560 (10 nm bandwidth)	590 nm (10 nm bandwidth)
Fluorescence (Filter)	535 nm (25 nm bandwidth)	615 nm (10 nm bandwidth)
Absorbance	570 nm	600 nm (reference wavelength for normalization)

Data Reporting

Higher fluorescence or absorbance values correlate to greater total metabolic activity.

- Calculate average the fluorescence values of the cell-free control wells
- Subtract the average cell-free fluoresecence from the fluorescence value of each experimental well (water samples and controls).
- Plot fluorescence vs. experimental condition (cell number, compound concentration).

GENEBLAZER ASSAY PROTOCOL (DA cells)

DAY ONE: Plate cells, add reference compounds and water extracts. Incubate overnight

1. Thaw DA cells by placing at 37°C in a water bath with gentle agitation for 1–2 minutes. Do not submerge vial in water.
2. Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet using sterile technique.
3. Calculate the volume of cell suspension needed for the assay. Add an extra ~ 50 µL of cell suspension to the amount calculated.

   ERα and GR – use 50,000 cells/well

   AR and PR – use 40,000 cells/well
4. Transfer the cells drop-wise into 10 mL of Assay Medium in a sterile 15-mL conical tube.
5. Refreeze the leftover cells following the instructions given in Box 1.
6. Centrifuge cells at 200 × *g* for 5 minutes.
7. Aspirate supernatant and resuspend the cell pellet in 6 mL fresh Assay Medium.
8. Count the cells using a hemocytometer (see **Appendix A**).
9. Dilute cells in assay medium to the appropriate density

   ERα and **GR**: 5.5×10^5 cells/mL (for **50,000** cells/ well)

   AR and **PR**: 4.4×10^5 cells/mL (for **40,000** cells/ well)
10. Plate cells in black, clear bottom 96-well plate (Corning 3603). See **Appendix C** for plate layout. Do not touch bottom or use dusty plate.
11. Add 90 µL of Assay Medium to the cell-free control wells only.
12. Add 90 µL of cell suspension to the other wells.
13. Prepare dilutions for the reference compounds (see **Appendix D**) and water extracts (see **Appendix E**).
14. Add 10 µL of appropriate compound or water extract dilution to each well.
15. Incubate overnight (~ 16 hours) at 37°C with 5% CO_2 .

DAY TWO: Add substrate and read GeneBLAzer assay

1. Remove assay plate from the incubator and allow to equilibrate to room temperature. ****DO NOT TOUCH BOTTOM OF THE PLATE****

   Please do steps 2 to 5 with indirect light and turn off overhead lights.

2. Prepare LiveBLAzer™-FRET B/G substrate mixture (CCF4-AM) in the absence of direct strong lighting.

   a) **Solution A**: Add 64 µL of DMSO to one vial of 70 µg FRET B/G dry substrate (CCF4-AM) (i.e. 912 µL of DMSO per mg of dry substrate). This is sufficient for 4 plates. ***Solution A is VERY light sensitive.***

   b) Prepare aliquots of 8 µL of Solution A in labeled microcentrifuge tubes with screw cap lids. Each plate uses 2 tubes (16 µL), the other aliquots should be stored in the -20°C.

   c) Prepare **6X Loading Solution** (protocol below is for one plate)
   - Use two 8 µL-aliquots of Solution A and add 80 µL of Solution B to each tube. Vortex.
   - Then add 1,245 µL of Solution C to each tube and vortex. If foaming occurs, centrifuge for 1 min in a microcentrifuge to remove foam.
   - After centrifuging, mix both aliquots into a multi-pipet reservoir for a total volume of approx. 2,666 µL. Only 1,920 µL is needed for one plate, but this gives an extra 740 µL to cover the bottom of the multi-pipet reservoir.

3. Add 20 µL of the 6X Loading Solution to each well.

4. Cover the plate with an aluminum adhesive film to protect it from light and evaporation.

5. Incubate at room temperature for 2 hours (in the dark).

6. During incubation, set up plate reader*
   - Set the fluorescence plate reader to bottom-read mode with optimal gain and 5 reads.
   - Before reading the plate allow the fluorescent lamp to warm up for at least 10 minutes and make sure that the plate is free of dust (use compressed air) and fingerprints.
   - Use the following filter selections to read the the GeneBLAzer Assay:

	Scan 1	Scan 2
Purpose	Measure fluorescence in the Blue channel	Measure FRET signal in the Green channel
Excitation filter	409 / 20 nm	409 / 20 nm
Emission filter	460 / 40 nm	530 / 30 nm

*Follow proper plate-reader set-up instructions. For compatibility of other plate readers, follow this link: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Drug-Discovery/DD-Misc/instrument-compatibility-portal/compatible-instruments-by-assay-type/compatible-geneblazer.html. Invitrogen Technical Support team can help you through the set-up if needed.

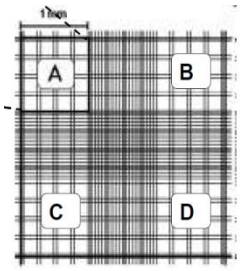
GeneBLAzer data reporting

1. Cell-free background subtraction
    - For each plate, identify the cell-free control wells.
    - Determine the average emission from the cell-free control wells at both 460 nm (average Blue background) and 530 nm (average Green background).
    - Subtract the average cell-free Blue background from all of the Blue emission data.
    - Subtract the average cell-free Green background from all of the Green emission data.
2. Blue/Green Ratio
    - For each well divide the background-subtracted Blue emission value by the background-subtracted Green emission value.
3. DMSO background subtraction
    - For each plate, identify the DMSO control wells.
    - Determine the average Blue/Green ratio for DMSO control.
    - Subtract the average DMSO ratio from all the B/G ratio data (wells for reference toxicants and water extracts).

An Excel spreadsheet is provided to report GeneBLAzer data. Enter the raw blue and green values in the first two tables. This will automatically calculate the different ratios and plot the graphs.
Please note that the spreadsheet follows the plate layout described in Appendix C. If any changes are made to the layout (e.g. order of samples), remember to change the formula accordingly.

Appendix A. Cell counting protocol using hemocytometer

- Clean the hemocytometer using 70% ethanol and place a coverslip.
- Add 150 µL of Trypan Blue (0.4% in PBS) into a 1.5 mL microcentrifuge tube, then add 50 µL of cell suspension (dilution factor of cell suspension is 4).
- Mix the tube content and add 16 µL of the diluted cell suspension (in Trypan Blue) to the v-shaped well on the side of the counting chamber.
- Let the cell suspension fill the chamber under the coverslip by capillary action.
- Count the number of cells in section A (see figure below) using the 10X objective of the microscope.
- Repeat this for section B, C and D.
- Calculate the amount of cells per mL using the equation given below.
- Clean the counting chamber and the coverslip with 70% ethanol.



$$\text{Cells per mL} = \frac{(A + B + C + D)}{4} \times Dilution\ Factor \times 10^4$$

Appendix B. 96-well plate layout for cytotoxicity assay

I)

	1	2	3	4	5	6	7	8	9	10	11	12
A	+ve control (10% DMSO) -->			DMSO control (0.5%) ----->			DMSO free control ------->			Cell free control ---------->		
B												
C	Sample - 1 - dilu 1 -------->			Sample - 2 - dilu 1 ------->			Sample - 3 - dilu 1 ------->			Sample - 4 - dilu 1 ------->		
D	Sample - 1 - dilu 2 -------->			Sample - 2 - dilu 2-------->			Sample - 3 - dilu 2-------->			Sample - 4 - dilu 2-------->		
E	Sample - 1 - dilu 3 -------->			Sample - 2 - dilu 3-------->			Sample - 3 - dilu 3-------->			Sample - 4 - dilu 3-------->		
F	Sample - 5 - dilu 1 -------->			Sample - 6 - dilu 1 -------->			Sample - 7 - dilu 1 ------->			Sample - 8 - dilu 1 ------->		
G	Sample - 5 - dilu 2 -------->			Sample - 6 - dilu 2-------->			Sample - 7 - dilu 2-------->			Sample - 8 - dilu 2-------->		
H	Sample - 5 - dilu 3 -------->			Sample - 6 - dilu 3 --------->			Sample - 7 - dilu 3-------->			Sample - 8 - dilu 3-------->		

II)

	1	2	3	4	5	6	7	8	9	10	11	12
A	+ve control (10% DMSO) -->			DMSO control (0.5%) ----->			DMSO free control ------->			Cell free control ---------->		
B												
C	Sample - 9 - dilu 1 -------->			Sample - 10 - dilu 1 ------->			Sample - 11 - dilu 1 ------>			Sample - 12 - dilu 1 ------->		
D	Sample - 9 - dilu 2 -------->			Sample - 10 - dilu 2 ------->			Sample - 11 - dilu 2 ------>			Sample - 12 - dilu 2 ------->		
E	Sample - 9 - dilu 3 -------->			Sample - 10 - dilu 3 ------->			Sample - 11 - dilu 3 ------>			Sample - 12 - dilu 3 ------->		
F	Sample RR LEV - dilu 1 ---->											
G	Sample RR LEV - dilu 2 ---->											
H	Sample RR LEV - dilu 3 ---->											

Appendix C. 96-well plate layout for GeneBLAzer assay

Stagger the start of plate 2 and 3 plates by 15 min when adding 6X loading solution.

I)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Tox - 1	Tox - 2	Tox - 3	Tox - 4	Tox - 5	Tox - 6	Tox - 7	Tox - 8	Tox - 9	DMSO	DMSO free	Cell free
B	Tox - 1	Tox - 2	Tox - 3	Tox - 4	Tox - 5	Tox - 6	Tox - 7	Tox - 8	Tox - 9	DMSO	DMSO free	Cell free
C	Tox - 1	Tox - 2	Tox - 3	Tox - 4	Tox - 5	Tox - 6	Tox - 7	Tox - 8	Tox - 9	DMSO	DMSO free	Cell free
D												
E	Sample - 1 - dilu 1 -------->			Sample - 1 - dilu 2 ------>			Sample - 1 - dilu 3 ------->			Sample - 1 - dilu 4 -------->		
F	Sample - 2 - dilu 1 -------->			Sample - 2 - dilu 2 ------->			Sample - 2 - dilu 3 ------->			Sample - 2 - dilu 4 -------->		
G	Sample - 3 - dilu 1 -------->			Sample - 3 - dilu 2 ------->			Sample - 3 - dilu 3 ------->			Sample - 3 - dilu 4 -------->		
H	Sample - 4 - dilu 1 -------->			Sample - 4 - dilu 2 ------->			Sample - 4 - dilu 3 ------->			Sample - 4 - dilu 4 -------->		

II)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Tox - 2	Tox - 3	Tox - 4	Tox - 5	Tox - 6	Tox - 7	Tox - 8			DMSO	DMSO free	Cell free
B	Tox - 2	Tox - 3	Tox - 4	Tox - 5	Tox - 6	Tox - 7	Tox - 8			DMSO	DMSO free	Cell free
C										DMSO	DMSO free	Cell free
D	Sample - 5 - dilu 1 -------->			Sample - 5 - dilu 2 ------->			Sample - 5 - dilu 3 ------->			Sample - 5 - dilu 4 -------->		
E	Sample - 6 - dilu 1 -------->			Sample - 6 - dilu 2 ------->			Sample - 6 - dilu 3 ------->			Sample - 6 - dilu 4 -------->		
F	Sample - 7 - dilu 1 -------->			Sample - 8 - dilu 2 ------->			Sample - 7 - dilu 3 ------->			Sample - 7 - dilu 4 -------->		
G	Sample - 8 - dilu 1 -------->			Sample - 8 - dilu 2 ------->			Sample - 8 - dilu 3 ------->			Sample - 8 - dilu 4 -------->		
H	Sample - 9 - dilu 1 -------->			Sample - 9 - dilu 2 ------->			Sample - 9 - dilu 3 -------->			Sample - 9 - dilu 4 -------->		

III)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Tox - 2	Tox - 3	Tox - 4	Tox - 5	Tox - 6	Tox - 7	Tox - 8			DMSO	DMSO free	Cell free
B	Tox - 2	Tox - 3	Tox - 4	Tox - 5	Tox - 6	Tox - 7	Tox - 8			DMSO	DMSO free	Cell free
C										DMSO	DMSO free	Cell free
D												
E	Sample - 10 - dilu 1 -------->			Sample - 10 - dilu 2 ------->			Sample - 10 - dilu 3 -------->			Sample - 10 - dilu 4 -------->		
F	Sample - 11 - dilu 1 -------->			Sample - 11 - dilu 2 ------->			Sample - 11 - dilu 3 -------->			Sample - 11 - dilu 4 -------->		
G	Sample - 12 - dilu 1 -------->			Sample - 12 - dilu 2 ------->			Sample - 12 - dilu 3 -------->			Sample - 12 - dilu 4 -------->		
H	Sample RR LEV - dilu 1 -->			Sample RR LEV - dilu 2 -->			Sample RR LEV - dilu 3 -->			Sample RR LEV - dilu 4 -->		

Appendix D. Preparation of reference compound dilutions

Table C1. Reference compounds for GeneBLAzer assays

Assay	Reference Compound	CAS #	Molecular weight (g/mol)	Source and Cat #
ERα	17β Estradiol	50-28-2	272.38	Sigma #E1024
AR	R1881 (Methyltrienolone)	965-93-5	284.393	Perkin-Elmer #NLP005005MG
GR	Dexamethasone	50-02-2	392.461	Sigma #D-4902
PR	Levonorgestrel	797-63-7	312.446	Sigma #N2260- Fluka

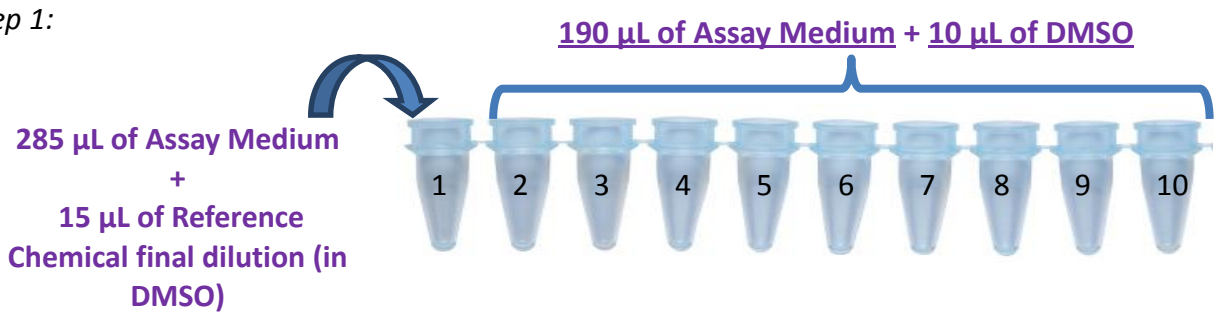
Table C2. Stock solutions of reference compounds

Assay	Reference Compound	Protocol
ERα	17β Estradiol (E2) Final dilution = 2 µM	• **20 mM E2 stock:** Dissolve 5.4476 mg E2 in 1 mL DMSO • **1 mM E2:** Add 50 µL 20 mM E2 to 950 µL DMSO • **0.1 mM E2:** Add 100 µL 1 mM E2 to 900 µL DMSO • **0.002 mM E2:** Add 20 µL 0.1 mM E2 to 980 µL DMSO
AR	R1881 (Methyltrienolone) Final dilution = 20 µM	• **20 mM R1881 stock:** Dissolve 5.6878 mg R1881 in 1 mL DMSO • **1 mM R1881:** Add 50 µL 20 mM R1881 to 950 µL DMSO • **0.02 mM R1881:** Add 20 µL 1 mM R1881 to 980 µL DMSO
GR	Dexamethasone (DEX) Final dilution = 0.1 mM	• **20 mM DEX stock:** Dissolve 7.849 mg DEX in 1 mL DMSO • **1 mM DEX:** Add 50 µL of 20 mM DEX to 950 µL DMSO • **0.1 mM DEX:** Add 100 µL of 1 mM DEX to 900 µL DMSO
PR	Levonorgestrel (LEVO) Final dilution = 20 µM	• **20 mM LEVO stock:** Dissolve 6.2489 mg LEVO in 1 mL DMSO • **1 mM LEVO:** Add 50 µL 20 mM LEVO to 950 µL DMSO • **0.02 mM LEVO:** Add 20 µL of 1 mM LEVO to 980 µL DMSO

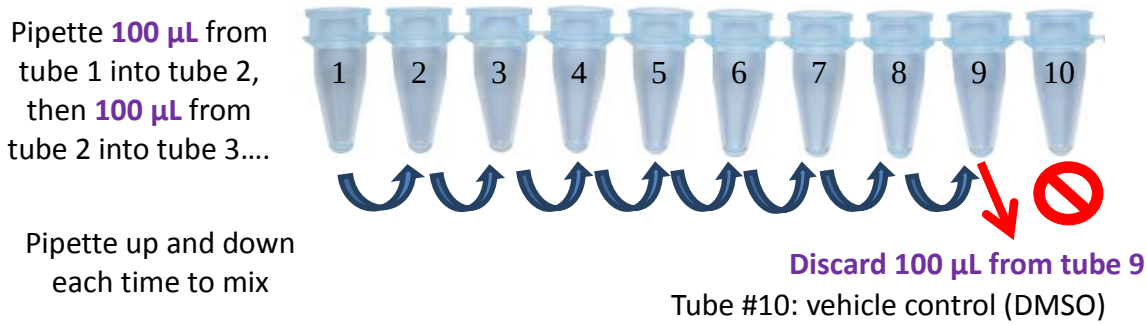
Dilution protocol for reference compounds

Obtain a strip of 12 PCR tubes with at least 350 µL capacity.

Step 1:



Step 2:



Step 3: With multichannel pipette add 10 µL to the appropriate well (see plate layout) containing 90 µL of cell suspension. Pipette up and down 3 times to mix.

Table C3. Final concentrations of reference compounds in wells

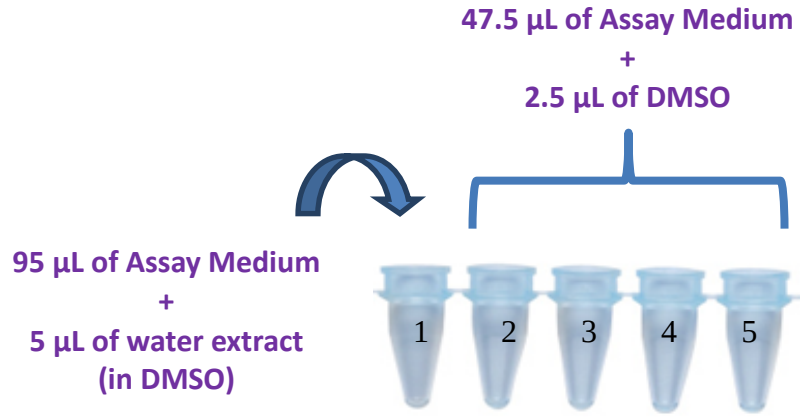
Assay	Reference Compound	Tube #									
		1	2	3	4	5	6	7	8	9	10
ERα	E2	1E-8	3.33E-9	1.11E-9	3.7E-10	1.23E-10	4.12E-11	1.37E-11	4.57E-12	1.52E-12	DMSO
AR	R1881	1E-7	3.33E-8	1.11E-8	3.7E-9	1.23E-9	4.11E-10	1.37E-10	4.57E-11	1.52E-11	DMSO
GR	DEX	5E-7	1.67E-7	5.56E-8	1.85E-8	6.17E-9	2.06E-9	6.86E-10	2.29E-10	7.62E-11	DMSO
PR	LEVO	1E-7	3.33E-8	1.11E-8	3.7E-9	1.23E-9	4.11E-10	1.37E-10	4.57E-11	1.52E-11	DMSO

The final DMSO level is 0.5% in wells.

<u>Appendix E. Dilution protocol for water extracts</u> (two-fold serial dilution)

Obtain a strip of 8 PCR tubes with at least 150 µL capacity (or use a 96-well PCR plate).

Step 1:



Step 2:

Pipette **50 µL** from tube 1 into tube 2, then **50 µL** from tube 2 into tube 3….

Pipette up and down each time to mix

Tube #5 : vehicle control (0.5% DMSO)

Note: Results of the PrestoBlue cytotoxicity assay will determine the dilution series to use for the GeneBLAzer assay (i.e. the dilutions used should be not cause any cytotoxicity to either ERa or PR cells).

Step 3: With a multichannel pipette add 10 µL per well to a 96-well plate containing 90 µL of cell suspension (in assay medium). Pipette up and down few times to mix.

The final DMSO level is 0.5% in wells.

APPENDIX 4.5 – RESULTS OF THE LABORATORY INTERCALIBRATION EXERCISE

Environmental Science & Technology

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Benchmarking Organic Micropollutants in Wastewater, Recycled Water and Drinking Water with In Vitro Bioassays

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Supporting Information

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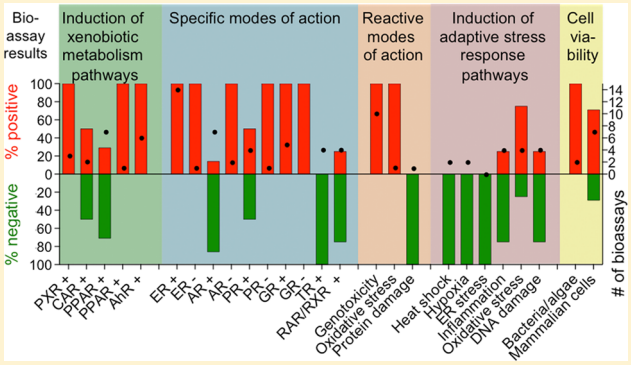
ABSTRACT: Thousands of organic micropollutants and their transformation products occur in water. Although often present at low concentrations, individual compounds contribute to mixture effects. Cell-based bioassays that target health-relevant biological endpoints may therefore complement chemical analysis for water quality assessment. The objective of this study was to evaluate cell-based bioassays for their suitability to benchmark water quality and to assess efficacy of water treatment processes. The selected bioassays cover relevant steps in the toxicity pathways including induction of xenobiotic metabolism, specific and reactive modes of toxic action, activation of adaptive stress response pathways and system responses. Twenty laboratories applied 103 unique in vitro bioassays to a common set of 10 water samples collected in Australia, including wastewater treatment plant effluent, two types of recycled water (reverse osmosis and ozonation/activated carbon filtration), stormwater, surface water, and drinking water. Sixty-five bioassays (63%) showed positive results in at least one sample, typically in wastewater treatment plant effluent, and only five (5%) were positive in the control (ultrapure water). Each water type had a characteristic bioanalytical profile with particular groups of toxicity pathways either consistently responsive or not responsive across test systems. The most responsive health-relevant endpoints were related to xenobiotic metabolism (pregnane X and aryl hydrocarbon receptors), hormone-mediated modes of action (mainly related to the estrogen, glucocorticoid, and antiandrogen activities), reactive modes of action (genotoxicity) and adaptive stress response pathway (oxidative stress response). This study has demonstrated that selected cell-based bioassays are suitable to benchmark water quality and it is recommended to use a purpose-tailored panel of bioassays for routine monitoring.



INTRODUCTION

The Tox21, a joint program of the National Institutes of Health, U.S. Environmental Protection Agency (EPA), and U.S. Food and Drug Administration[1] and the U.S. EPA ToxCast Program[2] aim to advance molecular toxicology, systems biology, and computational toxicology to overcome shortcomings of traditional in vivo toxicity testing of chemicals. Parallel initiatives exist in Europe, for example, the EU project ChemScreen.[3] Jointly these programs bring a paradigm shift in toxicity testing as in vitro methods help elucidate mechanisms of toxicity, prioritize chemicals for further testing and develop predictive models in order to refine, reduce, or replace future in vivo testing. These programs rely heavily on high-throughput screening (HTS) using cell-based and cell-free in vitro bioassays of large numbers of chemicals to elucidate their toxicity pathways. While Tox21 focuses on the bioanalytical profiling of individual chemicals, these tools should also be applicable to environmental samples of unknown and complex composition, and this study brings together for the first time selected bioassays from Tox21 with established bioassays for water quality assessment.[4]

Cell-based bioassays have been developed to target all steps of the toxicity pathway (Figure 1).[5] While the induction of xenobiotic metabolism may not lead to cytotoxicity, it is an indicator of the presence of pollutants. Some bioassays provide measures of mechanisms of toxicity by visualizing the interaction of stressors, for example, chemicals, with specific biological targets, for example, binding to endocrine receptors or reaction with DNA. The exposed cells may respond through induction of adaptive stress response pathways, a feature that can be used for the assessment of environmental pollutants, although it is not an adverse effect per se. Cell viability, growth and/or proliferation are indicators of adverse effects on a cellular level. If the cell represents a specialized tissue, this may give an indication of tissue-specific impairment.

Cellular responses will not always imply higher-level effects but are a prerequisite for whole organism and population responses.[6] The direct detection of initiating key events in a bioassay only provides a measure of a potential adverse effect because repair and defense mechanisms may ultimately prevent toxicity. From a precautionary perspective, however, the potential to cause an adverse effect is a crucial assessment endpoint. Cell-based bioassays are not suitable to replace regulatory in vivo tests but provide hazard information for screening and prioritization of chemicals.[7]

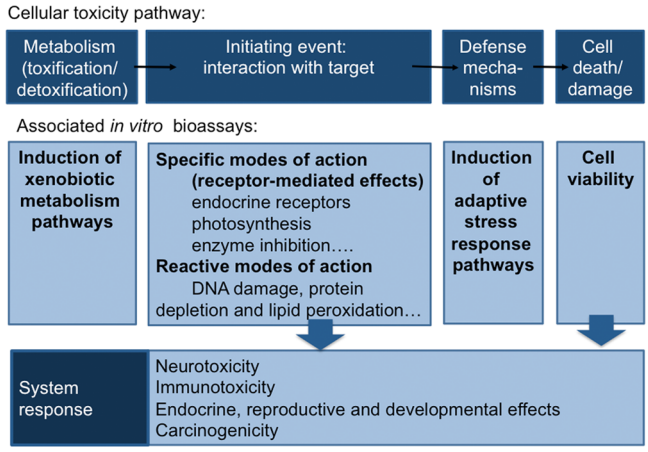


Figure 1. Classification of in vitro bioassays according to cellular toxicity pathways (adapted from refs 4, 5, and 117).

A large variety of cell-based bioassays have been applied for water quality assessment in the past[4] but many of the end points evaluated under HTS toxicology programs[8] are yet to be included. Many studies rely on a small set of bioassays and each study uses different types of water samples, sample preparation methods, bioassays, and data evaluation methods. Only a few cell-based bioassays have standardized protocols, such as the OECD or ISO guidelines. The goals of this study were to evaluate as many bioassays as practically achievable using one set of water samples with one sample preparation method and to recommend a screening test battery for water quality testing.

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We selected cell-based bioassays using three criteria. First, we selected bioassays that have previously been used for water quality assessment. Second, a comprehensive literature review allowed us to identify cell-based bioassays that responded to environmentally relevant organic micropollutants but had not been used for water quality assessment prior to this study. Third, for final selection of endpoints, we screened 25 nuclear receptors (NR) and 48 transcription factor (TF) response elements in HepG2 human liver carcinoma cell lines[9] to ensure inclusion of endpoints relevant for the particular samples tested.

We evaluated 10 samples, including nine ambient water samples ranging from effluent, recycled water to drinking water, plus one procedural blank. The samples were extracted and concentrated with an optimized solid phase extraction (SPE) method and sent to a total of 20 worldwide laboratories applying 103 bioassays for bioanalytical testing. All experimental data were evaluated using a common method specifically developed for this study to harmonize the different approaches to data evaluation. The results were used not only to validate and compare the different bioassays for application with water samples but also to benchmark water quality. The data may also serve to compare the efficacy of different water treatment processes for removal of organic micropollutants.

It was not the goal of this study to directly compare bioassay protocols and performance of bioassays but rather to obtain an overview of the biological endpoints responsive to typical water contaminants. One goal was to cover the major health-relevant toxicity pathways introduced in Figure 1 and to evaluate which pathways were relevant for water quality testing. The study was further expected to identify the most robust and responsive bioassays, thus, not only mammalian but also bacterial assays were included. The outcome includes recommendations on the makeup of a screening test battery and on indicator bioassays that appear to be particularly relevant for further investigation in water quality monitoring programs.

MATERIALS AND METHODS

Samples. Ten grab samples of water were collected in December 2011 and January 2012 (Supporting Information (SI), Section S1 and Table S1). Sample Eff1 is a secondary treated sewage effluent (activated sludge treatment) that serves as influent to a Water Reclamation Plant (WRP) that produces high quality recycled water for indirect potable reuse. Three samples were taken at different stages of treatment: after microfiltration (MF), reverse osmosis (RO) and advanced oxidation (AO) using H_2O_2/UV. Bioanalytical assessments had been previously undertaken at this WRP.[10,11]

The second investigated WRP treats secondary sewage effluent (Eff2) by ozonation followed by biologically activated carbon filtration (O_3/BAC) to produce recycled water for irrigation and industrial usage. The fate of micropollutants in this plant has been previously characterized in more detail.[12−14]

River water (RW) and drinking water (DW) samples were collected at the inlet and outlet of a metropolitan drinking water treatment plant applying chlorination and chloramination, which was also previously assessed with bioanalytical tools.[10,15]

The stormwater sample (SW) was collected from a stormwater drain in Brisbane, Australia, that receives runoff from a residential catchment.[16] The laboratory blank consisted of ultrapure water (Milli-Q water) run through the same SPE process as the samples. Fourteen liters were collected for each of Eff1, MF, Eff2, and SW, while 28 L were collected for the remaining samples.

Sample Preparation and Distribution to the Participating Laboratories. The SPE was performed according to Macova et al.[10] using the sorbent materials Oasis HLB (Waters) followed by Supelclean coconut charcoal (Sigma-Aldrich), a combination that was confirmed previously to extract a broad range of organic micropollutants.[17] Details and information on sample preparation and the logistics of sample distribution are summarized in the SI, Section S1. All distributed samples were labeled with codes for blind sample processing.

Sample Characterization. 293 organic micropollutants were previously characterized in these samples.[18] Dissolved organic carbon concentrations are reported in the SI, Table S1.

Bioassays. The majority of selected bioassays was based on mammalian, bacterial or yeast cells. A zebrafish embryo test was included because fish embryos are considered as nonprotected life stages and as ethically accepted alternative to the testing of (adult) animals.[19] Only one bioassay employed a naked enzyme (acetylcholinesterase inhibition assay).

All applied bioassays and their associated experimental methods are listed in Table 1 and categorized according to the toxicity pathways outlined in Figure 1. For bioassays where the protocol was modified or applied for the first time, Section S2 in the SI and Table S2 give additional information on the experimental procedures.

Concentration-Effect Assessment. A critical aspect when working with diverse biological endpoints is a consistent data evaluation process. It was the goal of the present study to harmonize the data evaluation as much as possible, which is challenging given the different types of endpoints measured but a prerequisite for quantitative comparison between different bioassays.

The concentrations of samples were expressed in units of relative enrichment factor REF. The REF is the product of the enrichment factor of the SPE process and the dilution of the extract in the bioassay (for derivation of equations, see SI, Section S3). A REF > 1 means that the sample is enriched in the bioassay (e.g., a REF of 10 means the sample was concentrated 10-fold in the bioassay), a REF < 1 means it was diluted in the bioassay, and a REF of 1 is equivalent to the organic micropollutants in the ambient (undiluted and unconcentrated) sample stripped from inorganics, metals and most colloidal organic matter by SPE.

For each bioassay, the measured responses were plotted against the sample concentration expressed as REF (Figure 2). For assays with a known maximum response, responses were converted to percent of maximum effect. For all endpoints that relate to cell viability and cell population growth, the controls can be expressed as 0% effect, while 100% relates to "no growth" or "all cells dead". For reporter gene assays that measure the binding to a receptor or the transactivation of a receptor, 0% refers to the basal activity of the receptor, while 100% is defined using an appropriate reference compound that can saturate the receptor without causing cytotoxicity.

The ideal case would be a full concentration-effect curve covering 0% to 100% of effect (Figure 2A), which typically has a sigmoidal shape that can be described with a log−logistic equation. Any log−logistic concentration-effect curve will be linear with respect to (nonlogarithmic) concentrations at low effect level (up to 20−30% effect). As the water samples investigated in the present study often showed only very low effect levels, the linear form of the concentration-effect curves was used for derivation of the effect concentration causing 10% of maximum effect (EC_{10}) of the samples

Table 1. Bioassays Used and Their Toxicity Pathway Classifications[a]

#	laboratory	bioassay	literature reference for method development	experimental approach (literature reference/more information in SI)
Xenobiotic Metabolism				
1	ATG	PXR-cisFACTORIAL	46	9
2	ATG	PXR-transFACTORIAL	46	9
3	IRCM	HG5LN PXR	47	48
4	ATG	CAR-transFACTORIAL	46	9
5	CAPIM	CAR-yeast	49	see SI, Section S2-A.
6	ATG	PPARα-transFACTORIAL	46	9
7	ATG	PPARγ-transFACTORIAL	46	9
8	IRCM	HELN-PPARγ	50	28
9	BDS	CALUX-PPARα	51	52
10	BDS, CSIRO	CALUX-PPARγ2	53	52
11	HK	MCF7-PPAR	54	54
12	GU	PPARγ-GeneBLAzer	27	55
13	GU	Anti-PPARγ-GeneBLAzer	27	see SI, Section S2-B.
14	CAPIM	AhR-yeast	56	57 and SI, Section S2-B.
15	UQ, RECETOX	CAFLUX	58	RECETOX: 59 UQ: 60
16	RECETOX	H4IIEluc	61	59
17	HK	MCF7DRE	54	54
18	ATG	AhR-cisFACTORIAL	46	9
19	UFZ	DART cyp1a induction	62	63
Specific Modes of Action				
20	UQ, SWISS	algae photosynthesis inhibition	31	64
21	UQ	acetylcholinesterase inhibition	65	30
Specific MOA: ER				
22	GU, CSIRO, BDS, IWW	ER-CALUX	66	33
23	UQ	E-SCREEN	67	10
24	SWISS, CSIRO, UA	YES	68	69
25	CAPIM	hER yeast	49	70
26	CAPIM	medER yeast	49	70
27	IRCM	HELN-ERα	71	72
28	IRCM	HELN-ERß	71	73
29	ATG	ERE-cisFACTORIAL	46	9
30	RECETOX	hERα-HeLa-9903	74	74
31	HK	MCF7-ERE	54	54
32	ATG	ERα-transFACTORIAL	46	9
33	NJU	Steroidogenesis (estrogens)	75	76
34	UFZ	DART cyp19a1b	77	63
35	UF, USF, UCR, SCCWRP	ERα-GeneBLAzer	27	55 see SI, Section S2-C.
36	CSIRO, GU	Anti ER-CALUX	66	17
Specific MOA: AR				
37	GU, BDS, CSIRO	AR-CALUX	66,78	33
38	IRCM	HELN-AR	79	80
39	HK	MCF7-ARE	54	54
40	UA, CSIRO	YAS	81	82
41	UF, USF, UCR, SCCWRP	AR-GeneBLAzer	27	55 see SI, Section S2-C.
42	ATG	AR-transFACTORIAL	46	9
43	RECETOX	MDA-kb2	83	84
44	RECETOX	Anti-MDA-kb2	83	84
45	CSIRO, GU	Anti-AR-CALUX	66,78	17
Specific MOA: GR				
46	GU, BDS, CSIRO	GR-CALUX	66	33
47	UA	GR Switchgear		see SI, Section S2-D.
48	ATG	GR-transFACTORIAL	46	9
49	RECETOX	GR-MDA-kb2	83	84
50	GU, UF, USF, SCCWRP	GR-GeneBLAzer	27	55
51	GU	Anti-GR-GeneBLAzer	27	
52	GU	Anti-GR-CALUX	66	33
Specific MOA: PR				
53	UF, USF, UCR, SCCWRP	PR-GeneBLAzer	27	55 see SI, Section S2-B.
54	GU, BDS, CSIRO	PR-CALUX	66	33

Table 1. continued

#	laboratory	bioassay	literature reference for method development	experimental approach (literature reference/more information in SI)
Specific MOA: PR				
55	GU	Anti-PR-CALUX	66	
56	NJU	steroidogenesis (progesterone)	75	76
57	NJU	steroidogenesis (17α OH-progesterone)	75	76
Specific MOA: TR				
58	BDS, GU	TR-CALUX	66	17
59	UQ	T-SCREEN	85	
60	ATG	THRα1-transFACTORIAL	46	9
61	IRCM	HELN-TR		86
Specific MOA: Reproductive and Developmental Effects				
62	HK	MCF7-RARE	54	54
63	UQ	P19/A15	87	see SI, Section S2-E.
64	ATG	RORß-transFACTORIAL	46	9
65	CAPIM	hRAR-Yeast Assay	49	88
Reactive MOA				
66	UQ, RCEES	umuC TA1535/pSK1002	89	UQ: 90 RCEES: 89
67	UQ	umuC TA1535/pSK1002 +S9	89	90
68	RCEES	umuC NM5004	91	89
69	RECETOX	SOS chromotest	92	93
70	UA, IWW	Ames TA98	94	IWW: 95
71	UA, IWW	Ames TA98+ S9	94	IWW: 95,96
72	UA	Ames TAmix	94	
73	UA	Ames TAmix +S9	94	
74	UQ, IWW	Ames TA100	94	UQ: 97 IWW: 95
75	AWQC	micronucleus assay	98	99
76	CSIRO	ROS formation RTG2	100	101
77	UQ	protein damage *E.coli*	102	38
Adaptive Stress Response				
78	ATG	HSE-cisFACTORIAL	46	9
79	UFZ	hspb11 induction DART	63	63
80	ATG	HIF-1a-cisFACTORIAL	46	9
81	UA	Hypoxia-Switchgear		see SI, Section S2-F.
82	ATG	NF-kB-cisFACTORIAL	46	9
83	UQ	NF-kB-Geneblazer	27	55 see SI, Section S2-C.
84	BDS	NF-kB-CALUX	103	[33]
85	GU	Jurkat E6.1 IkB	none	see SI, Section S2-G.
86	UQ	AREc32	104	39
87	UA	Nrf2-keap	105	see SI, Section S2-H.
88	ATG	Nrf2/ARE-cisFACTORIAL	46	9
89	BDS	Nrf2-CALUX	106	52
90	ATG	p53-cisFACTORIAL	46	9
91	BDS	p53-CALUX	106	52
92	BDS	p53-CALUX +S9	106	52
93	UF	p53-GeneBLAzer	27	55 see SI, Section S2-C.
Cytotoxicity and Indicators of System Response				
94	UQ	AREc32 cell viability	39	39
95	GU	Caco 2 NRU	107	17 see SI, Section S2-I.
96	CSIRO	RTG2MTT	108	
97	UFZ	DART 48h lethality	109	110
98	UFZ	DART 120h sublethal	111	110
99	GU	SK-N-SH cytotoxicity	112	see SI, Section S2-J.
100	GU	THP1 cytokine	113	17 see SI, Section S2-K.
101	UQ	algae growth inhibition	114	64
102	UQ, SWISS	*Vibrio fischeri* (Microtox)	115	SWISS: 69 UQ: 18
103	RCEES	*Photobacterium phosphoreum*	116	

[a]Assay numbers (#) are equivalent to the numbers in Figure 3. Literature references are provided for the method development and for how the assay was performed. Modifications of the assay methods are summarized in the Supporting Information, SI, Table S2.

(Figure 2C). A detailed derivation of EC_{10} is provided in the SI, Section S3-A.

For assays where the maximum response was unknown or unachievable, the responses were normalized to growth medium

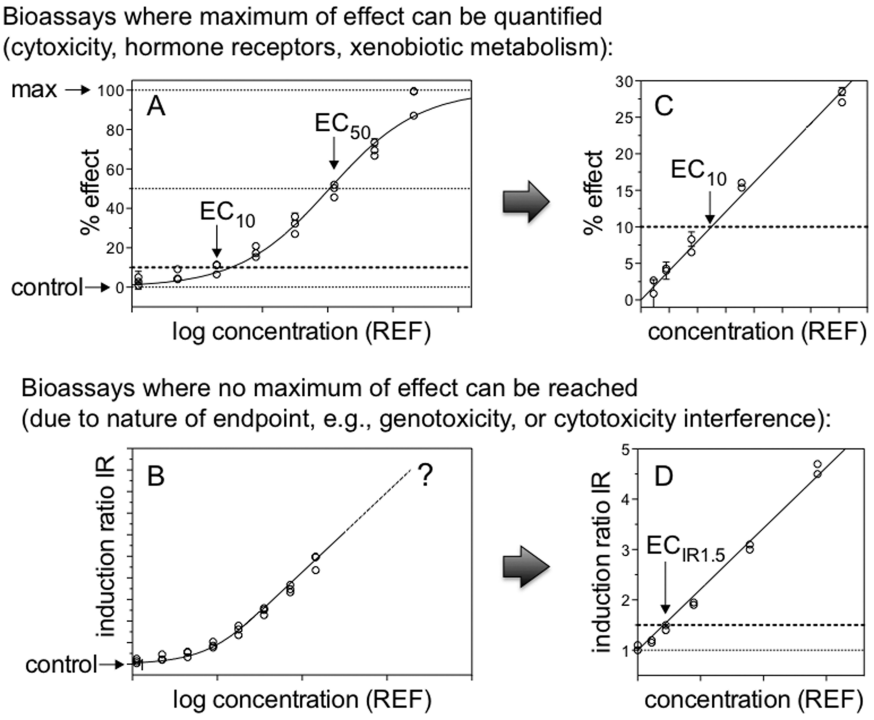

Figure 2. Overview of the concentration-effect models applied to derive benchmark effect concentrations (EC).

controls (including the same percentage of solvent as in the corresponding samples) and assessed via the induction ratio (IR; that is, fold induction relative to control). The maximum response was unknown if no appropriate reference compound existed, or if cytotoxicity quenched the reading of the reporter activity. The problem also arose if the endpoint is inducible or, by nature, there is no clear upper limit, for example, for DNA damage or adaptive stress response. In practice, linear regression through the control effect level (IR = 1) was used for derivation of the effect concentration that causes an IR of 1.5, or $EC_{IR1.5}$ (Figure 2D). A detailed derivation of $EC_{IR1.5}$ is provided in the SI, Section S3-B together with a discussion of the comparability of EC_{10} versus $EC_{IR1.5}$ (SI Table S3).

A bioassay can be run in antagonistic mode if the receptors are occupied with a constant concentration of a known and potent agonist. Varying concentrations of sample are added and if the signal of the control is suppressed the sample can be considered to exhibit an antagonistic effect. The effect concentration causing a suppression ratio SR of 0.2, $EC_{SR0.2}$, was used to describe all antagonistic effects and correspondingly an $EC_{CD0.2}$ was defined for endpoints that are based on chaperone dissociation (e.g., IκB dissociation from NFκB). A detailed derivation is provided in the SI, Section S3-C and Figure S1.

Data Presentation. A heatmap presenting all measured EC values was generated using the R Software package gplots (www.r-project.org/). Hierarchical clustering was performed using the "complete linkage" method to find similar clusters of water samples.

RESULTS

Repeatability of Bioassays. A number of bioassays were performed simultaneously in multiple laboratories. As discussed in detail in the SI, Section S4 and Table S4, the results were consistent between laboratories and therefore the results of the same bioassays were averaged.

Initial Screening of Nuclear Receptors and Transcription Factors. The FACTORIAL bioassays[9] were used for an initial profiling of the water samples after enrichment by SPE to a REF of 4. As discussed in the SI, Section S5 and Figure S2, an IR of 1.5 is regarded as the threshold for positive effects. In the SI, Figure S3, the activity profiles of all samples are depicted.

The blank did not induce any of the tested endpoints indicating that the sample extraction and enrichment process did not negatively influence the test outcome. The Eff1 and Eff2 samples caused an activation of five of 25 nuclear receptors (NR) and 5 of 48 transcription factors (TF) tested (SI Figure S3A and C). The active NRs were the pregnane X receptor (PXR), the peroxisome proliferator-activated receptor (PPARγ), the estrogen receptor (ERα) and marginally the glucocorticoid receptor (GR) and the liver X receptor (LXR). The active TFs were related to the aryl hydrocarbon receptor (AhR), PXR, the oxidative stress response (nrf2/ARE), the estrogen response element (ERE), and the RAR-related orphan receptor (RORE).

The screening provides strong support to expand the test battery to include additional endpoints to those routinely employed.[4] Particularly the PXR and AhR, which are related to xenobiotic metabolism, warrant more attention as these had the highest activity in the prescreening assay. The LXR is relevant as its activation induces the PPAR.[20] The PPAR pathway is related to obesity[21] and has gained much attention in recent years and therefore various PPAR-related endpoints have been included in the test battery if not specifically LXR. The oxidative stress response pathway appeared to be of high relevance and has rarely been investigated with water samples prior to this study.

In response to the findings of this screening, the active three NRs and five TFs and two others associated to relevant pathways (CAR, PPARα, AR, GR, THRα1, RORβ, HSE, HIF1a, NFκB, p53) were included in the detailed dose−response analysis.

Responsiveness of the Bioassays. Figure 3, Table 2, and Table S5 in the SI give an overview of all results of the 101 different bioassays tested plus the two bacterial cytotoxicity assays.

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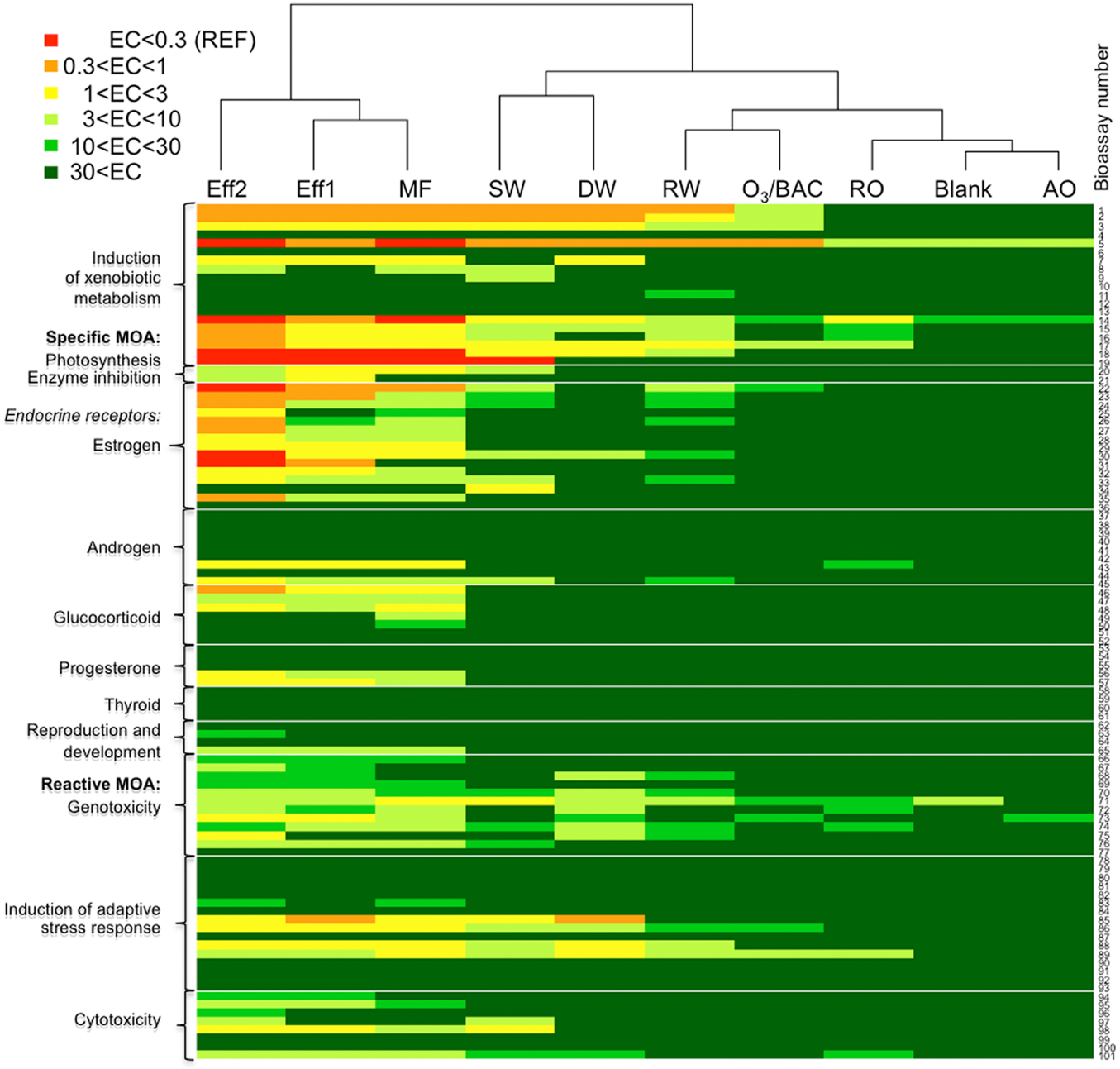

Figure 3. Summary of results in 101 bioassays (excluding inactive FACTORIAL and the bacterial cytotoxicity assays). Plotted are the effect concentrations (EC_{10}, $EC_{IR1.5}$, or $EC_{SR0.2}$) in units of REF (relative enrichment factors). The colors encode for the magnitude of the EC. Green stands for high effect concentrations (low potency) and transitions to red for low effect concentrations (high potency). Dark green are EC values that were >30 REF (which means that the sample that is enriched 30 times still does not show an effect), green from 10 to 30 REF, light green from 3 to 10 REF. A sample that has its EC at concentrations of the native sample up to three times enriched is denoted in yellow. Samples that have to be diluted for the EC are orange for up to 3 times diluted (REF 1 to 0.3) and red for over 3 times diluted. Numbers on the right refer to bioassay numbers in Table 1.

A positive response is defined here as $EC_{IR1.5}$ or EC_{10} lower than the highest tested REF, thus no extrapolations were performed.

The first two questions that we have to answer to judge the suitability of bioassays for water quality assessment are (A) "do "polluted" samples induce a response?" and (B) "is the response acceptably low in control samples?" (A) Sixty-five bioassays showed a response in at least one of the water samples. For Eff2, which can be considered as a moderately "polluted" sample, the number of positive results was 60. (B) No solvent blank caused any effect and the procedural blank with ultrapure water only produced effect in five bioassays (4.9%).

Procedural Blank. Even small impurities leaching out of the material or present in the solvent would likely contribute to the nonspecific effect of the blank. Here we applied two different SPE sorbent materials (HLB and coconut charcoal), which were eluted separately and required twice the amount of solvent. This consideration rationalizes the low but positive results of the blanks in the two bioluminescence inhibition assays with marine luminescent bacteria, which were higher than previously seen when only one type of solid-phase material were applied.[10]

Furthermore, the yeast-based assays AhR-yeast and CAR-yeast showed responses in the blanks but only at much higher REF

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Table 2. Summary of Responsive and Non-Responsive Bioassays (Total = Number of Bioassays, In Parentheses Number of Replicates), + = Number of Positive Responses, − = Number of Negative Responses)

toxicity pathway	MOA	inducing chemicals/ *positive controls*	total	+	−	positive response	negative response
xenobiotic metabolism	pregnane X receptor (PXR)	steroids/	3	3	0	PXR-cisFACTORIAL, PXR-transFACTORIAL, HG5LN PXR	-
	constitutive androstane receptor (CAR)	phenobarbitol, various pharma-ceuticals	2 (1)	1	1	CAR-yeast	CAR-transFACTORIAL
	peroxisome proliferator-activated receptor (PPAR)	phthalates, fibrate pharmaceuticals	7 (1)	2	5	PPARγ-transFACTORIAL, HELN-PPARγ	PPARα-transFACTORIAL, CALUX-PPARα, CALUX-PPARγ, PPARγ GeneBLAzer, MCF7-PPAR
	PPAR suppression		1	0	1	-	Anti-PPARγ GeneBLAzer
	aryl hydrocarbon receptor (AhR)	PAHs, PCDDs, coplanar PCBs	6 (1)	6	0	AhR-yeast, CAFLUX, H4IIEluc, MCF7-DRE (transient), AhR-cisFACTORIAL, DART cyp1a induction	-
specific MOA	acetylcholinesterase (AChE)	insecticides	1	0	1	-	AChE enzyme inhibition
	photosystem II	herbicides	1 (1)	1	0	IPAM	-
specific receptor-mediated MOA	estrogen receptor (ER)	human hormones and industrial chemicals (xenoestrogens), *17β-estradiol*	14 (9)	14	0	ER-CALUX, E-SCREEN, YES, HELN_ERα, HELN_ERß, ERE-cisFACTORIAL, hERα-HeLa-9903, MCF7-ERE, ERα-transFACTORIAL, Steroidogenesis, DART cyp19a1b (aromatase), ER-GeneBLAzer, hER yeast, medER yeast	
	ER suppression	*4-Hydroxy-tamoxifen*	1 (1)	1	0	Anti-ER-CALUX	-
	androgen receptor (AR)	*(Dihydro)-testosterone*	7 (6)	1	6	MDA-kb2 (but coexpression with GR)	AR-CALUX, HELN-AR, MCF7-ARE, Yeast Androgen Screen (YAS), AR-GeneBLAzer, AR-transFACTORIAL
	AR suppresion	*Flutamide*	2 (1)	2	0	Anti-AR-CALUX, anti MDA-kb2	
	progesterone receptor (PR)	*Levonorgestrel*	4 (5)	2	2	Steroidogenesis, induction of progesterone and of 17α OH-progesterone	PR-CALUX, PR-GeneBLAzer
	PR suppression	*Mifepristone*	1	1	0	Anti-PR-CALUX	-
	glucocorticoid receptor (GR)	*Dexamethasone*	5 (6)	5	0	GR-CALUX, GR Switchgear, GR-transFACTORIAL, GR-MDA-kb2 (AR suppressed), GR-GeneBLAzer	-
	GR suppression	*Mifepristone*	2	2	0	Anti-GR-CALUX, anti-GR-GeneBLAzer	-
	thyroid receptor (TR)	*3,3′5-Triiodo-thyronine*	4 (1)	0	4	-	TR-CALUX, T-SCREEN, THRα1-trans-FACTORIAL, HELN-TR
	RAR/RXR (Reproductive and developmental effects)	*Retinoic acid*	4	1	3	hRAR-yeast assay	MCF7-RARE, P19/A15, RORß-trans-FACTORIAL,
reactive modes of action	genotoxicity	*4-Nitroquinoline-N-oxide*	11 (4)	11	0	umuC ±S9, SOS chromotest, Ames ±S9, micronucleus assay	
	oxidative stress	PAH, electrophilic chemicals, *t-butyl hydroquinone*	1	1	0	oxidative stress in RTG2 cells	-
	protein damage	*Sea-Nine*	1	0	1	-	protein damage *E.coli* GSH±
adaptive stress response pathway	heat shock response	oxygen depletion (can be caused by metals)	2	0	2	-	HSE-cisFACTORIAL, hspb11 induction in DART after 120h
	hypoxia	tunicamycin, caplain	2	0	2	-	HIF-1a-cisFACTORIAL, Hypoxia-Switchgear
	endoplasmic reticulum stress	high salt, glycol	0	0	0	-	-
	inflammation	metals, PCBs, smoke, particles	4	1	3	Jurkat E6.1 IkB	NF-kB-CALUX, NF-kB-GeneBLAzer, NF-kB-cisFACTORIAL
	oxidative stress	reactive oxygen species, *t-butyl hydroquinone*	4	3	1	AREc32, Nrf2/ARE-cisFACTORIAL, Nrf2-CALUX	Nrf2-keap

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Table 2. continued

toxicity pathway	MOA	inducing chemicals/ *positive controls*	total	+	−	positive response	negative response
	DNA damage	electrophilic chemicals, UV radiation, *nutlin-3*	4 (1)	0	4		p53-cisFACTORIAL, p53-CALUX-S9, p53-CALUX +S9, p53-GeneBLAzer
cytotoxicity indicative of system response	bacterial cytotoxicity	all	2 (1)	2	0	*Vibrio fischeri* (Microtox), *Photobacterium phosphoreum*	-
	algal growth	all (+herbicides)	1 (1)	1	0	algae growth inhibition	-
	cytotoxicity (mammalian cells)	all	4	4	0	AREc32 cell viability, Caco 2 NRU, RTG2MTT, DART 48h lethality	-
	development	all	1	1	0	DART 120h sublethal	
	neurotoxicity	insecticides	1	0	1	-	SK-N-SH cytotoxicity
	immunotoxicity	immunosuppressive chemicals	1	0	1	-	THP1 cytokine

than the samples. A positive blank value was observed in one of the various Ames assays and is most likely due to measurement uncertainty as this value was derived from only one data point.

Bacterial Cytotoxicity Screening Assays. The bacterial cytotoxicity assays with *Vibrio fischeri* and *Photobacterium phosphoreum* were used as quick bioanalytical assessment tools, as these tests are rapid with only 15−30 min of exposure. Bioluminescence tests are nonspecific assays, as all stressors can impair the energy production and thus decrease bioluminescence. They provide a high responsiveness, that is, often the indicate effects for the "diluted" sample at REFs of less than 1. However, the high sensitivity if compared to other cellular assays may result from a higher bioavailability, that is, absence of serum proteins typically used in in vitro assay. This increased bioavailability could result in a detection of trace amounts of coextracted dissolved organic carbon (DOC). In particular the low-molecular fraction of assimilable organic carbon can add to the observed effects as previously demonstrated.[22] However, the high sensitivity may also result from specific interactions with bacterial physiology. Therefore, and because the control sample exhibited some effects as well, the luminescence assays have been excluded from the heatmap (see below).

Heatmap. The summary of 101 EC values (excluding the two bacterial cytotoxicity assays) in each of the 10 samples is presented in form of a heatmap (Figure 3, data summarized in SI, Table S5). The similarity of bioanalytical fingerprints between different water samples was characterized by hierarchical clustering.

Evidently, quantitative comparison is difficult because EC were expressed as EC_{10} or $EC_{IR1.5}$ and these two values are only directly comparable if the maximum IR is around 6 (see discussion in SI, Section S3-B). Therefore hierarchical clustering was only performed on samples and not on bioassays.

The closest similarity existed between the blank and the highly treated AO sample, while the RO sample clustered with this group on the next level of hierarchy (Figure 3). Surface water and ozonated recycled water clustered together. Both of these groups (cluster RW + O_3/BAC and cluster RO + Blank + AO) clustered closely on the next level of similarity. Of the more polluted samples, Eff1 and MF were highly similar. This is not unexpected, as microfiltration, the only treatment step separating the two samples, is ineffective at removing micropollutants. Slightly higher effects were, however, observed in the MF sample likely due to disinfection by chloramination of the membrane to avoid biofouling.[10] On the next level of hierarchical clustering, the two WWTP effluents Eff1 + MF and Eff2 showed high similarity.

The largest separation was observed between the cluster of Eff1 + Eff2 + MF and all other samples (Figure 3), clearly demonstrating that cell-based bioassays can distinguish between wastewater and reclaimed water samples.

The bioanalytical fingerprints can also help distinguish between different water types: WWTP effluents not only showed the highest effects but also distinct responses related to known environmental pollutants, including pesticides, industrial chemicals, pharmaceuticals, and personal care products, for example, the activation of the aryl hydrocarbon receptor by PAHs or methylmercaptoaniline,[23] the activation of the estrogen receptor by natural hormones and xenoestrogens, the activation of the glucocorticoid receptor by dexamethasone and organotin compounds,[24] or photosynthesis inhibition by herbicides. The specific effects, caused by chemicals that bind to receptors, were decreased substantially in the WRPs.

Stormwater had a slightly different pattern to WWTP effluents but was also dominated by pesticides, as represented for example by herbicidal activity that was absent in other samples of its cluster.

In other studies on stormwater one could even identify sewer cross-contamination by bioanalytical profiling.[16]

In contrast, disinfection by chlorination lead to disinfection byproducts, which showed distinct bioassay response patterns with clearly increased genotoxicity and oxidative stress responses in DW as compared to its source water (RW), while specific-receptor mediated effects were low in RW and almost fully disappeared in DW (Figure 3). This is consistent with previous bioanalytical profiling of the drinking water treatment process.[15]

DISCUSSION

Multiplexed Assays As a Screening Tool. The FACTORIAL assay was applied here for the first time to water samples and yielded interesting fingerprints of effects that were consistent with the results of the other bioassays. However, more work is required to implement reference chemicals and include a more rigorous dose−response assessment. The effect fingerprints were qualitatively consistent with responses seen in the initial ToxCast I screening of 320 pesticides, where 73% of the pesticides were responsive in PXR, 52% in Nrf2/ARE and 46% in PPARγ.[9]

Benchmarking Water Quality. A detailed discussion of the responses of each bioassay is provided in the SI, Section S6. Here follows a summary of the responsive and nonresponsive endpoints in relation to the associated step in the toxicity pathway (Table 2). It should be noted that, although responsiveness is related to assay sensitivity, even the most sensitive assay will not respond in the absence of chemicals capable of activating the bioassay endpoint. In other words, the absence of a measurable effect does not necessarily prove an assay insufficiently sensitive. Relative assay sensitivity can be assessed by comparing the effect concentrations and limits of detection of reference chemicals between assays. While such comparison was not possible for all assays tested in the present study, the results obtained can give some indication regarding the suitability of bioassays for monitoring purposes.

Induction of Xenobiotic Metabolism Pathways. Induction of metabolic pathways is not per se an indicator of toxicity but gives an indication of exposure to bioactive chemicals. Metabolism can detoxify or bioactivate chemicals. Omiecinski et al.[25] stressed the relevance and the toxicological implications of a number of xenobiotic metabolism pathways and associated NR, including the PXR, PPARα, β and γ, AhR, and CAR.

Three and six bioassays were evaluated for the PXR and AhR, respectively, and all showed positive responses in less treated samples and negative responses in recycled water and the blank (Table 2, SI, Section S6-A, Figure S5).

CAR plays a role in both phase I and II metabolism and plays a protective role against toxicity induced by bile acids as well as regulation of physiological functions. The target chemicals for CAR are less clearly defined than for AhR and while a few pesticides (e.g., methoxychlor, carbaryl propazine, 6-deisopropylatrazine) induced the CAR in the CAR-trans-FACTORIAL assay in previous work,[9] no response was detected in the water samples in the CAR-trans-FACTORIAL assay up to an REF of 4. In contrast, the CAR-yeast showed a response in all samples ($EC_{IR1.5}$ from 0.1 to 9.4) (Table 2, SI, Section S6-A, Figure S5).

For PPAR, only two of seven bioassays (PPARγ-trans-FACTORIAL and HELN-PPARγ) gave signals in the four most polluted samples (Table 2, SI, Section S6-A, Figure S5). PPAR is strongly linked to the regulation of glucose and lipid metabolism as well as inflammation, and is less important for xenobiotic metabolism.[26] In a high throughput study of 3000 environmentally relevant chemicals, roughly 1% of the tested chemicals were PPARγ agonists and 8% were PPARγ antagonists.[27] Organotins[28] and polyhalogenated bisphenol A[29] were found to induce PPARα and γ. The higher activity of PPARγ over PPARα for water samples is consistent with the finding that 146 of 309 ToxCast phase I chemicals were active in PPARγ-transFACTORIAL, while the other isoforms were less responsive.[9]

Specific Modes of Toxic Action. Most specific modes of action involve binding to receptors or inhibition of enzymes. In the past, direct enzyme inhibition assays have been popular tools for water quality testing. Recent work on the influence of dissolved organic matter (DOM) on the acetylcholinesterase assay has demonstrated that DOM nonspecifically impacts the assay at relatively low concentrations.[30] The implication of these findings is that for most tests with naked enzymes, water samples cannot be concentrated above a REF of 2. In the present study, only the two wastewater samples produced a valid response in this assay. Despite the high relevance of this biological endpoint for many insecticides, it thus proves unsuitable to investigate recycled water samples.

Photosynthesis Inhibition. An important group of environmental contaminants are herbicides that inhibit photosynthesis. While they are specifically designed to target photosynthesis inhibition, herbicides can nevertheless be toxic to humans and are regulated in recycled and drinking water guidelines. The most sensitive species to detect herbicides are algae, for which the inhibition of photosystem II by triazines and phenylurea herbicides can be specifically measured by pulse-amplitude modulated fluorometry.[31] This endpoint was very responsive in the water samples Eff1, Eff2, MF, SW that were suspected to contain herbicides (SI, Table S5).

Estrogen Receptor. The most relevant receptor-mediated effects are related to endocrine disruption (SI, Section S6-B, Figure S6). Estrogenic effects are by far the most prominent and environmentally relevant endocrine effects for aquatic species but they are overshadowed by other endocrine end points when it comes to human health. Fourteen different bioassays indicative of estrogenic effects were evaluated and all were active in four to five samples (Table 2). The absolute responsiveness was highest for ER-CALUX and MCF7-ERE but the effect pattern across the different samples was similar for all bioassays (SI, Table S5). No antiestrogenic activity could be detected in any of the samples, which is typical for samples that contain estrogenic chemicals.[17]

Androgen Receptor. Of seven bioassays (bioassay nos. 37−43, Table 1), only the MDA-kb2 produced positive results in the wastewater samples. Both GR and AR are expressed in this cell line and they share the same DNA response element, so it is unclear if the activity in this assay is purely AR-mediated, although incubation with flutamide indicates that the contribution of AR to the overall effect is higher than of GR.

Both bioassays for antiandrogenicity (anti-AR-CALUX and anti-MDA-kb2) were positive in some samples although only at very high REF. The WWTP effluents, which typically have highest antiandrogenic effects, were not responsive in anti-MDA-kb2, presumably due to the interfering agonistic response of effluent, similar to what has been observed with YAS and anti-YAS in an earlier study.[32]

Progesterone Receptor. The two transactivation assays for the PR, PR-CALUX, and PR-GeneBLAzer, did not exceed the 10% effect threshold in all samples. However, progestagenic activity has been detected previously in aquatic samples.[17,33,34] The anti-PR (anti-PR-CALUX) assay was also negative with samples tested to a REF of 2. The increased levels of progesterone and 17α-hydroxyprogesterone in the H295R bioassay

for steroidogenesis were most likely due to an inhibitory effect on CYP21A.

Glucocorticoid Receptor. ER, AR, and PR are important for the development and functioning of the reproductive system. The GR is more abundant and found in all cell types. Given that the GR has important functions in glucose metabolism and the immune feedback mechanisms, it has been linked to a wide spectrum of diseases, including cardiovascular, inflammatory and immune disease, diabetes, and obesity, and is therefore of high potential relevance. Five bioassays targeting activation of the GR were included in this study, all of which were active in one or more samples (SI, Figure S7). The observed GR activity is in agreement with previous studies on similar water types.[17,33]

Thyroid Receptor. No assay indicative of modulation of the thyroid hormone system showed response to any of the water samples (SI, Table S5), which comes as no surprise because the most commonly observed thyroid agonists and goitrogens are oxyanions such as the perchlorate and nitrate.[35] These inorganic ions do not act via TR binding and are not extracted with SPE.

RAR/RXR. The retinoic acid signaling pathway is crucial for reproduction and development as well as for cell homeostasis and immune function.[36] Two receptors are key to this pathway, the retinoic acid receptor (RAR) and the retinoic X receptor (RXR). RXR is a heterodimer partner not only for the RAR but also for other nuclear receptors including PPAR, PXR, CAR, and TR.[37]

We tested four bioassays that are connected to the retinoic acid-signaling pathway but only the two-hybrid assay, where RARγ is inserted into yeast with lacZ as reporter gene, showed activity in three samples. The RORβ-transFACTORIAL did not show any response with the water samples tested, although this endpoint tested positive in 30% of the ToxCast I chemicals.[9] Clearly, the role of RXR for water quality assessment should be further explored in the future.

Reactive Toxicity. Testing for reactive toxicity focused on genotoxicity and mutagenicity (Table 2, SI Figure S8). Only one bioassay, the micronucleus assay, detects DNA damage directly; the Ames test relies on reverse mutations and the umuC assay on detection of DNA repair. Three samples were active in the micronucleus assay, Eff2, RW and DW (SI, Figure S8). The SOS chromotest and umuC assays gave consistent results and were responsive at lower REFs but the Ames assay gave more variable responses and even false-positive responses (presumably due to the high inherent degree of endogenous gene mutation in bacteria).

Tests for genotoxicity can be run in the presence and absence of a rat liver metabolic enzyme mix (S9 fraction) to differentiate between chemicals that require metabolic activation and those that are detoxified by metabolism. In the umuC and the Ames assay, there was no discernible difference between response with and without S9.

The *E. coli* assay for protein damage relies on growth inhibition differences between a strain that is glutathione-deficient (GSH-) and the corresponding parent strain (GSH+).[38,39] These assays were found to be unsuitable for samples with high organic matter content.[38] In the present study no effects could be detected although there appeared to be a qualitative difference in growth inhibition between the GSH- and the GSH+ strains.

Only one assay attempted to quantify reactive oxygen species formation in RTG2 cells and results were positive and consistent with the activation of oxidative stress response pathway discussed below.

Induction of Adaptive Stress Response Pathways. Both the heat shock response and the hypoxia induction were negative in all assays tested (SI, Table S5). No bioassay for endoplasmic reticulum stress could be identified and therefore this potentially relevant endpoint had to be omitted.

Response to inflammation was tested by enzyme-linked immunosorbent assay (ELISA) in the human T-lymphoblast cell line Jurkat E6.1 by quantifying IκB, which is a chaperone for NF-κB that keeps NF-κB inactive and prevents it from entering the nucleus. Five samples tested positive in this assay (SI, Table S5). In contrast, the NF-κB-CALUX, NF-κB-GeneBLAzer and the NF-κB-cisFACTORIAL did not respond to any of the samples. These latter assays are relatively new, have not yet been applied for water quality assessment and possibly require further validation work to improve their detection limits.

Three of four bioassays indicative of the oxidative stress response were active in six to eight samples, highlighting the potential importance of this stress response pathway. The AREc32, Nrf2/ARE-cisFACTORIAL and the Nrf2-CALUX were all able to detect effects at low sample enrichment. The data also showed a wide dynamic range between different samples, which makes them ideal water quality indicators, although their relevance to health effects is less evident than for other bioassays.

The p53 protein plays an important role as a tumor suppression factor but all evaluated assays did not show any effect, both, in presence and absence of S9.

General Cytotoxicity and Models for System Response. The overarching effect overlying each of the cellular toxicity pathways is cytotoxicity (Figure 1). As cytotoxicity normally manifests at higher concentrations than induction of response pathways, this endpoint is best implemented as a quality control measure for all induction assays to verify that cell vitality is not adversely affected. We did not complete full dose−response curves for cytotoxicity in all mammalian reporter gene assays apart from AREc32 for which cytotoxicity was similar to the targeted cytotoxicity assays in a human colon cancer cell line Caco 2 NRU. The fish cell line RTG2 was of relatively low responsiveness. Acute toxicity in the zebrafish embryo (DART 48h lethality) was only observed in two samples Eff2 and SW but at high enrichment factors (REF 5−6).

Cytotoxicity assays may also give information about system toxicity if appropriate cell lines are used, although a recent report by the ACuteTox project suggests little difference in response using different cell lines in vitro.[40] Here we considered the sublethal endpoint in the zebrafish embryo toxicity test after 120 h of incubation as an indicator of developmental and potentially long-term apical effects. This effect was clearly more responsive than the 48 h acute lethality endpoint in the zebrafish embryo.

The SK-N-SH neuroblastoma cell line[41] is sensitive to chemicals that block the sodium channels and similar cell lines have been used previously in an assay to evaluate paralytic shellfish poisons caused by neurotoxic freshwater cyanobacteria.[42] We used a simpler version of this assay in this study, evaluating cytotoxicity as a coarse measure of cellular neurotoxicity. This endpoint was not active for our water samples tested to a REF of 2.

Expression of various cytokines in the human acute monocytic leukemia cell line THP-1 gives an indication of potential immunotoxicity.[17] Although a previous study reported

detectable inhibition of IL1β secretion in chlorinated waters,[17] we did not observe detectable effects in our study up to a REF of 2.

Benchmarking Treatment Efficacy. The bioassay results can be used to assess and monitor treatment processes (see SI, Section S7 for more detail). Best suited for this purpose are bioassays that show a clear decrease in response with increasing treatment and will not fall below the detection limit after treatment. We refer to these assays as "indicator bioassays" from hereon. Reverse osmosis (RO) is known as highly efficient in removing trace constituents, and only 13 indicator bioassays remained above detection limit after RO (but below LOD after AO, SI, Figure S10). After ozonation and BAC treatment, (another) 13 indicator bioassays remained above detection limit (SI, Figures S11 and S12). Between the two water reclamation plants, 18 suitable indicator bioassays were identified, including those indicative of AhR, PXR, CAR, ER, algal toxicity, genotoxicity and oxidative stress (SI, Figures S11 and S12).

In contrast, chlorination and chloramination increased the response in 15 of the 101 bioassays in drinking water samples (SI, Figure S13). The increased effect was most pronounced in the induction of xenobiotic metabolism and the reactive modes of action and oxidative stress response, which is consistent with the formation of chlorinated disinfection byproducts that cause genotoxicity and oxidative stress.[15,43] This comparison demonstrates that there is no single battery of bioassays that can be applied universally, but rather that a panel of assays should be tailored to fit the needs of each application.

A Routine Test Battery of Indicator Bioassays. Because a single bioassay is not capable of assessing water quality comprehensively, a set of relevant biological endpoints that are sensitive to micropollutants typically encountered in water samples can be used collectively as indicators of water quality. A battery of bioassays should include integrative endpoints such as cytotoxicity as well as endpoints specific to relevant steps in the cellular toxicity pathway. As a minimum, indicator bioassays should cover examples found responsive to and representative of "induction of xenobiotic metabolism", "endocrine disruption" and "adaptive stress responses". Relevant endpoints are proposed in the following:

1. Induction of xenobiotic metabolism. Our results confirmed that activation of the aryl hydrocarbon receptor, already one of the most widely applied endpoints in water quality assessment, is a relevant indicator of the presence of chemicals and should be included in any routine test battery. The pregnane X receptor showed high responsiveness to water samples and responds to a wide range of chemicals and should be further explored for routine application.
2. Endocrine disruption. Specific receptor-mediated modes of action including estrogenic and androgenic effects showed the most promise for routine water quality screening applications. Recent work using GR-CALUX applied to various environmental chemicals and water samples[33] also support our findings that GR activity is present and could be detected in secondary treated effluent with the current battery of GR bioassays. In addition, given the co-occurrence of progestins and synthetic estrogens in hormone replacement therapy, PR activity remains of interest, despite the negative findings here. Lastly, it is vital to test for antagonistic as well as agonistic effects.
3. Reactive modes of action. Genotoxicity as measured by well-established bioassays such as umuC or SOS chromotest served the purpose well. Bioassays derived from mammalian cells would be more relevant for human health and thus preferable to bacterial assays. As the p53 assays did not show the hoped-for responsiveness it is recommended to further evaluate alternative bioassays.
4. Adaptive stress response pathways. Oxidative stress response appears to be a highly sensitive and yet selective indicator of environmental pollution that responds to a wide range of chemicals as well as to transformation products and disinfection byproducts.[44] This is consistent with previous chemical testing in Nrf2/ARE-cisFACTORIAL, where almost 50% of the ToxCast chemicals were active.[9] Thus this mode of action is recommended to be included in any routine test battery, especially if transformation reactions are expected.
5. Cytotoxicity and systemic response. The bacterial cytotoxicity assays (*V. fischeri* and *P. phosphoreum*) are very fast and sensitive screening assays but their high sensitivity and effects caused by controls indicated that the responses may not be of human health relevance. In contrast, cytotoxicity assays with mammalian cells are comparatively less sensitive and clearly the bioassays for toxicity pathways are more relevant. With limitations, specific cell lines may be used as indicators of organ/systemic response. Nevertheless, further work has to be invested in the selection of appropriate tests systems and protocols for cell-based bioassays for organ/systemic responses as these are much less developed than nonspecific cytotoxicity assays and bioassays targeting cellular toxicity pathways. The enzymatic AChE inhibition assay to test for one aspect of neurotoxicity failed completely but there is the potential to implement neurotoxicity endpoints in the zebrafish embryo toxicity test.[45] A whole organism in vitro assay, such as the zebrafish embryo assay may help to link specific responses from the cellular assays to systemic responses by the observed phenotypes.

We also recommend that more attention be paid to the basal activities of cell lines in use. As metabolism is the most crucial modifier of toxicity, detoxifying many chemicals but activating others, the metabolic capacity of bioassay cell lines needs to be considered when selecting or designing a bioassay. Many available cell lines have low metabolic activity and for these it is advisable to run each experiment in parallel in the presence of an exogenous metabolic mixture, for example, liver S9 fraction.

In summary, an ideal battery of bioassays for water quality assessment and testing should contain sensitive bioassays that cover a wide range of cellular toxicity pathways (Figure 1). For induction of xenobiotic metabolism pathways, we recommend AhR and PXR. For specific modes of action, the receptor-mediated hormonal effects related to the estrogenic, glucocorticoid and antiandrogenic pathways appear to be most relevant as most are responsive to water samples. The oxidative stress response clearly stands out as a highly responsive defense mechanism. Cell viability ("cytotoxicity") assays should be further developed with a focus on those representative of systemic responses.

■ ASSOCIATED CONTENT

Supporting Information

Additional information on the water samples, the bioassay methods, the data evaluation, initial screening, bioassay results (including a large table with all detailed results), and a text on monitoring the treatment efficacy at WRPs is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Attagene and BDS are companies that market the bioassays applied by them in the present study.

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■ ABBREVIATIONS

AO	advanced oxidation
AR	androgen receptor
ASR	adaptive stress response
ATG	Attagene
AWQC	Australian Water Quality Centre
BDS	BioDetection Systems
BEQ	bioanalytical equivalent concentration
CAPIM	Centre for Aquatic Pollution Identification and Management
CAR	constitutive androstane receptor
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CT	cytotoxicity
CYP	cytochrome P450 monoxygenase
DART	embryo toxicity test with the zebrafish *Danio rerio*
DW	drinking water
EC	effect concentration
EEQ	estradiol equivalent concentration
Eff	effluent
ER	estrogen receptor
GU	Griffith University
GR	glucocorticoid receptor
HK	Hong Kong Baptist University
HTS	high-throughput screening
IR	induction ratio
IRCM	Cancer Research Institute of Montpellier
ISO	International Organization for Standardization
IWW	Institute for Water Research in North-Rhine Westfalia, Germany
MF	microfiltration
MOA	mode of action
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NJU	Nanjing University
NIH	National Institutes of Health
NRU	neutral red uptake
OECD	Organisation for Economic Co-operation and Development
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PXR	pregnane-X-receptor
RAR	retinoic acid receptor
RCEES	Research Center for Eco-Environmental Sciences
RECETOX	Research Centre for Toxic Compounds in the Environment
REF	relative enrichment factor
RFU	relative fluorescence units
RLU	relative light units
RO	reverse osmosis
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
RW	river water
SCCWRP	Southern California Coastal Water Research Project
SPE	solid phase extraction
SW	stormwater
SWISS	Swiss Centre for Applied Ecotoxicology
TEQ	toxic equivalent concentration
UA	University of Arizona
UCR	University of California Riverside
UF	University of Florida
UFZ	Helmholtz Centre for Environmental Research
UQ	The University of Queensland
USF	University of South Florida
WRP	water reclamation plant
WWTP	wastewater treatment plant
XM	xenobiotic metabolism

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Supporting Information

Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays

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Section S6. Additional information on bioassay results

Section S7. Monitoring treatment efficacy

Section S1. Additional information on collected water samples

The water samples were collected across a subset of sites in Southeast Queensland, Australia (Table S1), where previous water collections and bioanalytical characterization have taken place (Macova *et al.*, 2011).

Table S1. Description of samples and concentration of dissolved organic carbon (DOC).

Sample	Site description	DOC ($mg_C\ L^{-1}$)	Collected amount of water
Water Reclamation Plant 1			
Eff1	WWTP effluent from a municipal sewage treatment plant that uses activated sludge treatment, taken at the influent of the WRP	8.0 ± 0.1	14 L
MF	Water sample taken after microfiltration (MF) using filters disinfected by chloramination to avoid biofouling	7.7 ± 0.3	14 L
RO	Permeate after the reverse osmosis (RO) process	0.3 ±0.1	28 L
AO	Final recycled water after RO and treatment with UV/H_2O_2 (indirect potable reuse quality)	0.2 ± 0.2	28 L
Water Reclamation Plant 2			
Eff2	WWTP effluent from a municipal sewage treatment plant that uses activated sludge treatment, taken at the influent of the WRP	11.5 ±0.2	14 L
O_3/ BAC	Recycled water after ozonation and biologically activated carbon filtration (for industrial reuse and irrigation)	4.8 ±0.4	28 L
RW	River water taken at the influent of a metropolitan drinking water treatment plant	5.6 ± 0.2	28 L
DW	Drinking water treated by coagulation, chlorination and chloramination	3.0 ± 0.4	28 L

SW	Collected after a rain event on the 25th of January 2012 from a stormwater drain that receives runoff from a residential catchment.	4.2 ± 2.0[a]	14 L
H_2O	Ultrapure water (milliQ water) run through the same SPE as all other water samples		28 L

[a]Average of eight measurements collected at the same site during 16/03/2011 to 17/04/2012, as SW collected 25/01/2013 yielded invalid DOC results. SPE = solid phase extraction, WRP = water reclamation plant, WWTP = wastewater treatment plant.

The water samples were collected in 1 L amber glass bottles and transported to the laboratory within 2 h where they were acidified to pH 3 with concentrated hydrochloric acid (HCl). Samples containing chlorine were quenched with sodium thiosulphate (1 g/L). Most samples underwent solid phase extraction (SPE) immediately. As the sample volume was high, some samples could not be processed immediately and these were cooled to 4°C and stored for less than a week before SPE was performed.

The SPE was performed according to Macova *et al.* (2011) with the sorbent material validated in (Leusch *et al.*, 2013). All samples were filtered with a 1.6 µm glass fiber filter (GF/A Whatman) before extraction. Fourteen one-liter batches of Eff1, MF, Eff2 and SW and 28 one-litre batches of RO, AO, O_3/BAC, RW, DW and Blank (defined in Table S1) were extracted by passing each through two 6 cc solid phase cartridges in series, first an Oasis® HLB (500 mg, Catalogue Number 186000115, Waters) followed by a Supelclean coconut charcoal cartridge (2 g, Catalogue Number 57144-U, Sigma-Aldrich). Both types of cartridges were individually preconditioned prior to extraction with 10 mL of 1:1 acetone:hexane mixture, followed by 10 mL methanol and 10 mL of 5 mM HCl in MilliQ water. This resulted in 28/14 pairs of cartridges per sample (28 pairs for the cleaner samples and controls, 14 pairs of the WWTP effluent samples, MF and SW).

All cartridges were sealed individually and kept at -20°C until elution. Before elution the cartridges were defrosted and dried completely under vacuum, then elution was carried out with 10 mL of methanol and 10 mL of acetone:hexane. The eluate of 8/4 pairs of cartridges per sample were combined and evaporated under purified nitrogen gas before being solvent exchanged to methanol at a final volume of 1 mL.

The SPE extracts were aliquoted and tested in four laboratories (ATG, GU, UA, UQ). The extracts were dried as described below to send to ATG and were sent as methanolic extracts to UA. After the initial positive results, the remaining 20/10 pairs of cartridges, which had been stored for 5 months, were eluted. The extracts were combined and aliquoted for the remaining 16 laboratories, and were evaporated under purified nitrogen gas before being solvent exchanged to dimethyl sulfoxide (DMSO) at a final volume of 2 µL for shipping. The 2 µL samples in Agilent high-recovery HPLC vials (Catalogue Number 5183-2030) were flushed with purified argon gas. The samples were shipped at room temperature with express mail to all laboratories, where they were reconstituted upon arrival (after 1 day (Australia) to 3-5 days (overseas)) with appropriate solvent and stored at -20 °C until bioanalysis.

For practicality while all water samples were collected and enriched on SPE cartridges together, the cartridges were eluted in two batches. The first batch was used in four laboratories (UQ, UA, GU and ATG). Only after the appropriateness of the 10 samples were assessed in this initial stage by comparison with historic data, the second batch was extracted, aliquoted and sent out to the remaining 16 laboratories. To assure that the storage of cartridges had not changed the samples, the Microtox assay (see below) was performed on both batches and there was good agreement (paired t-test, paring was effective with a P=0.0001 and r=0.9351 and a log-log linear regression with a r^2 of 0.9162).

Section S2. Additional information on the bioassay methods

In Table 1 of the main article, all bioassay methods are referenced. In some cases, small modifications were made to the protocols and these are listed in Table S2. If modifications were more extensive or the protocols were unpublished, these are detailed in the following paragraphs and referred to in Table S2.

Table S2. Modifications in the bioassay methods in comparison to the literature references. Only modified assays are included in this Table.

#	**Labora-tories**	**Bioassay**	**Method modification**
5	CAPIM	CAR-yeast	Section S2-A.
9	BDS	CALUX-PPARα	BDS: Assay performed at 1% DMSO and in 384-well format.
10	BDS, CSIRO	CALUX-PPARγ	BDS: Assay performed at 1% DMSO and in 384-well format, CSIRO: Cells were lysed in 50 µL of Triton lysis buffer. Luciferase assay substrate was prepared according to Brasier and Fortin (2001).
12	GU	PPARγ-GeneBLAzer	Section S2-B.
13	GU	Anti-PPARγ-GeneBLAzer	Section S2-B.
15	UQ, RECETOX	CAFLUX	RECETOX: seeded at 30000/well, 24h of exposure; cells washed with PBS, 100 µl PBS added to each well, measured by fluorometer.
19	UFZ	DART cyp1a induction	Different exposure times (0-120 hpf) were used.
22	GU, CSIRO, BDS, IWW	ER-CALUX	BDS: Assay performed at 1% DMSO and in 384-well format, CSIRO: Cells were lysed in 50 µL of Triton lysis buffer. Luciferase assay substrate was performed according to Brasier and Fortin (2001). IWW: (Richard, 2012).
30	RECETOX	hERα-HeLa-9903	Medium DMEM-F12 (Sigma Aldrich, USA), 10% dialyzed fetal calf serum treated with dextran coated charcoal. Each plate exposed to: medium, solvent control, 17β-estradiol (1-500 pM) in triplicates, for 24h, 37° C.
34	UFZ	DART cyp19a1b	Different exposure time (0-120 hpf) was used.
35	UF, USF, UCR, SCCWRP	ERα-GeneBLAzer	96-Well format, Section S2-C.
37	GU, BDS,	AR-CALUX	BDS: Assay performed at 1% DMSO and in

#	**Labora-tories**	**Bioassay**	**Method modification**
	CSIRO		384 well format.
41	UF, USF, UCR, SCCWRP	AR-GeneBLAzer	96-Well format, Section S2-C.
43	RECETOX	MDA-kb2	Cells seeded at 50000/well; solvent control, medium and dihydrotestosterone (DHT 1 pM - 0.1 μM) tested on each plate.
44	RECETOX	Anti-MDA-kb2	Competing androgenic ligand: 0.1 nM DHT, agonist controls (0.01 μM and 0.1 nM DHT), medium, solvent control, and standard anti-androgen flutamide (10 nM - 10 μM) tested at each plate.
46	GU, BDS, CSIRO	GR-CALUX	BDS: Assay performed at 1% DMSO and in 384-well format, CSIRO: Cells were lysed in 50 μL of Triton lysis buffer. Luciferase assay substrate according to Brasier *et al.* (2001).
47	UA	GR Switchgear	Section S2-D.
49	RECETOX	GR-MDA-kb2	10 μM Flutamide was added to each sample dilution and agonist control (10 μM DHT) to inhibit androgenic activity; solvent control, medium and DHT (1 pM - 0.1 μM) tested on each plate.
50	GU, UF, USF, SCCWRP	GR-GeneBLAzer	96-Well format, see SI, Section S2-C; GU: ran as 384-well plate format, no change from original protocol.
51	GU	Anti-GR-GeneBLAzer	Agonist 0.4 nM mifepristone.
53	UF, USF, UCR, SCCWRP	PR-GeneBLAzer	96-Well format, Section S2-C.
54	GU, BDS, CSIRO	PR-CALUX	BDS: Assay performed at 1% DMSO and in 384-well format, CSIRO: Cells were lysed in 50 μL of Triton lysis buffer. Luciferase assay substrate according to Brasier *et al.* (2001).
58	BDS, GU	TR-CALUX	BDS: Assay performed at 1% DMSO and in 384-well format.
59	UQ	T-SCREEN	BDS: Assay performed at 1% DMSO and in 384-well format.
63	UQ	P19/A15	Section S2-E.
70	UA, IWW	Ames TA98	According to Xenometric manual (http://www.xenometrix.ch).
71	UA, IWW	Ames TA98+ S9	IWW: (Richard, 2012).
74	UQ, IWW	Ames TA100	UQ: none, IWW: (Richard, 2012).
79	UFZ	hspb11 induction	Different exposure time (0-120 hpf) was used.

#	Labora-tories	Bioassay	Method modification
		DART	
81	UA	Hypoxia-Switchgear	Section S2-F.
83	UQ	NFκB - Geneblazer	96-well format, Section S2-C.
84	BDS	NFκB -CALUX	Assay performed at 1% DMSO and in 384-well format.
85	GU	Jurkat E6.1 IkB	Section S2-G.
87	UA	Nrf2-keap	Section S2-H.
89	BDS	Nrf2-CALUX	Assay performed at 1% DMSO and in 384-well format.
91	BDS	p53-CALUX	Assay performed at 1% DMSO and in 384-well format.
92	BDS	p53-CALUX +S9	Assay performed at 1% DMSO and in 384-well format.
93	UF	p53-GeneBLAzer	96-well format, Section S2-B.
97	GU	Caco 2 NRU	Section S2-I.
98	CSIRO	RTG2 MTT	Exposure media were exchanged with media containing 0.5 mg/mL MTT rather than adding MTT solution directly to the wells. Incubation was for 3 hours at 22degC. MTT was solubilized with DMSO and absorbance determined at 540 nm.
101	GU	SK-N-SH cytotoxicity	Section S2-J.
102	GU	THP1 cytokine	Section S2-K.

S2-A. Two-hybrid CAR yeast assay

The experiments were performed according to Shiraishi *et al.* (2000) with the following modifications: Yeast cells that were introduced human constitutive androstane receptor (CAR) were cultured (30^{o}C, overnight; Sanyo Incubator, Tokyo, Japan) in a modified SD medium supplemented with 0.88% glucose, lacking tryptophan and leucine. After centrifuge at 2000 rpm for 20 minutes, the medium was replaced by a fresh MSD medium. The yeast solution cell density was measured (595 nm), and, if necessary, cell density adjusted by diluting with MSD medium to readings to a constant 1.75 -1.85. MSD solution (60 µL) was added to each well of the first row of a 96-well culture plate (Sumilon 96F disposable plates; Sumilon Bakelite Co., Tokyo, Japan). Thereafter, 2% DMSO / MSD solution (60 µL) was automatically added (Nichiryo NSP-7000 Multi-

channel Auto Sampling System, Nichiryo Co., Tokyo, Japan) to each well of the 2nd - 8th rows of the plate. Six samples were run on each plate, with aliquots of each sample (60 μL) added to two neighboring wells of the 1st row of the plate. An aliquot was removed from each well of row 1 and added to row 2 to dilute 2-fold. This process was repeated from rows 2–7. No sample solution from row 7 was added to the 8th row. Thereafter, yeast solution (60 μL) was added to all wells, the plate shaken (30s; Taiyo S-2000 Automatic Mixer, Taiyo, Tokyo, Japan) and then incubated (30°C, 4 h).

After incubation, a mixed solution (80 μL) for inducing chemiluminescence and for enzymatic digestion (Aurora GAL-XE Reaction Buffer containing GalactaLux substrate, MP Biomedicals Inc., CA, USA and Zymolyase 100T diluted with Z buffer (a mixture of 21.5 g $Na_2HPO_4 \cdot 12H_20$; 6.2 g $Na_2HPO_4 \cdot 2H_20$; 0.75g KCl; 0.246 g $MgSO_4 \cdot 7H_20$ in 1 L deionised water)) was then added to each well, and the plate incubated (37° C, 1 h; Ikemoto Scientific Technology Co, Tokyo, Japan). Thereafter, a light emission accelerator solution (50 μL; Aurora Accelerator, MP Biomedicals Inc., CA, USA) was added to each well, and the chemiluminescence produced by released β-galactosidase measured with a 96-well plate luminometer (Luminescencer-JNR AB-2100, ATTO Bioinstruments, Tokyo, Japan). 4-tert-octylphenol (Wako Pure Chemical Industries Ltd, Osaka, Japan) was used as positive control. A solvent (vehicle) control (DMSO, Nakalai Tesque Co., Kyoto, Japan) was also used.

S2-B. PPARγ-GeneBLAzer assay

The commercially available PPARγ-GeneBLAzer assay (Life Technologies, Vic, Australia) is based on a human embryonic kidney cell line (HEK 293H cells) modified to express a fusion protein combining the ligand binding domain of the human peroxisome proliferator-activated receptor γ (PPARγ) fused with the DNA binding domain of the GAL4 gene, and stably transfected with a β-lactamase reporter gene downstream of a GAL4 activator sequence. When an agonist binds to the ligand-binding domain of the PPARγ-GAL4 fusion protein, the protein binds to the activator sequence and stimulates expression of β-lactamase.

The division arrested (DA) kit was used here (cat no K1419, Life Technologies, Vic, Australia). In brief, the DA cell aliquot was thawed quickly in a 37ºC water bath,

transferred to 10 mL of assay medium, and centrifuged at 200 ×g for 5 min. The supernatant was discarded, and the cell pellet reconstituted to a cell density of 9.4×10^5 cells/mL (determined using a Millipore Scepter Handheld Automated Cell Counter). Using a multi-channel pipette, 32 µL of assay medium was added to the "cell-free control" wells, and 32 µL of the cell suspension was added to all the other wells (30,000 cells/well) of a black wall clear bottom poly-D-lysine coated 384-well plate (cat no 354663, BD, NSW, Australia). In agonist mode, 8 µL of 5× 0.5% DMSO (solvent control), 5× rosiglitazone (reference compound, final concentration range from 7 pM to 2 µM) or 5× test samples were added to their respective wells (maximum 0.1% solvent in the final well for all test samples). In antagonist mode, 10× solutions of 0.5% DMSO (solvent control), 10× GW9662 (reference compound, final concentration range from 13 pM to 3.6 µM) or 10× test samples were pre-mixed 1:1 with 10× rosiglitazone agonist (for a final concentration in the well of 32 nM), and 8 µL of the resulting mix added to the respective wells for solvent control, reference compound or sample (maximum 0.1% solvent in the final well for all test samples).

The plate was then incubated for 16 h in a humidified 37ºC/5% CO_2 incubator. At the end of incubation, 8 µL of 6× substrate mixture (provided in the kit) was added and the plate incubated for a further 2 h in the dark at room temperature. Fluorescence was then read with a plate reader (BMG Fluostar Omega; BMG Labtech, Vic, Australia) at 460 and 530 nm after excitation at 409 nm. Background fluorescence (determined in the cell-free control wells) was subtracted from all readings, and a β-lactamase expression ratio calculated by dividing the net fluorescence at 460 by net fluorescence at 530 nm. Samples were deemed as positive in agonist mode when they exceeded the 10 % effect concentration (EC_{10}; determined from the rosiglitazone standard curve) and in antagonist mode when they exceeded the 20 % inhibitory concentration (IC_{20}; determined from the GW9662 standard curve).

S2-C. GeneBLAZER assay panel

Reference chemicals were 17β-estradiol (E2), levonorgestrel (LEV), dexamethasone (DEX), and R1881 for the estrogen α, progesterone, glucocorticoid and androgen receptors (ER-α, PR, GR and AR), respectively. Chemicals were purchased from Sigma

Aldrich (E2, LEV, DEX) or PerkinElmer (R1881). Reference chemicals were diluted in assay-specific assay medium from a stock solution (E2: 2 μM, LEV: 20 μM, DEX, 0.1mM, R1881: 20 μM) prepared in DMSO. Nine dilutions, plus a DMSO-only control were made, and were based on previous optimization of reference compounds to include the entire linear range of fluorescent induction. Final DMSO concentration in diluted reference chemicals was 0.5%.

Water extracts were reconstituted in a total of 300 μL of DMSO. Four dilutions of each of the 10 water extracts were prepared by adding 5 μL of reconstituted water extract to assay-specific assay medium to the first dilution and then serial diluting 50 μL of first dilution and adding it to 47.5 μL of assay media and 2.5 μL of DMSO to the second and so forth. Final DMSO concentration in diluted reconstituted water extract was 0.5%.

Plate set-up was uniform across all laboratories, except in the case of USF, who did not have DMSO-control wells on the second plate. Reference chemicals and water extract dilutions were assayed simultaneously across two 96 well plates. Each reference chemical and water extract dilution was assayed in triplicate. Cell-free media, DMSO-control, and DMSO-free control were assayed in triplicate on each plate (except at USF).

The four reporter assays used in this assessment were GeneBLAzer® ER-Alpha, PR, GR and AR Division Arrested Assay Kits (Life Technologies, Carlsbad, CA). All kits were commercially bought except for the AR assay, which was manufactured for this assessment. Assays were bought as kits and optimized for use in a 96-well plate format, rather than the manufacturer suggested 384-well format. All procedures were performed in a Class II biological safety cabinet using sterile techniques. All media, chemicals and materials used in these assays were from manufacturer recommended sources. Modifications to the manufacturer's protocol were made to cell number, and media/dose volume to optimize the assay for 96-well format.

Cells stored under liquid nitrogen vapor were quickly thawed and transferred, drop-wise, into 10 mL of assay medium in a sterile 15-mL conical tube and centrifuged at 200 × *g* for 5 minutes. Supernatant was aspirated and cell pellet was resuspended in 6 mL fresh assay medium. Cells were counted and diluted in assay medium to a density of 5.5 × 10^5 cells/mL. Ninety μL (50,000 cells) of cell suspension or assay medium (cell-free control wells) were added to each well. Ten μL of appropriate 10X reference chemical,

diluted water extract, or DMSO-added assay media were added to corresponding wells. Cells were incubated overnight (~ 16 h) at 37°C with 5% CO_2 .

On the following day, 20 µL of six times concentrated loading solution, prepared according to the manufacturer's protocol, was added to each well. The plate was covered to protect from light and evaporation, and incubated at room temperature for 2 h in the dark. Fluorescent measurements were made according to manufacturer's instructions.

S2-D. Switchgear Assay for the Glucocorticoid Receptor

A commercially available GR assay kit (Switchgear Genomics, California) was used to evaluate the GR activity in water samples. The GR-Switchgear assay integrates the signal from four validated pathway-specific reporter vectors using the RenSP reporter gene. This is important, and unique to this assay, since there are numerous endogenous promoters for the gene and no single promoter can respond to all potential agonists and antagonists. Multiple validated house-keeping reporters, using the CLuc reporter gene, are also applied to monitor cell "health" during the assay, also unique to this particular assay. The assay is a transient-transfection assay, which means that plasmids containing the reporter genes are freshly transfected each time the assay is performed.

A human fibrosarcoma cell line (HT1080) was maintained in standard growth medium composed of 500 mL minimal essential media (MEM), 5 mL GlutaMax, 50 mL Fetal Bovine Serum (FBS) (Heat inactivated) and 5 mL Pen/Strep. Cells were thawed from liquid nitrogen and passaging was carried out in 75 cm^2 flasks every 2-3 days. The 2^{nd} generation cells were used in this assay when they reached greater than 80% confluence. Cell density was controlled at 1×10^5 cells/mL in stripped growth medium (charcoal stripped FBS, without antibiotics). The transfection reagent containing the 4 GR plasmid constructs and housekeeping constructs were thawed, mixed, and incubated for 30 min at room temperature. The transfection mix was then added to cell medium, thoroughly mixed, and 100 µL added to each well of a 96-well white tissue culture (TC) plate. In a separate 96-well clear TC plate, an aliquot of 100 µL was added to cells in 12 wells for visual monitoring of cell viability and growth. Both plates were incubated at 37°C in a CO_2 incubator for 12-16 h.

After overnight culture, the medium was replaced by 90 µL fresh stripped FBS growth medium and 10 µL of water sample extract diluted in 10% of stripped medium. After 18-24 h of exposure, 10 µL of the cell supernatant was transferred to a secondary white 96-well TC plate and both plates were frozen at -80ºC. Substrate and buffer solutions were added after the plates were thawed. Luminescence was measured to determine for the luciferase reporter gene activity (LightSwitch Dual Assay System). DEX was used as the positive control and a negative control and solvent control were also included for quality control.

S2-E. p19/A15 assay for induction of the retinoic acid receptor (RAR)

p19/A15 Cells were grown in Dulbecco's MEM with sodium pyruvate and L-glutamine, high glucose, 10% FBS, 1% penicillin-streptomycin, 1.6% non essential amino acid (NEAA) was obtained from Gibco, Australia. Cells were grown in T75 flasks in 11 mL Dulbecco modified minimal essential medium (DMEM) and incubated at 37ºC and 5% CO_2 and passaged every 2-3 days when cells were 70% confluent.

For an exposure experiment, cell concentration was adjusted to 100,000 cells/mL and 100 µL was transferred in each well of a white polystyrene tissue culture treated 96-well microplate (Corning). The plates were then incubated for 24h at 37ºC and 5% CO_2 and dosed with the appropriate amount of chemical or extract. Each plate should include one serial dilution of atRA ($3.23 \cdot 10^{-11}$ M to $3.85 \cdot 10^{-18}$ M) or 9-cis RA ($2.43 \cdot 10^{-07}$ M to $2.89 \cdot 10^{-14}$ M) as positive control and one row of medium only. The plates were then covered with PCR-SP plate sealer from Axygen and incubated for 24 h before cytotoxicity or induction was assessed.

A typical experiment consists of two steps, where each step is performed in duplicate. First, a range finder with a serial (2-fold) dilution series was performed, where induction of RAR pathway and cytotoxicity were evaluated. Interference by cytotoxicity causes a suppression of the induction signal and such concentrations cannot be used for the induction data evaluation. Second, non-cytotoxic concentrations/dilutions of the water sample were selected and a linear dose-response curve was measured for induction only. Often the window between induction and cytotoxicity is small and no maximum

induction can be reached, therefore concentrations should be selected in a way that the maximum induction ratio is 5.

As a control, the cell viability was assessed with the MTS assay. MTS (tetrazolium) is bioreduced by cells into an aqueous, soluble formazan product by dehydrogenase enzymes found in metabolically active cells (Mosmann, 1983). When cells die, the ability to reduce these products is rapidly lost due to mitochondrial dysfunction. The absorbance of the formazan product at 490 nm can be measured directly from 96-well assay plates without additional processing, and the amount is directly proportional to the number of living cells in culture. After 24 h incubation the medium in each plate was replaced by 120 µL MTS (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) with MTS and phenazine methosulfate as the electron coupling reagent) in Hyclone DMEM without phenol red (Thermo Scientific) and absorbance at 492 nm was read after 2 h incubation.

S2-F. Switchgear Assay for Hypoxia

The commercially available hypoxia assay kit (Switchgear Genomics, California) uses the HT1080 cell line with transient transfection of three reporter constructs including lactate dehydrogenase (LDHA) promoter, H1F1a promoter, housekeeping gene ACTB (ACTB_PROM) from Switchgear, in which H1F1a is a well-known hypoxia inducible transcription factor.

In brief, transfection reagent, which contained the three plasmid constructs (LDHA, H1F1a, and ACTB), were first thawed from -20ºC and incubated for 30 min at room temperature. The human fibrosarcoma cell line HT1080 was thawed quickly in a 37ºC water bath from the -80ºC freezer, and the thawed cells were immediately added to the growth medium which was composed of Eagle's minimum essential medium (EMEM, ATCC #30-2003), 10% normal FBS, 1% of GlutaMax and 1% of PenStrep. To get 20,000 cells per well, cell density was maintained at 2.1×10^5 cells/mL. Then the transfection reagents were mixed with the cell and medium solution at a ratio of 5:95. Using a multi-channel pipette, 100 µL of the transfected cell mixture was aliquoted to each well of a white 96-well tissue culture plate. In a separate clear 96-well tissue culture plate, 100 µL of cells were aliquoted to 12 wells for visual monitoring of cell viability

and growth. Both plates were incubated at 37 ºC in a CO_2 incubator for 12-16 h. After overnight culture, the medium was replaced by 90 µL of fresh charcoal-stripped FBS growth medium and 10 µL of sample, which was diluted in 10% of stripped medium in advance.

After 24 h of exposure, 10 µL of the supernatant was transferred to a secondary white 96-well tissue culture plate, and both of the plates were frozen in -80ºC for better sensitivity. Substrate and buffer solution were then added after the plates were thawed, and luminescence was quantified as a measure of luciferase activity (LightSwitch Dual Assay System, available in the kit). Desferrioxamine (DFO) was used as the positive control. Negative control and solvent control were included for quality control.

S2-G. Jurkat E6.1 IkB

In the assay Jurkat E6.1, cells were resuspended in Roswell Park Memorial Institute medium (RPMI without phenol red supplemented with 5% charcoal-stripped fetal bovine serum) at 1×10^6 cells/mL (determined using a Millipore Scepter Handheld Automated Cell Counter). Cells were then seeded at 200,000 cells/well by adding 200 µL of cell suspension to the 48 inner wells of a flat bottom standard 96-well plate, and the test samples were added in 50 µL of white media (maximum 0.1% final solvent concentration). The remaining wells were filled with 250 µL of phosphate buffered saline (PBS) to act as a humidity barrier, and the plate incubated for 24 h in a humidified 37ºC/5% CO_2 incubator. A geometric dilution series of phorbol-12-myristate-13-acetate (PMA) was used as a reference compound, with final concentrations in the well ranging from 0.2 nM to 0.2 µM.

After incubation, the content of each well was gently mixed and 200 µL was transferred to a v-bottom 96-well plate. The plate was centrifuged at 300 ×g for 5 min, and 150 µL of the supernatant was discarded (paying particular attention not to disturb the cell pellet). The pellet was rinsed with 100 µL of warm sterile PBS and the plate centrifuged again at 300 ×g for 5 min. After centrifugation, 100 µL of the supernatant was discarded (again paying particular attention not to disrupt the cell pellet). IκB concentration in the cell pellet was then determined using a commercially available ELISA kit (IκBα Total InstantOne ELISA; cat no 85-86061, eBioscience), with minor modifications. In brief, cells were lysed with 1.5× lysis mix added in the v-bottom 96-

well plate directly and mixed by aspirating/dispensing with a multi-channel pipette, then placed on an orbital shaker at 300 RPM for 10 min at room temperature. Then, 50 µL of cell lysate were transferred into the InstantOne assay plate (provided with the kit) followed by 50 µL of IκB antibody cocktail (provided with the kit). A negative and positive IκB control, provided with the kit, were also tested with every ELISA run.

The plate was covered with an adhesive seal and incubated for 1 h at room temperature on a microplate shaker at 300 RPM. The wells were washed with 200 µL of wash buffer (provided with the kit), all liquid removed by inverting on a paper towel, and 100 µL of detection reagent (provided with the kit) was added to each well. The plate was incubated for 10 min at room temperature on a microplate shaker at 300 RPM, and the reaction stopped by adding 100 µL of stop solution. The absorbance of each well was then measured with a plate reader (BMG Fluostar Omega; BMG Labtech, Vic, Australia) at 450 nm.

S2-H. Nrf2-keap cell line

The human breast cancer cell line MDA-MB-231-745, which was transfected with the antioxidant response element (ARE) luciferase plasmid (Villeneuve *et al.*, 2008), was donated by Prof. Donna Zhang at Department of Pharmacy, the University of Arizona. The standard growth medium was composed of minimal essential medium (MEM, Life Tech, #11095-080), 10% FBS, 1% L-glutamine, 0.1% Gentamycin, 6 ng/mL Insulin, 2 mM HEPES and 1.5 µg/mL puromycin. Cells were thawed from liquid nitrogen and passaging was carried out in 75 cm^2 flasks every fourth day. The 4^{th} generation cells at more than 80% confluence were used in this assay. Cell density was controlled at 2×10^5 cells/mL. Using a 8-channel pipette, 100 µL of the cell solution were seeded into one white 96-well plates and one clear 96-well plate (cytotoxicity test).

After overnight culture in a CO_2 incubator for 16 h (5% CO_2, 90% humidity), the medium was replaced by 90 µL of fresh growth medium and 10 µL of sample, which was diluted in 10% of growth medium in advance. All samples were tested in triplicate including the medium blank and solvent blank. Tert-butylhydroquinone (tBHQ) was used as the positive control and the solvent used was methanol. After another 16 h of exposure in the CO_2 incubator, the medium in the white plates was removed, and washed with PBS. Twenty-five microlitres of Passive Lysis Buffer (PLB) was then added and the plates

were shaken for 15 min before luciferase analysis. Gen5 micro-plate reader with a delivery pump was used for the measurement and the luminescence was read directly by well after luciferase buffer (pH=7.8) was added. For the cytotoxicity test, after 16 h exposure the medium was replaced by 100 µL of clear fresh medium (without phenol red) and 20 µL of MTS solution (Promega, #G3580). Absorbance at 492 nm was read after 2 h of incubation.

S2-I. CaCo2 NRU assay

The Caco2-NRU (neutral red uptake) test is a measure of non-specific cytotoxicity. It is used to determine if the test sample impacts the viability of Caco2 (human epithelial colorectal adenocarcinoma) cells after 21h of exposure. Cell viability at the end of the incubation period is determined by adding neutral red, a dye that stains only live cells, and measuring the amount of dye taken up by the cell culture. The method was adapted from Konsoula and Barile (2005). In brief, Caco2 cells were grown in DMEM/F12 with phenol red supplemented with 8% FBS and 100 µM non-essential amino acids.

For the assay, cells were seeded at 20,000 cells/well in 100 µL assay medium (DMEM/F12 medium without phenol red supplemented with 5% stripped FBS (CD-FBS) and 100 µM non-essential amino acids) in 96-well plates and incubated for approximately 24h at 37ºC 5% CO_2. When the cells reached confluence (usually about 24h), the medium was removed and replaced by 150 µL of fresh assay medium and 50 µL of assay medium containing the model compound or water extract to be tested (final methanol concentration in the assay plate ≤0.1%). After 21 h of incubation at 37º C 5% CO_2, the medium was removed, the wells rinsed with 150 µL PBS, and the PBS was replaced by 150 µL of neutral red media (50 µg/mL neutral red in assay media, made fresh). After a further 3h incubation at 37ºC 5% CO_2, the medium was aspirated and replaced with 150 µL of neutral red desorbing fixative (1% acetic acid, 50% ethanol, in deionized water). The plate was placed on an orbital shaker at 600 rpm for 10 min at room temperature and absorbance was read in a plate absorbance reader (BMG FluoSTAR Omega) at 540 nm.

S2-J. Human neuroblastoma (SK-N-SH) cytotoxicity

Human neuroblastoma cells (SK-N-SH cells) were resuspended in white medium (DMEM/F12 without phenol red supplemented with 5% FBS, 1× non-essential amino

acids and 2 mM Glutamax; Life Technologies, Vic, Australia) at 1×10^5 cells/mL (determined using a Millipore Scepter Handheld Automated Cell Counter). Using a multi-channel pipette, 200 µL of cell suspension was added to every well (20,000 cells/well) of a standard flat bottom 96-well plate, and the plate was incubated for 24 h in a humidified 37ºC/5% CO_2 incubator.

The medium was then removed by aspiration and replaced with 200 µL of fresh white media containing the test sample (maximum solvent concentration of 0.5%), and the plate incubated 21 h in a humidified 37ºC/5% CO_2 incubator. The media was then aspirated, the wells rinsed with 150 µL of PBS, the PBS aspirated, and 150 µL of neutral red media (50 µg/mL neutral red solution, prepared fresh) was added. The plate was then incubated a further 3 h in a humidified 37ºC/5% CO_2 incubator.

At the end of the incubation, the medium was aspirated, the wells rinsed with 150 µL PBS, the PBS was aspirated and 150 µL of neutral red desorbing fixative (1% acetic acid, 50% ethanol, prepared in ultrapure water) was added. The plate was placed on an orbital shaker at 600 rpm for 10 min and the absorbance was read at 540 nm in a plate reader (BMG Fluostar Omega; BMG Labtech, Vic, Australia). DMSO was used as a reference compound, with an IC_{10} and IC_{50} of approximately 50 and 500 mM, respectively. Samples were deemed as "neurotoxic", when cytotoxicity exceeded IC_{10} (determined from the DMSO standard curve).

S2-K. THP1 cytokine assay

The THP1 cytokine assay provides a measure of immunotoxicity. For this assay, we monitored interleukin 1β (IL1β). The assay was run in antagonist mode, by measuring the inhibition of the normal production of IL1β by THP1 cells exposed to *E. coli* lipopolysaccharide (LPS) after exposure to the sample for 24h. The methods were adapted from Baqui *et al.* (1998). In brief, THP1 cells were cultured in growth medium (DMEM/F12 medium with phenol red supplemented with 8% FBS and 100 µM non-essential amino acids).

For the assay, cells were seeded at 200,000 cells/well in 200 µL of growth media (with 1 µg/mL LPS in antagonist mode) and 50 µL of assay medium containing the model compound or water extract to be tested (final methanol concentration in the assay plate ≤0.1%). After 24 h incubation at 37ºC 5% CO_2, cells were transferred to a V-bottom

96-well plate, centrifuged at 300×g for 5 min, and the supernatant was transferred to a fresh 96-well plate. IL1β concentration in the supernatant was assayed by ELISA (Human IL1β quantikine ELISA, RnD Systems), following the supplier's instruction.

Section S3. Additional information on data evaluation

The dose-metric of the concentration-effect curves is the relative enrichment factor REF, which is the combination of the enrichment of the extraction and the dilution in the bioassay (Eq. 1), thereby representing the enrichment (REF > 1) or dilution (REF < 1) of the original sample in each bioassay. The REF is expressed in the units of $[L_{water\ sample}/L_{bioassay}]$.

$$\mathrm{REF} = \mathrm{dilution\ factor}_{bioassay} \cdot \mathrm{enrichment\ factor}_{SPE} \quad (1)$$

The enrichment factor of the SPE enrichment factor_{SPE} was calculated using Eq. 2 from the volume of extracted water to the volume of resulting extract (in solvent).

$$\mathrm{enrichment\ factor}_{SPE} = \frac{V_{water}}{V_{extract}} \quad (2)$$

The dilution factor of each bioassay was calculated using Eq. 3.

$$\mathrm{dilution\ factor}_{bioassay} = \frac{\mathrm{volume\ of\ extract\ added\ to\ bioassay}}{\mathrm{total\ volume\ of\ bioassay}} \quad (3)$$

S3-A. EC_{10} (10% effect concentration)

EC_{10} values were reported for the cytotoxicity bioassays and for receptor-mediated effects and were obtained from a log-logistic fit of the concentration-effect curves (Figure 2A in the main article). The % effect was calculated with Eq. 4

$$\%effect = \frac{signal_{sample} - signal_{control}}{signal_{max} - signal_{control}} \quad (4)$$

Adjustable parameters were the slope and the effect concentration causing 50% reduction of maximum effect, EC_{50}.

$$\%effect = \frac{1}{1 + 10^{slope \cdot (\log EC_{50} - \log concentration)}} \quad (5)$$

The EC_{10}, the effect concentration causing 10% reduction of cell viability, was derived from the EC_{50} and the slope s (Eq. 6).

$$\log EC_{10} = \log EC_{50} + \frac{1}{s}\log\left(\frac{1}{9}\right) \quad (6)$$

In many cases, no full concentration-effect curves were obtained for the sample extracts. Partial concentration-effect curves can only be fitted if the slope is fixed at 1 or at the slope of the reference compound. Alternatively, because the lower portion of the log-logistic concentration effect curves is linear with respect to non-logarithmic concentrations, the EC_{10} can also be derived from a linear concentration-effect curve with intercept zero up to 20% of maximum effect (Eq. 7, Figure 2C in the main article).

$$\%effect = slope \cdot concentration \quad (7)$$

$$EC_{10} = \frac{10\%}{slope} \quad (8)$$

The EC_{10} values derived with the linear method agreed well with the log-logistic derivation and the final results of the samples were derived from the linear concentration-effect curves, although the EC_{10} values of the reference compounds were from the full log-logistic fit.

S3-B. $EC_{IR1.5}$ (effect concentration causing an induction ratio IR of 1.5)

The $EC_{IR1.5}$ was derived for all reporter gene assays where no maximum response could be obtained. By nature of the endpoint the IR approach applies to genotoxicity and most

adaptive stress responses such as the oxidative stress response. In addition, a few of the endpoints assessed had no reference compounds, e.g., the FACTORIAL. For all endpoints where no reference compounds were tested, the $EC_{IR1.5}$ was deduced.

The IR is the ratio of the measured signal (*e.g.*, absorbance, relative light units (RLU), relative fluorescence units (RFU)) to its control value (Eq. 9). An analogous equation can be used for the number of revertants in the Ames assay, hence called revertant ratio (RR).

$$IR = \frac{signal_{sample}}{signal_{control}} \quad (9)$$

Concentration-effect (IR) curves would show the typical log-logistic form but the maximum is hard to establish due to cytotoxicity interference or it may not even exist (Figure 2B in the main article). Therefore only the linear portion of the concentration-effect curves was evaluated up to an IR of 5 (Eq. 10, Figure 2D in the main article).

$$IR = 1 + slope \cdot concentration \quad (10)$$

The assessment endpoint is the concentration that induces an IR of 1.5 ($EC_{IR1.5}$). The $EC_{IR1.5}$ can be derived using the linear regression function with Eq. 11 (and analogously for the revertant ratio in the Ames test with Eq. 12).

$$EC_{IR1.5} = \frac{0.5}{slope} \quad (11)$$

$$EC_{RR1.5} = \frac{0.5}{slope} \quad (12)$$

The threshold of 1.5 was selected because (a) it is employed in several guideline documents, *e.g.*, umuC genotoxicity assay, (b) it is very close to the limit of detection in many cases (control plus 3 standard deviations) (Escher *et al.*, 2012), (c) it is an interpolation not an extrapolation such as the EC_{50}, and (d) it can be applied if the maximum of the dose-response curve is not known. The disadvantage of using IR is that depending on the bioassay, maximum response can be at IR of 2 up to over 100. If the maximum IR reaches 6, then the $EC_{IR1.5}$ is equivalent to the EC_{10}, if the maximum ER is 2, the $EC_{IR1.5}$ is equivalent to the EC_{50}, and for IRs that level off at 100 or more, the $EC_{IR1.5}$ is often close to the limit of detection.

For the 22 assays for which the maximum effect and EC_{10} were derived, it was possible to calculate what % effect would be equivalent to IR 1.5 (Table S3). For 13 of these bioassays, the max IR fell between 3 and 15 allowing the EC_{10} and the $EC_{IR1.5}$ to be directly compared. For nine, the maximum IR was well above 15 up to 940 and in these cases the EC_{10} would be inherently less responsive than the $EC_{IR1.5}$.

Table S3. Comparison of maximum IR with effect level at IR 1.5.

#	Laboratories	Bioassay	Maximum IR	Effect level at IR 1.5
9	BDS	CALUX-PPARα	5	13%
10	BDS, CSIRO	CALUX-PPARγ	20	3%
12	GU	PPARγ GeneBLAzer	3	25%
15	UQ, RECETOX	AhR-CAFLUX	13, 2	4%, 50%
16	RECETOX	H4IIEluc	5 to 11	5 to 13 %
22	GU, CSIRO, BDS, IWW	ER-CALUX	15, 15, 5, 15	3, 3, 11, 3%
23	UQ	E-SCREEN	3 to 26	2 to 23%
24	Swiss, CSIRO, UA	YES	100	0.5%
30	RECETOX	hERα-HeLa-9903	3 to 5	13 to 25%
31	HK	MCF7-ERE	6	10%
35	UFL, USF, UCR, SCCWRP	ER-GeneBLAzer	24, 27, 5	3, 3, 13%
37	GU, BDS, CSIRO	AR-CALUX	10 to 60, 45, 20	1 to 60, 1, 3%
40	UA, CSIRO	YAS	100, 54	1, 1%
41	UFL, USF, UCR, SCCWRP	AR-GeneBLAzer	3	26%
43	RECETOX	MDA-kb2	6	10
46	GU, BDS, CSIRO	GR-CALUX	15 to 40, 15, 20	1 to 4, 4, 3%
47	UA	GR Switchgear	15	4%
50	GU, UFL, USF, UCR, SCCWRP	GR-GeneBLAzer	22, 25, 20, 50, 22	2, 3, 3, 1, 2%
53	UFL, USF, UCR, SCCWRP	PR-GeneBLAzer	6, 9, 3, 5	10, 6, 25, 15%
54	GU, BDS, CSIRO	PR-CALUX	50, 110, 940	1, 0.5, 0.05%
58	BDS, GU	TR-CALUX	940, 40	0.05, 1%
59	UQ	T-Screen	5	12%

S3-C. $EC_{SR0.2}$ (effect concentration causing a suppression ratio SR of 0.2) for all antagonistic effects and chaperon dissociation.

A receptor-binding bioassay is run in antagonistic mode if the receptors are saturated or occupied with a constant concentration of an agonist (positive control). In an antagonistic mode experiment, varying concentrations of sample are added, while the concentration of the agonist is kept constant. If the signal of the agonist is suppressed the sample has an antagonistic effect (Figure S1). The suppression ratio SR is defined by Eq. 13. The analogous equation was used for endpoints that are based on chaperon dissociation (CD).

$$SR = 1 - \frac{signal_{sample} - signal_{control}}{signal_{agonist} - signal_{control}} \quad (13)$$

$Signal_{agonist}$ refers to the signal (relative fluorescence/light units (RFU, RLU), *etc*.) measured in presence of the agonist (positive control), which is normally the highest signal obtained unless the agonist was not added at saturating concentrations and the sample also had an agonistic effect. If $signal_{agonist} >> signal_{control}$, Eq. 13 simplifies to Eq. 14.

$$SR = 1 - \frac{signal_{sample}}{signal_{agonist}} \quad (14)$$

In most cases no full concentration-effect curves were obtained for antagonistic effects. Therefore, we used only the initial linear part of the concentration-effect curves up to a suppression ratio of 0.3 (Figure S1). The $EC_{SR0.2}$ is calculated from a linear regression through the zero point (Eq. 15). The 20% suppression level (SR 0.2) was chosen to derive the $EC_{SR0.2}$ (Eq. 16) because the variability is typically larger than in the agonist mode and the 10% suppression level (SR 0.1) is often not above the variability of the controls, which would produce false-positive results.

$$SR = slope \cdot concentration \quad (15)$$

$$EC_{SR0.2} = \frac{0.2}{slope} \quad (16)$$

Analogously, an $EC_{CD0.2}$ was defined for chaperone dissociation.

$$EC_{CD0.2} = \frac{0.2}{slope} \quad (17)$$

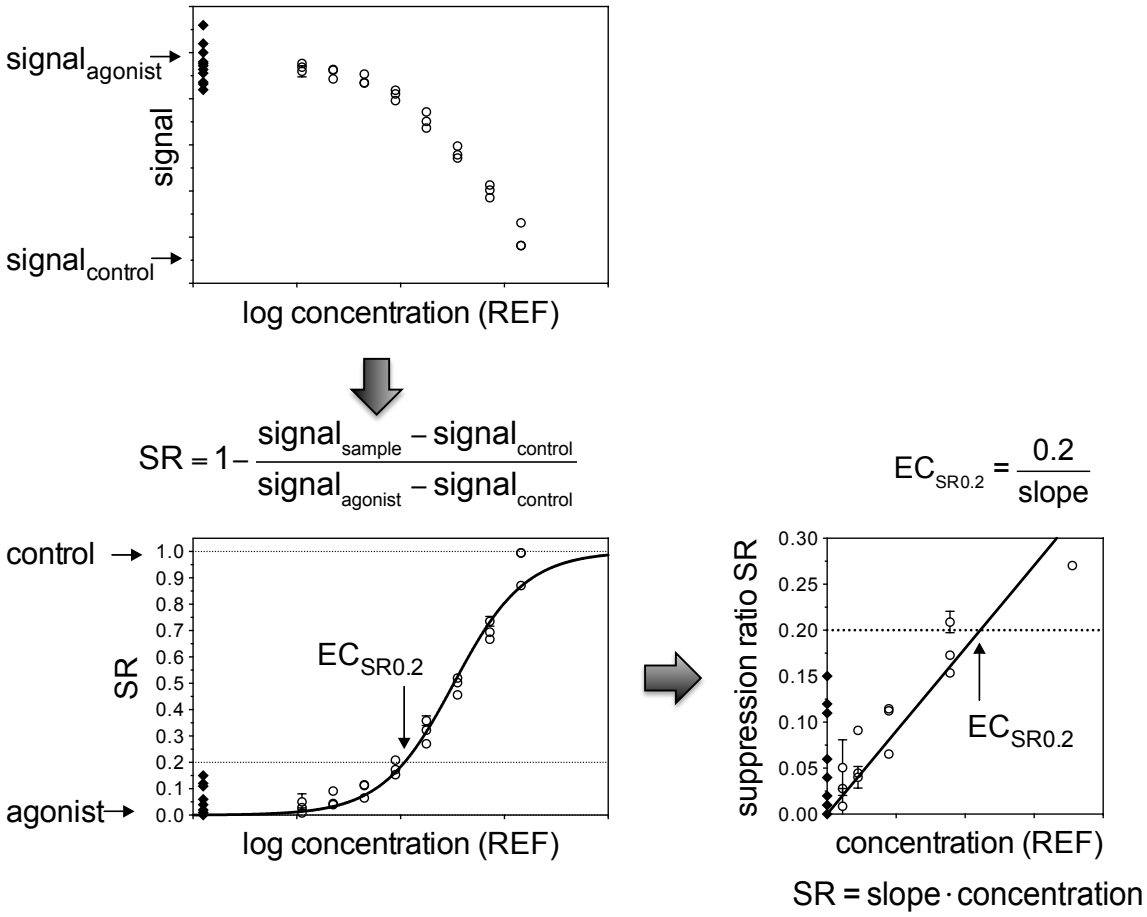


Figure S1. Derivation of $EC_{SR0.2}$.

Section S4. How robust were bioassays performed in different laboratories?

A number of bioassays were performed in multiple laboratories. In the following sections only mean results per bioassay are reported. For bioassays that had some positive and some negative results in two or more laboratories, the reported mean was obtained as follows:

(a) If no effects were observed until the highest REF tested in one laboratory but the maximum experimental REF used in that laboratory was lower than the REF in another laboratory, then the results from the laboratory with lower maximum enrichment was ignored.

(b) If the maximum REF were similar but some laboratories reported effects, others not, then the data without observed effect was not used to calculate the mean if it was only one laboratory out of three or four. If the test was only performed by two laboratories, and one was ">highest REF tested", the other one was not, then the positive data were used.

The bioassays listed in Table S4 were performed multiple times and a repeated measures one-way ANOVA followed by the Bonferroni multiple comparison post-test was performed to assess if the results matched between the different laboratories and if it was legitimate to report mean values for each endpoint only (if only two laboratories were involved, then a paired t-test was used). We did not use ANOVA *per se* to quantify the distribution of the ten samples. Clearly, we cannot assume that these 10 samples follow a Gaussian distribution, and we did not target a true mean of all samples, but we can assume that if thousands of water samples of these types were tested they would follow a Gaussian distribution. Therefore a non-parametric test was not suitable for this analysis. ANOVA is suitable in this case as with the independent testing in two or more laboratories the circularity assumption is fulfilled.

Table S4. Comparison of the bioassay results for the same bioassay performed in different laboratories.

Bioassay	Labo-ratory	Test	# Samples com-pared (>LOD)	Is there signi-ficant pair-ing?	P	r2 (r for t-test)	Bonferroni post test
Photo-synthesis inhibition	UQ, Swiss	Paired t-test	4	yes	0.014	0.973	
Algae growth inhibition	UQ, Swiss	Paired t-test	3	yes	0.039	0.993	
AhR-CAFLUX	UQ, RECETOX	Paired t-test	6	yes	0.008	0.894	
ER-CALUX	GU, BDS, CSIRO, IWW	Repeated measures ANOVA	3	yes	0.001	0.694	CSIRO vs. BDS signifi-cantly different
ER-GeneBLAzer	UF, USF, UCR, SCCWRP	Repeated measures ANOVA	4	yes	0.001	0.812	
GR-GeneBLAzer	UF, USF, UCR, SCCWRP	Repeated measures ANOVA	2+8 <LOD[a]	yes	<0.00 1	0.957	All laboratories not significantly different; the non-detects were included in the analysis because there were too few detects
GR-CALUX	BDS, CSIRO	Paired t-test	2	no	0.451	0.153	GU used lower REF[b] (and results were below LOD), therefore only BDS and CSIRO compared.
Microtox	UQ (1+2) Swiss	Repeated measures ANOVA	8	yes	0.046	0.531	All laboratories not significantly different

[a]LOD = limit of detection, [b]REF = relative enrichment factor.

Also, data were log-transformed before the test was performed to assure that small values had equal weight. We used this test to obtain P values that test the null hypothesis of the population row means being equal.

Although the maximum amount of sample extract to be dosed was not prescribed, which lead to different laboratories testing different highest doses, we found generally good consistency between the results of the different laboratories.

Four laboratories performed the ER-CALUX and their results paired up although the CSIRO vs. BDS results were significantly different but the two other datasets lay well in the middle and therefore all four were averaged.

For the YES assay, two laboratories were below detection limits in all samples but the dataset of a third laboratory showed clear and differentiated results and high quality raw data. In this case therefore only the positive results of the third laboratory were used despite violation of the above rules.

Working with a semi-standardized protocol was beneficial as the four laboratories that performed the ER-GeneBLAzer assay achieved consistent results despite the fact that the assay was newly established in all laboratories. All ER-GeneBLAzer results were averaged.

The bioassays for androgenicity, *e.g.*, the AR-CALUX and the YAS, as well as for the progesterone receptor, PR-CALUX, and for the thyroid receptor, TR-CALUX, did not the effect threshold of 10% at the highest REF in any of the laboratories where the test was applied. The AR-GeneBLAzer and the PR-GeneBLAzer were performed in four and three laboratories, respectively. In a few cases individual samples were just above detection limit but in each case they were below detection limit in all other laboratories and they were therefore assigned as non-detects. It must be noted that this study was limited by the amounts distributed to each laboratory. Therefore it was not possible to do more repetitions, which one might normally do with results close to the detection limit. The GR-GeneBLAzer assay was consistent between four laboratories showing positive results in the same two samples and with all other samples below the significance threshold of effect of 10%. The results were averaged.

The GR-CALUX was one of the few assays where the different laboratories did not agree although the relative effect pattern was consistent. One laboratory tested much lower concentrations that the others and did not detect any effect, the two other

laboratories showed two samples that were positive (MF and Eff2) but a third sample (Eff1) that was cytotoxic in one laboratory and inducing in another lab. The effects of the two positive samples differed more than in other bioassays and pairing could not be established. Therefore only the data set from the laboratory that reported highest activation and no cytotoxicity was reported in the final table.

For reactive toxicity, we tested a large variety of Ames strains with slightly different properties and therefore were unable to narrow down specific patterns, although similar samples caused induction. IWW duplicated some of the tests but obtained no responses due to smaller REFs applied and these datasets were omitted as other laboratories had positive results with the same strains. For adaptive stress response pathways, the only assay performed in duplicate was the p53-CALUX, which was not responsive.

Section S5. Initial screening of nuclear receptors and transcription factors

The FACTORIAL bioassays were applied here for the first time to screen water samples. The raw water samples did not show any effects (data not shown), the responses discussed in this section relate to water samples after enrichment by SPE to a REF of 4. As no reference compounds were measured and the maximum response was not known, only induction ratios could be calculated from the raw response data. For more than 90% of the tested endpoints, the limit of detection (LOD; IR of control plus 3 times standard deviation) fell below an IR of 1.5 (Figure S2).

A fixed threshold has the advantage that datasets of variable size can be compared, while the LOD is dependent on the number of datapoints from which it is derived. For example, in Figure S2 the LOD calculations for the nuclear receptors (NR) was based on 24 control data points while the LOD for the transcription factors (TF) was based on 48 datapoints. The latter yielded much lower variability and lower LODs. Therefore we opted to use the IR 1.5 as a threshold of effect for the FACTORIAL bioassays but also for all other bioassays where no % effect could be calculated.

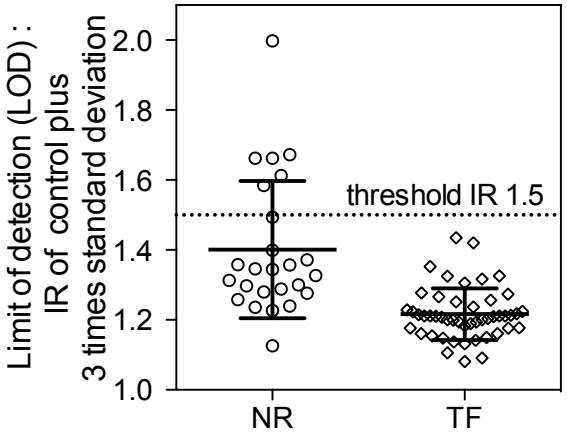


Figure S2. Limit of detection (LOD) calculated from the induction ratio (IR) of control plus 3 times standard deviation for the 25 nuclear receptors (NR) and 48 transcription factors (TF) of the FACTORIAL bioassay.

All samples were screened with the FACTORIAL bioassays and the results are depicted in Figure S3. The radar plots in Figure S3 depict the induction ratios IR directly

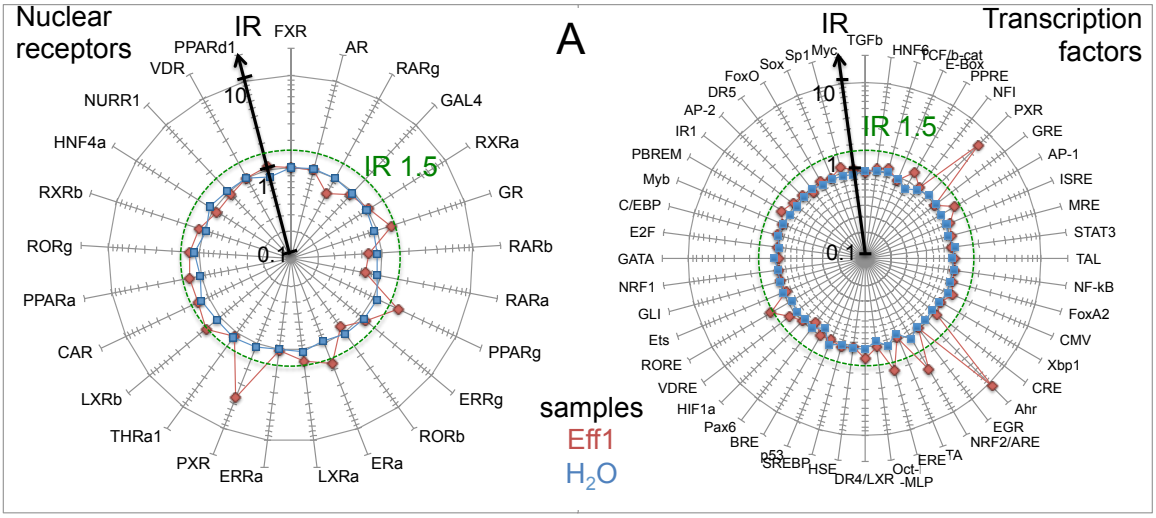
measured after incubation of the HepG2 cells with water samples at an REF of 4. A high IR relates to a high effect, the controls are IR = 1 and the threshold of effect was defined as IR = 1.5 as this is also the IR for the derivation of the $EC_{IR1.5}$.

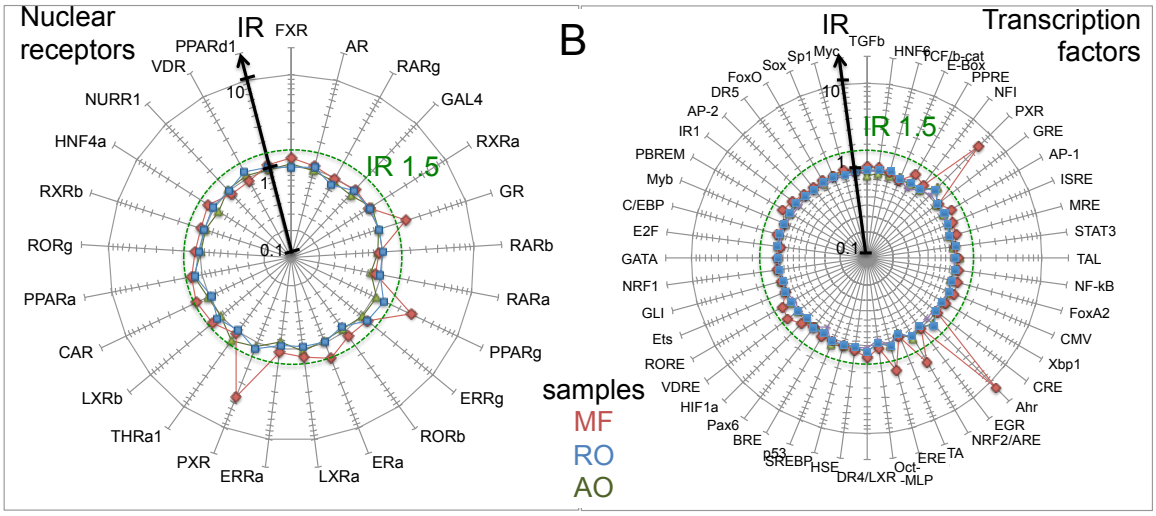
The highest induction was seen for the pregnane X receptor PXR both in the NR and TF assay and in all samples but the blank (H_2O) (Figure S3). As expected the estrogen receptor ERα was activated but not the estrogen related receptors ERRα and ERRγ in the NR assay and the estrogen response element ERE was activated in the TF assay (Figure S3).

In the NR assay, the peroxisome proliferator activated receptor PPARγ was active but with a lower IR around or below 2 in the samples Eff1, Eff2, MF. The glucocorticoid receptor responded weakly in the NR assay (IR of 1.4 at an REF of 4, i.e. just below the threshold of effect) but showed no response in the TF assay.

The highest induction in the TF assay was observed for the arylhydrocarbon response element, which does not come as a surprise as a large number of chemicals activate this xenobiotic metabolism pathway. Activity measured in Effl1, Eff2 and MF disappeared after further treatment, and drinking water treatment marginally increased the effect.

The next highest activity was caused by the response element associated with the PXR and this is consistent with the high activity in the NR assay. Third in activity was the antioxidant response element (ARE) that is activated through the Keap-Nrf2 pathway. Then came the response elements for ER (ERE), the retinoic acid receptor (RAR)-related orphan receptor (RORE) and response element for the PPAR (PPRE; IR 1.4 just below effect threshold).


Nuclear receptors
IR
FXR
AR
RARg
GAL4
RXRa
GR
RARb
RARa
PPARg
ERRg
RORb
ERa
LXRa
ERRa
PXR
THRa1
LXRb
CAR
PPARa
RORg
RXRb
HNF4a
NURR1
VDR
PPARd1
10
1
0.1
IR 1.5
A
Transcription factors
TGFb
HNF6
TCF/b-cat
E-Box
PPRE
NFI
PXR
GRE
AP-1
ISRE
MRE
STAT3
TAL
NF-kB
FoxA2
CMV
Xbp1
CRE
Ahr
EGR
NRF2/ARE
TA
ERE
Oct-MLP
DR4/LXR
HSE
SREBP
p53
BRE
Pax6
HIF1a
VDRE
RORE
Ets
GLI
NRF1
GATA
E2F
C/EBP
Myb
PBREM
IR1
AP-2
DR5
FoxO
Sox
Sp1
Myc
samples
Eff1
H2O


Nuclear receptors
IR
FXR
AR
RARg
GAL4
RXRa
GR
RARb
RARa
PPARg
ERRg
RORb
ERa
LXRa
ERRa
PXR
THRa1
LXRb
CAR
PPARa
RORg
RXRb
HNF4a
NURR1
VDR
PPARd1
10
1
0.1
IR 1.5
B
Transcription factors
TGFb
HNF6
TCF/b-cat
E-Box
PPRE
NFI
PXR
GRE
AP-1
ISRE
MRE
STAT3
TAL
NF-kB
FoxA2
CMV
Xbp1
CRE
Ahr
EGR
NRF2/ARE
TA
ERE
Oct-MLP
DR4/LXR
HSE
SREBP
p53
BRE
Pax6
HIF1a
VDRE
RORE
Ets
GLI
NRF1
GATA
E2F
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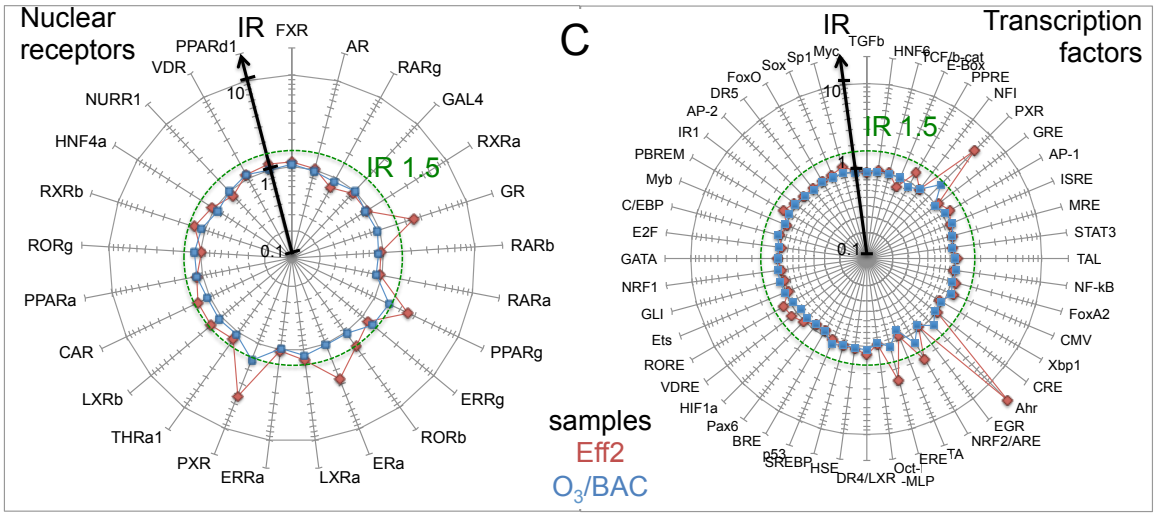
Myb
PBREM
IR1
AP-2
DR5
FoxO
Sox
Sp1
Myc
samples
MF
RO
AO


Nuclear receptors
IR
FXR
AR
RARg
GAL4
RXRa
GR
RARb
RARa
PPARg
ERRg
RORb
ERa
LXRa
ERRa
PXR
THRa1
LXRb
CAR
PPARa
RORg
RXRb
HNF4a
NURR1
VDR
PPARd1
10
1
0.1
IR 1.5
C
Transcription factors
TGFb
HNF6
TCF/b-cat
E-Box
PPRE
NFI
PXR
GRE
AP-1
ISRE
MRE
STAT3
TAL
NF-kB
FoxA2
CMV
Xbp1
CRE
Ahr
EGR
NRF2/ARE
TA
ERE
Oct-MLP
DR4/LXR
HSE
SREBP
p53
BRE
Pax6
HIF1a
VDRE
RORE
Ets
GLI
NRF1
GATA
E2F
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Myb
PBREM
IR1
AP-2
DR5
FoxO
Sox
Sp1
Myc
samples
Eff2
O3/BAC

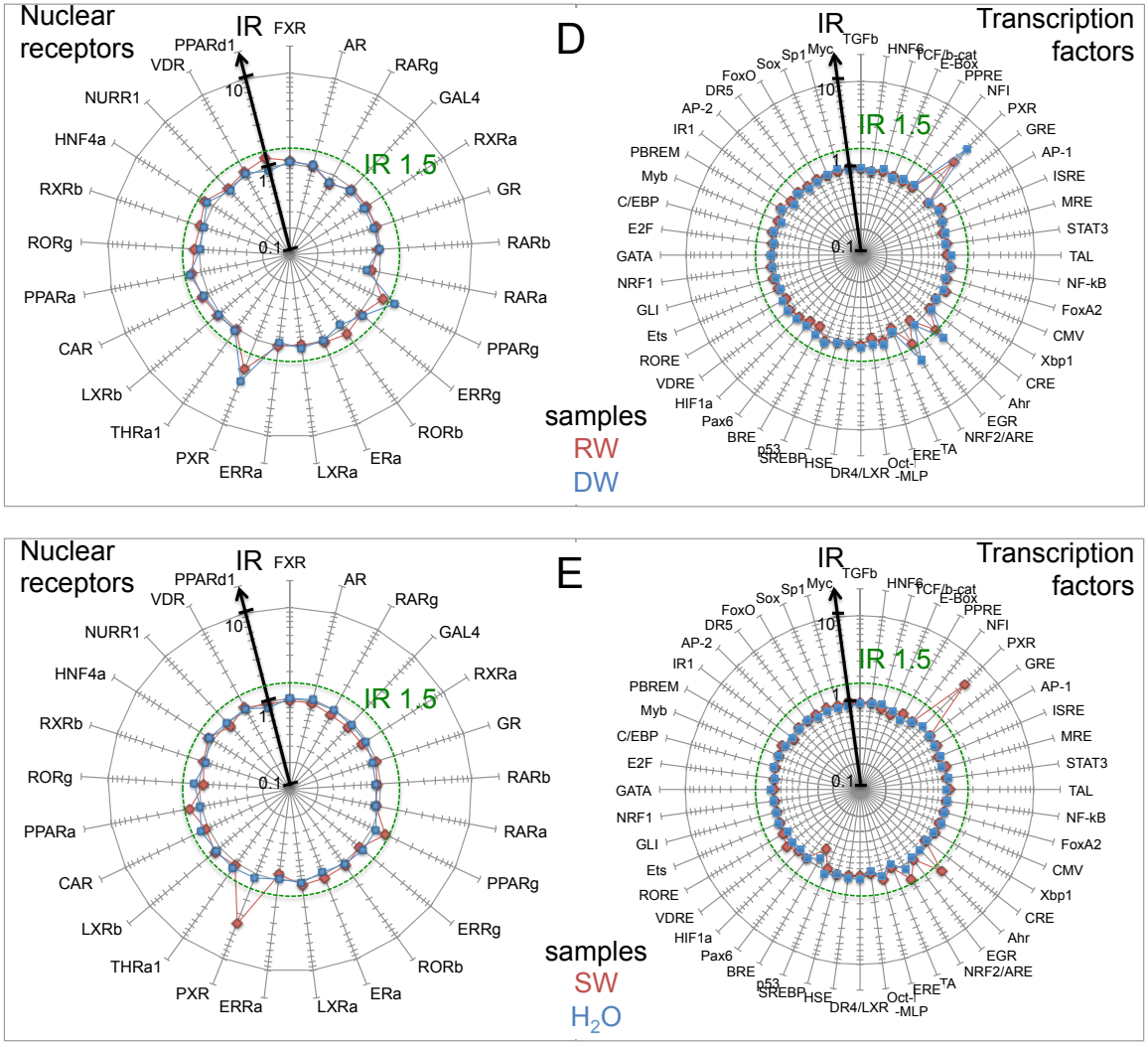

Figure S3. Screening of 25 nuclear receptors and 48 transcription factors with the FACTORIAL bioassay. The induction ratios (IR) are depicted on the radar scale and the effect threshold of IR 1.5 is depicted with a green dashed circle. Activity profile of the induction ratios IR of nuclear receptors (left) and transcription factors (right) in HepG2 cells incubated for 24h with water samples at a REF of 4; (A) wastewater treatment plant effluent (Eff1) and the blank (milliQ water) (B) microfiltration (MF), reverse osmosis (RO), advanced oxidation (AO), (C) Eff2 and ozone/biologically activated carbon (O_3/BAC), (D) river water (RW) and drinking water (DW), (E) stormwater (SW) and laboratory blank (H_2O).

Section S6. Additional information on the bioassay results

Details of the bioassay results are depicted in plots that are structured as shown in Figure S4. The EC values were plotted in an inverse scale so that the most toxic ECs were on the top and the least toxic ECs on the bottom (Figure S4-A). The samples were grouped according to the treatment processes (Figure S4-B). Sensitivity cannot be compared directly because of the two different endpoints (EC_{10} and $EC_{IR1.5}$) but these plots give an indication about the responsiveness and thus the suitability of the bioassays for water quality assessment.

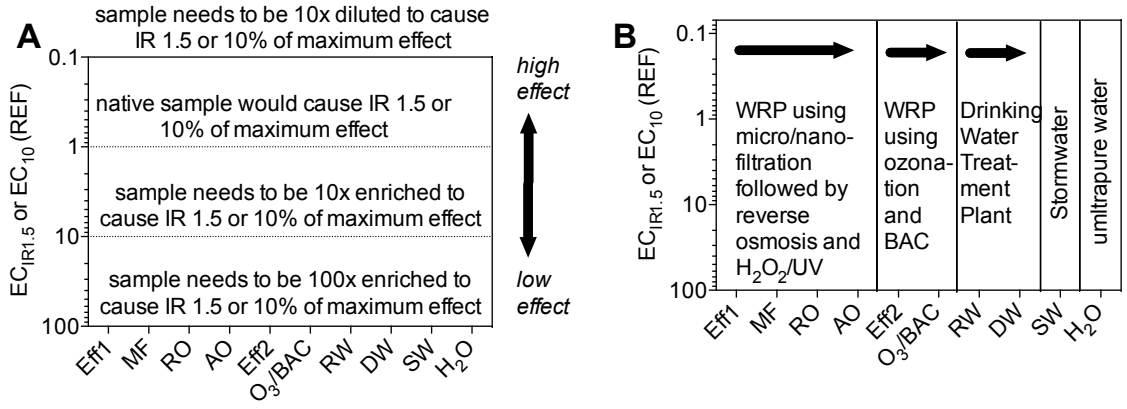

Figure S4. Presentation of bioassay results (EC = effect concentration, IR = induction ratio, REF = relative enrichment factor, for sample abbreviation see Table S1).

S6-A. Induction of xenobiotic metabolism pathways

The three bioassays for PXR and the six bioassays for AhR all showed positive responses in less treated samples and negative responses in recycled water and the blank (Figure S5). For the PXR (Figure S5-A), the FACTORIAL assays were most responsive with an $EC_{IR1.5}$ below a REF of 1, *i.e.*, this effect would be observable in the ambient water sample. The HG5LN-hPXR (Seimandi *et al.*, 2005; Lemaire *et al.*, 2006) reporter gene assay has been applied widely in water quality monitoring including for testing of wastewater, surface water and reclaimed water (Mahjoub *et al.*, 2009; Creusot *et al.*, 2010; Kinani *et al.*, 2010; Mnif *et al.*, 2010; Mnif *et al.*, 2011). This assay was responsive to the same samples as the two PXR-FACTORIAL endpoints.

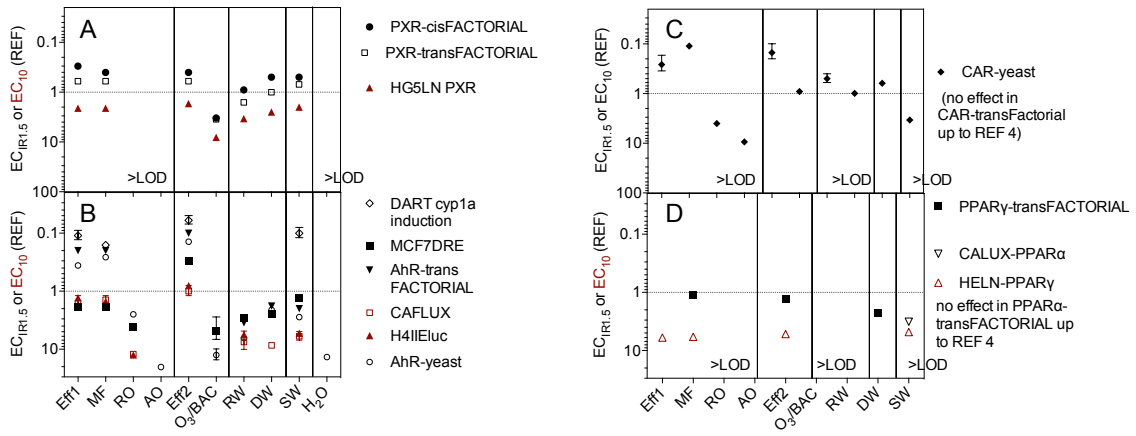

Figure S5. Results of bioassays indicative of induction of xenobiotic metabolism pathways. The red symbols are EC_{10} values the black symbols are $EC_{IR1.5}$ values.

The six AhR-related bioassays all showed consistent effect-patterns for the different samples and similar range of sensitivity although quantitative comparison is not possible because two bioassays yielded EC_{10} and four $EC_{IR1.5}$ values (Figure S5-B). The AhR-yeast had a remarkable dynamic range of EC over more than two orders of magnitude. A very responsive endpoint for induction of AhR was the CYP1a transcription in zebrafish embryos measured by RT-PCR. Only four samples (Eff1, MF, Eff2 and SW) were tested with RT-PCR but they all responded at low REF. The resulting $EC_{IR1.5}$ ranged from 0.06 to 0.16 REF (*i.e.*, responsive even in diluted samples).

CAR was tested in two bioassays but only the CAR-yeast gave a response already at low REFs (Figure S5-C). For PPAR, only two of seven bioassays gave signals in the four most polluted samples (Figure S5-D). Both active PPAR assays related to PPARγ (PPARγ-transFACTORIAL and HELN-PPARγ). We detected no PPAR antagonism but samples were only tested up to a REF of 2.

S6-B. Endocrine disruption

All bioassays for estrogenicity were active in four to five samples. Blanks, RO and AO did not induce any estrogenic *in vitro* effects, and only two bioassays gave very low effects for samples DW and O_3/BAC (Table S5, Figure S6). The results are consistent with a previous interlaboratory comparison study of five different bioassays for estrogenicity (GWRC, 2008), where the effects observed in the ER-CALUX, the YES, the E-SCREEN and the T47KBluc (not assessed here) were highly correlated. Leusch *et*

al. (2010) also showed that the yeast-based assays have higher detection limits and therefore are not suitable for highly treated water but for the present applications they could still be used as the REF could be increased without cytotoxicity occurring and EC values obtained for MF and Eff2 were in the same range as for other endpoints. Again it must be cautioned that a quantitative comparison between EC_{10} and $EC_{IR1.5}$ is not possible but as Figure S6 demonstrates they are in the same range of relative sensitivity.

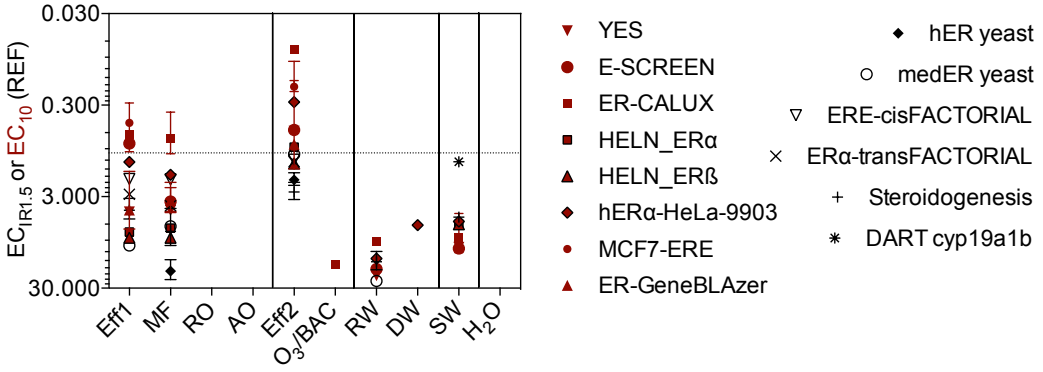

Figure S6. Results of bioassays indicative of estrogenicity. The red symbols are EC_{10} values the black symbols are $EC_{IR1.5}$ values.

The anti-ER test quantifies how the sample influences the effect of a constant concentration of estradiol that is typically spiked at concentrations that would elicit 50 to 80% of maximum effect. If the effect of the constant concentration of estradiol was suppressed and the sample was not cytotoxic, the sample can be considered to act as an anti-estrogen. No anti-estrogenic activity could be detected with the anti-ER-CALUX in any of the samples. It should be noted that agonistic and antagonistic activity may be occurring simultaneously in these assays, masking any such individual response of these activities.

The steroidogenesis pathway represents the biosynthesis route of steroid hormones from cholesterol via a battery of oxidative enzymes (Zhang *et al.*, 2005). The H295R steroidogenesis assay is an OECD validated bioassay used to evaluate the endocrine disrupting effects by chemicals via non-receptor mediated mechanisms. As this pathway directly affects the hormone system function, we have classified it with the receptor-mediated hormonal effects rather than with xenobiotic metabolism. The steroidogenesis assay showed increased concentrations of estrone and estradiol, which could be associated with a decreased estradiol metabolism, as well as increase in

progesterone and 17α-hydroxyprogesterone, which is most likely due to an inhibitory effect on CYP21A. A similar effect was observed when oil sand product water was assessed with the steroidogenesis assay: the raw water increased the estradiol levels and the effect disappeared after ozonation (He *et al.*, 2010). In the same way that the effect of sample Eff2 went below the limit of detection when it was ozonated. The effect pattern of the sample Eff1 was similar to what has been observed when dosing with bisphenol A (Zhang *et al.*, 2011).

In relation to activation of the glucocorticoid receptor, GR-CALUX was the most responsive of these assays, followed by GR-transFACTORIAL (Figure S7). Their EC values were roughly ten times lower than the GR-Switchgear and GR-MDA-kb2 assays. The GR-GeneBLAzer was positive in MF, Eff2 and SW but the potency did not correlate well with the other assays.

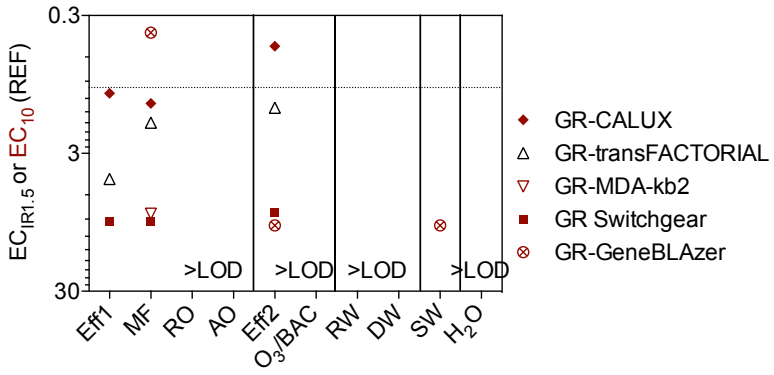

Figure S7. Results of bioassays indicative of glucocorticoid receptor (GR) activation. The red symbols are EC_{10} values the black symbols are $EC_{IR1.5}$ values.

No assay indicative of modulation of the thyroid hormone system showed response to any of the water samples, even at high REF. The T-Screen is a cell proliferation assay where the cells only proliferate in the presence of thyroid hormones (Gutleb *et al.*, 2005). This assay has been mainly applied for chemical dose-response tests and only few of the environmentally relevant chemicals showed activity. Accordingly it was not surprising that no effects were detected in the water samples. Many of the thyroid-active chemicals appear to need metabolic activation, and thus a combination with a system for metabolic activation may be beneficial (Taxvig *et al.*, 2011). Inoue *et al.* (2009) observed activity lower than 10% of maximum effect at a REF of 100 with a two-hybrid yeast assay in surface waters; a greater response (>10%) was observed for a

WWTP influent but these effects disappeared in the effluent (Inoue *et al.*, 2011). In a different yeast-based assay, Li *et al.* (2011) did not observe any TR agonistic effect in water samples, and attributed anti-TR activity to phthalates (Li *et al.*, 2010).

The P19/A15 cell line was developed by transfecting an embryonic mouse carcinoma cell line with a plasmid carrying the retinoic acid response element (Novak *et al.*, 2007). This cell line has not been tested with water samples prior to this study and did not show any effects with water samples but the water samples enhanced the effect of constant concentrations of 9-cis retinoic acid slightly (data not shown). This effect could be caused by mixture effect or by the organic micropollutants acting as solubilizer for the very lipophilic RA.

S6-C. Reactive toxicity

Three samples were active in the micronucleus assay, Eff2, RW and DW (Figure S8). This is a different profile as compared to the receptor-mediated modes of action, where the DW typically did not show any response and the activity in the DW sample is presumably due to disinfection by-products formed during chlorination.

Both the SOS chromotest, based on induction of SOS repair in *Escherichia coli* (Quillardet *et al.*, 1982), and the umuC assays with *Salmonella typhimurium* (Oda *et al.*, 1985) are reporter gene assays, while the Ames test uses histidine-deficient *S. typhimurium* that can only grow if a reverse mutation occurs. The umuC and Ames tests utilize different strains of *S. typhimurium*, so the seven genotoxicity assays in Table 1 (main article) in fact only represents four different assay types. All umuC assays showed activity only at high REFs around 20 (Figure S8). The SOS chromotest gave very similar responses as the umuC. The Ames assay responded generally at lower REFs but suffered from high variability between the different bacterial strains (Figure S8). One problem was that several samples (*e.g.*, RO, AO, SW and H_2O) showed detectable yet inconsistent activity in the Ames assay, which was not apparent in the other genotoxicity assays. The dynamic range of these genotoxicity assays was relatively small and effects were only observed at relatively high enrichments (REF up to 20).

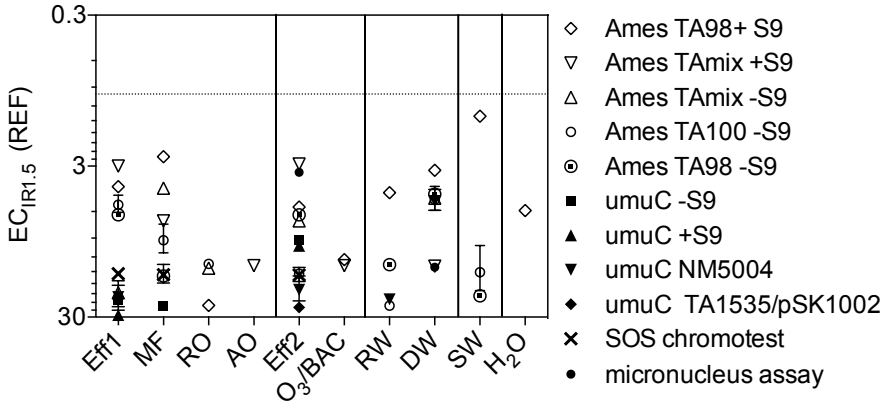

Figure S8. Results of bioassays indicative of reactive modes of action.

S6-D. Adaptive stress response

These bioassays are discussed in more detail in the main manuscript.

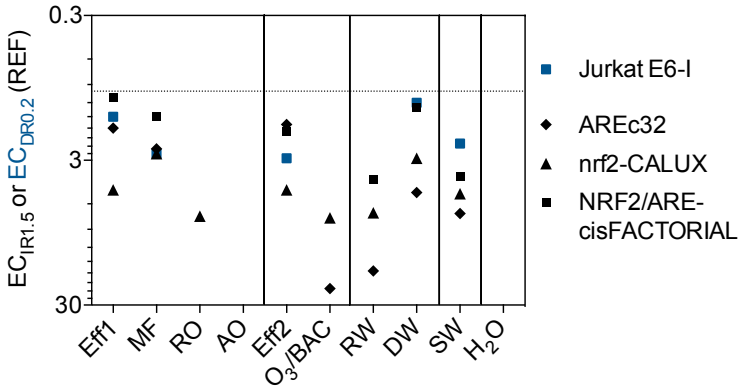

Figure S9. Results of bioassays indicative of adaptive stress response pathways.

Table S5. Summary of all EC values measured in all water samples (see Table S1 for definition of sample abbreviations). The errors represent the propagated standard error of the concentration-effect curve if only one laboratory performed the assay, and standard deviation of the mean results from different laboratories if the bioassay was performed by several laboratories (see also Section S4 for details on treatment of bioassays that were measured by multiple laboratories).

#	Class of MOA[a]	Lab(s)	Bioassay	EC (REF)	Eff1	MF	RO	AO	Eff2	O_3/ BAC	RW	DW	SW	H_2O
1	XM	ATG	PXR-cisFACTORIAL	$EC_{IR1.5}$	0.3	0.4	>4	>4	0.4	3.3	0.9	0.5	0.5	>4
2	XM	ATG	PXR-transFACTORIAL	$EC_{IR1.5}$	0.6	0.6	>4	>4	0.6	3.5	1.6	1.0	0.7	>4
3	XM	IRCM	HG5LN PXR	EC_{10}	2.1 ±0.01	2.1 ±0.01	>12	>12	1.7 ±0.01	8.1 ±0.08	3.4 ±0.01	2.5 ±0.02	2.0 ±0.01	>12
4	XM	ATG	CAR-transFACTORIAL	$EC_{IR1.5}$	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
5	XM	CAPIM	CAR-yeast	$EC_{IR1.5}$	0.3 ±0.1	0.1±0.01	4.0 ±0.2	9.4 ±0.6	0.2 ±0.1	0.9 ±0.1	0.5 ±0.1	1.0 ±0.1	0.6 ±0.1	3.4 ±0.2
6	XM	ATG	PPARα-transFACTORIAL	$EC_{IR1.5}$	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
7	XM	ATG	PPARγ-transFACTORIAL	$EC_{IR1.5}$	2.0	1.1	>4	>4	1.3	>4	>4	2.3	>4	>4
8	XM	IRCM	HELN-PPARγ	EC_{10}	>6	5.8 ±0.1	>12	>12	5.2 ±0.1	>12	>12	>12	4.8 ±0.1	>12
9	XM	BDS	CALUX-PPARα	EC_{10}	>30	>30	>30	>30	>30	>30	>30	>30	3.2	>30
10	XM	BDS, CSIRO	CALUX-PPARγ	EC_{10}	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30
11	XM	HK	MCF7-PPAR	$EC_{IR1.5}$	n.t.	n.t.	>20	>20	n.t.	n.t.	17.0 ±11.3	n.t.	n.t.	>20
12	XM	GU	PPARγ GeneBLAzer	EC_{10}	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2
13	XM	GU	Anti-PPARγ GeneBLAzer	$EC_{SR0.2}$	>2	>2	>1	>1	>2	>1	>1	>1	>2	>1

#	Class of MOA[a]	Lab(s)	Bioassay	EC (REF)	Eff1	MF	RO	AO	Eff2	O_3/ BAC	RW	DW	SW	H_2O
14	XM	CAPIM	AhR-yeast	$EC_{IR1.5}$	0.36 ±0.01	0.26 ±0.02	2.46 ±0.03	20.2 ±0.5	0.14 ± 0.01	12.6 ±2.7	6.4 ±0.6	2.14 ±0.13	2.81 ±0.13	13.5 ±0.3
15	XM	UQ, RECETOX	AhR-CAFLUX 24h	EC_{10}	1.6 ±0.4	1.4 ±0.2	12.1 ±0.5	>30	1.0 ±0.2	>30	7.5 ±2.6	8.6	6.0 ±1.0	>30
16	XM	RECETOX	H4IIEluc	EC_{10}	1.3 ±0.1	1.5 ±0.2	12.5 ±1.6	>14	0.8 ±0.1	>14	5.6 ±0.6	>14	5.3 ±0.7	>27
17	XM	HK	MCF7-DRE	$EC_{IR1.5}$	1.9 ±0.3	1.9 ±0.3	4.1 ±0.5	>20	0.3 ±0.1	4.8 ±2.0	2.9 ±0.3	2.5 ±0.3	1.3 ±0.2	>18
18	XM	ATG	AhR-cisFACTORIAL	$EC_{IR1.5}$	0.2	0.2	>4	>4	0.1	>4	3.5	1.8	2.0	>4
19	XM	UFZ	DART cyp1a induction	$EC_{IR1.5}$	0.11 ±0.02	0.16 ±0.02	-	-	0.06 ±0.01	-	-	-	0.10 ±0.02	-
20	Specific MOA	UQ, Swiss	Algae photosynthesis inhibition	EC_{10}	2.2 ±0.6	2.6 ±0.3	>20	>20	6.3 ±3.8	>20	>20	>20	6.5	>20
21	Specific MOA	UQ	Acetylcholin-esterase inhibition	EC_{10}	1.9±0.1	>2	>2	>2	3.2 ±0.2	>2	>2	>2	>2	>2
22	Specific MOA: ER	GU, CSIRO, BDS, IWW	ER-CALUX	EC_{10}[b]	0.63 ±0.34	0.69 ±0.33	>25	>25	0.07 ±0.06	16.6	9.3	>25	8.5 ±3.9	>25
23	Specific MOA: ER	UQ	E-SCREEN	EC_{10}	0.79 ±0.02	3.39 ±0.02	>30	>30	0.56 ±0.02	>30	18.6 ±0.4	>30	11.0 ±0.3	>30
24	Specific MOA: ER	Swiss, CSIRO, UA	Yeast Estrogen Screen (YES)	EC_{10}[c]	4.40 ±0.05	4.26 ±0.05	>30	>30	0.58 ±0.01	>30	22.0 ±0.1	>30	10.7 ±0.1	>30
25	Specific MOA: ER	CAPIM	hER yeast	$EC_{IR1.5}$	>30	19.5 ±4.8	>30	>30	2.0 ±0.3	>30	>30	>30	>30	>30
26	Specific MOA: ER	CAPIM	medER yeast	$EC_{IR1.5}$	10.3 ±1.0	6.3 ±0.6	>30	>30	1.0 ±0.1	>30	25.0 ±3.7	>30	>30	>30

#	Class of MOA[a]	Lab(s)	Bioassay	EC (REF)	Eff1	MF	RO	AO	Eff2	O_3/ BAC	RW	DW	SW	H_2O
27	Specific MOA: ER	IRCM	HELN-ERα	EC_{10}	7.29 ±0.13	6.61 ±0.05	>12	>12	0.86 ±0.01	>12	>12	>12	>6	>12
28	Specific MOA: ER	IRCM	HELN-ERβ	EC_{10}	8.42 ±0.15	8.42 ±0.11	>12	>12	1.30 ±0.02	>12	>12	>12	>6	>12
29	Specific MOA: ER	ATG	ERE-cisFACTORIAL	$EC_{IR1.5}$	1.91	1.94	>4	>4	1.18	>4	>4	>4	>4	>4
30	Specific MOA: ER	RECETOX	hERα-HeLa-9903	EC_{10}	1.26 ±0.01	1.73 ±0.02	>27	>27	0.28 ±0.01	>25	14.2 ±0.2	6.1 ±0.1	5.6 ±0.1	>27
31	Specific MOA: ER	HK	MCF7-ERE	EC_{10}	0.47 ±0.01	>10	>20	>20	0.19 ±0.03	>20	>20	>20	>10	>20
32	Specific MOA: ER	ATG	ERα-transFACTORIAL	$EC_{IR1.5}$	2.82	3.77	>4	>4	1.24	>4	>4	>4	>4	>4
33	Specific MOA: ER	NJU	Steroidogenesis	$EC_{IR1.5}$	4.2 ±1.1	7.2 ±3.1	>20	>20	2.7 ±0.6	>20	15.5 ±3.5	>20	5.0 ±0.7	>20
34	Specific MOA: ER	UFZ	DART cyp19a1b (aromatase)	$EC_{IR1.5}$	>1.2	>2.2	-	-	>2.2	-	-	-	0.89 ±0.31	-
35	Specific MOA: ER	UF, USF, UCR, SCCWRP	ER-GeneBLAzer	EC_{10}[b]	4.2 ±2.6	3.3 ±1.2	>20	>20	0.8 ±0.7	>20	>20	>20	>20	>20
36	Specific MOA: ER	CSIRO, GU	Anti-ER-CALUX	$EC_{SR0.2}$	>8	>8	>15	>15	>8	>15	>15	>15	>8	>15
37	Specific MOA:	GU, BDS,	AR-CALUX	EC_{10}	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30

#	Class of MOA[a]	Lab(s)	Bioassay	EC (REF)	Eff1	MF	RO	AO	Eff2	O_3/ BAC	RW	DW	SW	H_2O
	AR	CSIRO												
38	Specific MOA: AR	IRCM	HELN-AR	EC_{10}	>6	>6	>12	>12	>6	>12	>12	>12	>6	>12
39	Specific MOA: AR	HK	MCF7-ARE	$EC_{IR1.5}$	>10	>10	>20	>20	>10	>20	>20	>20	>10	>20
40	Specific MOA: AR	UA, CSIRO	Yeast Androgen Screen (YAS)	EC_{10}	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30
41	Specific MOA: AR	UF, USF, UCR, SCCWRP	AR-GeneBLAzer	EC_{10}	>10	>10	>20	>20	>10	>20	>20	>20	>10	>20
42	Specific MOA: AR	ATG	AR-transFACTORIAL	$EC_{IR1.5}$	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
43	Specific MOA: AR/GR	RECETOX	MDA-kb2	EC_{10}	2.3 ±0.1	1.7 ±0.1	24.6 ±0.4	>27	1.2 ±0.1	>27	>27	>27	>13	>27
44	Specific MOA: AR/GR	RECETOX	Anti-MDA-kb2	$EC_{SR0.2}$	>13	>13	>30	>30	>13	>30	6.2 ±7.0	>30	8.1 ±11.2	>30
45	Specific MOA: AR	CSIRO, GU	Anti-AR-CALUX	$EC_{SR0.2}$	7.7 ±2.0	6.4 ±1.0	>15	>15	2.9 ±0.2	>15	11.0 ±0.7	>15	6.6 ±1.1	>15
46	Specific MOA: GR	GU, BDS, CSIRO	GR-CALUX	EC_{10}[d]	1.1 ±0.1	1.3 ±0.1	>30	>30	0.5 ±0.1	>30	>30	>30	>30	>30
47	Specific MOA: GR	UA	GR Switchgear	EC_{10}	9.4 ±0.1	9.4 ±0.1	>20	>20	8.1 ±0.2	>20	>20	>20	>10	>20

#	Class of MOA[a]	Lab(s)	Bioassay	EC (REF)	Eff1	MF	RO	AO	Eff2	O_3/ BAC	RW	DW	SW	H_2O
48	Specific MOA: GR	ATG	GR-transFACTORIAL	$EC_{IR1.5}$	4.6	1.8	>4	>4	1.4	>4	>4	>4	>4	>4
49	Specific MOA: GR	RECETOX	GR-MDA-kb2 (AR suppressed with Flutamide)	EC_{10}	>14	8.2 ±0.1	>27	-	>14	-	-.	-	-	-
50	Specific MOA: GR	GU, UF, USF, UCR, SCCWRP	GR-GeneBLAzer	EC_{10}[b]	>10	12.6 ±3.0	>20	>20	>10	>20	>20	>20	>10	>20
51	Specific MOA: GR	GU	Anti-GR-GeneBLAzer	$EC_{SR0.2}$	>2	>2	>1	>1	>2	>1	>1	>1	>2	>1
52	Specific MOA: GR	GU	Anti-GR-CALUX	$EC_{SR0.2}$	>2	>2	>1	>1	>2	>1	>1	>1	>2	>1
53	Specific MOA: PR	UF, USF, UCR, SCCWRP	PR-GeneBLAzer	EC_{10}	>10	>20	>20	>20	>20	>20	>20	>20	>10	>20
54	Specific MOA: PR	GU, BDS, CSIRO	PR-CALUX	EC_{10}	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30
55	Specific MOA: PR	GU	Anti-PR-CALUX	$EC_{SR0.2}$	>2	>2	>1	>1	>2	>1	>1	>1	>2	>1
56	Specific MOA: PR	NJU	Steroidogenesis, induction of progesterone	$EC_{IR1.5}$	5.4 ±2.6	4.2 ±1.6	>10	>10	2.0 ±0.3	>10	>10	>10	>10	>10
57	Specific MOA: PR	NJU	Steroidogenesis, induction of 17α OH-progesterone	$EC_{IR1.5}$	2.7 ±0.3	6.3 ±0.8	>10	>10	1.1 ±0.1	>10	>10	>10	>10	>10

#	Class of MOA[a]	Lab(s)	Bioassay	EC (REF)	Eff1	MF	RO	AO	Eff2	O_3/ BAC	RW	DW	SW	H_2O
58	Specific MOA: TR	BDS, GU	TR-CALUX	EC_{10}	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30
59	Specific MOA: TR	UQ	T-Screen	EC_{10}	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30
60	Specific MOA: TR	ATG	THRα1-transFACTORIAL	$EC_{IR1.5}$	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
61	Specific MOA: TR	IRCM	HELN-TR	EC_{10}	>6	>6	>12	>12	>6	>12	>12	>12	>6	>12
62	Repro	HK	MCF7-RARE	$EC_{IR1.5}$	>10	>10	>20	>20	>20	>20	>20	>20	>20	>20
63	Repro	UQ	P19/A15	$EC_{IR1.5}$	>30	>30	>30	>30	25.3 ±4.7	>30	>30	>30	>30	>30
64	Repro	ATG	RORβ-transFACTORIAL	$EC_{IR1.5}$	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
65	Repro	CAPIM	hRAR-Yeast Assay	$EC_{IR1.5}$	4.6 ±1.0	3.6 ±0.3	>30	>30	8.0 ±1.2	>30	>30	>30	>30	>30
66	Reactive MOA	RCEES, UQ	umuC TA1535/pSK1002	$EC_{IR1.5}$[b]	22.6 ±0.7	25.3 ±1.6	>30	>30	17.5 ±11.8	>30	>30	>30	>30	>30
67	Reactive MOA	UQ	umuC TA1535/pSK1002 +S9	$EC_{IR1.5}$	29.0 ±1.4	31.3 ±2.2	>30	>30	10.1 ±0.2	>30	>30	>30	>30	>30
68	Reactive MOA	RCEES	umuC NM5004	$EC_{IR1.5}$	22.1 ±3.6	>30	>30	>30	19.8 ±3.7	>30	23±3	5.1 ±0.8	>30	>30
69	Reactive MOA	RECETOX	SOS chromotest	$EC_{IR1.5}$	15.5 ±1.3	15.7 ±2.2	>27	>27	15.8 ±0.1.7	>27	>27	>27	>13	>27
70	Reactive MOA	UA, IWW	Ames TA98 -S9	$EC_{RR1.5}$[e]	6.3	16.0	>30	>30	6.3	>30	13.5	4.6	21.7	>30
71	Reactive MOA	UA, IWW	Ames TA98+ S9	$EC_{RR1.5}$[e]	4.1	2.6	25.1	>30	5.6	12.5	4.5	3.2	1.4	5.9
72	Reactive MOA	UA, IWW	Ames TAmix -S9	$EC_{RR1.5}$	20.7	4.2	14.2	>30	6.9	>30	>30	4.9	>30	>30

#	Class of MOA[a]	Lab(s)	Bioassay	EC (REF)	Eff1	MF	RO	AO	Eff2	O_3/ BAC	RW	DW	SW	H_2O
73	Reactive MOA	UA, IWW	Ames TAmix +S9	$EC_{RR1.5}$[e]	3.0	6.9	>30	13.7	2.9	13.7	>30	13.8	>30	>30
74	Reactive MOA	UQ	Ames TA100 -S9	$EC_{RR1.5}$[e]	5.4 ±0.7	9.3 ±2.0	13.3 ±0.01	>30	15.5 ±23.2	>30	25.2 ±0.01	5.0 ±0.9	15.1 ±5.0	>30
75	Reactive MOA	AWQC	Micronucleus assay	$EC_{RR1.5}$[e]	>20	>20	>30	>30	2.6	>30	20.9	9.0	>20	>30
76	Reactive MOA	CSIRO	ROS formation in RTG2 cells	$EC_{RR1.5}$	9.0 ±0.2	9.4 ±0.2	>30	>30	4.9 ±0.1	>30	>30	>30	11.9 ±0.7	>30
77	Reactive MOA	UQ	Protein damage *E.coli* GSH+/-	$EC_{IR1.5}$	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30
78	ASR	ATG	HSE-cisFACTORIAL	$EC_{IR1.5}$	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
79	ASR	UFZ	hspb11 induction in DART after 120h	$EC_{IR1.5}$	>1.2	>4.2	-	-	>2.2	-	-	-	>2.2	-
80	ASR	ATG	HIF-1a-cisFACTORIAL	$EC_{IR1.5}$	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
81	ASR	UA	Hypoxia-Switchgear	$EC_{IR1.5}$	>10	>10	>20	>20	>10	>20	>20	>20	>10	>20
82	ASR	ATG	NFκB-cisFACTORIAL	$EC_{IR1.5}$	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
83	ASR	UQ	NFκB-Geneblazer	$EC_{IR1.5}$	>20	13.8 ±1.3	>20	>20	17.1 ±2.2	>20	>20	>20	>20	>20
84	ASR	BDS	NFκB-CALUX	$EC_{IR1.5}$	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30
85	ASR	GU	Jurkat E6.1 IκB	$EC_{CD0.2}$	0.8	1.4	>2	>2	1.6	>2	>2	0.7	1.3	>2
86	ASR	UQ	AREc32	$EC_{IR1.5}$[f]	1.8 ±0.1	2.5 ±0.1	>30	>30	1.7 ±0.1	23.1 ±1.3	17.4 ±0.5	5.0 ±0.2	7.0 ±0.3	>30
87	ASR	UA	Nrf2-keap	$EC_{IR1.5}$	>10	>10	>20	>20	>10	>20	>20	>20	>10	>20
88	ASR	ATG	Nrf2/ARE-cisFACTORIAL	$EC_{IR1.5}$	1.1 ±0.1	1.5 ±0.1	>4	>4	1.9 ±0.1	>4	4.1 ±0.7	1.3 ±0.1	3.9 ±1.1	>4
89	ASR	BDS	Nrf2-CALUX	$EC_{IR1.5}$	4.8 ±0.1	2.7 ±0.1	7.3 ±0.4	>30	4.8 ±0.4	7.5 ±0.5	6.9 ±0.4	2.9 ±0.2	5.1 ±0.3	>30
90	ASR	ATG	p53-	$EC_{IR1.5}$	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4

#	Class of MOA[a]	Lab(s)	Bioassay	EC (REF)	Eff1	MF	RO	AO	Eff2	O_3/ BAC	RW	DW	SW	H_2O
			cisFACTORIAL											
91	ASR	BDS, IWW	p53-CALUX	$EC_{IR1.5}$	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30
92	ASR	BDS	p53-CALUX +S9	$EC_{IR1.5}$	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30
93	ASR	UF	p53-GeneBLAzer	$EC_{IR1.5}$	>10	>10	>20	>20	>10	>20	>20	>20	>10	>20
94	CT	UQ	AREc32 cell viability	EC_{10}	15.5	>30	>30	>30	28.8	>30	>30	>30	>30	>30
95	CT	GU	Caco 2 NRU	EC_{10}	7.20	10.80	>20	>20	3.90	>20	>20	>20	>20	>10
96	CT	CSIRO	RTG2 MTT	EC_{10}	>30	>30	>30	>30	17.6 ±0.2	>30	>30	>30	>30	>30
97	CT	UFZ	DART 48h lethality	EC_{10}	>10	>10	>10	>30	5.0	>30	>30	>30	6.5	>30
98	CT	UFZ	DART 120h sublethal	EC_{10}	1.2	4.9	>10	>30	2.3	>30	>30	>30	2.2	>30
99	CT	GU	SK-N-SH cytotoxicity	EC_{10}	>1	>1	>2	>2	>1	>2	>2	>2	>2	>2
100	CT	GU	THP1 cytokine	EC_{10}	>1	>1	>2	>2	>1	>2	>2	>2	>2	>2
101	CT	UQ	algae growth inhibition	EC_{10}	5.4 ±0.6	4.5 ±0.2	17.3	>20	7.7 ±1.0	>20	>20	14.1	15.4	>20
102	CT	UQ, Swiss	*Vibrio fischeri* (Microtox)	EC_{10}[b,g]	1.3 ±2.1	0.7 ±0.6	2.7 ±4.5	10.7 ±19.1	0.4 ±0.3	1.4 ±1.6	1.8 ±1.8	0.7 ±0.7	1.7 ±2.1	14 ±22
103	CT	RCEES	*Photobacterium phosphoreum* T3	EC_{10}	0.4	0.5	2.7	6.6	0.2	0.5	0.5	0.2	0.3	1.7

 # refers to the number in the heatmap. [a]MOA= mode of action, XM = xenobiotic metabolism, Repro = reproductive and developmental effects, ASR = adaptive stress response, CT = cytotoxicity. [b]Bioassays that were performed by several laboratories and the error denotes the standard deviation of the mean of different laboratories' results; [c]only CSIRO results; [d]only BDS; [e]only UA, [f]previous published (Escher et al., 2013). [g]Previously published (Tang et al., 2013).

Section S7. Monitoring treatment efficacy

In this section we discuss the bioassays in the light of their suitability to serve as process monitoring tools. Of course one cannot say *a priori* that a bioassay is "good" or "sensitive" if it still measures an effect in treated waters. Effects can disappear if all chemicals that are responsive in this endpoint are well removed in the particular treatment process. The dynamic range between the effect of the product water and the blank is decisive for the suitability of an assay for assessing treatment efficacy (provided that reproducibility, repeatability and sensitivity have been already established with reference chemicals).

S7-A. Advanced water treatment plant using reverse osmosis

The investigated water reclamation plant (WRP) uses microfiltration followed by reverse osmosis and finished with advanced oxidation. The micropollutant flow in this plant has been characterized in detail in previous work by both chemical and bioanalytical tools (Escher *et al.*, 2011; Macova *et al.*, 2011). In the present study, we selected only four sampling points before and after critical treatment steps, the WRP inflow (WWTP effluent, sample Eff1), after microfiltration (sample MF), after reverse osmosis (sample RO) and after advanced oxidation combining hydrogen peroxide and UV irradiation (sample AO).

Effects were detected in Eff1 in 51 of 101 bioassays (Figure S10, red symbols and line, excluding the bacterial cytotoxicity assays). Subsequent treatment steps greatly reduced the effect burden caused by micropollutants. After MF (Figure, S10, blue symbols and line) 52 bioassays tested positive (not exactly the same ones), but RO decreased the number of positives sharply to 11. After AO, only three bioassays tested positive and these also tested positive in the ultrapure water blank.

In 12 bioassays, the effect after MF increased by more than 20%, *i.e.*, more than the variability of the assay response. In 20 bioassays the effect remained constant (±20%) and in 15 bioassays the effect decreased substantially already in the MF step. The five times increase in effect in the Ames TA_{mix}–S9 is presumably an artifact of the large variability of the results of this endpoint. To avoid biofouling, the MF membranes are

chloraminated, which causes the formation of disinfection by-products that can cause effects in some of the bioassays for reactive modes of action (Neale *et al.*, 2012).

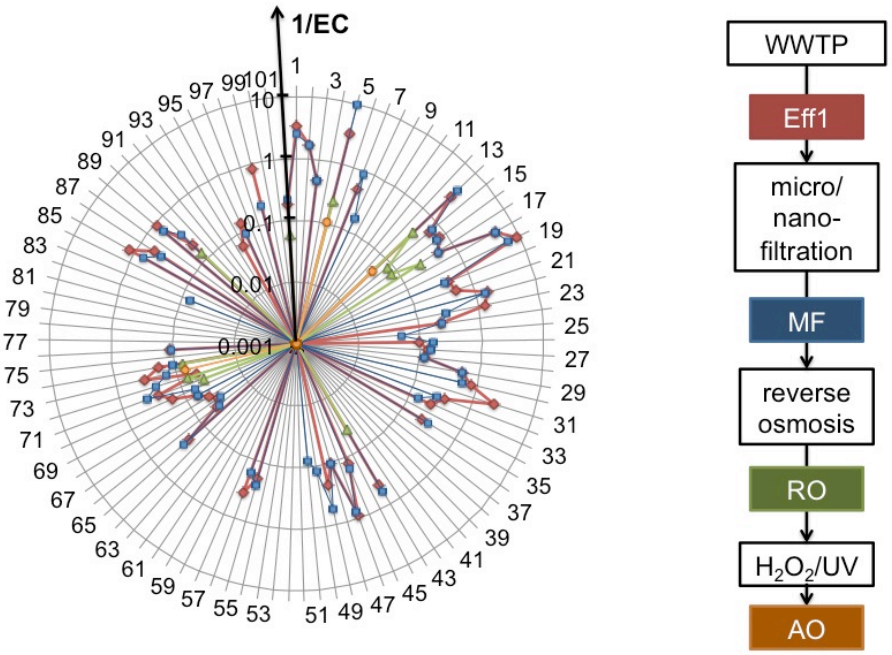

Figure S10 Bioanalytical fingerprint of the water from the WRP process using reverse osmosis. The red diamonds represent the Eff1, blue squares are MF, green triangles RO and yellow circles AO. The numbers refer to the bioassay numbers in Table 1 or Table S5.

The effects were greatly reduced after reverse osmosis (Figure S10). In 31 of 52 bioassays the effect disappeared to below detection limit and in most bioassays the effective response was reduced by an order of magnitude. There was no preferred type or group of effects removed. The bioassays with high variability, *e.g.*, the Ames assay, seem unsuitable for reliable assessment. The bioassays that showed reduction of effect but are sufficiently sensitive to respond after RO, are best suited as indicator bioassays. These include algae growth inhibition, the xenobiotic metabolism indicators, AhR-CAFLUX, H4IIEluc and MCF7-DRE. Of specific receptor-mediated modes of action, MDA-kb2 and hERα-HeLa-9903 were able to show the dynamics of treatment. In the group of adaptive stress responses, the AREc32 and Nrf2-CALUX showed a distinct reduction

pattern but were still above the LOD in RO water and are thus suitable as sensitive screening tool for process control.

S7-B. Water reclamation plant using ozonation and biologically activated carbon filtration

The second investigated WRP produces recycled water from secondary treated wastewater plant effluent using ozonation and activated carbon filtration (van Leeuwen *et al.*, 2003). The WRP has a capacity of 10 ML d^{-1} and provides water to industry for non-potable uses. Whilst the plant provides water for non-potable applications, it has been designed to meet drinking water standards. The treatment process incorporates biological denitrification, pre-ozonation, coagulation/ flocculation/dissolved air flotation-sand filtration (DAFF), ozonation, biological activated carbon treatment and ozone disinfection.

The removal efficiency of micropollutants has been analysed in detail in a series of studies that combined chemical analysis with bioanalytical tools (Macova *et al.*, 2010; Reungoat *et al.*, 2010; Reungoat *et al.*, 2011; Reungoat *et al.*, 2012a; Reungoat *et al.*, 2012b). In the present study, two points in the treatment train were targeted: the WRP influent (secondary treated effluent, Eff2) and the product water after ozonation and biological activated carbon treatment (O_3/BAC).

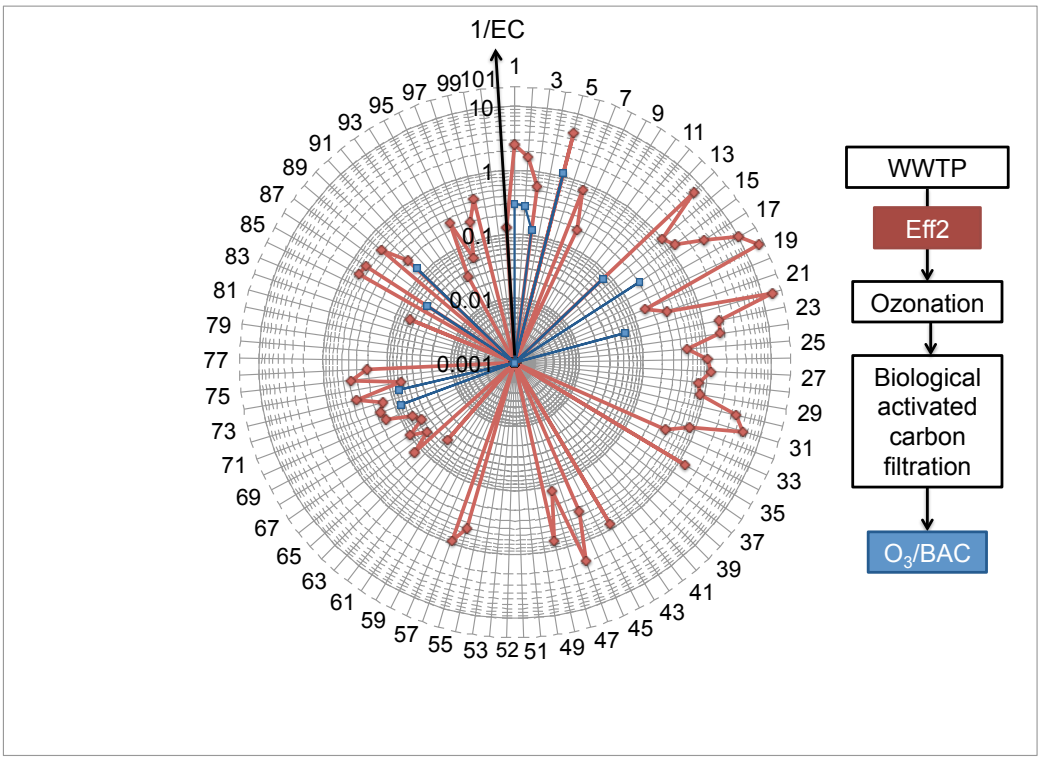

Figure S11. Bioanalytical fingerprint of the water treated with ozonation and biological activated carbon.

As is shown in Figure S11, 58 bioassays were above detection limit in the Eff2, similar to what was found with Eff1. The treatment reduced the number of responses to 11 and the effects in these positive bioassays were also greatly reduced (Figure S12). As was the case for the other facility, the bioassays that still showed an effect in the treated water are suitable as indicator bioassays to benchmark treatment efficiency.

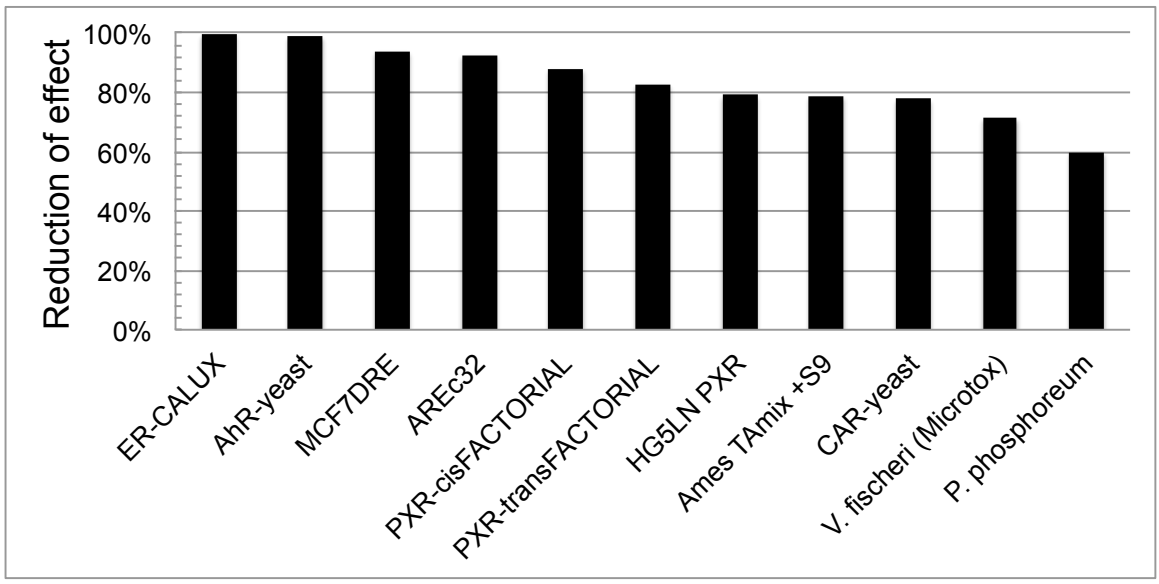

Figure S12. Percent treatment efficiency in the bioassays that did not fall below limit of detection (LOD) after treatment.

S7-C. Drinking water treatment plant

For comparison, we assessed treatment in a drinking water (DW) treatment plant. This plant has also been evaluated previously and applies coagulation and filtration followed by chlorination and finishing with chloramination (Macova *et al.*, 2011; Neale *et al.*, 2012). Here, only the feed water and the final drinking water were evaluated. The feed water is drawn from a river (RW) and the levels of micropollutants and effects (Figure S13) were low (Tang *et al.*, 2013). In RW and DW, 25 and 22 of 101 bioassays were positive but only 17 positive bioassays in DW were identical to those positive in the RW, for the remaining positives different biological endpoints were triggered in RW and DW.

The effects in the E-SCREEN, the AhR-CAFLUX and the MCF7-DRE remained the same or were reduced indicating that chlorination degraded or did not change existing micropollutants but did not produce specifically acting compounds. However drinking water treatment with chlorination and chloramination increased the non-specific and reactive toxicity (Figure S13) due to the formation of disinfection by-products, which is consistent with previous findings and chemical analysis of formed disinfection by-products (Neale *et al.*, 2012).

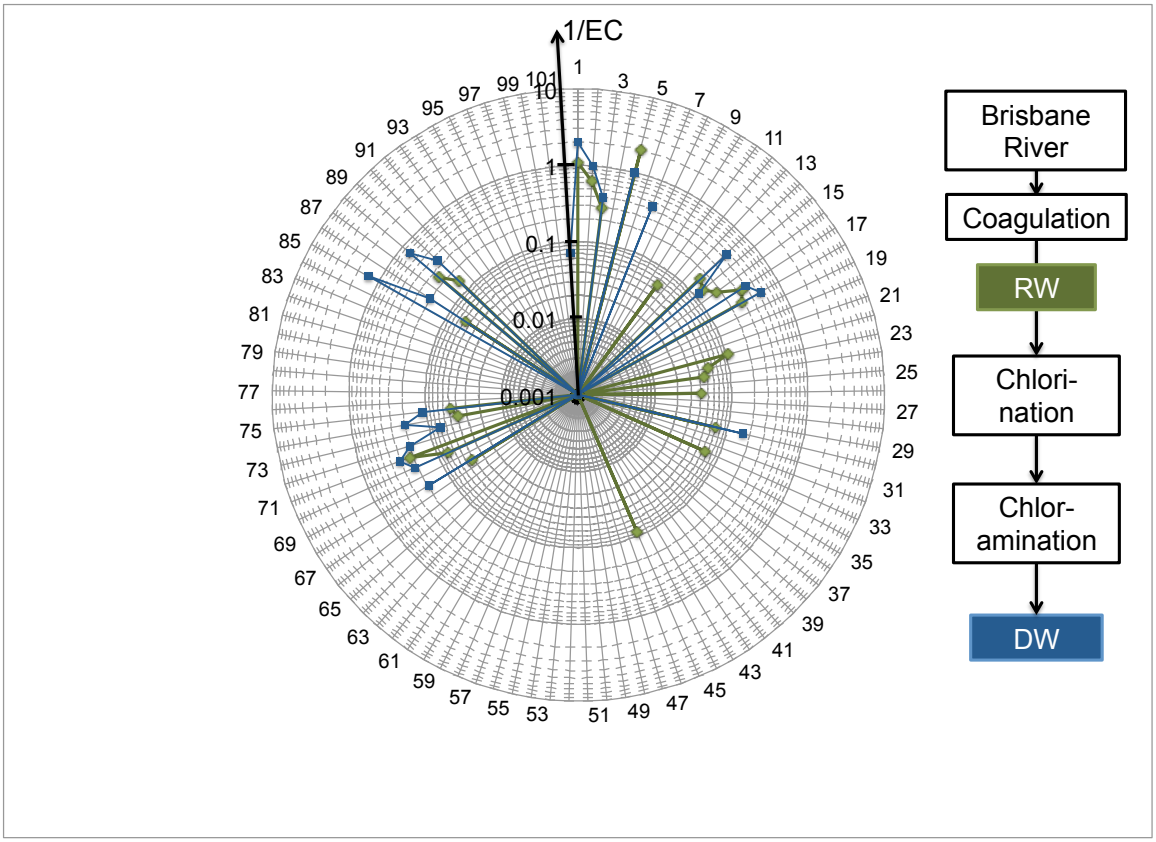

Figure S13. Bioanalytical fingerprint of the drinking water treatment.

Of the bioassays that increased in the toxicity, only one represents a specific mode of action, the hERα-HeLa-9903. As expected, the majority of these positive assays targeted xenobiotic metabolism, reactive modes of action and adaptive stress responses. Increase was most pronounced in the reactive modes of action (Ames TA98+ and –S9, Ames TA100 -S9, umuC NM5004 and micronucleus assay). There was a detectable but small increase by up to a factor of 2 for the bioassays indicative of xenobiotic metabolism, with a preference for the PXR (HG5LN PXR, PXR-transFACTORIAL, PXR-cisFACTORIAL). The responses in all three bioassays for the oxidative stress response (Nrf2-CALUX, Nrf2/ARE-cisFACTORIAL and AREc32) increased by a factor of 2.4 to 4.2.

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APPENDIX 5.1 – BIOASSAY RESULTS SPREADSHEET

GeneBLAzer assay - Excel spreadsheet for data reporting

1. Enter Blue and Green raw values generated by the plate reader.

Blue values

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Green values

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Note: Wells highlighted in yellow are for cell free control (media only)

2. Determine the average emission from the cell-free control wells

Cell free average for blue emission data
Cell free average for green emission data

3. Subtract the background of the cell free control from the samples data

Blue values - Cell free background removed

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Green values - Cell free background removed

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

4. Calculate the blue/green ratio for all the samples (example using layout of Plate I)

	Std- 1	Std - 2	Std- 3	Std -4	Std- 5	Std - 6	Std- 7	Std - 8	Std- 9	DMSO	DMSO free	
	Conc.	Conc.	Conc.	Conc.	Conc.	Conc.	Conc.	Conc.	Conc.			
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												
	dilution 1			dilution 2			dilution 3			dilution 3		

5. Calculate the blue/green ratio (B/G) by dividing the blue emission data by the green emission data

DMSO B/G average

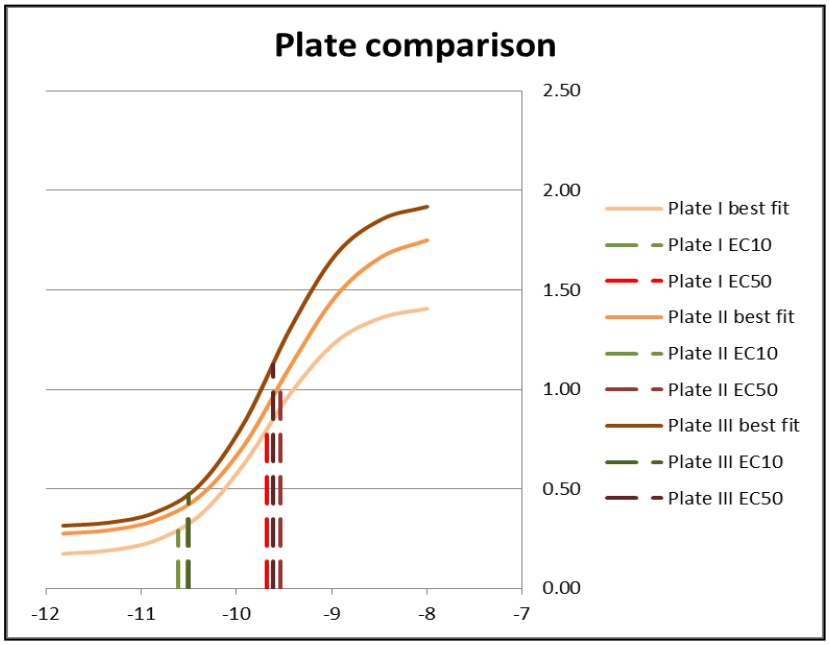
6. Subtract the DMSO background from the data of the experimental samples (example using layout of Plate I)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Summary - GeneBLAzer AR assay - Blue/Green ratio for reference toxicant on plates analyzed during the same batch

Sample	Conc. of ref. toxicant	Avg B/G value Plate I	St dev	B/G avg plate II	St dev	B/G avg plate III	St dev
Std - 9							
Std - 8							
Std - 7							
Std - 6							
Std - 5							
Std - 4							
Std - 3							
Std - 2							
Std - 1							

Example of analysis of the dose response curve for reference toxicant and calculation of EC50



Summary - GeneBLAzer AR Assay - Blue/Green ratios of environmental extracts

Sample ID	Average B/G value	St dev	Average B/G value	St dev	Average B/G value	St dev	Average B/G value	St dev
	Dilution 1		Dilution 2		Dilution 3		Dilution 4	
#1								
#2								
#3								
#4								
#5								
#6								
#7								
#8								
#9								
#10								
#								
#								
#								

Data are used to calculate the effect concentration relative to the reference toxicant expressed as bioanalytical equivalent concentration (ng/L)

APPENDIX 5.4 – DATA INTERPRETIVE GUIDELINES

INTEPRETIVE FRAMEWORK FOR MONITORING OF RECYCLED WATER USING IN VITRO BIOASSAYS

I. DATA ANALYSIS

Definitions/Scope

Effect concentration (EC_x) – concentration at which x% of the maximum response is achieved.

Bioassay equivalents (BEQs) – the bioassay response of a sample extract that corresponds to the equivalent concentration of a reference chemical, e.g. 17β-estradiol for the estrogen receptor – alpha (ERα) transactivation assay.

Relative enrichment factor (REF) – final concentration factor, a unitless parameter defined as the product of sample enrichment factor and the bioassay dilution factor (Escher et al. 2014)

Step 1. Confirm bioassay sensitivity (QA/QC)

a. Calculate EC_{10} and EC_{50} for the reference chemical using dose-response (log-logistic) curve fitting (e.g. as described in Escher et al. 2014).
b. Analyze procedural blank (DMSO and media without sample extract) contribution. If mean fold response of blank is < 20% of EC_{10}, proceed to step 1c. If mean blank response is > 20%, take appropriate corrective action (e.g. re-check raw data, analysis, and, if necessary, repeat analysis).
c. Compare EC_{10} and EC_{50} to pre-specified range based on historical data. If within the specified range, proceed to Step 2. If either EC_{10} or EC_{50} are outside of specified range, take appropriate corrective action (see 1b).

Step 2. Determine dose-responsivity for dilution series of sample

a. Test for difference in fold response (FR) among sample dilutions (ANOVA, t-test).
b. If dose response exists, calculate EC_{10} and EC_{50} (if range of response allows) using log-logistic curve fitting.
c. If no dose response exists, calculate mean ± sd of dilutions and compare to blank response (t-test). If no difference exists, report EC_x as maximum REF assayed * 2.

Step 3. Calculate bioassay equivalent concentration (BEQ)

a. Calculate BEQ (in units of ng/L) for sample using following equation:
$BEQ_{sample} = EC_x$ (reference chemical) / EC_x (sample)

Example: 1 L water is extracted using standardized protocol (Leusch et al. 2014; Escher et al. in press) and the resulting extract in organic solvent is exchanged and concentrated to 1 mL total volume in DMSO (concentration factor = 1000). Five microliters of sample extract is serially diluted in bioassay medium (100uL) to create a dilution series representing 0.01, 0.005, 0.0025 and 0.00125 of the sample extract aliquot. The dose response curve established for E2 results in an EC_{10} of 1.8e-12 M (0.49 ng/L as E2). The dose response curve for the sample results in an EC_{10} of 6 (in units of relative enrichment factor). Thus,

$$BEQ_{sample} = EC_{10}\,(reference) / EC_{10}\,(sample) = 0.49\ ng/L / 6 = 0.082\ ng/L\ (as\ E2)$$

II. ESTABLISHING CREDIBLE MONITORING THRESHOLDS

Definitions/Scope

Method Reporting Limit (MRL): mass or molar concentration that allows for achievement of monitoring goals; typically set below the effects threshold of interest, e.g. 10-fold lower than a consensus PNEC.

Action Level (AL): mass or molar concentration that serves as a benchmark for comparison of monitoring data. If monitoring data consistently exceeds the AL, an appropriate level of action is taken to rectify the exceedance.

Maximum Contaminant Level (MCL): a legal threshold limit on the amount of a substance that is allowed in public water systems under the Safe Drinking Water Act, usually expressed as a mass concentration.

Notification Level (NL): health-based advisory levels established by DPH for chemicals in drinking water that lack MCLs. When a chemical is found at a concentration greater than its NL, certain requirements and recommendations apply. The level at which DPH recommends removal of a drinking water source from service is called the "response level."

In California, the State Water Board (SWB) and Department of Public Health (DPH) share responsibility in setting and enforcing monitoring regulations (e.g. MRLs and ALs) for groundwater recharge applications using recycled water in accordance with the State's Recycled Water Policy (SWB 2013). The Office of Environmental Health Hazard Assessment (OEHHA) develops public health goals for contaminants in drinking water, which serve as the basis for establishing MRLs and ALs.

Step 1. Consult with SWB, DPH and other state agencies on existing or pending recycled and/or drinking water monitoring requirements, including, but not limited to:

a. Federal or state MCLs established for target CECs or analogs thereof
b. Current State or regional investigative requirements for target CECs
c. Non US published threshold based on target (human health) endpoints

Step 2. Investigate linkage of bioassay (in vitro) and higher order effect (in vivo)

a. Compile relative potency factors (PFs) linking in vitro responses to in vivo effects

e.g. PF = $EC_{50,\ \text{in vitro}}$ / $EC_{50,\ \text{in vivo}}$

b. Rank or weight PFs based on relevance and rigor of study/dataset (e.g. epidemiological > individual > organ > molecular)

Step 3. Apply margin of safety commensurate with goals of monitoring and degree of uncertainty associated with effects threshold development

AL = (PNEC or NOEC) / PF

Example: The published PNEC for 17β-estradiol (E2) in drinking/recycling water based on protection of human health has been recently reported as 1 ng/L (Caldwell et al. 2012).

Consensus values linking PFs for in vitro and in vivo effects for effects to humans exposed to estrogens in drinking water remain unknown, and is conservatively assumed to be unity.

AL = 1.0 ng/L / 1 = 1.0 ng/L

To allow for collection of monitoring data below this PNEC, scientists and expert panels have independently set MRLs for estrogenic chemicals (referenced to E2) at 0.09, 0.1 and 0.4 ng/L (Table II-1). The MRL achievable by current ERα bioassays is estimated to be 0.1 ng/L (Leusch et al. 2010). In this example, establishing an AL for estrogens at 1.0 ng/L allows for 10-fold measurement buffer.

Table II-1. Comparison of effects based- and bioassay method reporting limits (MRLs).

Constituent	Bioassay Endpoints	CA Expert Panel Effects-based MRL (ng/L)[a]	Other Effects-based Thresholds (ng/L)	Bioassay MRL (ng/L)
Estrogens (17β estradiol)	ERα-GeneBlazer ERα-CALUX	0.09	0.1[b], 0.4[c]	0.5[d] 0.1[e]
Androgens	AR-GeneBlazer AR-CALUX	n/a	10[b], 80[c]	20 (R1881)[d] 2.5 (DHT)[e]
Glucocorticoids	GR-GeneBlazer GR-CALUX	n/a		50 (DEX)[d] 430 (DEX)[f]
Progestins	PR-GeneBlazer PR-CALUX	n/a		50 (LEV)[d] 170 (LEV)[f]
Stimulants (caffeine)	n/a	35		n/a
Antibacterials (triclosan)	thyroid receptor (TR)	50		450 (T3)[f]

Nitrosamines (NDMA)	Genotoxicity, Mutagenicity	0.1		1.0e7 (MMS)[f] 9800 (NaN3)[f]
Non-specific	cytotoxicity	n/a		1.5e6 (MTX)[f]

[a] Anderson et al. (2010)

[b] environmental (non-human health) threshold (Caldwell et al. 2012, Leusch et al. 2014)

[c] Australian Guidelines for Water Recycling as cited in Leusch et al. (2014)

[d] estimated limit of detection (this study)

[e] method quantification limit (Leusch et al. 2010)

[f] EC50 for reference chemical (Leusch et al. 2014)

n/a – not available

III. DECISION MAKING FRAMEWORK

Definitions/Scope

Level of Concern: categorical classification of the risk to human health posed by recycled water monitored using in vitro bioassays based on the magnitude of response (BEQs), e.g. the degree of exceedance of a mass or molar concentration established as a MRL or AL.

Adaptive management: the ability to adjust and revise regulatory and monitoring requirements based on a body of scientific evidence in a timely fashion.

DPH has released draft monitoring requirements for recycled water applications (see Groundwater Replenishment with Recycled Water, DPH-09-009, June 26, 2013).

Step 1. Compare bioassay response (as BEQs) to established MRL or AL.

a. If BEQ > AL, GO TO STEP 2.
b. If BEQ < AL, continue with routine monitoring; GO TO STEP 3

Step 2. Define actions commensurate with degree and persistence of exceedance (see Fig. III-1).

a. Confirm a single exceedance within a specified period of time (e.g. 72 h).
b. If confirmed, initiate analysis of samples targeting chemicals known or suspected of eliciting responses consistent with the endpoint in question. Increase frequency of in vitro bioassay and/or chemical analysis of samples to determine the temporal variability and persistence of the exceedance. Notify the regulatory agency(s) responsible for oversight of monitoring.
c. Consider the degree of exceedance (i.e. magnitude of BEQ/AL) in determining the appropriate course of action.

Step 3. Review and evaluate monitoring data on a periodic (e.g. annual) schedule, using a tiered decision framework whose management actions are commensurate with the magnitude and consistency of bioassay results (see Fig. III-1)

a. For endpoints that consistently exhibit a BEQ/AL <0.1, consider decreasing monitoring frequency or eliminating requirement for inclusion of endpoint.
b. For endpoints that consistently exhibit responses at a minimal level of concern (0.1 < BEQ/AL < 10), continue monitoring to ensure that responses are not increasing over time.
c. For endpoints that consistently exhibit responses at an elevated level of concern (10< BEQ/AL < 1000), consider increasing frequency of monitoring, identifying sources and/or instituting alternative treatment options to reduce the bioactivity response into the minimal or no concern level.
d. For endpoints that consistently exhibit responses at a high level of concern (BEQ/AL > 1000), consider removing the RW supply while increasing frequency of monitoring, identifying sources and/or treatment alternatives.
e. Consult with independent experts to review monitoring data, update toxicological data (PNECs, NOECs, PFs) that may support revision of MRLs and ALs, and recommend improvements in the selection and performance of in vitro bioassays.

Example: Quarterly monitoring data for the ERα bioassay for a water reclamation plant regulated under the CA Recycled Water Policy show that the annual average BEQ is 0.23 ng/L, with a maximum of 0.88 ng/L and a minimum of 0.082 ng/L. According to the tiered response framework (Fig. III-1), an average BEQ/AL that is between 0.1 and 10 is not expected to impact the beneficial uses of the resource, but remains at a level where continued monitoring is warranted to ensure detectable levels do not increase. If the AL is set at 1.0 ng/L, the maximum monitoring value of 0.88 ng/L would not exceed the AL. If at any time the monitoring value exceeded the AL, the SWB/DPH may require confirmation within a specified time (e.g. 72 h). If the average of the original and confirmation values remains in exceedance, more frequent monitoring would be required, or depending on the magnitude and persistence of the exceedance, additional actions may be warranted (Fig. III-1). If the average after confirmation sampling does not exceed the AL, monitoring would continue at the baseline level.

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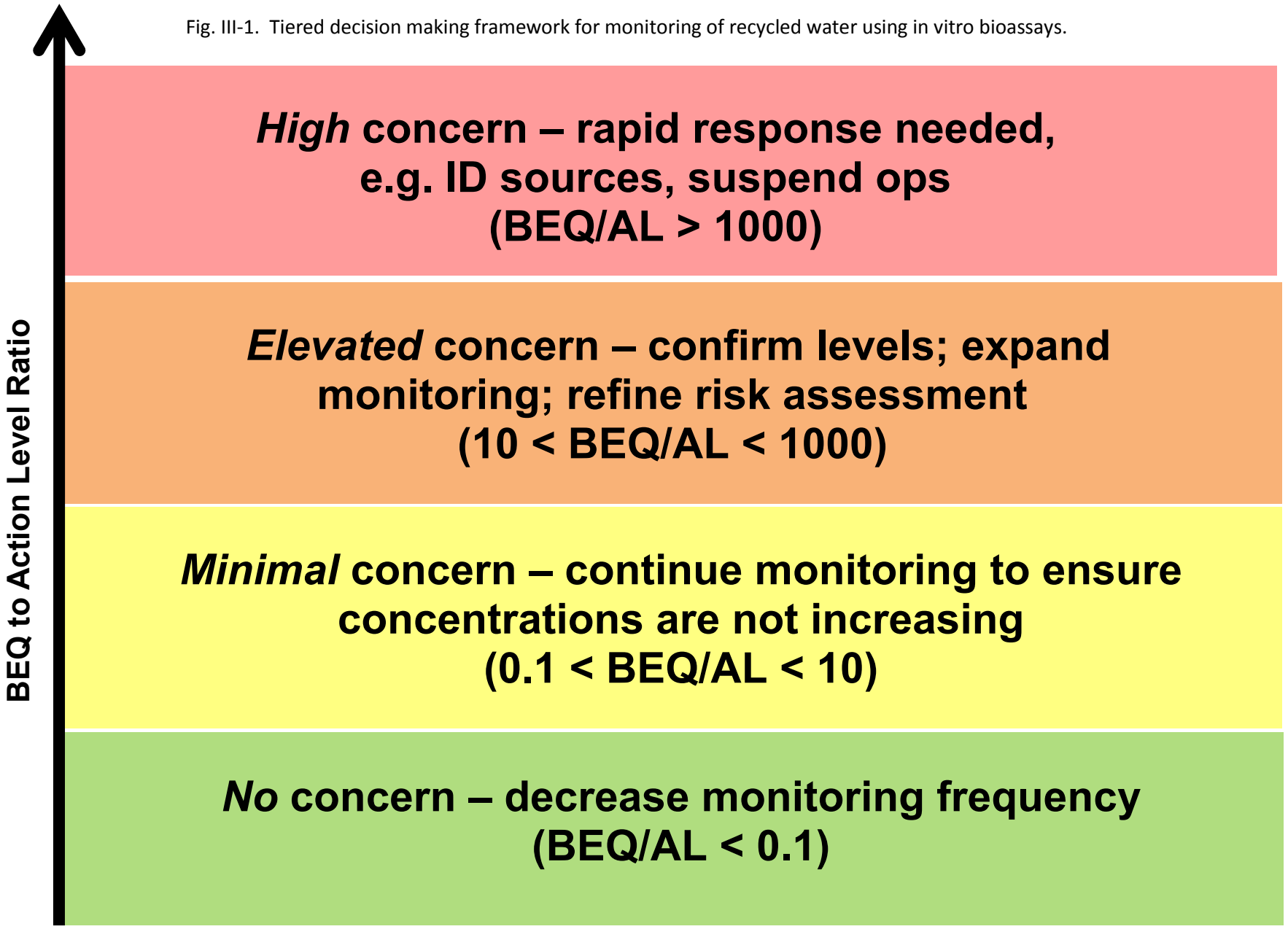
Fig. III-1. Tiered decision making framework for monitoring of recycled water using in vitro bioassays.



APPENDIX 6.1 – PROJECT ADVISORY COMMITTEE

BIOANALYTICAL METHOD DEVELOPMENT
FOR RECYCLED WATER APPLICATIONS IN CALIFORNIA

PROJECT ADVISORY COMMITTEE
June 2014

Charles Carter is Vice President of Quality and Technical Services for TestAmerica Laboratories, Inc. He is responsible for corporate QA and laboratory technical development, and he is involved with legal issues, contracts, health and safety, and IT efforts. Dr. Carter has a Ph.D. in environmental chemistry, has over 30 years experience in the environmental laboratory industry, and is a recognized industry expert in numerous areas of environmental and analytical chemistry. He has been active in various industry organizations and is a frequent speaker at environmental forums and conferences.

Melenee Emanuel is an Environmental Scientist in the Division of Water Quality of the California Water Resources Control Board. She currently serves as the Water Board's contract manager for the bioanalytical method development project. Melenee has worked on water quality issues for the Water Board including; surface water quality monitoring, listing of impaired water bodies, and non-point source pollution. More recently, she has worked on the adoption of the state's revised Recycled Water Policy that established monitoring requirements for contaminants of emerging concern (CECs) in recycled water used for groundwater recharge.

Dave Mazzera is a Research Scientist in the Division of Drinking Water and Environmental Management of the California Department of Public Health. He works as the lead scientist involved in setting the State's drinking water standards and is the CDPH liaison to the Water Board for the state's Recycled Water Policy. Dr. Mazzera has a Ph.D. in environmental science and a B.S. in environmental toxicology, and over the past 19 years has worked in both the private and public sectors assessing public health impacts and potential risks associated with exposure to chemicals in the environment.

Don McEnhill is Program Director for Russian Riverkeeper**.** Don is a Healdsburg resident with a business background who grew up on the Russian River spending his summers at the family cabin on Fitch Mountain. Working with citizen groups and attorneys, he has researched water supply, water quality, and land use issues in the Russian River for the past 9 years. Don is an avid canoer, white water paddler, and recreational fisherman - when he can get away from work!

Dave Smith is the Managing Director for the California Section of the WateReuse Association, a nonprofit organization with over 100 member agencies whose mission is to advance the beneficial and efficient use of water resources through education, sound science, and technology using reclamation, recycling, reuse and desalination. Dave is a water quality and regulatory expert with degrees in aquatic ecology (B.S.) and environmental engineering (Ph.D.).

Vickie Wilson is Chief of the Reproductive Toxicology Branch, Toxicity Assessment Division for the USEPA's Office of Research and Development. She has been active in reproductive

toxicology research for the past 11 years. Vickie's lab has developed targeted in vitro assays for androgen and estrogen signaling pathways, and has adapted and helped validate these assays to screen environmental water samples for bioactivity. She is active in several professional environmental and toxicological societies and is a frequent speaker at meetings and conferences.

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APPENDIX 6.2 – CROSS-PROJECT MEETING FINAL AGENDAS AND PRESENTATIONS

Development of bio-analytical techniques to assess the potential human health impacts of recycled water

Cross-Project Meeting#1

10/11 November 2011, SCCWRP, Costa Mesa, CA

The meeting will have three main components:

1. Seminar titled "Bioanalytical Tools in Water Quality Assessment" given by Drs. B. Escher and F. Leusch. Stakeholders, SCCWRP member agency and interested academic parties will be invited. Discussion to follow (Thurs am)
2. Cross-project meeting between California and WRRF project teams to brief each other, identify areas for collaboration and forge agreement for sharing of information/materials and division of tasks (~0.5 day; Thurs pm & Fri am)
3. Internal project team meetings as needed (~ 0.5 – 1 day; Thurs am & Fri)

1. "Bioanalytical Tools in Water Quality Assessment" – a seminar by Drs. Beate Escher & Fred Leusch

A 2 hour seminar followed by a one hour discussion period. The seminar will provide an overview on the current state of bioanalytical tools and their potential applications in water quality monitoring that is targeted for a diverse audience of practitioners and regulators. Invited participants will be from the scientific, regulatory and regulated communities. Seminar will be webcast for those who wish to view/participate remotely. A discussion period will follow the seminar that will be structured to address priority questions/comments from the CA and WRRF project participants (investigators and PAC members) and as time permits, questions from the larger audience. The primary goal of this session is to bring interested parties to the same level of understanding regarding the pros and cons associated with these tools.

Participants	Organisation	Participation
Discharger & regulator reps	SCCWRP Member Agencies	Live & remote
SCCWRP Staff	SCCWRP	
CA & WRRF Team Members	Various	
CA & WRRF Team Affiliates & Support Staff	Project Team Organizations	
Invited stakeholders	Various	

2. Cross-project Meeting of CA and WRRF teams

Objective: to delineate and understand individual project goals; explore possible synergistic interactions and collaborations between both projects, propose & approve collaborative approach (e.g. information and/or material sharing; division of research, method development and/or validation tasks)

Agenda items:

- Project goals and progress to date (60 min)
  - Literature review
  - Selection of candidate bioassays
  - Water extraction protocols
- Vendor presentations (45 min)
- Identification of commonality & areas of potential overlap & redundancy (60 min)
  - Bioassays targeted
  - Water quality and sharing of samples/extracts
  - Bioassay data interpretation & communication strategies
- Stakeholder issues and concerns (30 min)
- Discussion & agreement on collaboration and path forward (Friday late am session)

Participants	Role (Organisation)	Participation
Beate Escher; Fred Leusch; Shane Snyder; Erin Snyder*; Anita Poulsen; Brad Clarke*	WRRF Project PIs (various)	Yes
Keith Maruya; Nancy Denslow; Dan Schlenk; Shane Snyder; Sandy Westerheide	CA Project PIs (various)	Yes
Blythe Layton; John Griffith; Steve Bay	SCCWRP Staff	Invited
Julie Minton, Stefani McGregor	WRRF Project Administration	Yes
Charlie Carter; Gary Dickenson; Dave Mazzera*; Don McEnhill; Dave Smith; Vickie Wilson	CA PAC Members (various)	Yes
Michael Bartkow*; Denise Hosler*; Laura Kennedy; Menu Leddy; Max Zarate-Bermudez	WRRF PAC Members (various)	Yes
Jonathan Bishop	California Water Resources Control Board	Invited
Peter Behnisch (BDS); Shelley Force Aldred, Nathan Trinklein (SwitchGear); Gerry Pelanek, Kun Bi (Invitrogen)	Vendor representatives	presentation(s) & selected segments

*Remote participation

3. Internal Project Meetings

Objectives: discuss progress to date; finalize tasks/decisions that are due; identify wish lists and points of collaboration; plan, schedule and assign next steps/tasks

Agenda items: to be determined by Project Teams:

- WRRF
  - Final selection of Category 2 bioassays in all labs
    - Development of "SOP" of the validation of a category 2 bioassay
  - Plans for first small scale comparison study of core labs beginning in 2012
  - Points of collaboration with partner project
- CA
  - Discussion of literature search
  - Final selection of candidate bioassays
  - Future deliverables and milestones

Participants	Organisation	Participation
WRRF Project Team Members	Various	
CA Project Team Members	Various	
Vendor representatives		by invitation

DEVELOPMENT OF BIOANALYTICAL TECHNIQUES FOR MONITORING OF CHEMICALS OF EMERGING CONCERN IN RECYCLED WATER

JOINT MEETING BETWEEN CALIFORNIA AND WATER REUSE RESEARCH FOUNDATION PROJECT TEAMS

NOVEMBER 10 - 11, 2011
MEETING AGENDA

To be held at:
Southern California Coastal Water Research Project
3535 Harbor Blvd. Costa Mesa, CA 92626

Portions of Meeting will be webcast at conference.sccwrp.org

Thursday, November 10

8:30	Coffee & pastries	
9:00	Welcome & Introductions	Keith Maruya (SCCWRP) Julie Minton (WRRF)
9:15	Bioanalytical tools in water quality assessment, (available by webcast)	Beate Escher (Univ. Queensland) Frederic Leusch (Griffith Univ.)
11:15	Discussion/Q&A (webcast)	Moderators: Keith Maruya, Julie Minton
12:00	LUNCH (provided on site for $10)	
1:15	Cross-Project Meeting, Part 1	WRRF Project Team Members CA Project Team Members
3:30 - 3:45	BREAK	
3:45	Individual Project Meetings	
5:00	Adjourn for Day	

DINNER WITH WRRF AND CA PROJECT TEAM MEMBERS

Friday, November 11

8:00	Coffee & pastries	
8:30	Individual Project Meetings (cont.)	
10:15 – 10:30	BREAK	
10:30	Cross-Project Meeting, Part 2	WRRF Project Team Members CA Project Team Members
11:45	Action Items & Wrap Up	Beate Escher, Keith Maruya
Noon	Adjourn	

DEVELOPMENT OF BIOANALYTICAL TECHNIQUES FOR MONITORING OF CHEMICALS OF EMERGING CONCERN IN RECYCLED WATER

JOINT MEETING BETWEEN CALIFORNIA AND WATEREUSE RESEARCH FOUNDATION PROJECT TEAMS

JANUARY 23 - 24, 2014
MEETING AGENDA

To be held at:
Southern California Coastal Water Research Project
3535 Harbor Blvd. Suite 110, Costa Mesa, CA 92626

For Audio Access: Dial +1 (213) 493-0007
Access Code: 682-658-022
Audio PIN: Shown after joining the meeting

Thursday, January 23

8:30	Coffee & pastries	
9:00	Welcome & Introductions	Stephen Weisberg (SCCWRP) Julie Minton (WRF)
9:15	Opening Remarks	Jonathan Bishop (CA Water Board) David Smith (WateReuse CA) Vickie Wilson (EPA ORD) John Printen (Life Technologies)
9:30	WRF10-07 Project Summary (Goals, Key Results)	Beate Escher, Fred Leusch
10:15	BREAK	
10:30	CA Project Summary (Goals, Key Results)	Nancy Denslow, Sandy Westerheide
11:15	Discussion "*Which bioassays are ready for pilot implementation*?"	Moderators: N. Denslow, B. Escher
12:00	LUNCH	

1:00	Analysis & Interpretation of Bioassay Results WRF Intercalibration Exercise & Effect-Based Monitoring Trigger Development	B. Escher
2:00	Comparing Bioassay & Analytical Chemistry Results – CA Intercalibration Exercises	Shane Snyder
2:30	Standardization of Bioassay Protocols	Alvina Mehinto
3:00	BREAK	
3:15	Discussion - "*How do we implement bioassays for monitoring of recycled water*?"	Moderators: K. Maruya, B. Escher
4:00	Bioassays/MOA Wish List for CA	Dan Schlenk
4:30	Promising Endpoints in the Development Phase	F. Leusch
5:00	Discussion – "*What tools/data are needed to make monitoring more comprehensive and robust?"*	Moderators: V. Wilson, F. Leusch
5:45	Adjourn	

DINNER WITH THE GROUP

Friday, January 24

7:30	Coffee & pastries	
8:00	Summary of Day 1; Breakout Assignments	K. Maruya, B. Escher
8:30	Breakout Session (by Project)	
10:00 – 10:15	BREAK	
10:15	Meeting Summary and Consensus Building Bioassays to move forward Implementation Strategy Next Steps	Moderators: K. Maruya, B. Escher
11:30	Project Deliverables, Action Items & Wrap Up	
11:45	Adjourn	

WateReuse Research Foundation – SCCWRP Collaboration Meeting #2

Costa Mesa, CA

January 23-24, 2014

Julie Minton

Director of Research Programs

The Foundation's Mission

To conduct and promote applied research on the reclamation, recycling, reuse and desalination of water.

The Foundation's Vision

2011- 2020

- Annual Budget of $5-10 Million
- Funding Partners
  - DPR Initiative Donors
  - Utilities/Manufacturers
  - Bureau of Reclamation
  - CA SWRCB/DWR/CEC
  - Pentair Foundation
  - Subscribers
  - Partners: AWRCE/Singapore PUB
  - Multinational Corporations
  - Charitable Foundations

- A Global Presence and Reach
- The Respected Voice for Research on Water Reuse and Desalination

WATEREUSE RESEARCH

WateReuse Research Foundation : History

- Incorporated on **September 13, 1993** to:
  - Develop the Science & Technology Necessary to Support the Water Recycling Needs of the 21st Century
- Foundation Specializes in Conducting “Leading Edge” Applied Research
- Address Following: Chemical & Microbiological Agents, Treatment Technology, Economics, Marketing, Public Perception
- Push Back the Frontiers in Technology

WATEREUSE RESEARCH

Significant Events

- Hired FT Executive Director on August 1, 2000
- Secured $180,000 in Funding from USBR in September, 2000
- Secured “Earmark” of $1MM in FY 2001
- Received Matching Funding of $1MM from CA-SWRCB in 2002
- Expanded Mission to Include Desalination in 2003
- Reconstituted, Strengthened RAC in 2004
- Developed Equitable Sustainable Funding Model in 2007
- Changed Name in 2010
- Launched the CA DPR Initiative in June 2012
- New Executive Director to start March 1, 2014

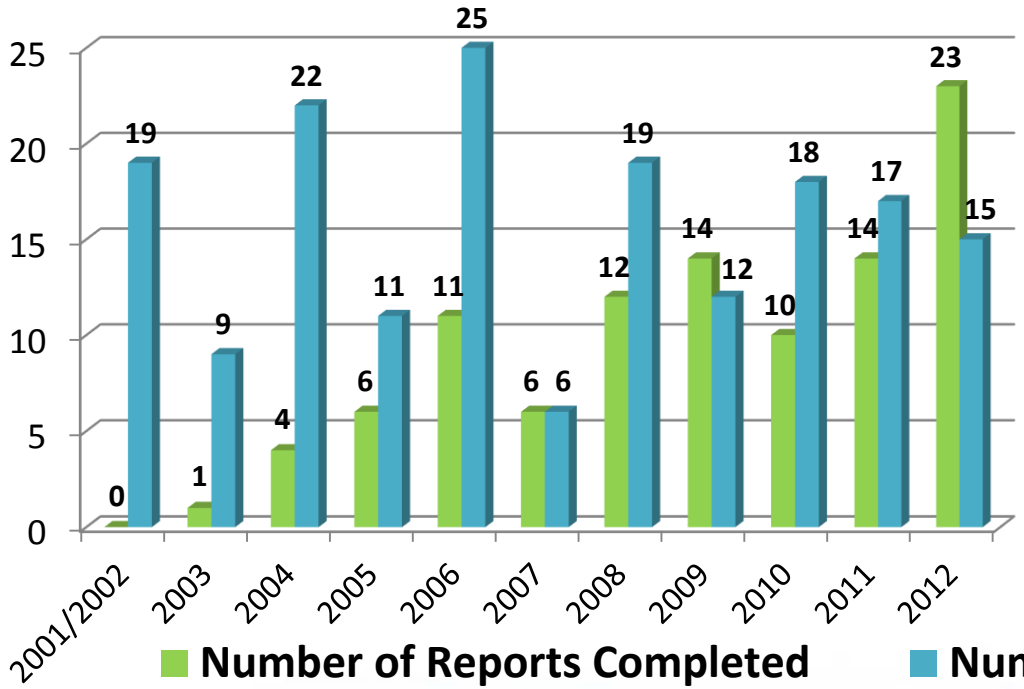
WATEREUSE RESEARCH

Outreach is an Important Element of Foundation Work

- Number of Outreach Pieces to Date: 500+
  (reports, presentations, proceedings, peer-reviewed publications)

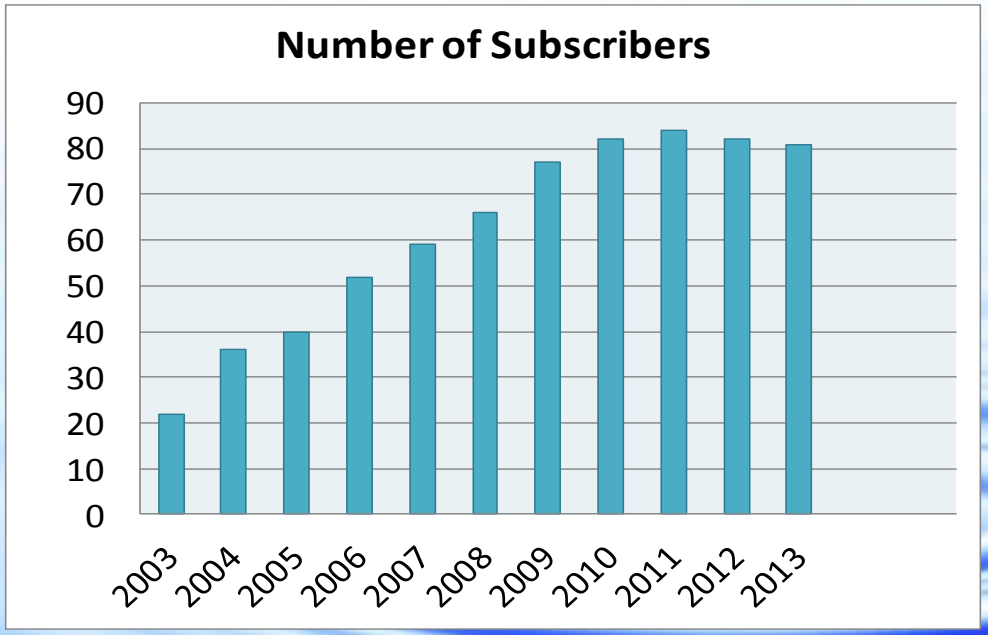
- New Journal Initiated in 2012– *WorldWater: Water Reuse and Desalination*

- Webcast Program initiated in 2011: 60-90 min program on hot topic held on the second Thursday of each month (free for Subscribers)



Since 2000…

172 projects commissioned
Over $50M in funding leveraged
120 published works
50 projects still active



In 2013…

12 projects launched
$1.9M in funding awarded
36 published reports

WATEREUSE RESEARCH

Annual WateReuse Research Foundation Conference

- First Conference held on June 5-6, 1997
- Theme was ”Merging Our Resources”
- Will Convene 18th Annual Conference
  - May 19-20, 2014 in Las Vegas, NV
- Conference provides opportunity to:
  - Showcase results of WRRF research
  - Hear presentations from federal agencies, researchers from partner organizations
  - Identify future research needs
- EPA’s OR&D and Water Research Foundation have been Conference Sponsors for 13 Consecutive Years

Research Categories

- Direct Potable Reuse
- Business Economics & Industrial Reuse
- Public Acceptance & Policy
- Desalination

Thank you!

Julie Minton

jminton@watereuse.org

703-548-0880 x 108

WATEREUSE'S FORWARD-LOOKING DIRECTION

- **RAC re-focused its framework for regular research to place more emphasis on socio-economic research angles, and public policy implications, to generate more ROI for subscribers – a sharpened focus**

- **The Foundation has made a major commitment to philanthropy, to replace previous government/agency funding lost – trying to attract more non-dues donors from within our community, but also from humanitarians/philanthropists**

- **Have raised $5.3 million in philanthropy since June 2012 towards DPR Adoption – tremendous opportunities**

- **Future research priorities will center around:**
  - **-- Potable reuse as a supply solution to water scarcity/availability across the US, not just in CA, TX, AZ and CO**
  - **-- Industrial reuse, especially the water-food-energy nexus**
  - **-- International water reuse, as it impacts the human condition**
  - **-- Championing innovation and new technology in reuse – for all water portfolios**

Goals and Key Results of the Project

Sandy Westerheide and Nancy Denslow
University of South Florida &
University of Florida

Goals of the project

- Characterize the response of selected *in vitro* bioassays for samples representing a range of recycled water quality
- Quantify the relationship, if any, between bioassay response and higher order impacts that are relevant to human health
- Identify the appropriate use and role for bioassays that exhibit acceptable performance in a recycled water monitoring program

Approach

Tasks

1. Literature review → identify most promising assays
2. Evaluate bioassays and optimize them. Validate with water samples of known chemistry
3. Compare bioassay response to reference doses – Predict BEQ's
4. Provide data interpretation and implementation guidance

Selecting Relevant Endpoints

Assay	Acronym	Mechanism	Potential Health Implications
Estrogen receptor activity	ER	Estrogen signaling	Reproduction, cancer
Androgen receptor activity	AR	Maintenance of male sexual phenotype	Androgen insensitivity syndrome
Progesterone receptor activity	PR	Embryonic development, cell differentiation, homeostasis	Cancer, diabetes, hormone resistance syndromes
Peroxisome proliferator-activated receptor gamma	PPARg	Fatty acid storage and glucose metabolism	Obesity, diabetes, atherosclerosis, and cancer
Glucocorticoid receptor	GR	cortisol, glucocorticoids	Development, metabolism, immune response, neuroendocrine integration
Genotoxicity		DNA mutations	Cancer
Cytotoxicity		General toxicity	Tissue integrity

Bioassay Comparison

- Relevance
  - specificity (MOA, CEC)
  - link to tox pathways, apical endpoints
- Robustness
  - specificity, sensitivity, precision
  - historical usage
- Simplicity
  - protocol complexity
- Time & Cost
  - set-up, incubation, data interpretation, reporting
  - capital & recurring costs
- Vendor support
  - co-investment, leveraging
  - ready resources & expertise

Commercial Assays Table

Vendor	Assay Name	Cell Type	Assay Description
Invitrogen	GeneBLAZER	293T cells (kidney)	FRET-based reporter assay
BioDetection Systems	CALUX	U2-OS (bone)	Luciferase reporter assay
SwitchGear Genomics	LightSwitch	HT1080 (fibrosarcoma)	Multiplexed luciferase reporter assay
Attagene	Factorial TM	Transfect into cells of choice	Multiplexed reporter assay using capillary electrophoresis
DiscoverX	PathHunter	MD453 (breast) U2OS (bone)	Split beta-Gal reporter assay
Indigo BioSciences-Axxora	Nuclear Receptor Assays	Unspecified	Luciferase reporter assay

Commercial Assays Table

Vendor	Assay Name	Cell Type	Assay Description
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SwitchGear Genomics	LightSwitch	HT1080 (fibrosarcoma)	Multiplexed luciferase reporter assay
Attagene	Factorial TM	Transfect into cells of choice	Multiplexed reporter assay using capillary electrophoresis
DiscoverX	PathHunter	MD453 (breast) U2OS (bone)	Split beta-Gal reporter assay
Indigo BioSciences-Axxora	Nuclear Receptor Assays	Unspecified	Luciferase reporter assay

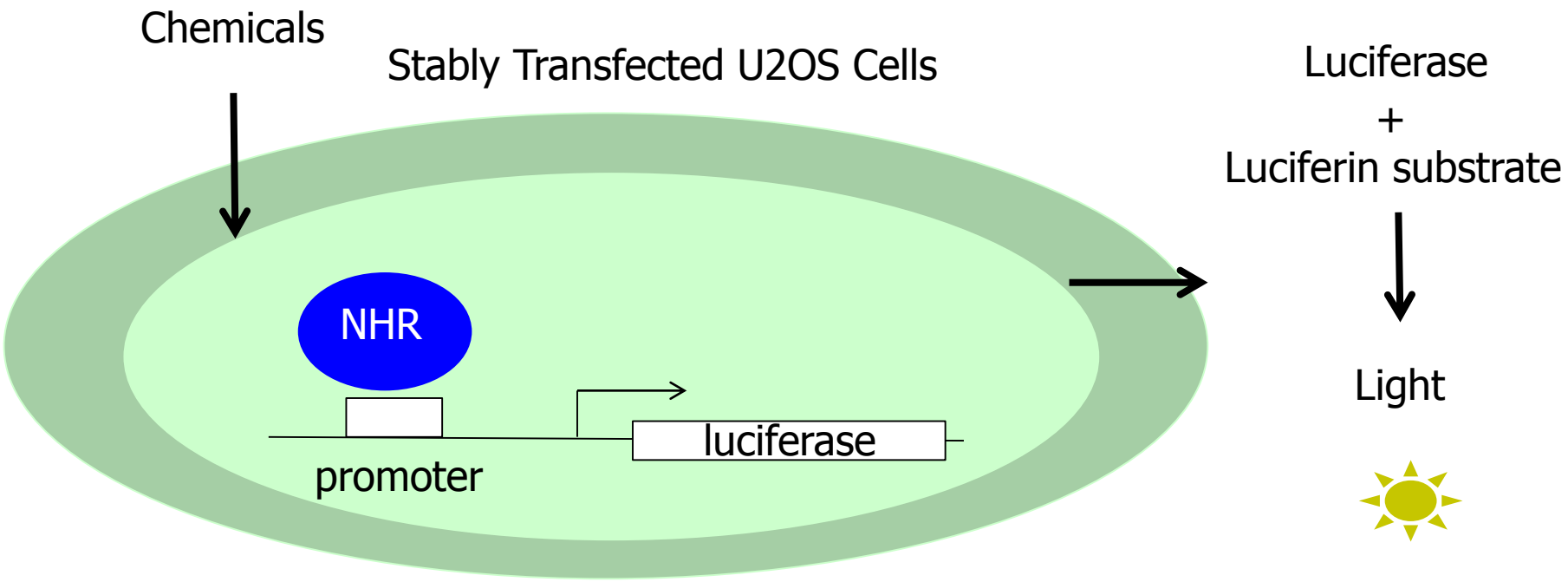
Vendor Assay Availability

Bioassay	Invitrogen	BDS-CALUX	SwitchGear
Estrogenicity- ER	Yes	Yes	Yes
Androgenicity- AR	Yes	Yes	Yes
Progesterone activity- PR	Yes	Yes	Yes
Genotoxicity- p53	Yes	Yes	Yes
Peroxisome proliferator activated receptor- PPARg	Yes	Yes	Yes
Glucocorticoid receptor activity- GR	Yes	Yes	Yes
Cytotoxicity	Yes-separate assay	No	Yes- integrated assay

BDS CALUX assays

- Stable U2OS (bone) cells
  - Express nuclear hormone receptor
  - Contain luciferase reporter with optimized DNA binding site for nuclear hormone receptor
- Cells are plated, treated with compounds, and then assayed for luciferase activity

BDS CALUX Assays



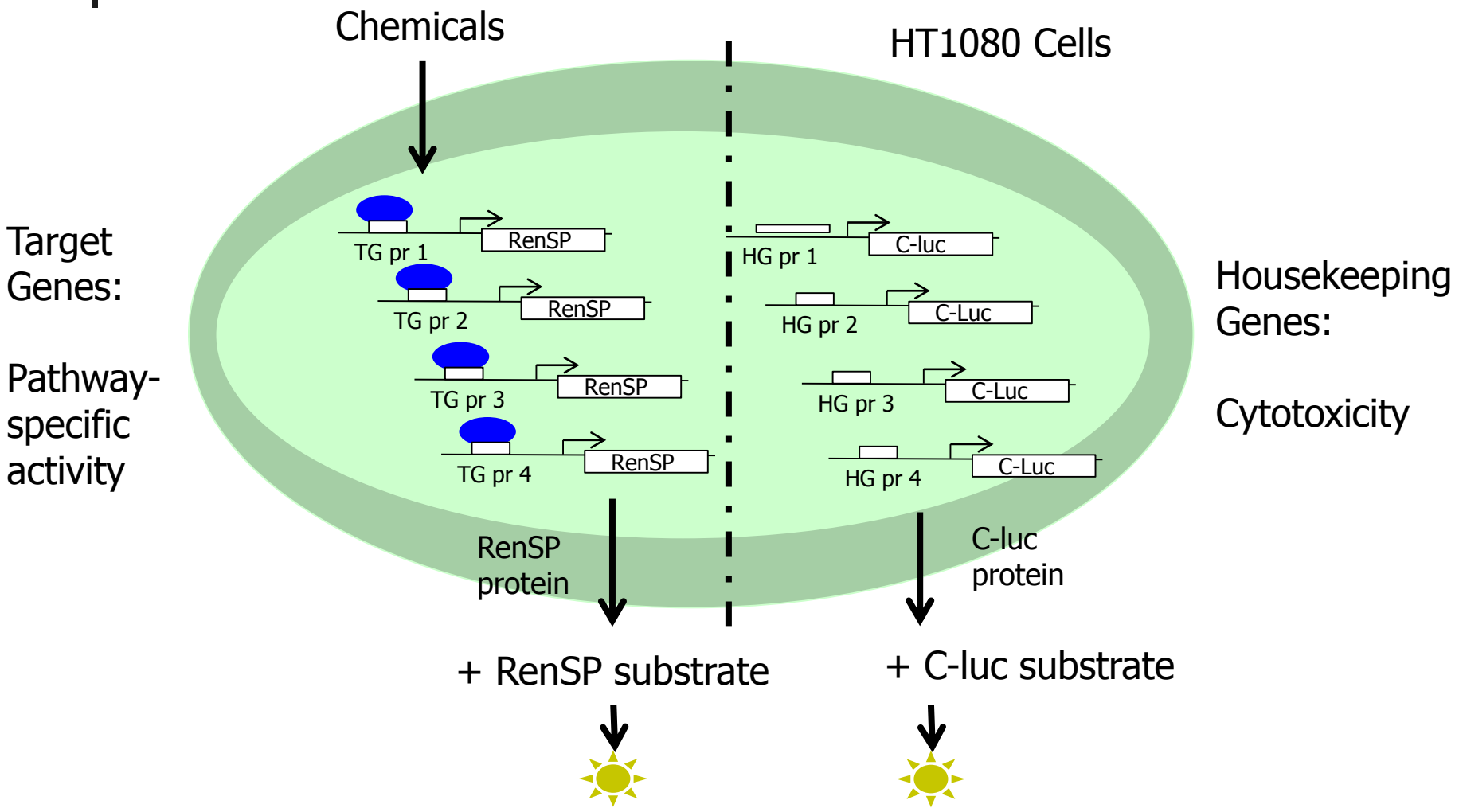
- Individual stable cell lines: ER, AR, PR, p53, PPARγ, GR
- No cytotoxicity assay

SwitchGear LightSwitch Assays

- HT1080 cells
  - Highly transfectable fibrosarcoma cells
  - Contain normal number of chromosomes
  - Can also use any other cell type of choice

- Cells are plated, transfected with pooled reporters, treated with compounds, and then assayed for dual luciferase activity

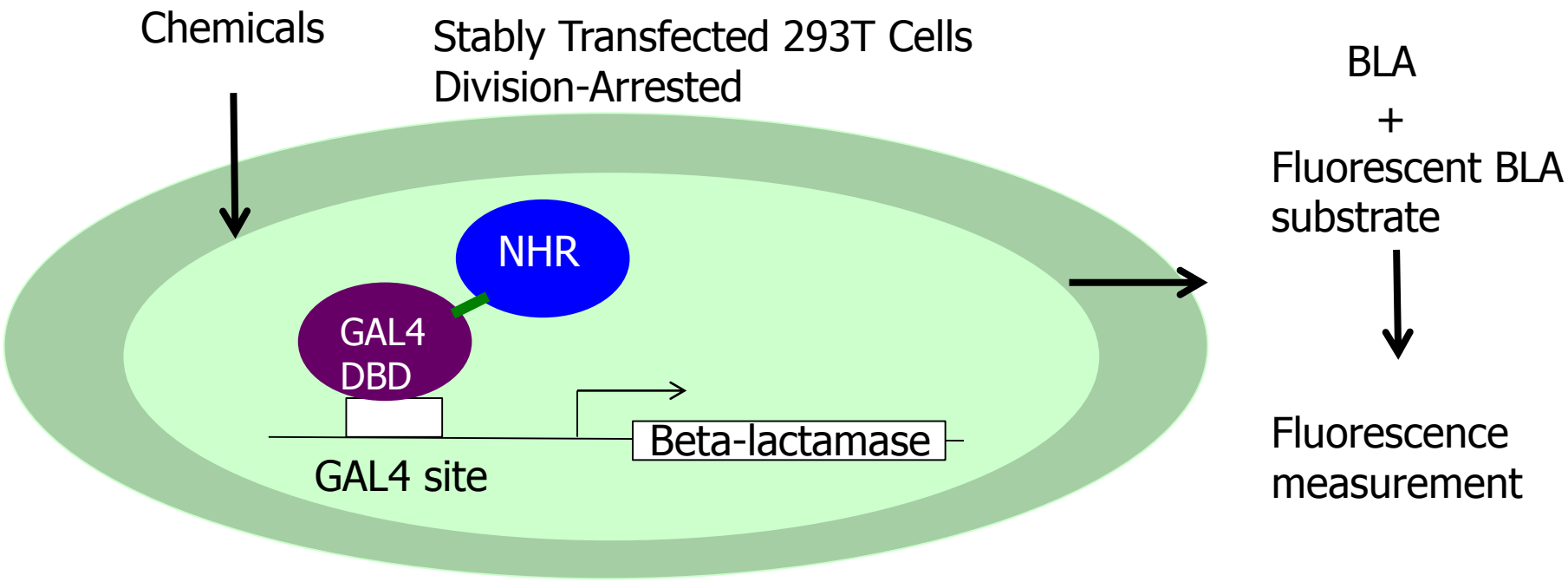
SwitchGear LightSwitch Assays



Invitrogen GeneBLAzer Assays

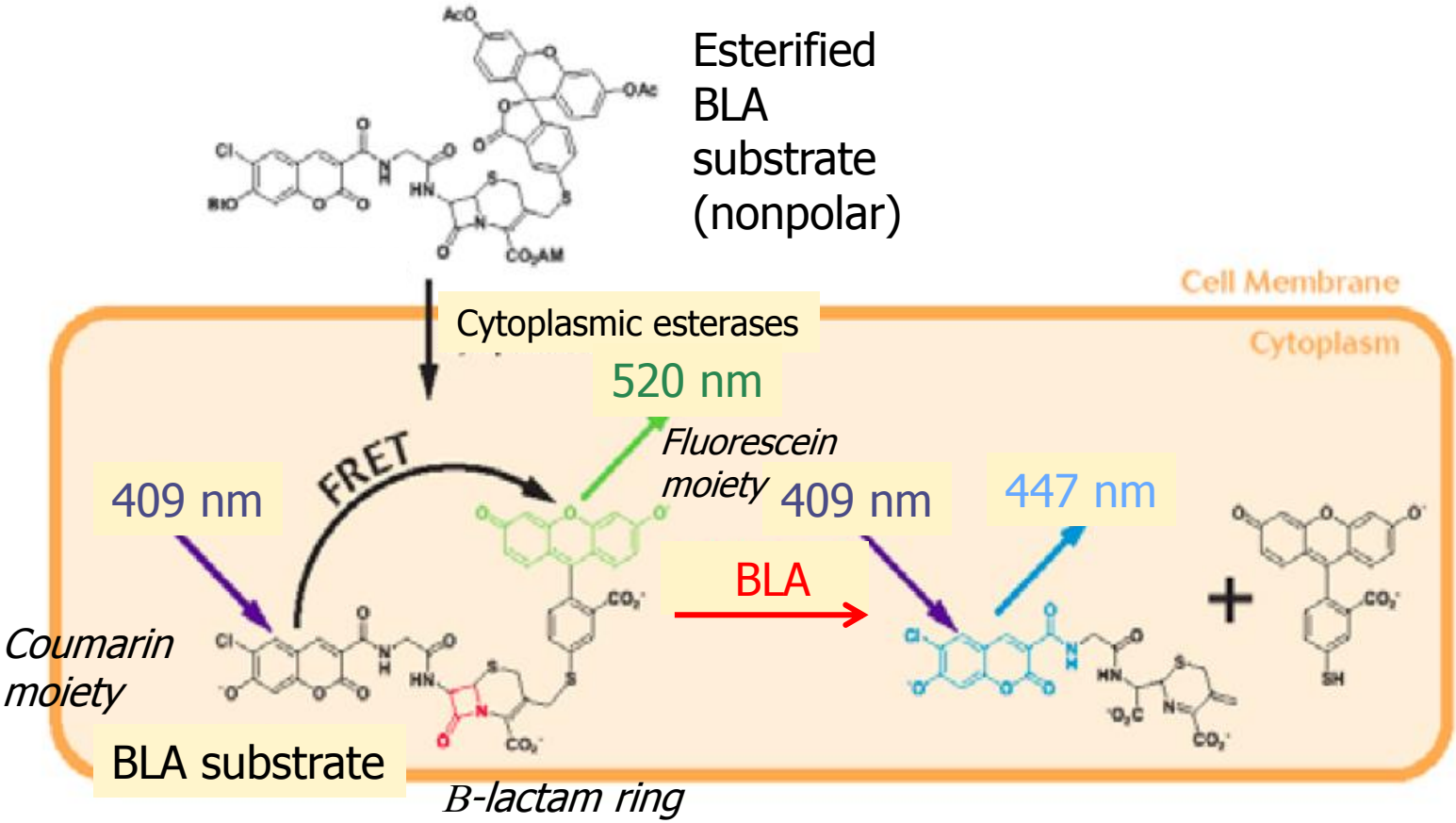
- Stable 293T cells
  - Transfected with GAL4-NHR and beta lactamase reporter containing GAL4 DNA binding site
- Cells are plated, treated with compounds, treated with fluorescent substrate, and then assayed for fluorescence activity

Invitrogen GeneBLAzer Assays



- Individual stable cell lines: ER, AR, PR, p53, PPARγ and GR
- Cytotoxicity measured separately (i.e. Presto Blue assay)

Fluorescence measurement



In the presence of beta lactamase expression (BLA), BLUE fluorescence is produced due to elimination of FRET

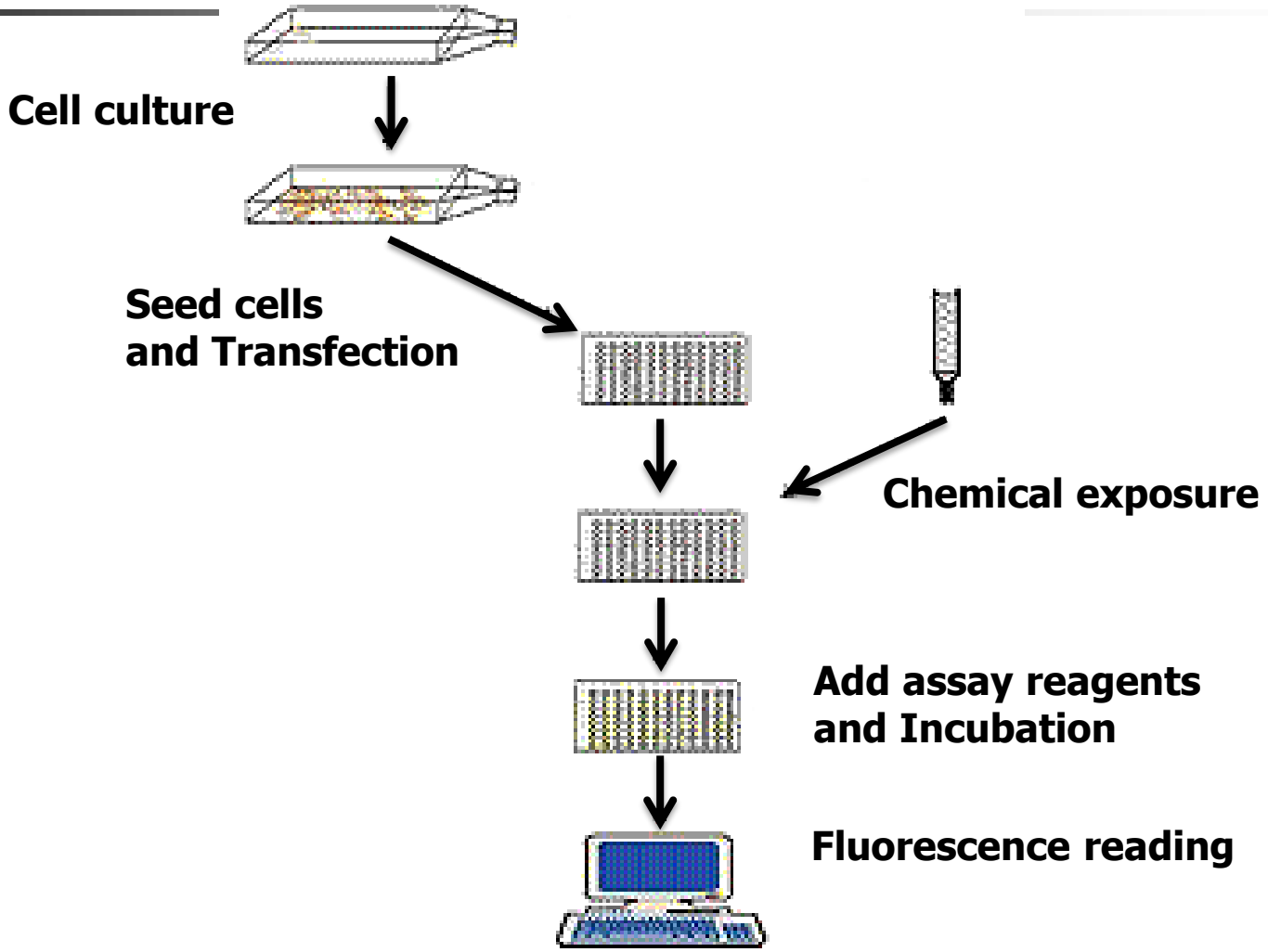
Characteristics of systems

- LightSwitch
  - Endogenous genes
  - Built-in cytotoxicity readout
  - Requires transfection
- CALUX
  - Artificial but sensitive
  - Widely used in Europe
  - Robust
  - Requires yearly license and MTA
- GeneBLAzer
  - Artificial but sensitive
  - Robust
  - Simplest and fastest
  - Best “kit” format

Optimization of GeneBLAzer Assays

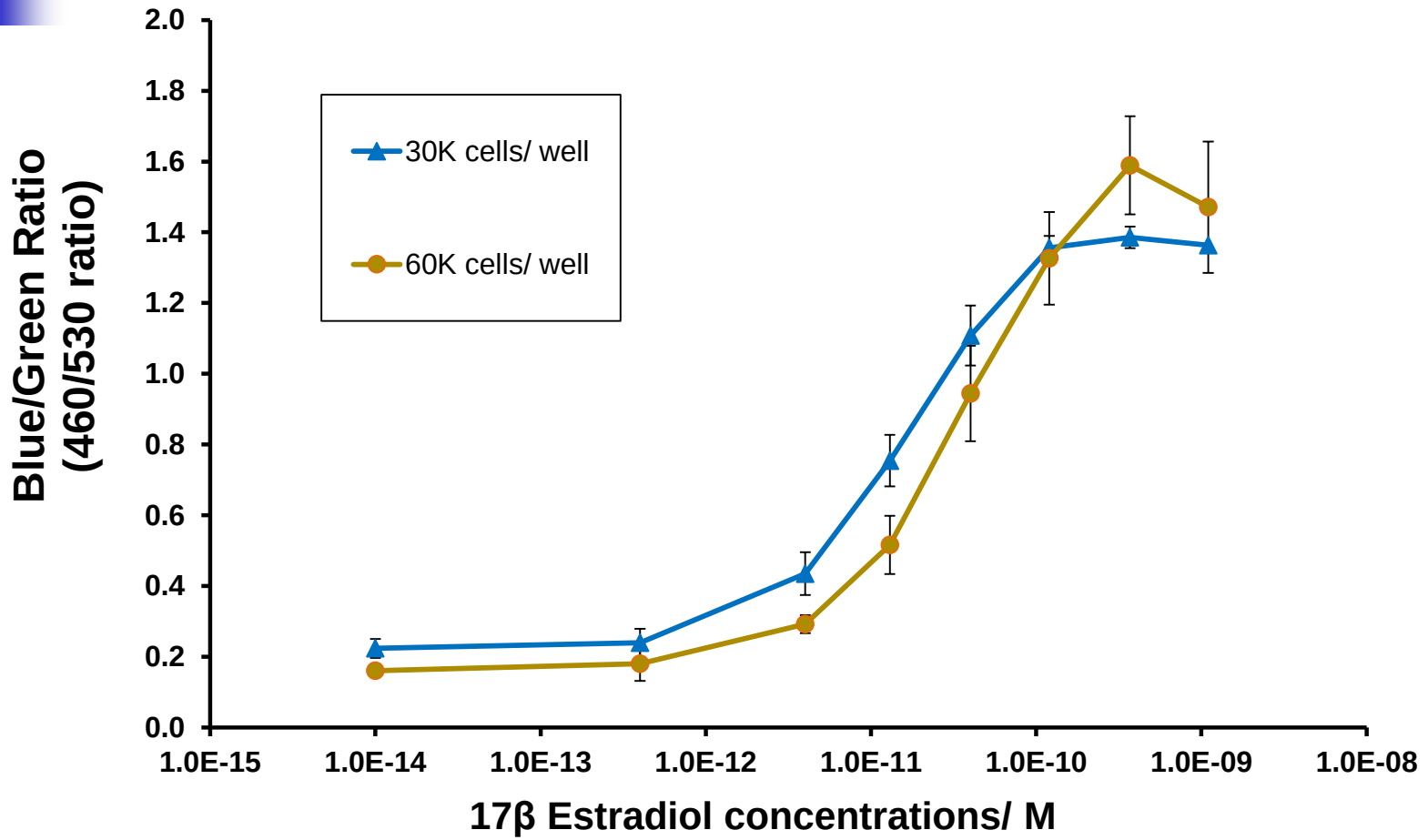
- Estrogen receptor -- ER
- Androgen receptor -- AR
- Progesterone receptor -- PR
- Glucocorticoid receptor -- GR
- Peroxisome proliferator activated receptor-- PPARα
- Peroxisome proliferator activated receptor --PPARγ
- Aryl hydrocarbon receptor -- AhR
- Cytotoxicity – Presto blue
- Genotoxicity – p53

In vitro assay protocol



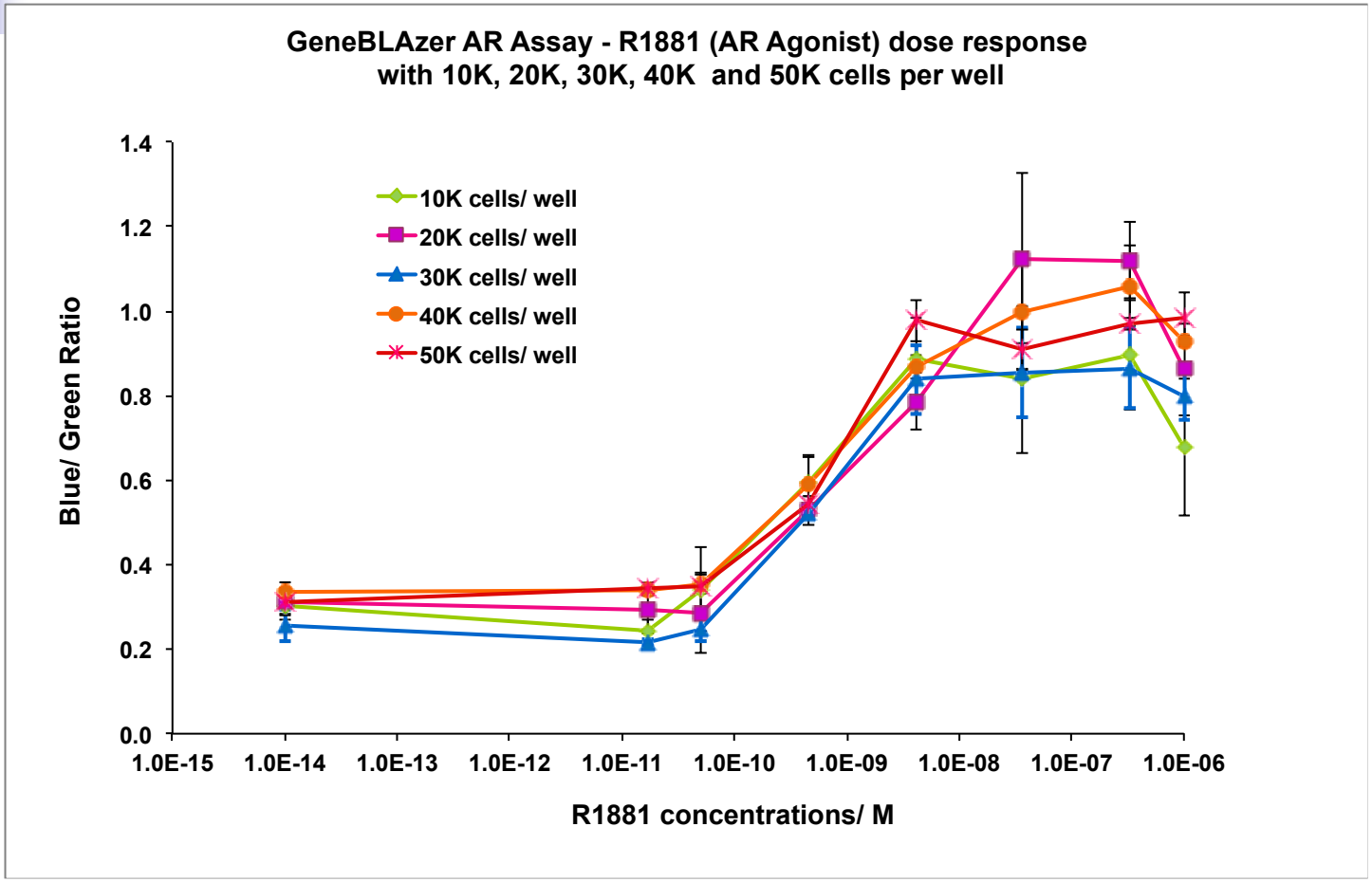
GeneBLAzer ERα Assay

E2 dose response with 30K and 60K cells per well

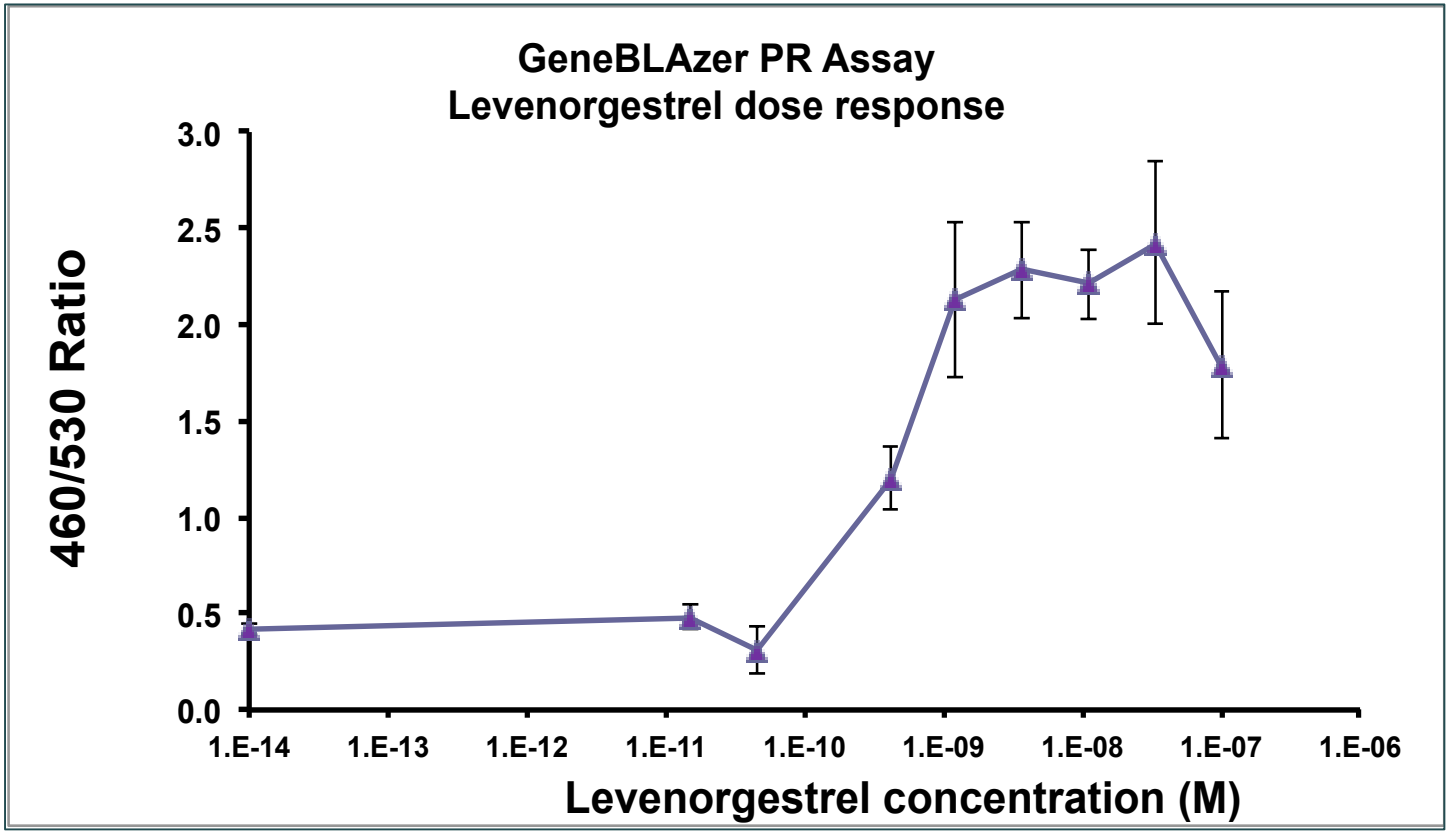


(Sumith Jayasinghe)

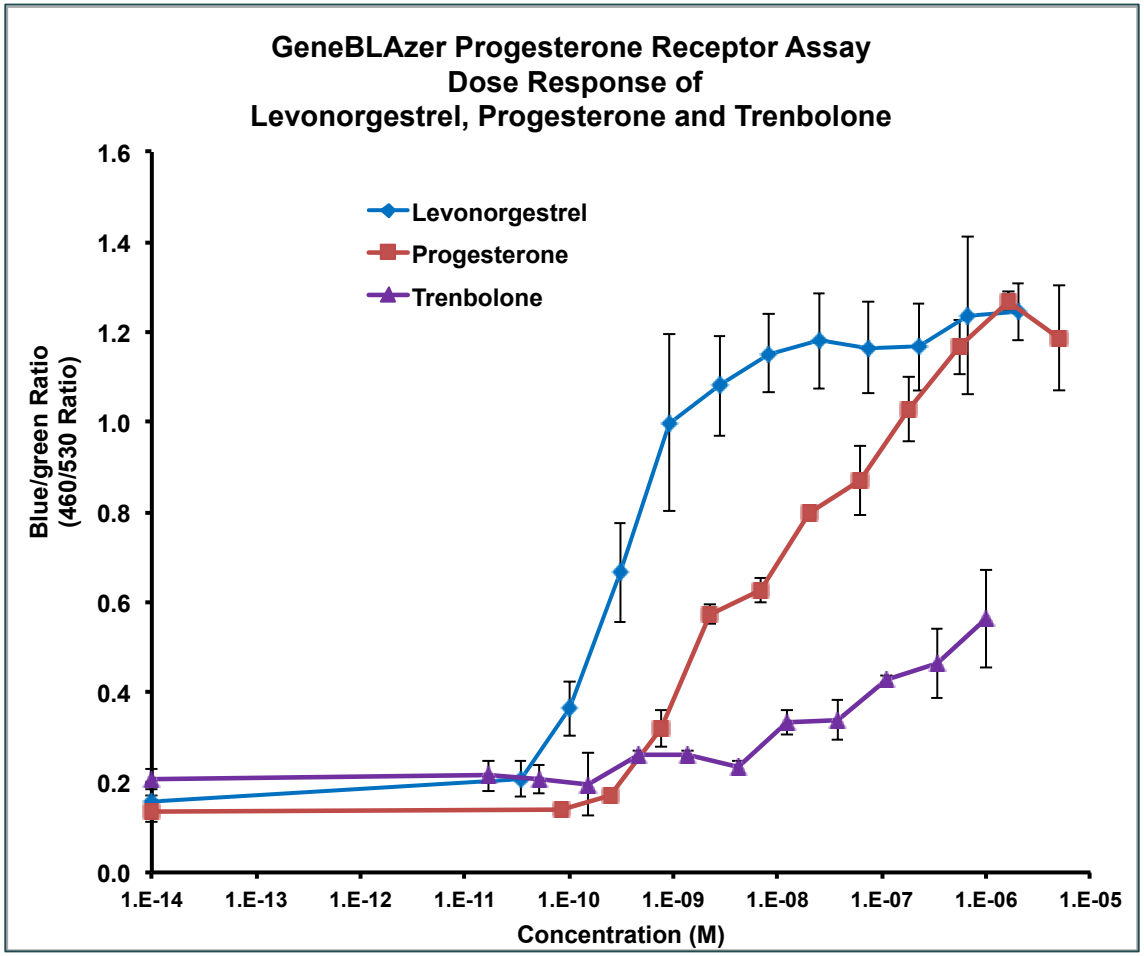
Invitrogen AR assay



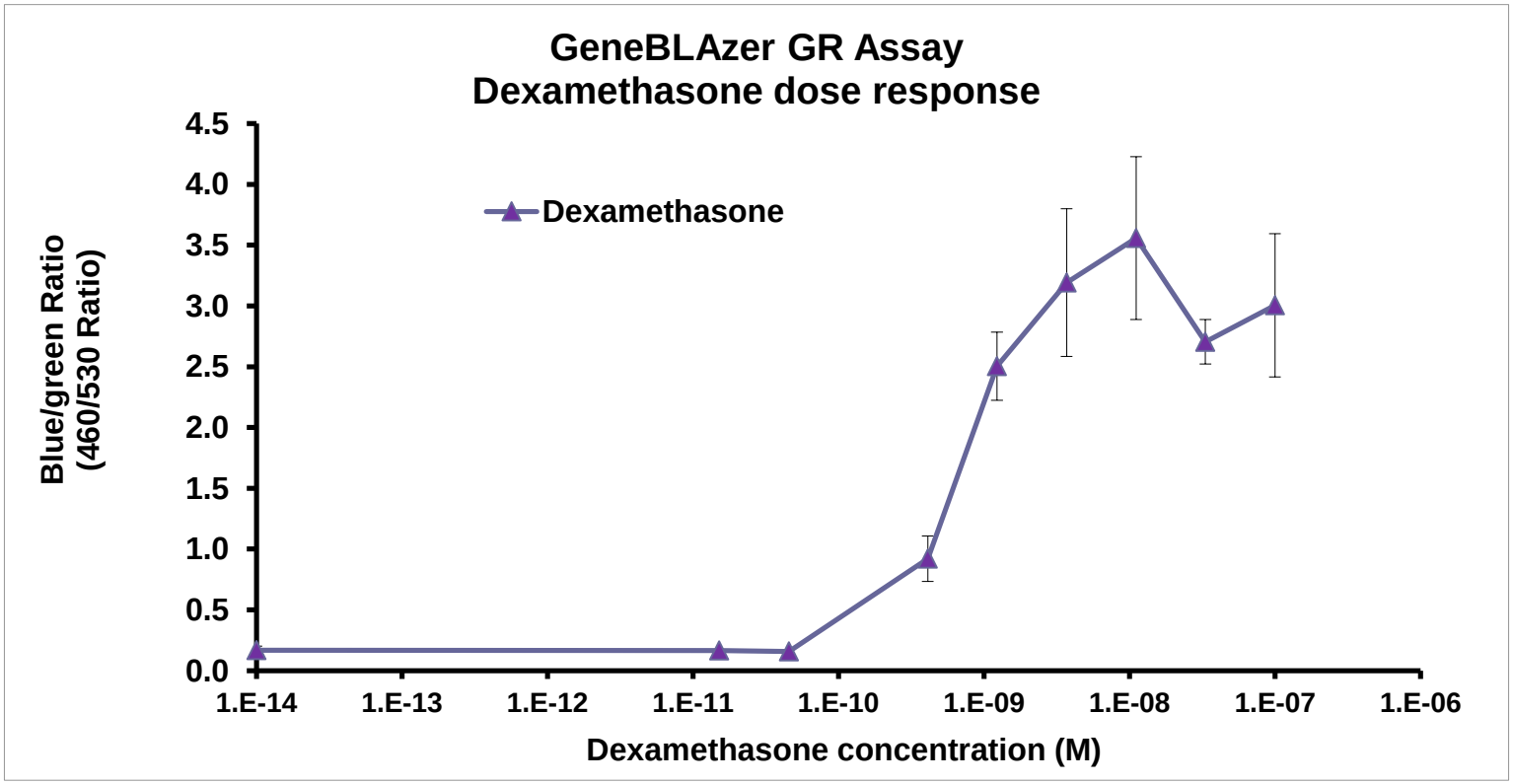
Invitrogen PR assay



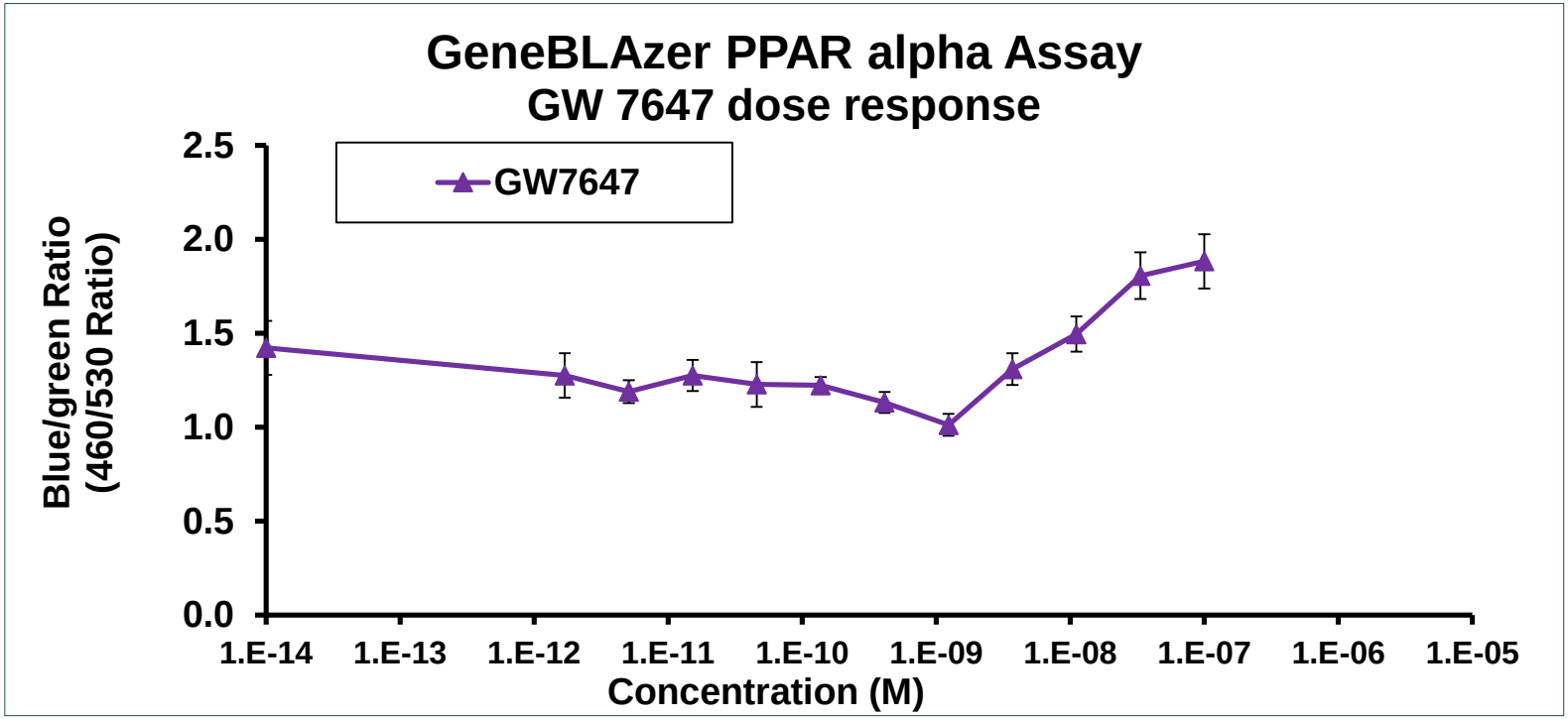
Progesterone receptor Levonorgestrel, progesterone, and trenbolone



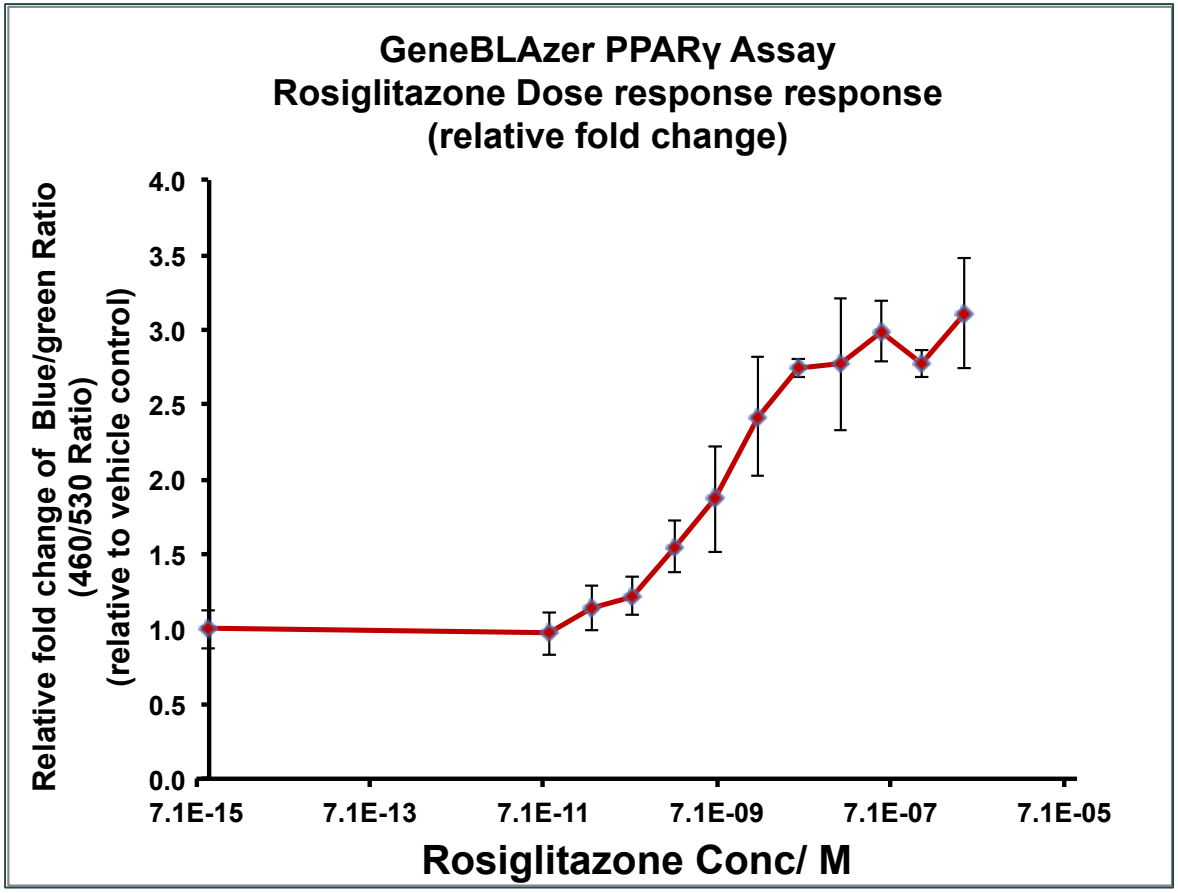
Invitrogen GR assay



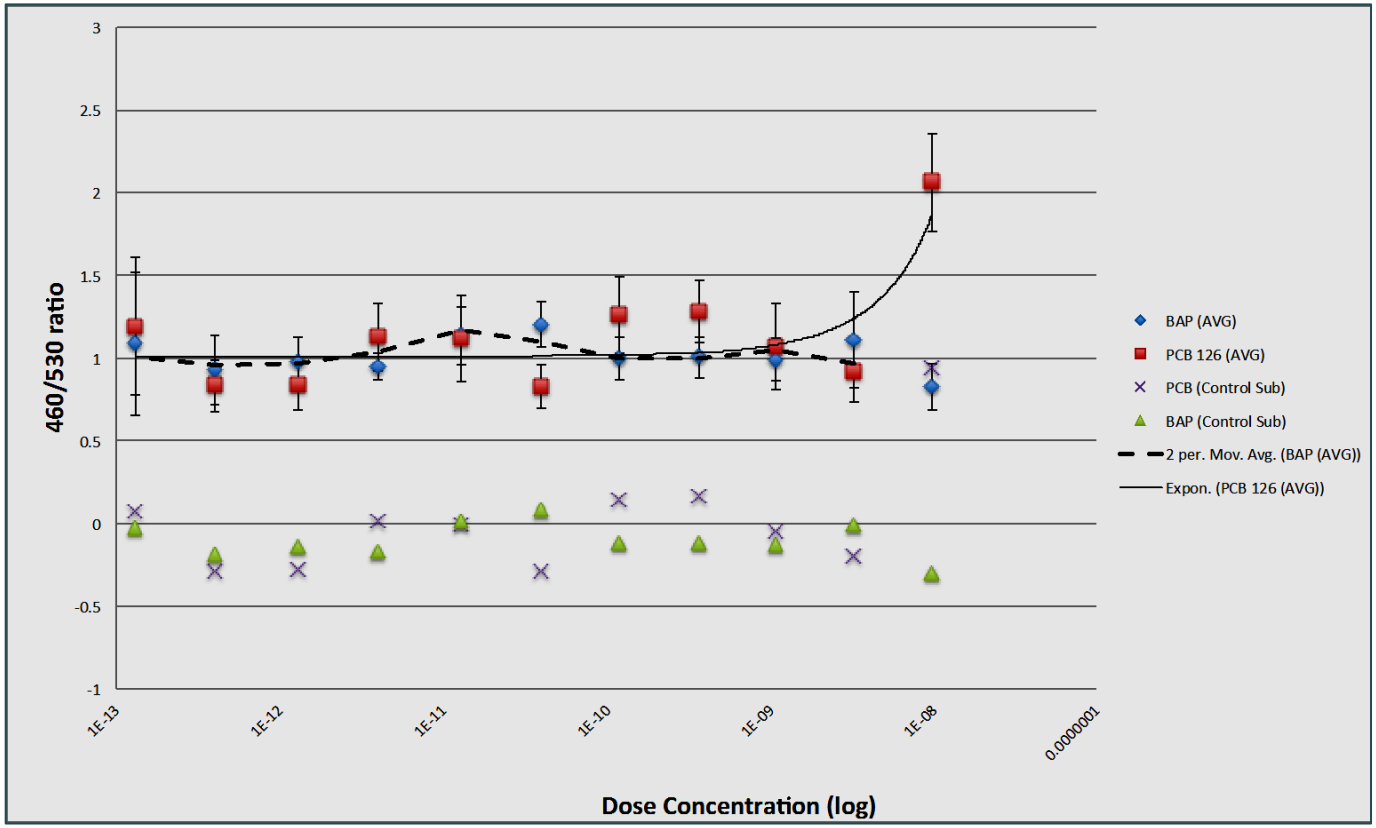
Invitrogen PPARα Assay



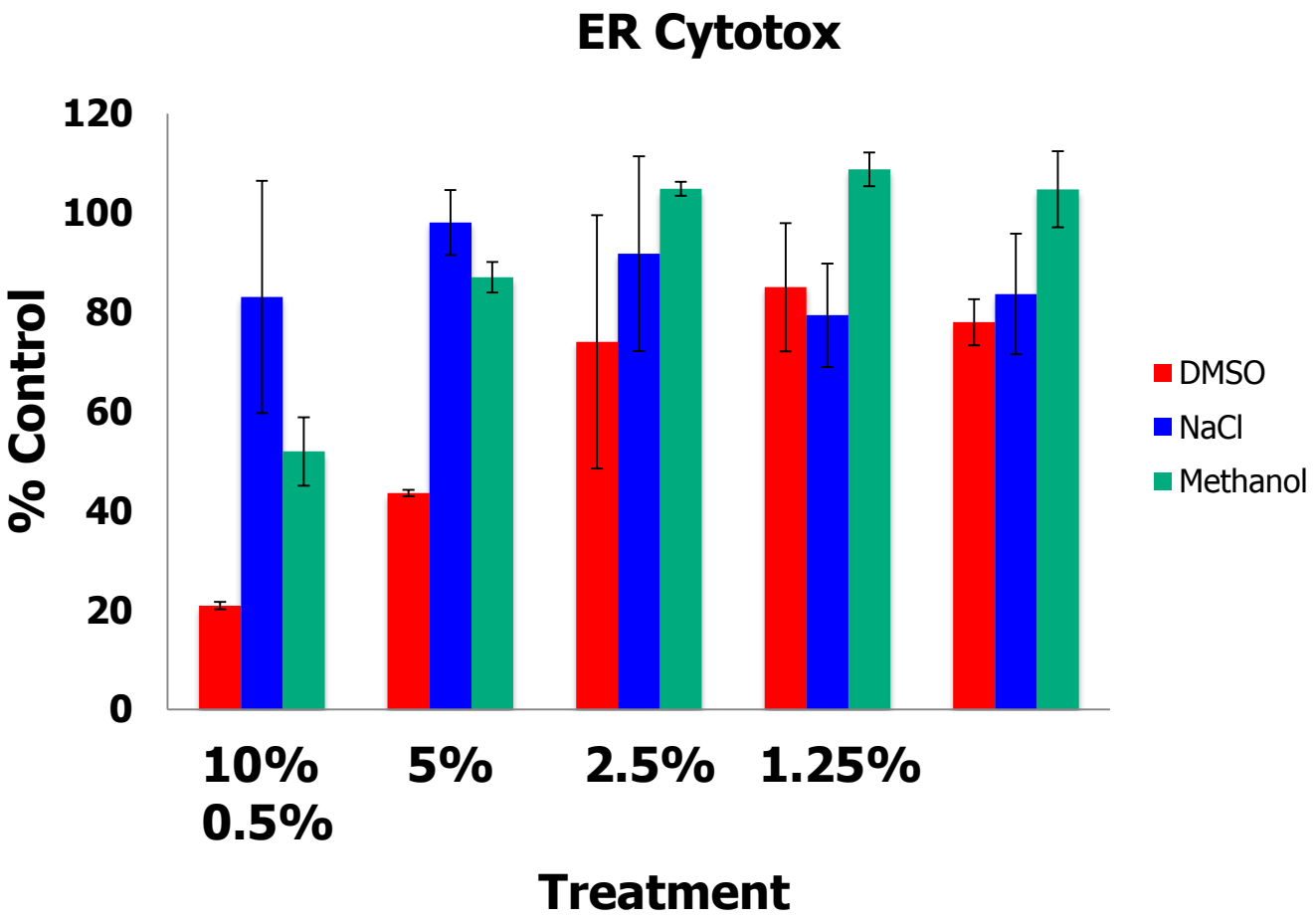
Invitrogen PPARγ Assay

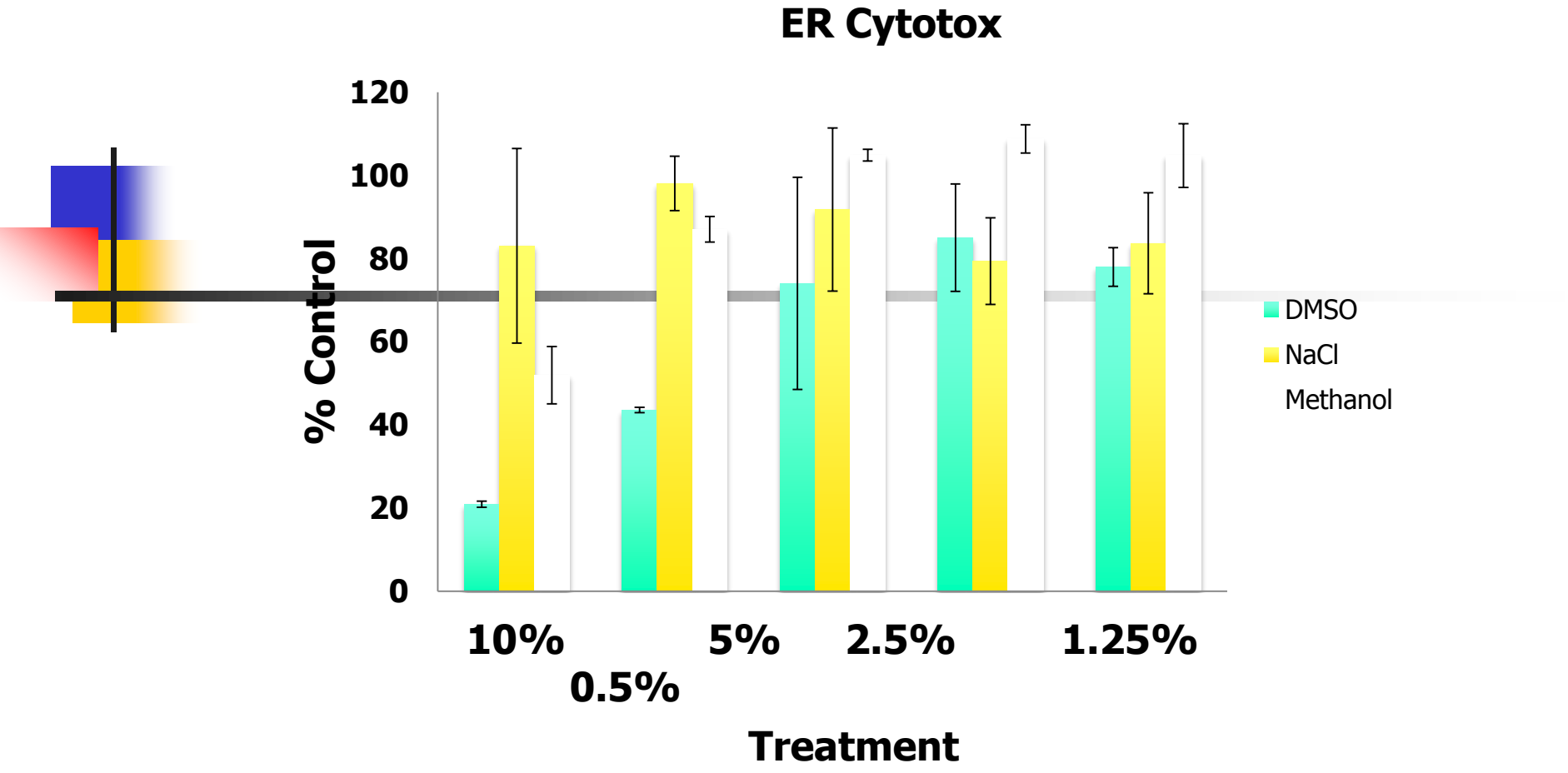


Invitrogen AhR assay

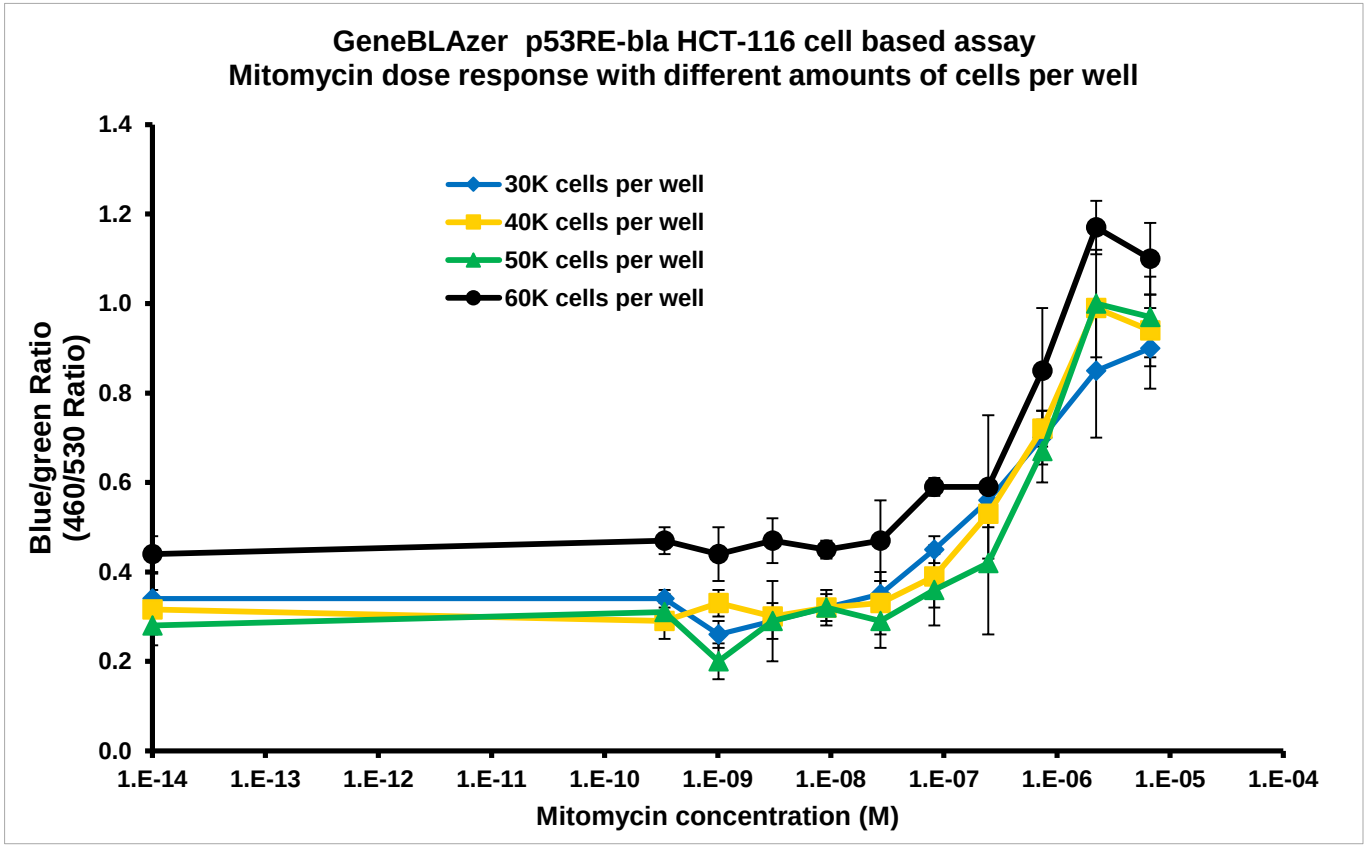


Invitrogen cytotoxicity

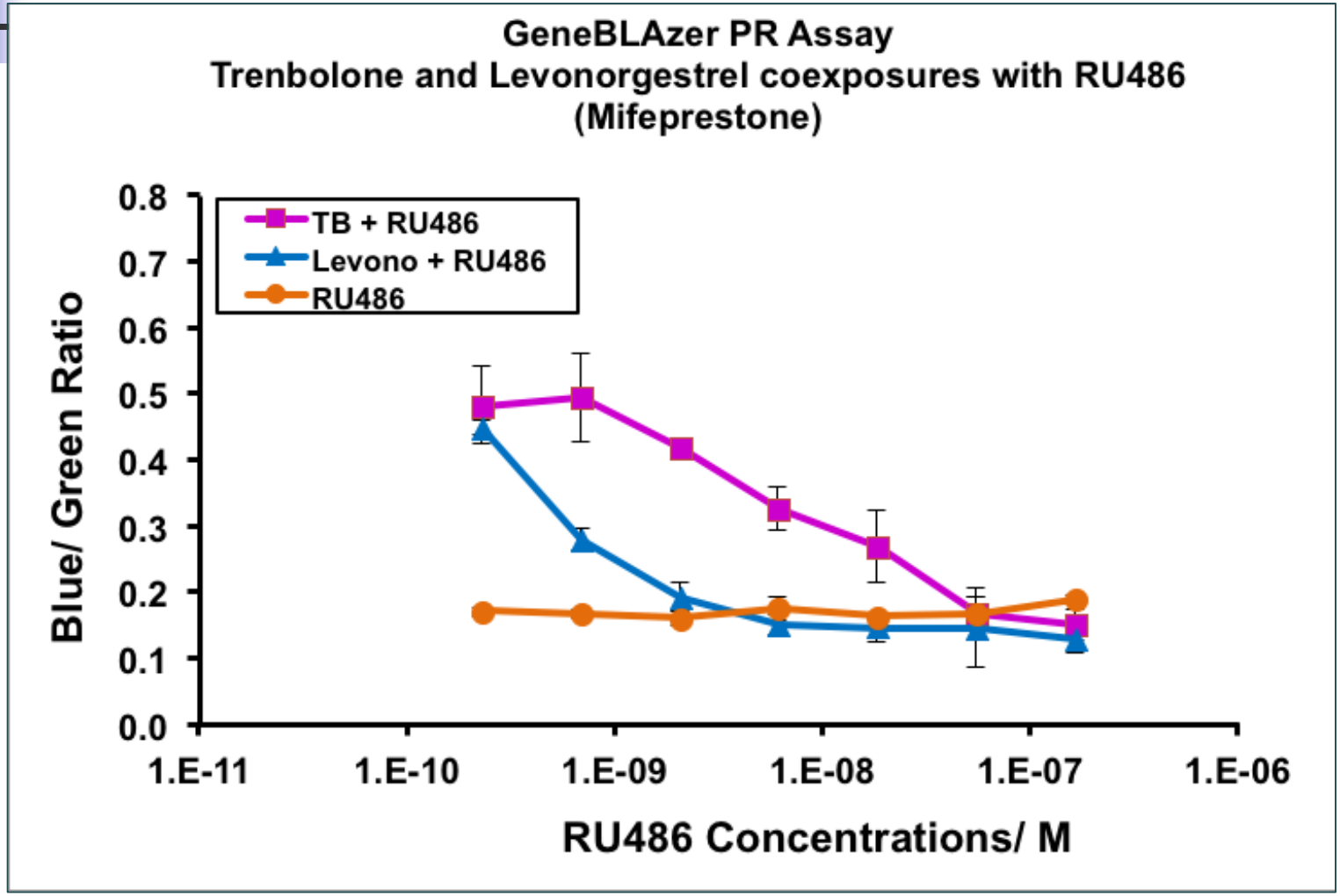



ER Cytotox
120
100
80
60
40
20
0
% Control
10%
5%
2.5%
1.25%
0.5%
Treatment
DMSO
NaCl
Methanol

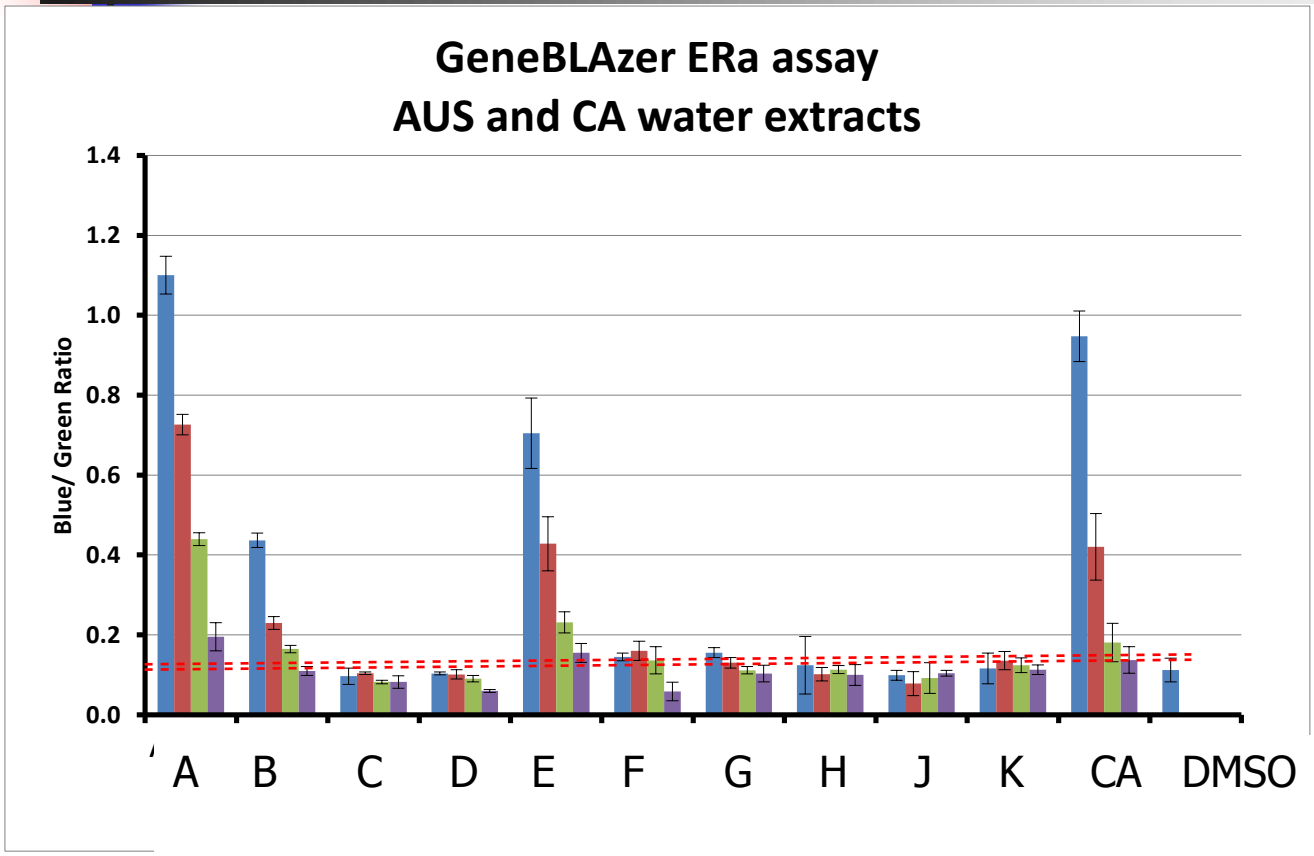
p53 assay using agonist mitomycin



Antagonism of PR Assay

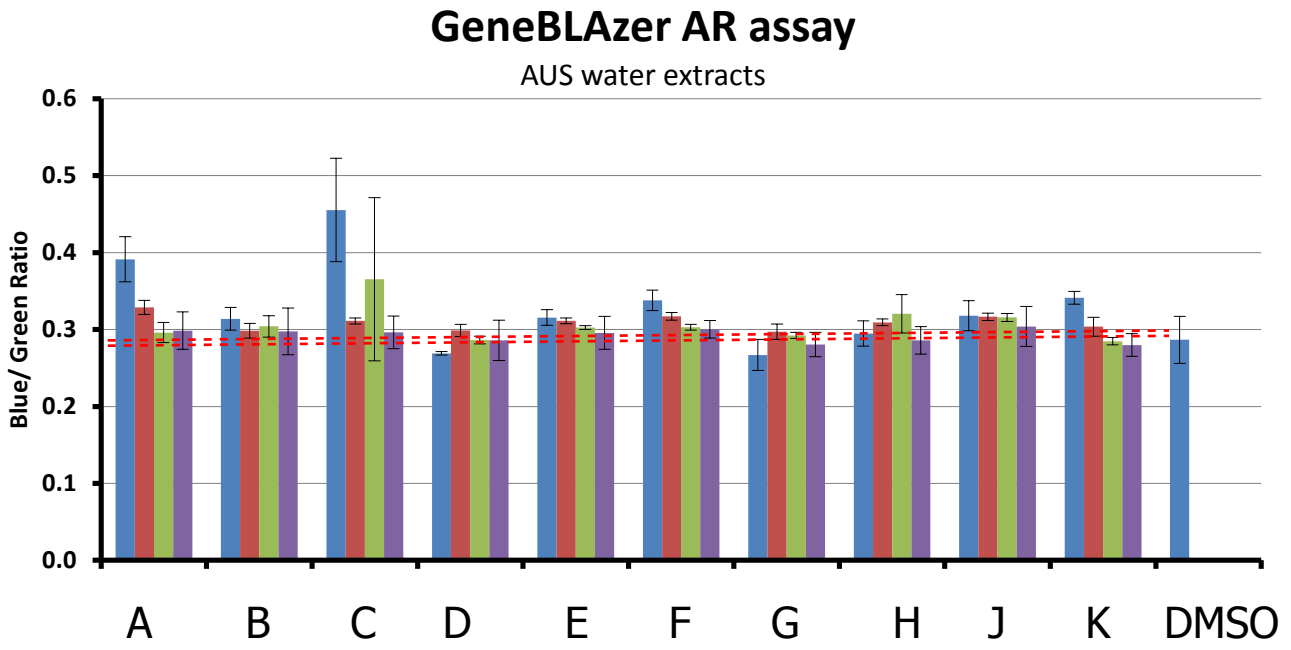


Round Robin Results -- ERα



Legend for samples
A= Effluent 2
B= Effluent 1
C= Ozonation
D= Storm water
E= Membrane
F= RO
G= River Water
H = AO
J= Blank
K= Drinking water
CA= SCCWRP proj

AR assay



Legend for samples

A= Effluent 2

B= Effluent 1

C= Ozonation

D= Storm water

E= Membrane

F= RO

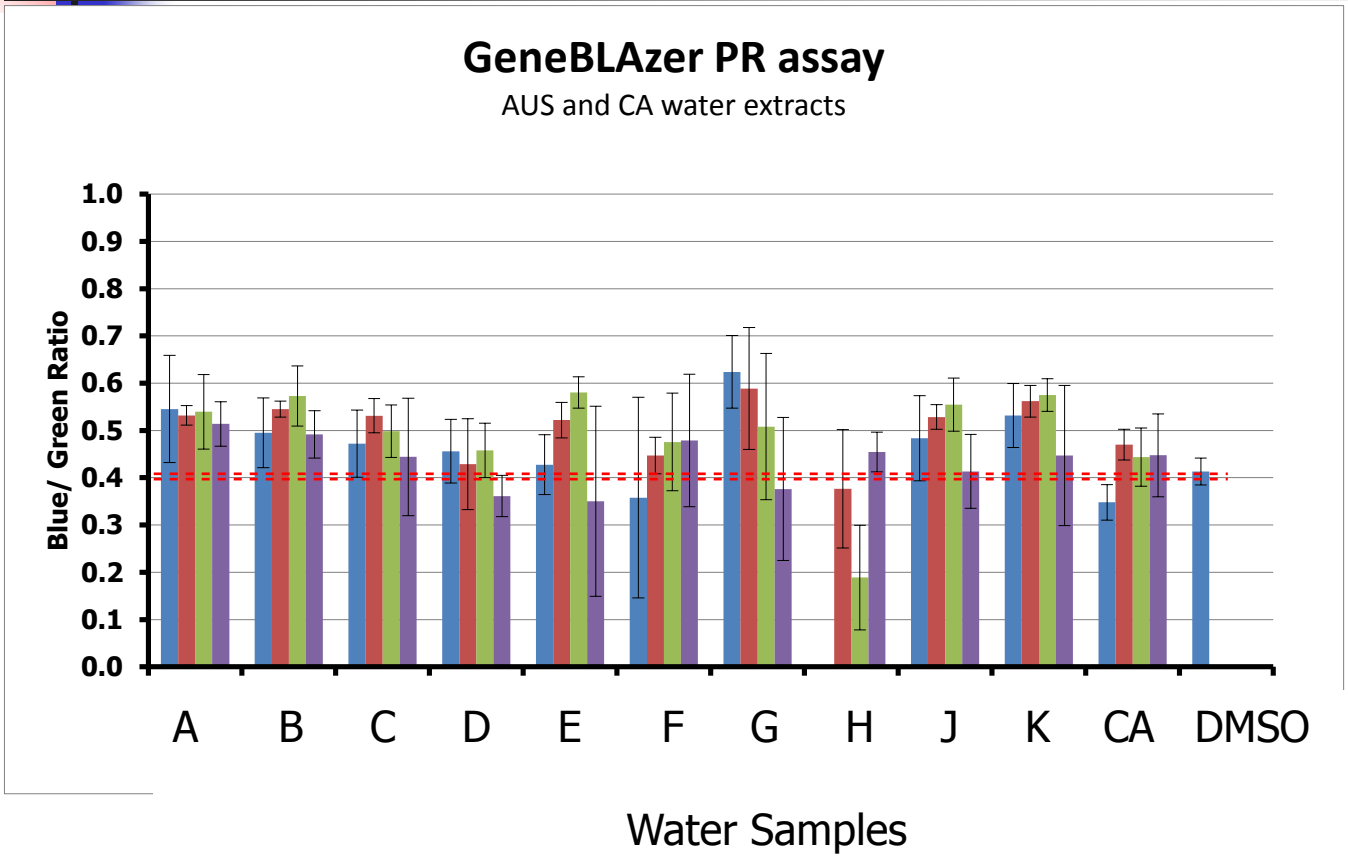
G= River Water

H = AO

J= Blank

K= Drinking water

PR Assay



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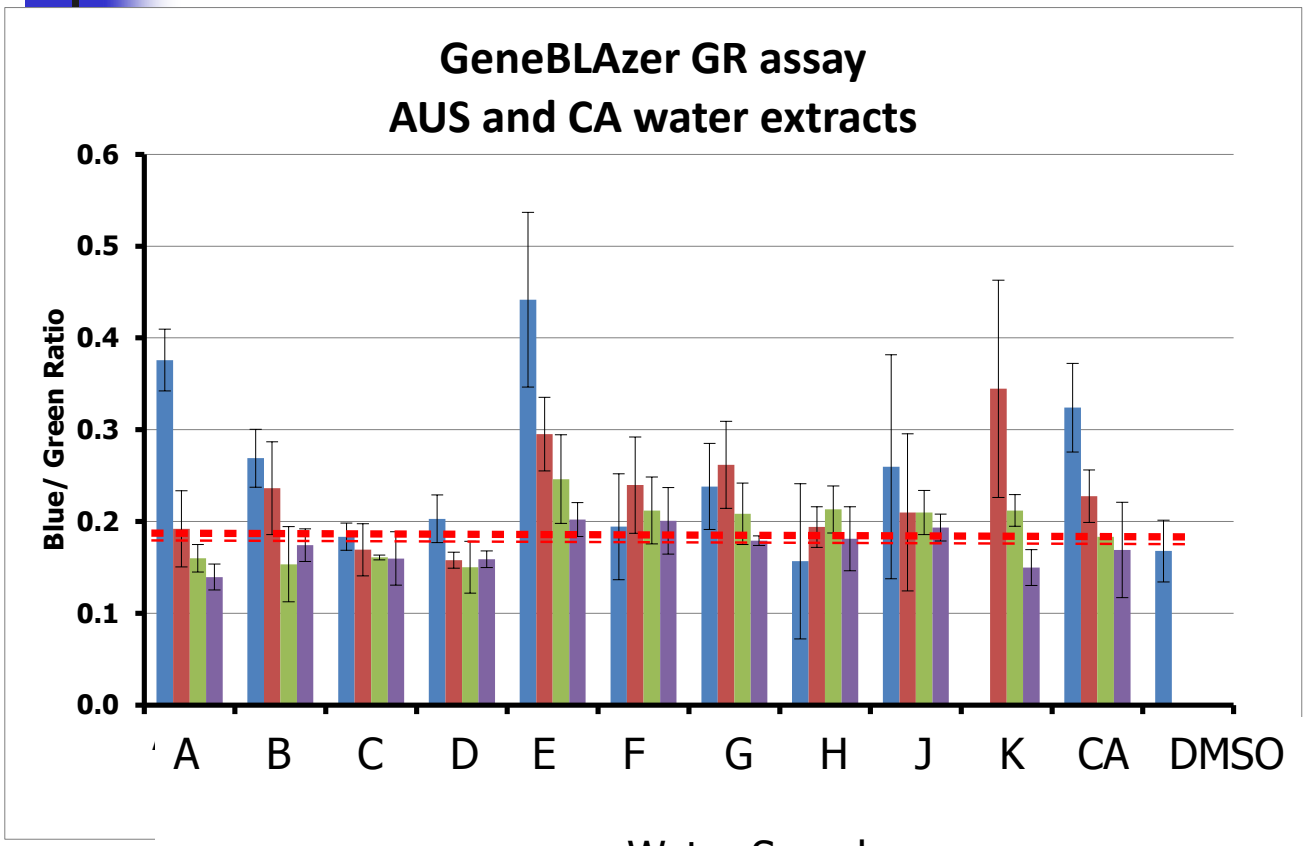
D= Storm water
E= Membrane
F= RO
G= River Water
H = AO
J= Blank
K= Drinking water
CA= SCCWRP proj

GR assay



Legend for samples

A= Effluent 2

B= Effluent 1

C= Ozonation

D= Storm water

E= Membrane

F= RO

G= River Water

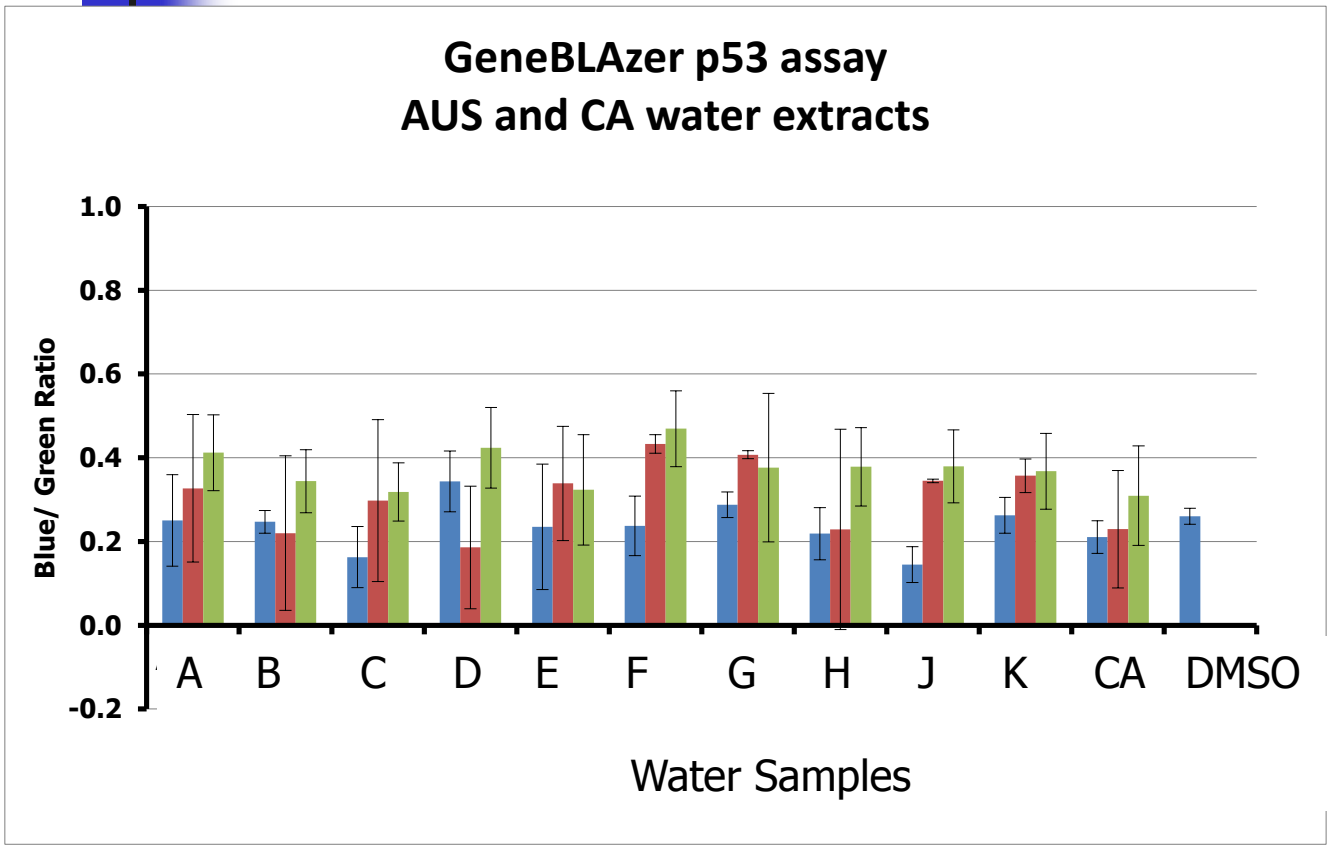
H = AO

J= Blank

K= Drinking water

CA= SCCWRP proj

P53 Genotoxicity Assay



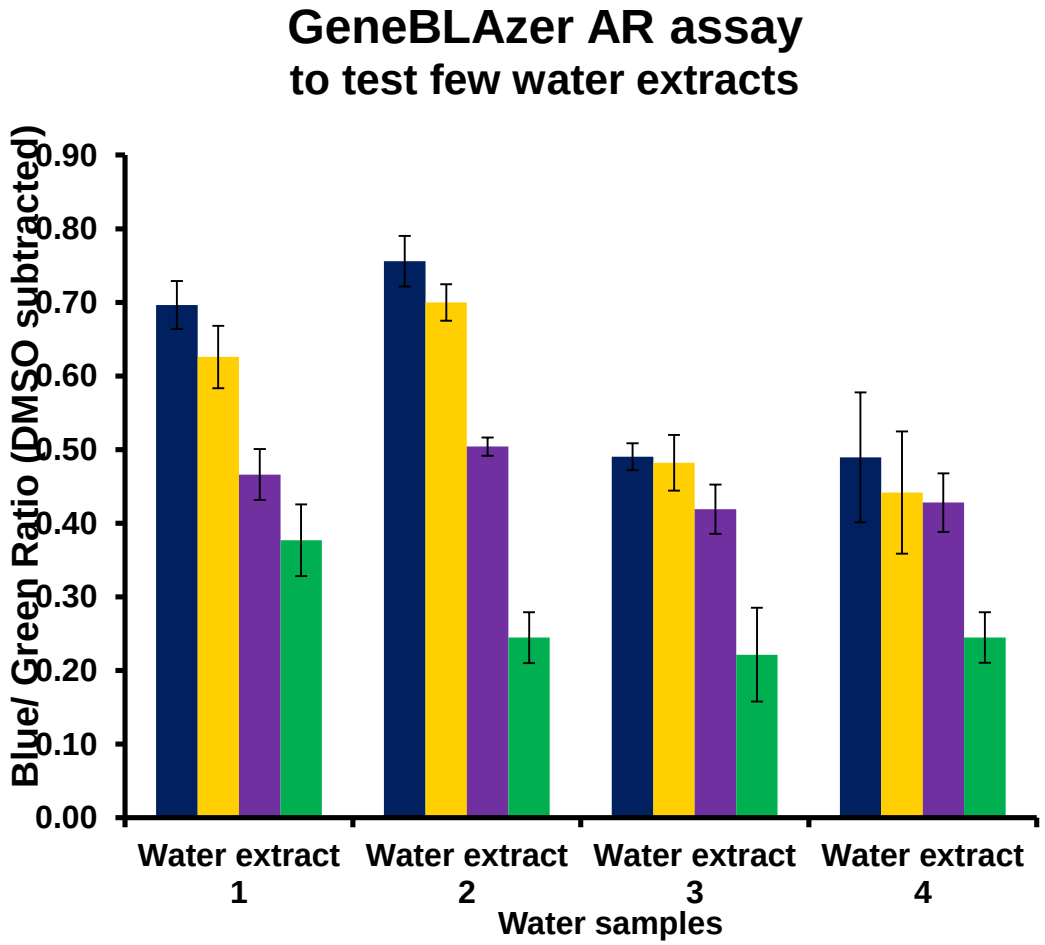
Legend for samples
A= Effluent 2
B= Effluent 1
C= Ozonation
D= Storm water
E= Membrane
F= RO
G= River Water
H = AO
J= Blank
K= Drinking water
CA= SCCWRP proj

Fenholloway river- Florida Androgens and progesterone

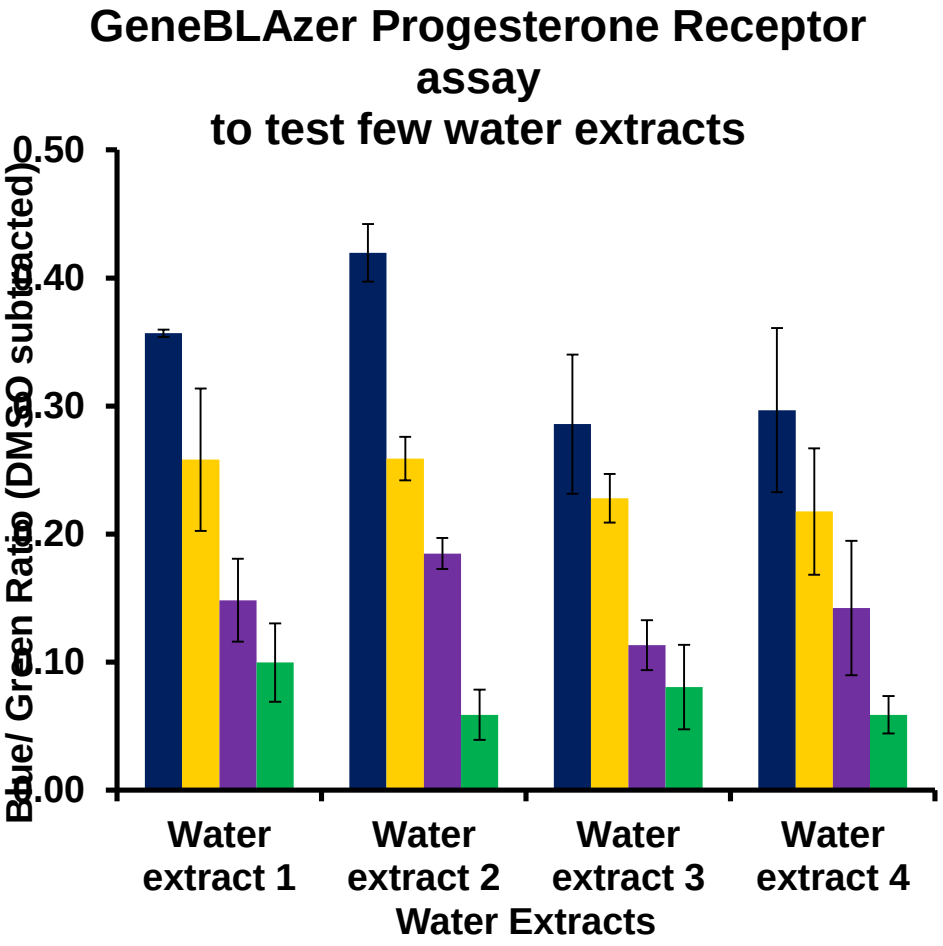
	Androstenedione	Progesterone
Water column	0.04 ±0.02 ug/L	2.06 ±0.38 ug/L
Sediments	0.7 ± 0.02 ug/L	48.8 ±7 ug/L

Jenkins, 2001

Fenholloway River and Econfina River in Florida



Fenholloway River and Econfina River in Florida



Conclusions

- Bioanalytical assays work well with standard chemicals and also work with water extracts
- Can be used to help inform the chemist about the analytes that should be investigated
- Multiple commercial assays are available
- Assays are relatively easy to perform – training required – mostly careful pipetting
- Still need to find a functional AhR assay

Acknowledgements

- UF team: Sumith Jayasinghe
- USF team: Jamie Mendez, Chris Menzie
- UCR team: Dan Schlenk, Jordan Crago
- UA team: Shane Snyder, Ai Jia
- SCCWRP team: Keith Maruya, Alvina Mehinto

Analytical Methods & Results

Ai Jia, Shimin Wu, Tarun Anumol, Bingfeng Dong,
Darcy VanDervort, & Shane Snyder

The University of Arizona

23rd January 2014

http://snyderlab.arizona.edu/

Challenges

- Extraction Method has limitations
  - Inappropriate for inorganics and highly-polar organics
  - Loss of highly-volatiles
  - Assumed recovery/stability for unknowns
  - Recovery not corrected for bioassays
- Analytical data from extracts less robust
  - No surrogates for recovery & suppression correction
  - Modern instrumental methods use <2 mL sample vol.
    - ➢ If mass balance good, instruments are faster/easier

Sample Collection-SCCWRP

Sampling Date:	**2012.6.18**	**Roger Road Effluent (1st round)**
	2012.8.28	**Green Valley AOP Pilot (2nd round)**
	2013.7.01	**West Basin recycle water (2nd round)**

+

Washed with MeOH and Milli-Q water

Ice inside

Sample Collection-1st round

Roger Road Wastewater Reclamation Facility (1st Round)

Treatment process consists of:

1) Headworks
2) Clarifiers
3) Biotowers
4) Chlorination

RR effluent is used for the irrigation of golf courses and also infiltrated.

Sample Collection-SCCWRP

Green Valley AOP Pilot Plant

1. GV-pilot influent (secondary eff)
2. GV-pilot UV (500mJ/cm2)
3. GV-pilot UV/H2O2 (500mJ/cm2, 10mg/L)
4. GV-pilot ozone (3mg/L)
5. GV-pilot ozone/UV (3mg/L, 500mJ/cm2)
6. GV-Chlorine (10mg/L HOCl, 2h contact)

Sample Collection-SCCWRP

West Basin Little Water Recycling Facility

1. Field Blank
2. WB-Influent
3. WB-Ozone
4. WB-MF
5. WB-RO
6. WB-UV AOP

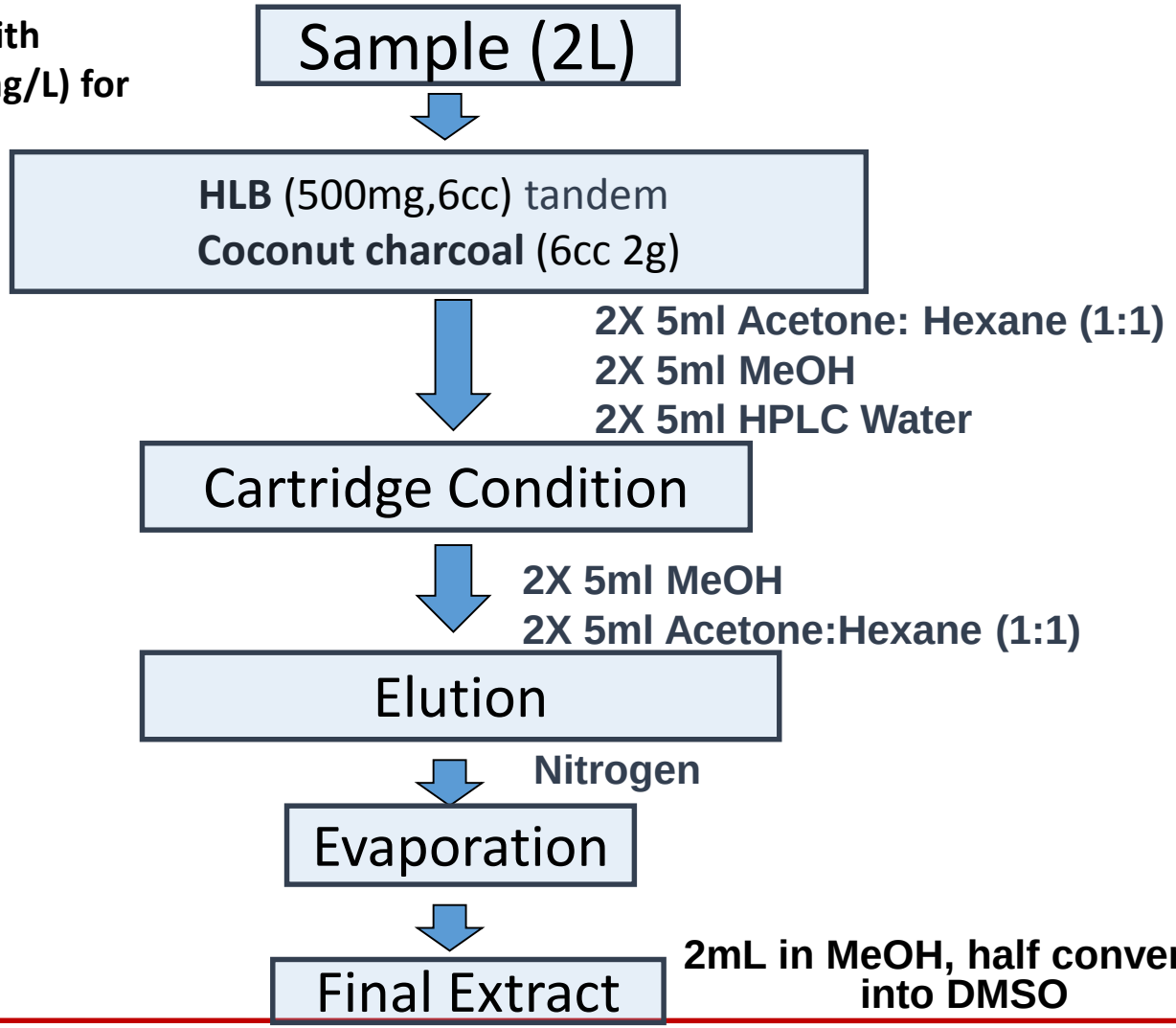
Sample Preparation

Samples as well as field blanks were moved into the lab and filtered immediately using the glass fiber filters (1.0um, Whatman)

Before SPE, all samples were stored at 4^{o}C.
Extraction was conducted within one week.

Sample Preparation

Dechlorinated with thiosulfate (50 mg/L) for specific samples.



Sample Preparation

1. Cartridge Conditioning

2. Loading Samples

3. Cartridge Elution

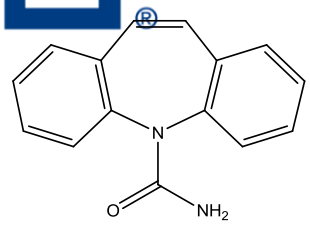
4. Evaporation

5. Transfer

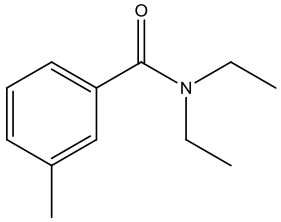
Target CECs

Acesulfame	Fluoxetine	PFBS	Sucralose
Atenolol	Gemfibrozil	PFDA	Sulfamethoxazole
Atrazine	Ibuprofen	PFDoA	TCEP
Benzophenone	Iohexol	PFHxA	TCPP
Benzotriazole	Iopamidol	PFHxDA	Testosterone
Caffeine	Iopromide	PFHxS	Triclocarban
Carbamazepine	Meprobamate	PFOA	Triclosan
DEET	Naproxene	PFOS	Trimethoprim
Diclofenac	Norethindrone	Primidone	
Diphenhydramine	Norgestrel	Propylparaben	
Ditiazem	PFBA	Simazine	

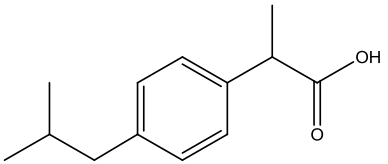
Target CECs



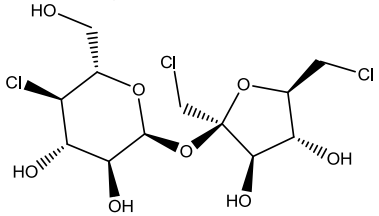
Carbamazepine (Anticonvulsant)



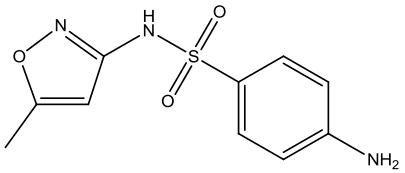
DEET (Insect Repellent)



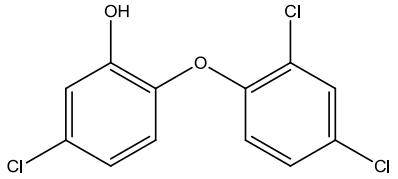
Ibuprofen (Anti-inflammatory Drug)



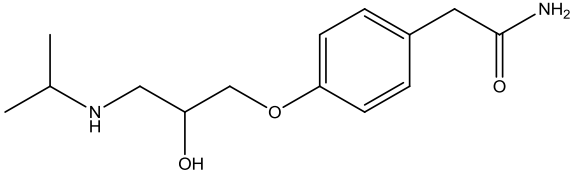
Sucralose (Artificial Sweetener)



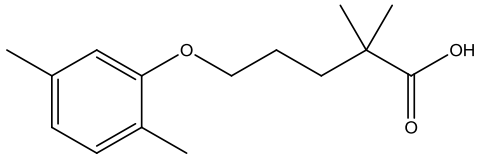
Sulfamethoxazole (Antibiotic)



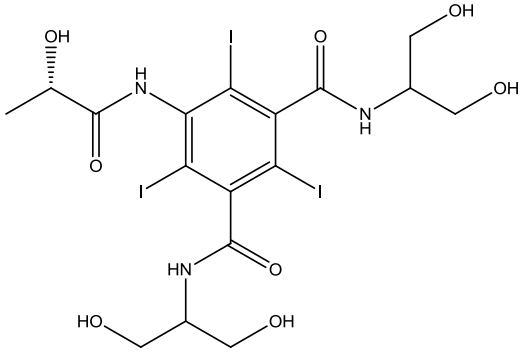
Triclosan (Antibacterial/Antifungal Agent)



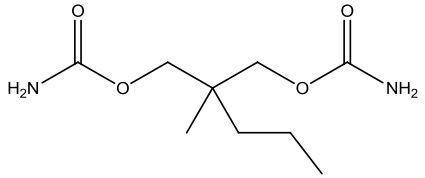
Atenolol (β-blocker)



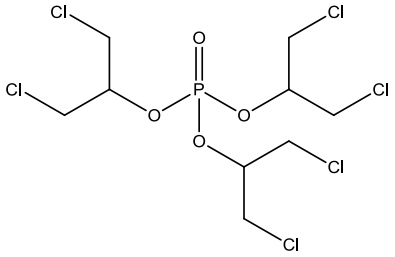
Gemfibrozil (Lipid-lowering Drug)



Iopamidol (Contrast Agent)



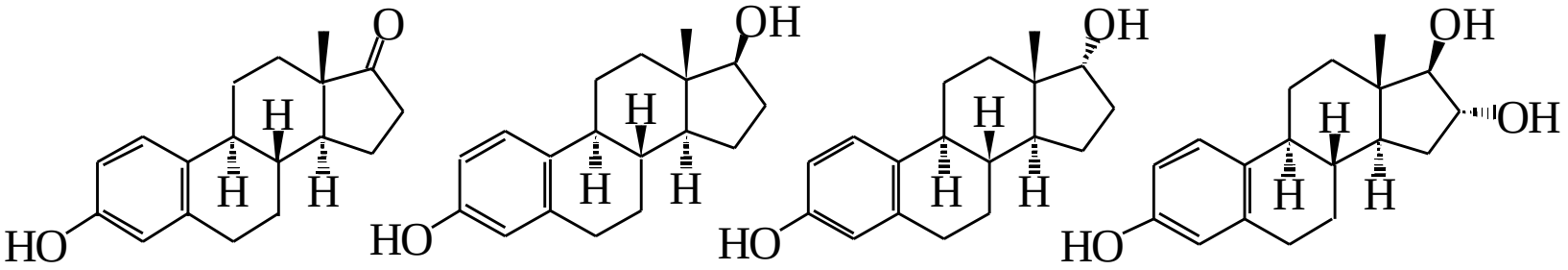
Meprobamate (Anxiolytic Drug)



TCPP (Flame Retardant)

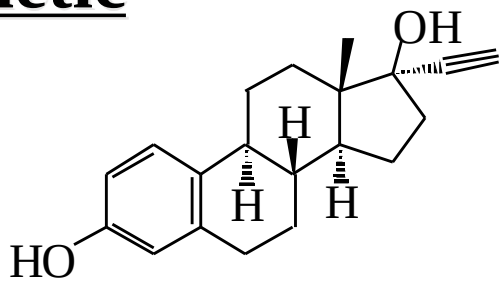
Target Hormones

Natural

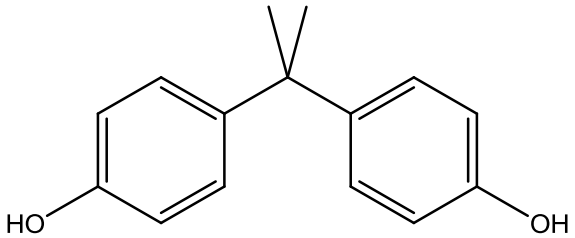


Estrone | 17β-estradiol (E2) | 17α-estradiol (E2) | Estriol (E3)

Synthetic



17α-Ethynylestradiol (EE2)



Bisphenol A (BPA)

Target Glucocortcoids

Target Analytes		
Aldosterone	Budesonide	Spironolactone
11-Deoxycorticosterone	Deflazacort	6-α-Methylprednisolone
Fludrocortisone	Flunisolide	Fluocinonide
Cortisone	Amcinonide	Betamethasone
Dexamethasone	Fluticasone Propionate	Fluorometholone
Triamcinolone	Mometasone Furoate	Triamcinolone Acetonide
Prednisone	Beclomethasone	Hydrocortisone
Prednisolone	Flumethasone	Fluocinolone Acetonide
Corticosterone	Clobetasol Propionate	Clobetasone Butyrate
Beclomethasone Dipropionate		
Surrogate		
Hydrocortisone-d_4	Dexamethasone-d4	Cortisone-d8
Prednisone-d4	Corticosterone-d8	Fludrocortisone-d5
Methylprednisolone-d2	Prednisolone-d6	

LC-MS/MS

Recovery: 88-122%

Method Performance for common CECs

Recoveries

(spike: 100 ng/L)

No Surrogates

Compounds	Recovery
Atrazine	63±4
TCPP	66±6
TCEP	66±2
Simazine	68±3
PFOS	71±2
Sulfamethoxazole	75±2
Sucralose	76±5
Caffeine	77±2
Primidone	78±4
PFBS	86±3
PFOA	88±2
Gemfibrozil	88±14
Carbamezapine	88±3
Trimethoprim	96±1
Sucralose	100±2
Triclosan	120±8
Sulfamethoxazole_13C6	97±2
Triclosan_d3	99±6
Sucralose_d6	79±17
Carbamezapine_d10	101±1
PFOA_C13	116±3

Compounds	Recovery
Fluoxetine	11±5
PFBA	28±2
DEET	38±9
Triclocarban	40±12
Fluoxetine d5	19±7

Method is good for common CECs

Detection Summary on 2nd round samples

- No compounds were detected in the field blank.
- Of the 12 samples analyzed, 29 of 41 (70%) target CECs were detected in the samples.
- 25 compounds were detected in more than 50% of Green Valley samples; while 24 were detected in more than 60% of West Basin samples (Raw, post ozone, post MF).
- Two compounds were detected in all of the samples except blank (Atenolol, Benzophenone).

CECs Concentration on 2nd round samples

<MRL

	Green Valley Pilot						West Basin					
ng/L	**Influent**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/UV**	**Cl_2**	**Influent**	**O_3**	**MF**	**RO**	**UV**	**FB**
Acesulfame	13.9	<6.7	<7.0	<7.3	<6.2	<6.7	191	167	141	<7.0	<7.4	<6.9
Atenolol	1730	1670	1210	994	568	547	514	310	325	3.1	3.0	<0.2
Atrazine	<0.3	<0.4	<0.4	<0.4	<0.4	<0.4	14.4	12.1	12.1	<0.4	<0.4	<0.4
Benzophenone	184	63.4	11.1	54.9	8.7	10.4	880	334	280	150	130	<0.5
Benzotriazole	120	191	67.2	76.0	52.9	77.4	<16	<14	<15	<9.1	<9.1	<9.1
Caffeine	<3.1	<3.5	<3.5	<3.6	<3.2	<3.3	73.6	61.4	66.2	32.4	31.5	<3.0
Carbamezapine	290	224	265	10.4	28.6	23.8	118	16.4	30.2	<0.4	<0.3	<0.3
DEET	54.5	32.6	49.7	27.0	24.2	23.5	96.9	60.8	74.5	<0.5	<0.5	<0.5
Diclofenac	1360	378	240	<2.2	<1.9	273	120	10.9	70.0	<2.1	<2.0	<1.8
Diphenhydramine	512	485	456	<0.1	196	35.9	470	<0.2	265	<0.1	<0.1	<0.1
Ditiazem	266	184	174	<0.1	<0.1	165	262	47.7	56.1	<0.1	<0.1	<0.1
Fluoxetine	199	173	164	130	112	89.2	<0.2	<0.2	<0.2	<0.1	<0.1	<0.1
Gemfibrozil	148	135	130	14.1	45.7	87.9	633	221	319	<1.0	<0.9	<0.9
Ibuprofen	58.2	55.9	30.8	33.5	28.7	52.4	180	77.4	99.6	<7.6	<8.3	<7.8

CECs Concentration on 2nd round samples

<MRL

	Green Valley Pilot						West Basin					
ng/L	**Influent**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/UV**	**Cl_2**	**Influent**	**O_3**	**MF**	**RO**	**UV**	**FB**
Iohexol	860	206	256	699	153	721	1830	1400	1320	<16	<15	<16
Iopamidol	294	79.8	52.8	168	40.3	147	387	277	324	<4.7	<4.5	<4.6
Iopromide	50.8	16.9	24.1	33.4	<15	37.4	44.3	54.1	39.9	<16	<15	<16
Meprobamate	540	402	417	324	313	404	370	300	336	<0.2	<0.1	<0.1
Naproxene	135	128	137	<3.5	19.2	40.3	854	163	267	<3.4	<3.2	<3.1
PFBA	6.8	6.0	6.4	4.9	4.7	4.7	<0.8	<0,8	<1.0	<0.8	<0.6	<0.4
PFOS	<0.7	<0.7	<0.7	<0.8	<0.7	<0.7	530	261	290	200	<0.6	<0.6
Primidone	709	812	711	449	471	595	49.0	33.9	42.1	<0.5	<0.5	<0.4
Sucralose	1810	1480	1610	282	346	216	12100	11100	19700	38.7	32.9	<8.5
Sulfamethoxazole	2270	537	129	41.4	27.3	<0.2	510	366	400	<0.2	<0.2	<0.2
TCEP	380	196	308	339	271	235	381	417	410	<0.3	<0.3	<0.3
TCPP	3960	1240	1930	1970	1230	693	731	718	859	<0.4	<0.4	<0.4
Triclocarban	185	99.7	93.9	60.5	42.5	37.0	30.8	15.1	18.3	<0.1	<0.1	<0.1
Triclosan	211	26.2	23.2	<2.5	<2.3	<2.3	346	<9.2	11.2	<2.4	<2.2	<2.2
Trimethoprim	288	269	269	<0.1	<0.1	<0.1	878	194	264	<0.1	<0.1	<0.1

CECs Concentration on 2nd round samples

Compounds not detected in any of the samples:

	Green Valley Pilot						West Basin					
ng/L	**Influent**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/UV**	**Cl_2**	**Influent**	**O_3**	**MF**	**RO**	**UV**	**FB**
Norethindrone	<1.8	<2.1	<2.1	<2.2	<1.9	<1.9	<7.1	<6.7	<5.9	<1.9	<1.7	<1.7
Norgestrel	<0.7	<0.8	<0.7	<0.9	<0.7	<0.7	<1.7	<1.6	<1.6	<0.7	<0.7	<0.7
PFBS	<3.4	<4.0	<3.9	<4.1	<3.6	<3.7	<4.1	<3.9	<3.9	<3.9	<3.7	<3.7
PFDA	<0.6	<0.5	<0.7	<0.8	<0.6	<0.7	<0.4	<0.4	<0.4	<0.6	<0.5	<0.4
PFDoA	<2.8	<1.3	<2.8	<2.9	<2.5	<3.7	<1.2	<1.4	<1.2	<2.3	<1.1	<1.1
PFHxA	<46	<31	<37	<37	<23	<37	<45	<32	<25	<34	<31	<21
PFHxDA	<2.6	<3.6	<2.5	<2.8	<2.4	<4.3	<1.9	<2.1	<1.2	<2.1	<2.6	<1.5
PFOA	<0.8	<0.7	<0.9	<1.0	<0.7	<0.9	<0.7	<0.7	<0.8	<1.0	<0.9	<0.9
Propylparaben	<0.3	<0.4	<0.3	<0.4	<0.3	<0.3	<0.3	<0.3	<0.3	<0.4	<0.3	<0.3
Simazine	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2	<0.2	<0.2	<0.1	<0.1	<0.1
Testosterone	<0.7	<0.8	<0.8	<0.8	<0.7	<0.7	<2.2	<1.9	<2.1	<0.7	<0.7	<0.7

<MRL

Steroid Hormone Concentration

- **Of the target estrogen compounds, only BPA was detected.**
- **Five glucocorticoid compounds were detected in some samples.**

<MRL

	Green Valley Pilot						West Basin					
ng/L	**Influent**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/UV**	**Cl_2**	**Influent**	**O_3**	**MF**	**RO**	**UV**	**FB**
Bisphenol A	<2.7	3.2	<2.1	2.5	<2.2	<2.4	35.3	6.5	7.6	0.5	1.2	<0.4
Prednisolone/ Cortisone	0.06	<0.02	<0.02	<0.02	<0.02	<0.02	<0.05	<0.02	<0.02	<0.02	<0.02	<0.02
Amcinonide	0.4	0.68	0.49	0.5	0.62	0.47	<0.5	<0.2	<0.1	0.36	0.48	<0.1
Hydrocortisone	<0.2	<0.05	<0.05	<0.05	<0.05	<0.05	<0.2	<0.05	<0.05	<0.05	<0.05	<0.05
Fluticasone Propionate	<0.5	<0.1	<0.1	0.57	<0.1	<0.1	<0.5	<0.1	<0.1	<0.1	<0.1	<0.1
Fluocinonide	0.37	0.46	0.47	0.35	0.32	0.29	<0.4	<0.1	<0.1	<0.1	<0.1	<0.1
Betamethasone/ Dexamethasone	0.07	0.13	0.09	0.08	0.07	0.06	<0.05	<0.02	<0.02	0.03	<0.02	<0.02

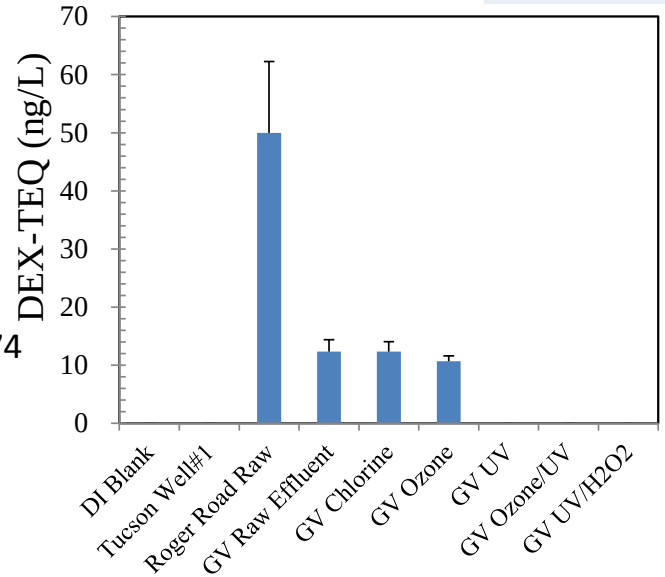
Chemistry & Bioassay

GR Analysis-TEQ value

compound	GR CALUX EC50 (nM)	REP[a]
aldosterone	112.2 ± 4.84	0.008 ± 0.06
amcinonide	0.49 ± 0.04	1.7 ± 0.09
betamethasone[b]	1.02 ± 0.05	0.8 ± 0.06
cortisol	11.4 ± 0.87	0.07 ± 0.08
cortisone	>1000[c]	<0.0008 ± 0.00006
desoximetasone	0.66 ± 0.03	1.3 ± 0.06
dexamethasone	0.84 ± 0.03	1 ± 0.05
flunisolide	0.49 ± 0.03	1.7 ± 0.07
fluorometholone	0.59 ± 0.03	1.4 ± 0.06
6α-methylprednisolone	2.25 ± 0.14	0.4 ± 0.07
paramethasone[b]	1.14 ± 0.04	0.7 ± 0.05
prednicarbate	4.75 ± 0.20	0.2 ± 0.06
prednisolone	3.68 ± 0.34	0.2 ± 0.1
prednisone	>500[c]	<0.002 ± 0.0004
rimexolone	0.83 ± 0.04	1 ± 0.06
triamcinolone	5.67 ± 0.23	0.2 ± 0.05
triamcinolone acetonide	0.37 ± 0.01	2.3 ± 0.04
triamcinolone hexacetonide	3.40 ± 0.17	0.3 ± 0.06

Environ. Sci. Technol. **2010,** *44,* 4766–4774

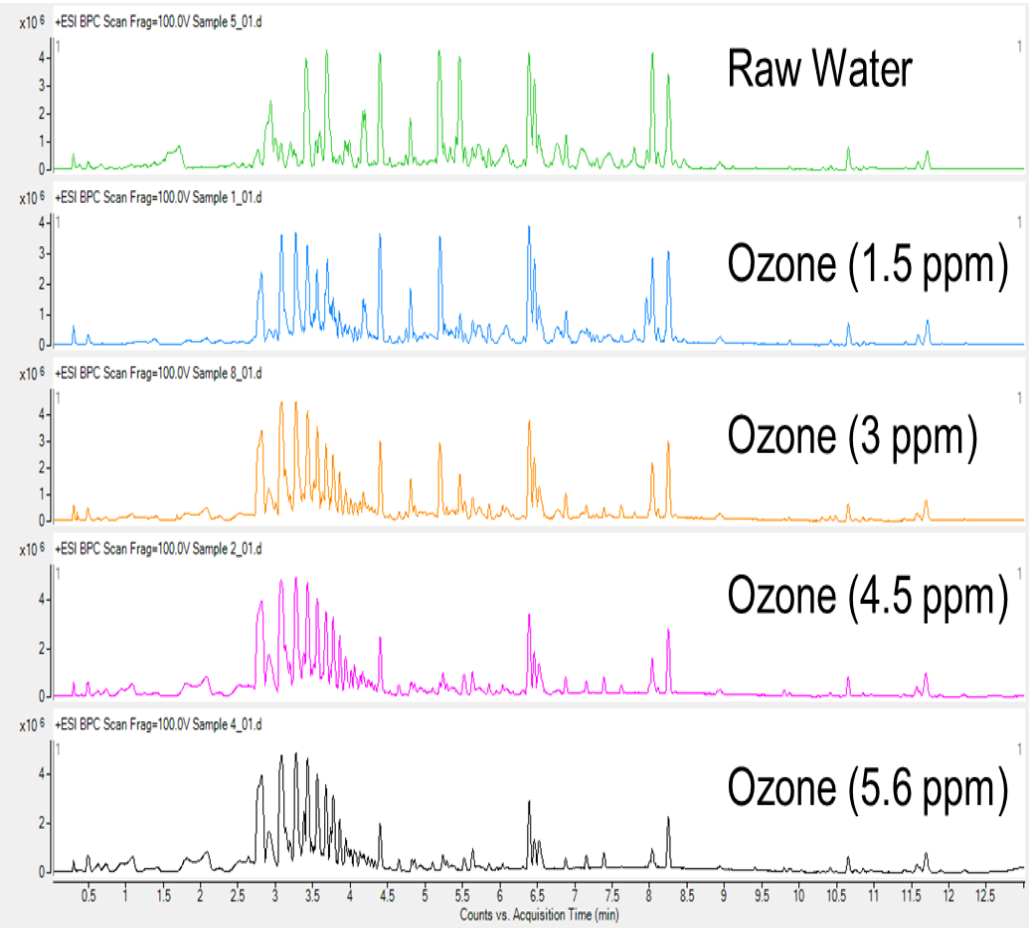
	DEX-EQ (ng/L)
GV Eff	1.1
UV	1.8
UV/H2O2	1.6
O3	2.3
O3/UV	2.0
Chlor	2.0



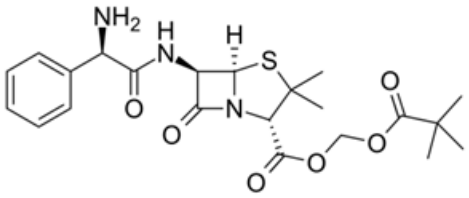
Chem-EQ << Bio-EQ

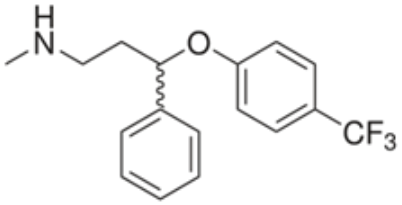
What if you don’t know the cause???

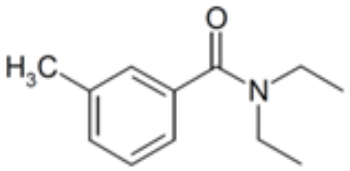
GC & LC QTOF for identification of unknowns



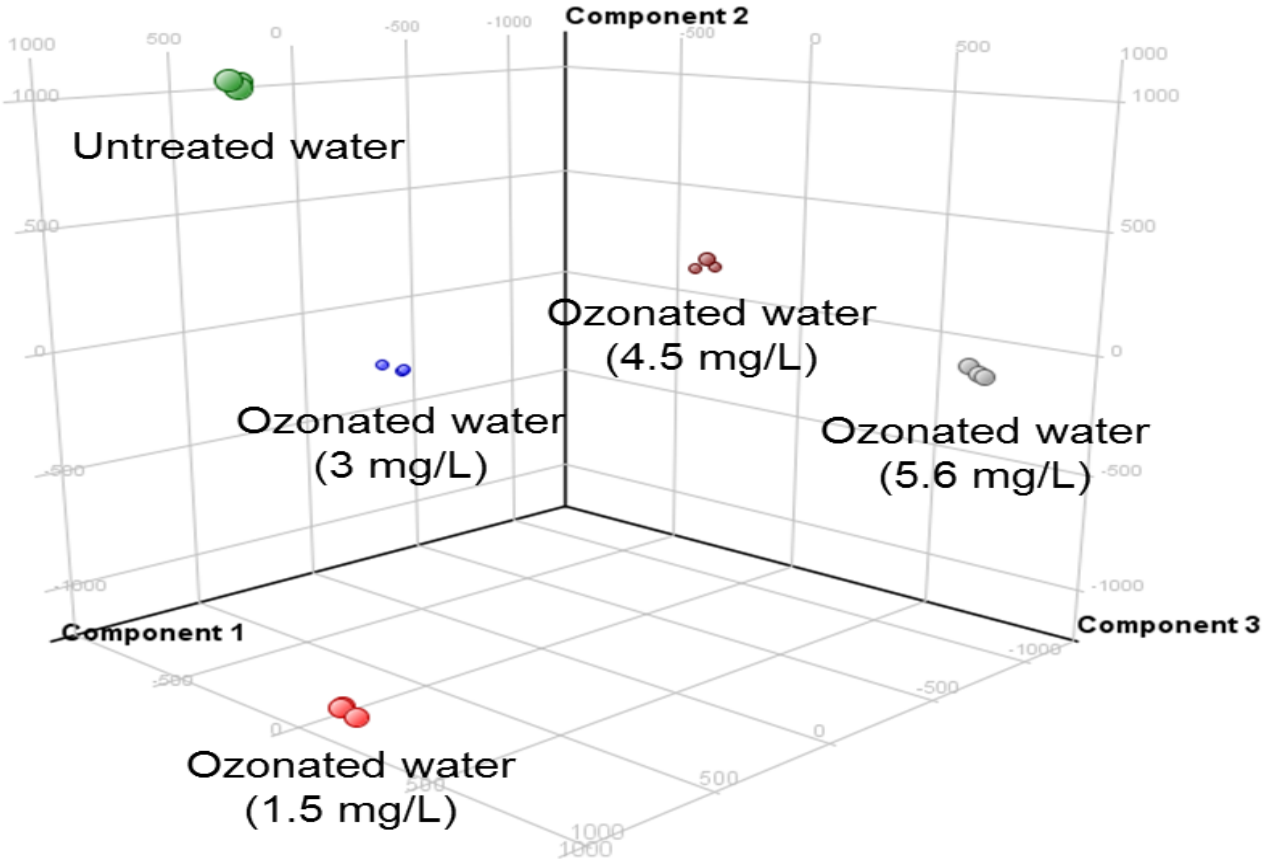
What if you don't know the cause???







PCA Plot for Different Ozone Doses

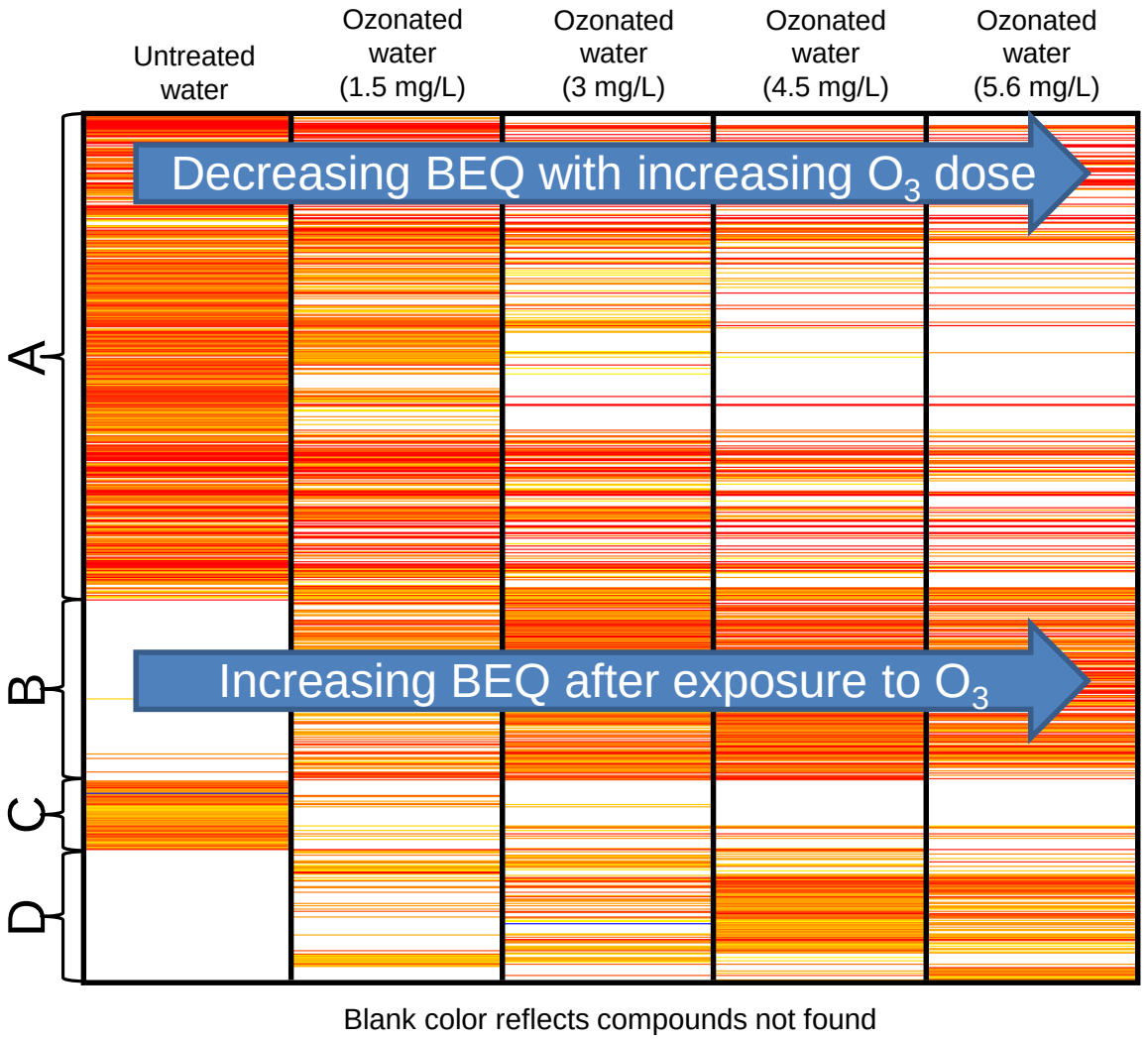


WATER TREATMENT & ANALYSIS OF UNKNOWNS

Although chromatograms were all similar for the analyst, clear differences appear on the heatmap

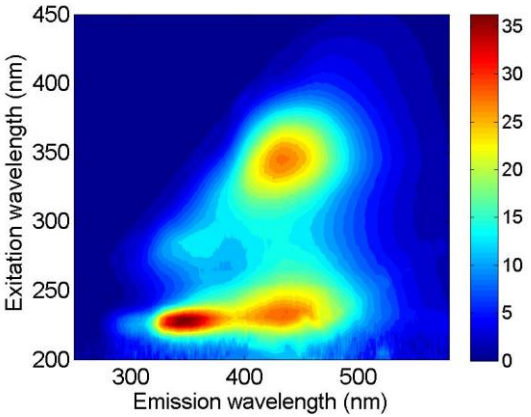
A & C are group of compounds in the raw water but at lower concentration or absent in ozonated water **(removed by ozone)**

B & D are compounds absent in raw water but present in treated water **(ozone by-products)**

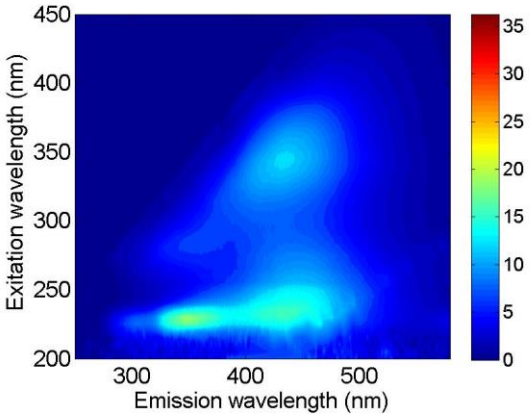


Application of Fluorescence indexes as surrogates for water quality

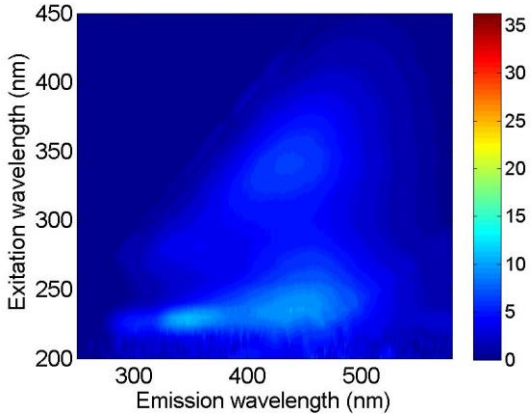
Wastewater Effluent on Ozone treatment



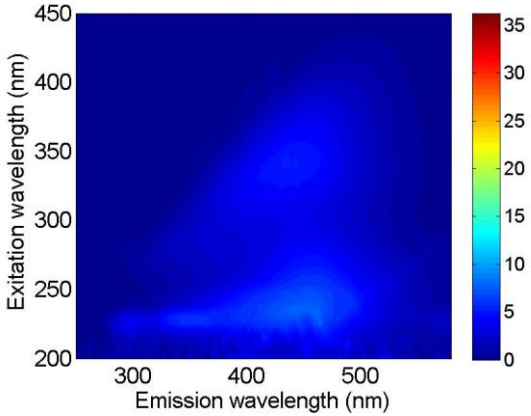
Control



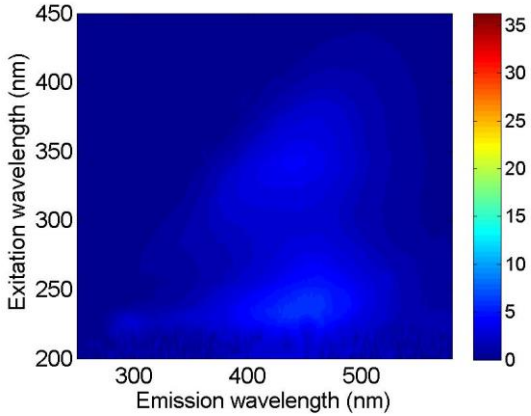
1.5 ppm



3 ppm

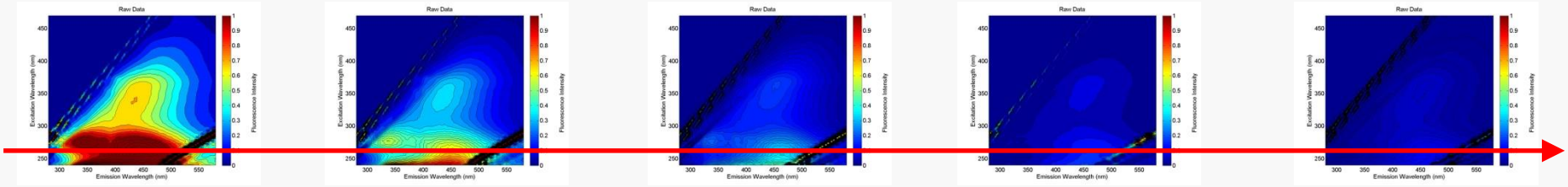


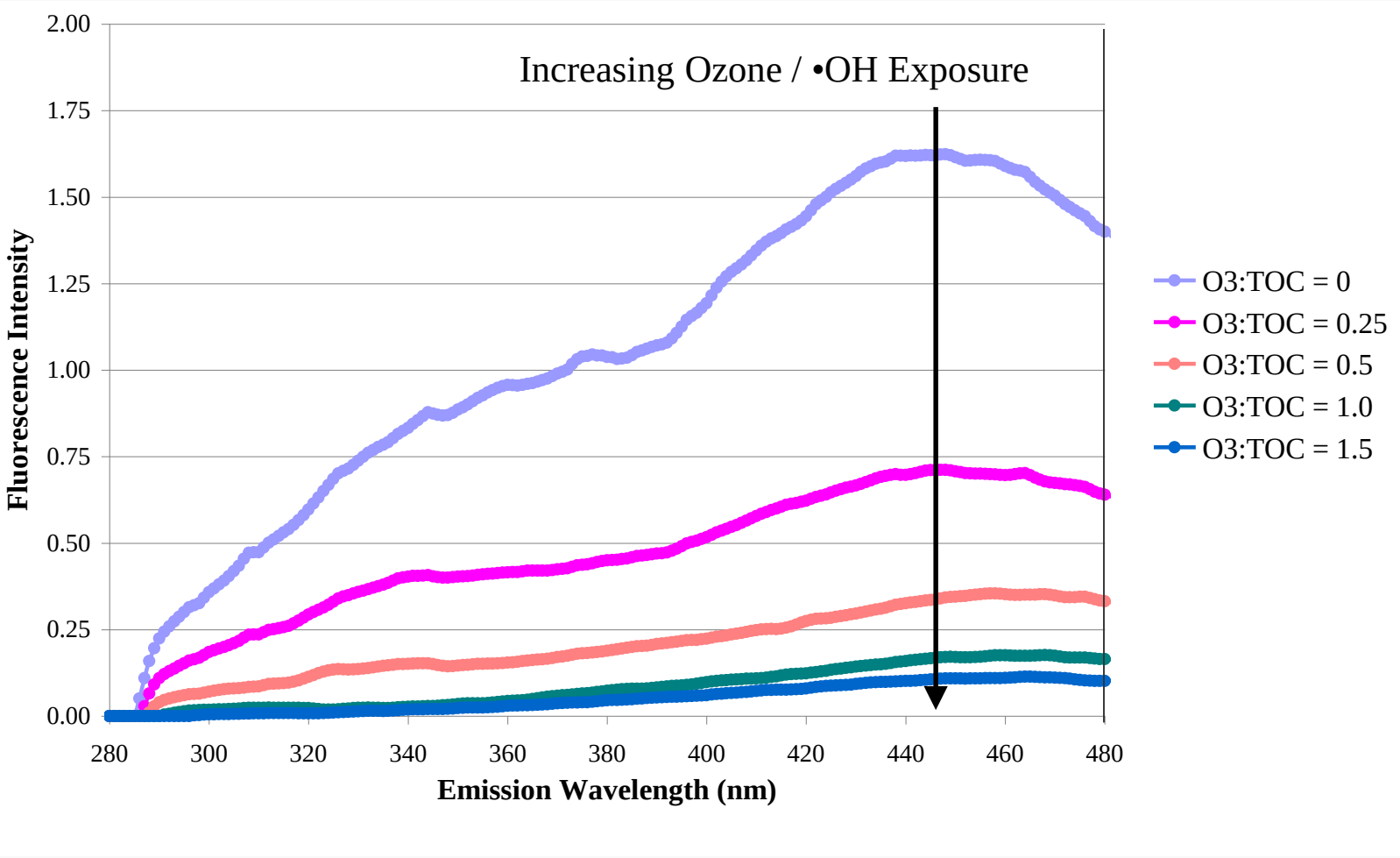
4.5 ppm



6 ppm

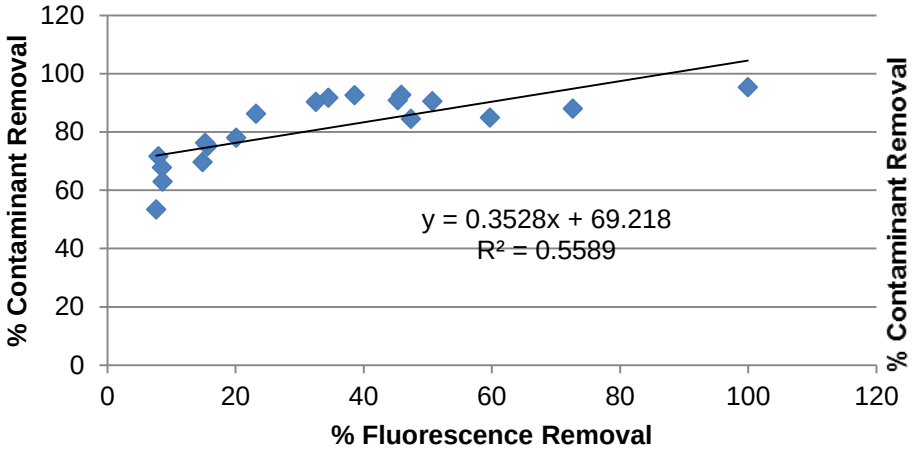
Fluorescence after O3 at 254 nm Excitation



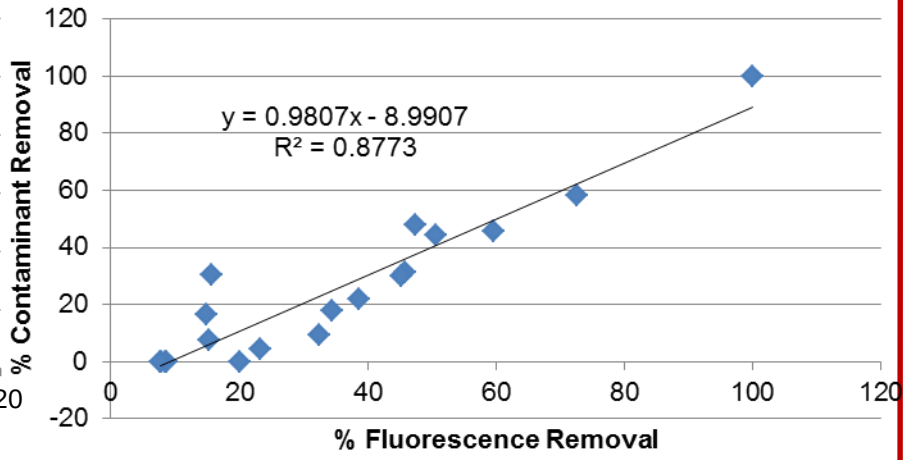


Fluorescence Excitation/Emission Pairs

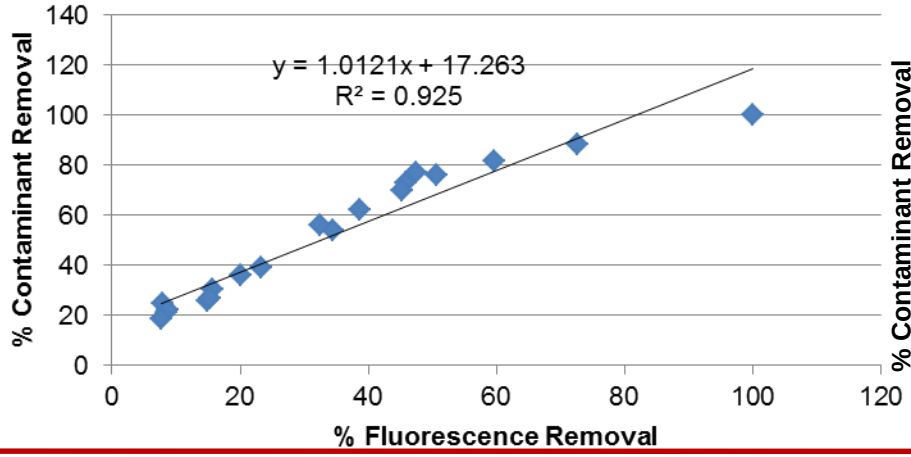
Group 1: Triclocarban



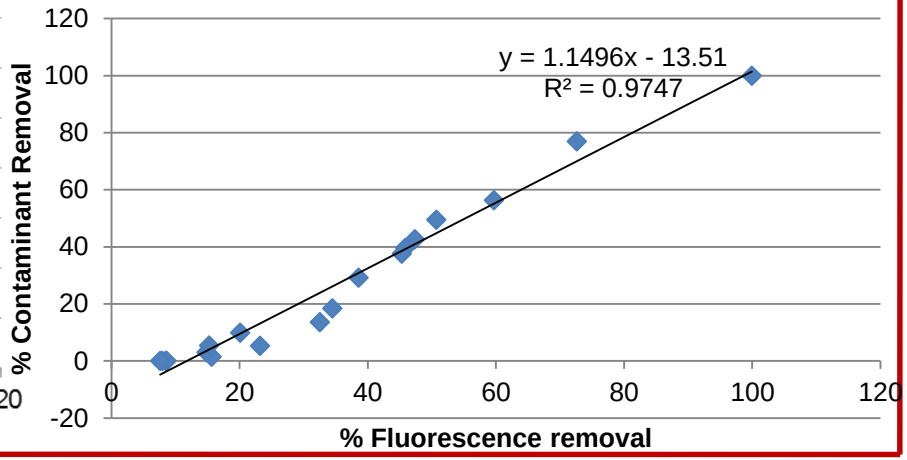
Group 2: PFOA



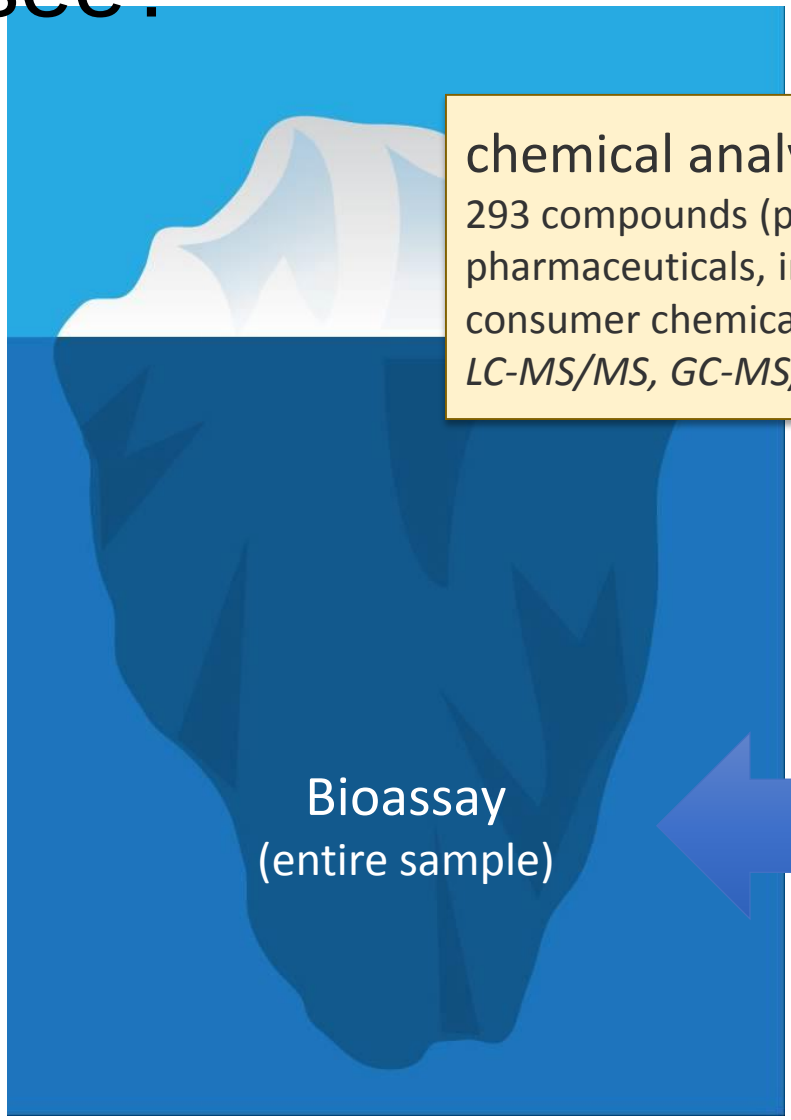
Group 3: Atenolol



Group 4: Primidone



Mixtures: how many micropollutants do we see?



chemical analysis
293 compounds (pesticides, pharmaceuticals, industrial and consumer chemicals)
LC-MS/MS, GC-MS/MS

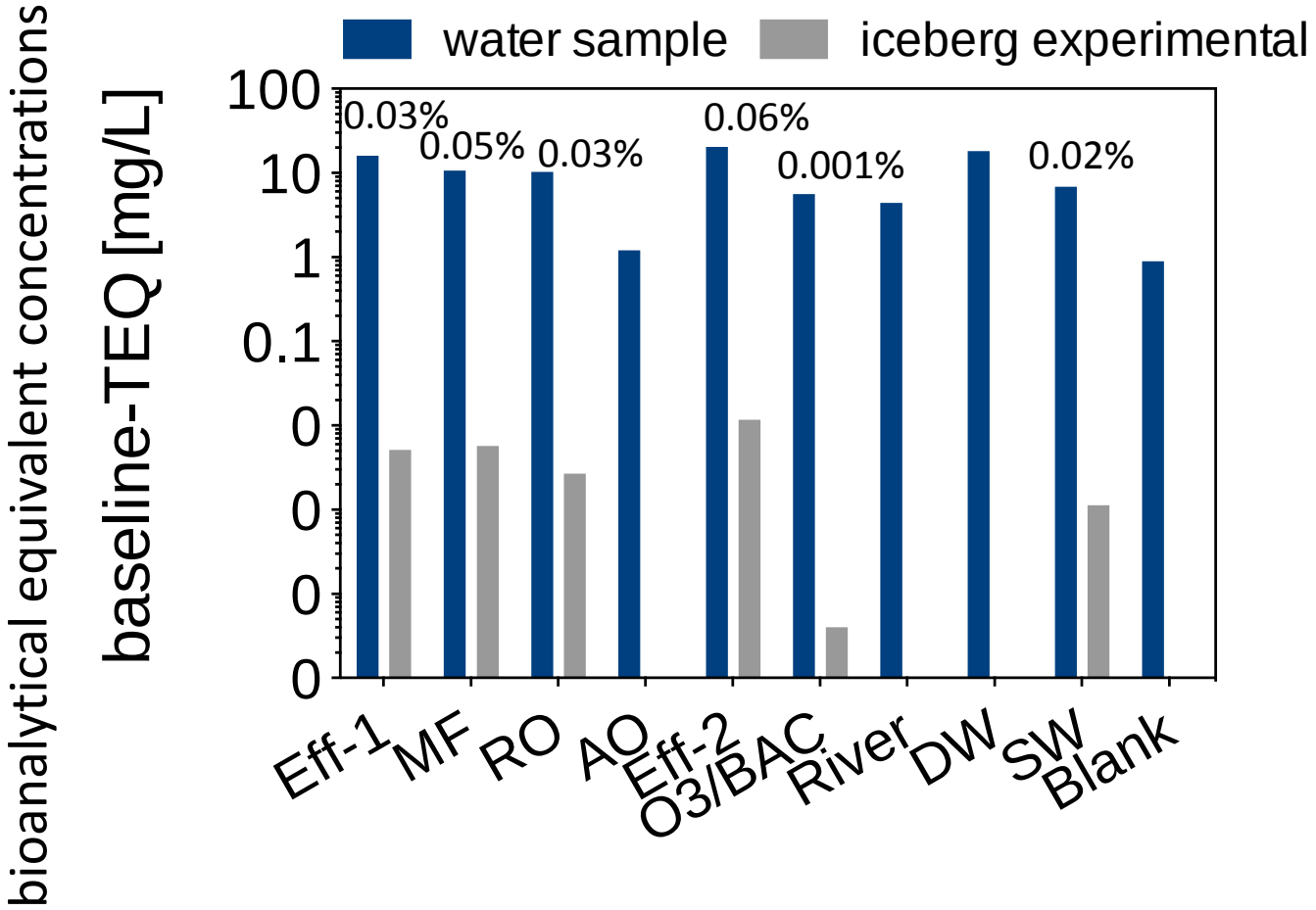
5 (SW) to 48 (Eff2) chemicals detected (<LOD in DW, blank)

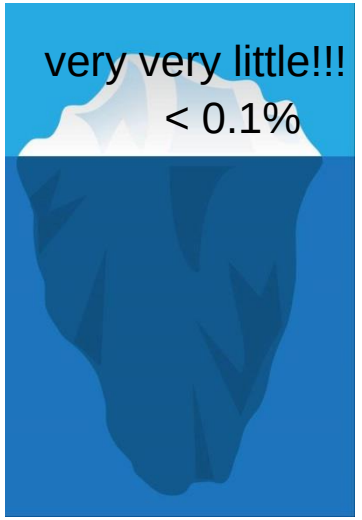
?

Bioassay
"tip of the iceberg"

Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S., Escher, B.I. 2013. Water Res., 47: 3300-3314.

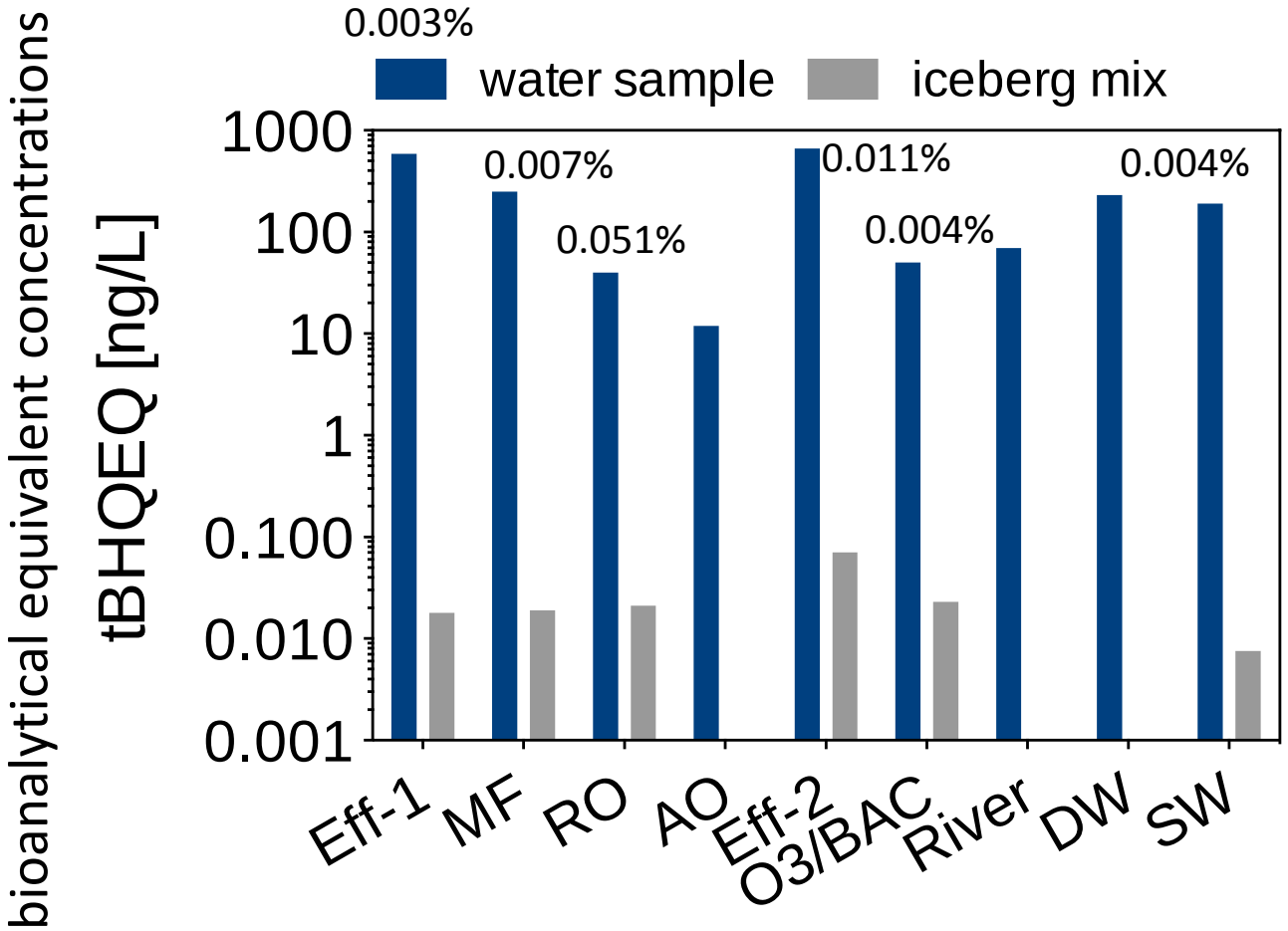
Which fraction of effect can be explained by known chemicals?
Example: Microtox





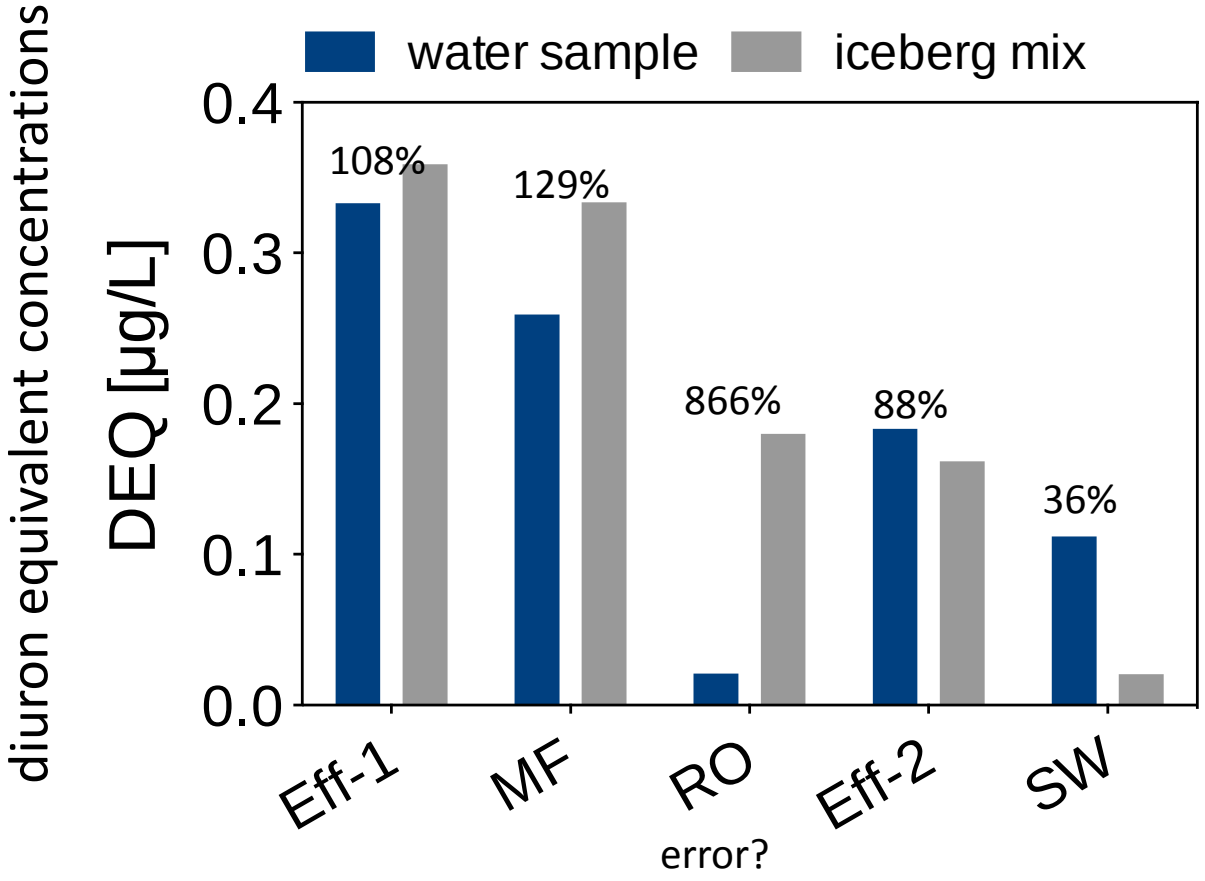
Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S.,Escher, B.I. 2013. Water Res., 47: 3300-3314.

Which fraction of effect can be explained by known chemicals?
Example: oxidative stress response



Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M. and Altenburger, R. (2013) Oxidative Stress Response Triggered By Pesticides, Pharmaceuticals And Their Mixtures Environmental Science & Technology, : 47(13): 7002-7011.

Which fraction of effect can be explained by known chemicals?
Example: photosynthesis inhibition



Tang, J.Y.M. and Escher, B.I. (2014). Realistic environmental mixtures of micropollutants in wastewater, recycled water and surface water: herbicides dominate the mixture toxicity towards algae. *Environmental Toxicology and Chemistry*: submitted 10 Oct 2013.

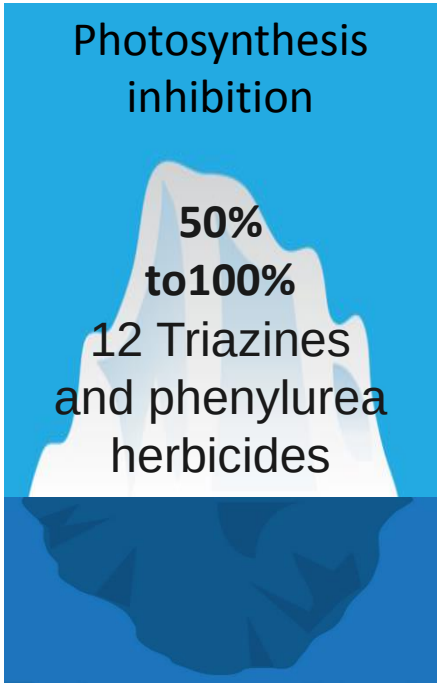
Which fraction of effect can be explained by known chemicals?

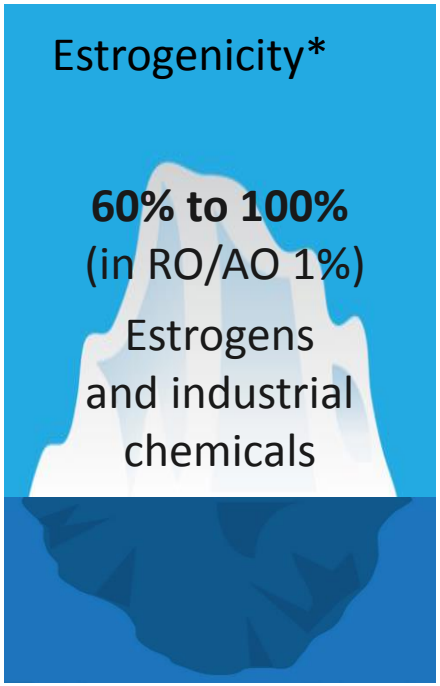
Cellular toxicity pathway

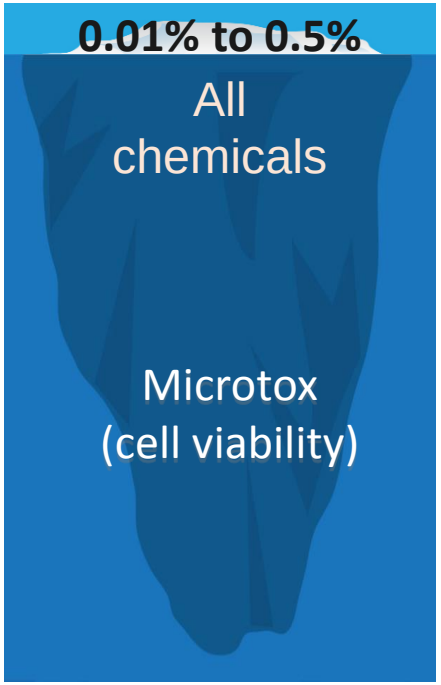
Metabolism → Interaction with target → Defense → Cell death

Receptor- mediated effects

Stress response and cytotoxicity







Escher, B.I., Lawrence, M., Macova, M., Mueller, J.F., Poussade, Y., Robillot, C., Roux, A.,Gernjak, W. 2011. Environ. Sci. Technol., 45: 5387-5394. Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S.,Escher, B.I. 2013. Water Res., 47: 3300-3314.

Standardization of Bioassay Protocols

ALVINA MEHINTO

SCCWRP

Development of SOP

1. Background (cell lines, mode of action)
2. List of laboratory equipment, consumables, cell kit
3. Assay protocol
4. Data reporting (e.g. standard data entry spreadsheet)
5. Appendices (e.g. plate layout, preparation of dilutions)
6. Expected results
7. Troubleshooting

Laboratory Set-up

Molecular laboratory (centrifuges, microscopes, multichannel pipets...)

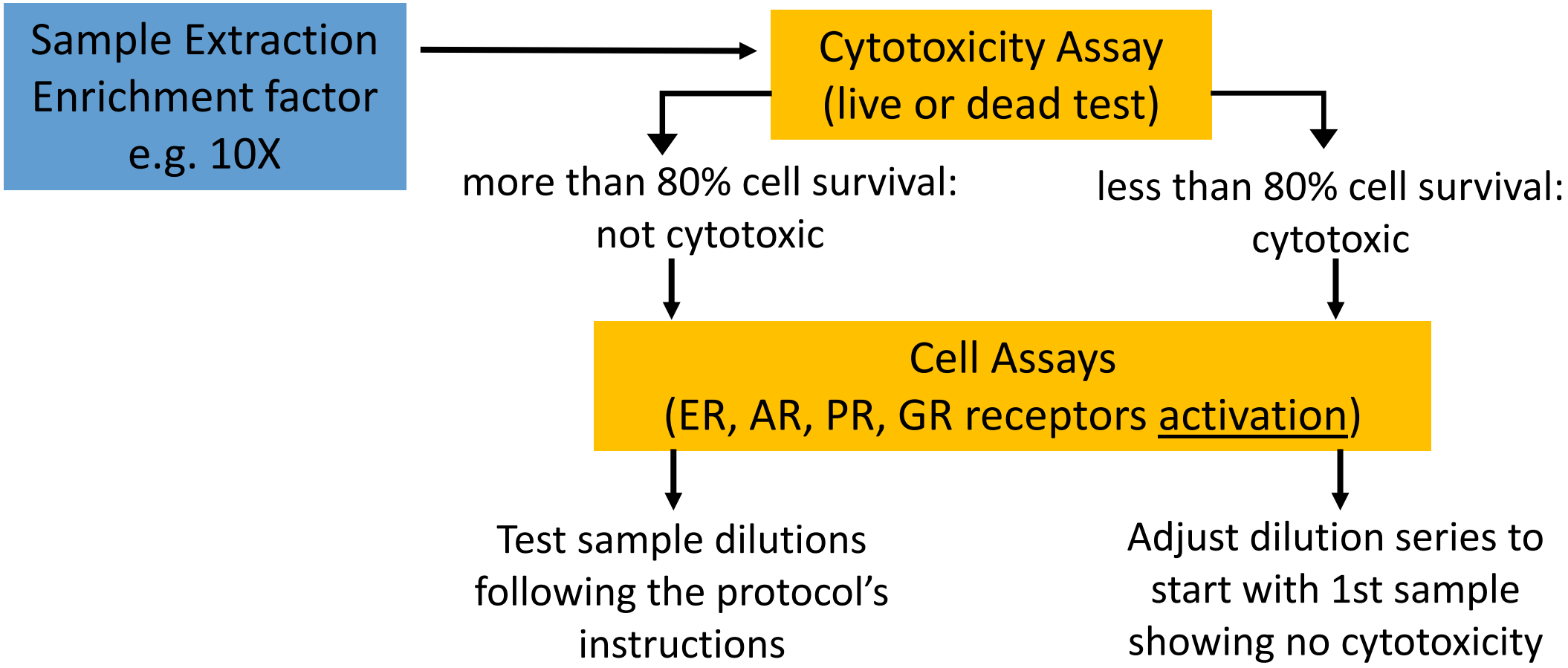
Specific equipment for bioassay:

- Biological safety cabinet class II
- Humidified cell culture incubator to maintain cells
- Cryogenic freezer
- Fluorescence plate reader, bottom read capabilities

Assay consumables

- Cell assay kit, assay media
- Cell culture plates
- Reference ompound

Bio-screening Workflow



Standardized Approach for CA Project

- Division arrested cells for ERa, AR, PR and GR
- Vehicle control: 0.5% DMSO
- Cell density: 50,000 cells/well (ERa, GR), 40,000 cells/well (AR, PR)
- Reference compounds: 9 concentrations for dose response curve
- Sample extracts: 4 dilutions in triplicate
- Set of QA/QC

Bioassay Preparation

Solutions:

- Assay media (different assay media may be required for different cell assays)
- Stock solutions for reference chemicals
- Working dilutions for reference chemicals and sample extracts

Cells:

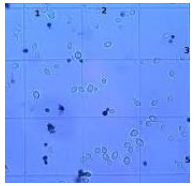
- Provided frozen, division-arrested
- Revived in assay media and plated the same day

Cell viability and count:

- Stain and count number of cells in known volume
- Dilute cell suspension to required cell density for the assay

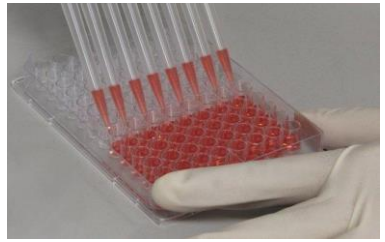
Cell Assay Protocol

Day 1



Cell count

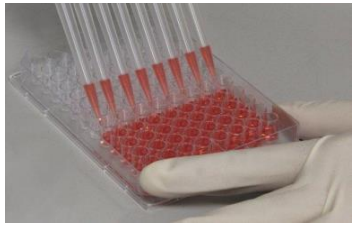
Plate cells @ specific density



Add diluted extracts

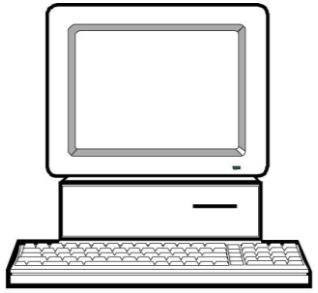
Overnight incubation (~16 hrs) at 37°C, 5% CO_2

Day 2



Add substrate

Incubation (~2 hrs) at room temperature



Measure fluorescence

QA/QC

Control for contribution of artifacts (blanks)

- Cell-free control – determine plate background
- Vehicle-free control – determine background of unstimulated cells
- Vehicle (e.g. DMSO) control – determine background caused by vehicle control
- Blank extract – chemical extraction blank sample
  - X3 replicates on EACH assay plate

➢ Control should not exceed e.g. 10% of EC_{10}

QA/QC – cont.

Calibrate assay response with reference compound

- Dose response curve with potent agonist (e.g. 17β-estradiol for ERα) to determine Bio-EQ
  - 9 dilutions X3 on first assay plate
  - 5 dilutions X2 on subsequent plates

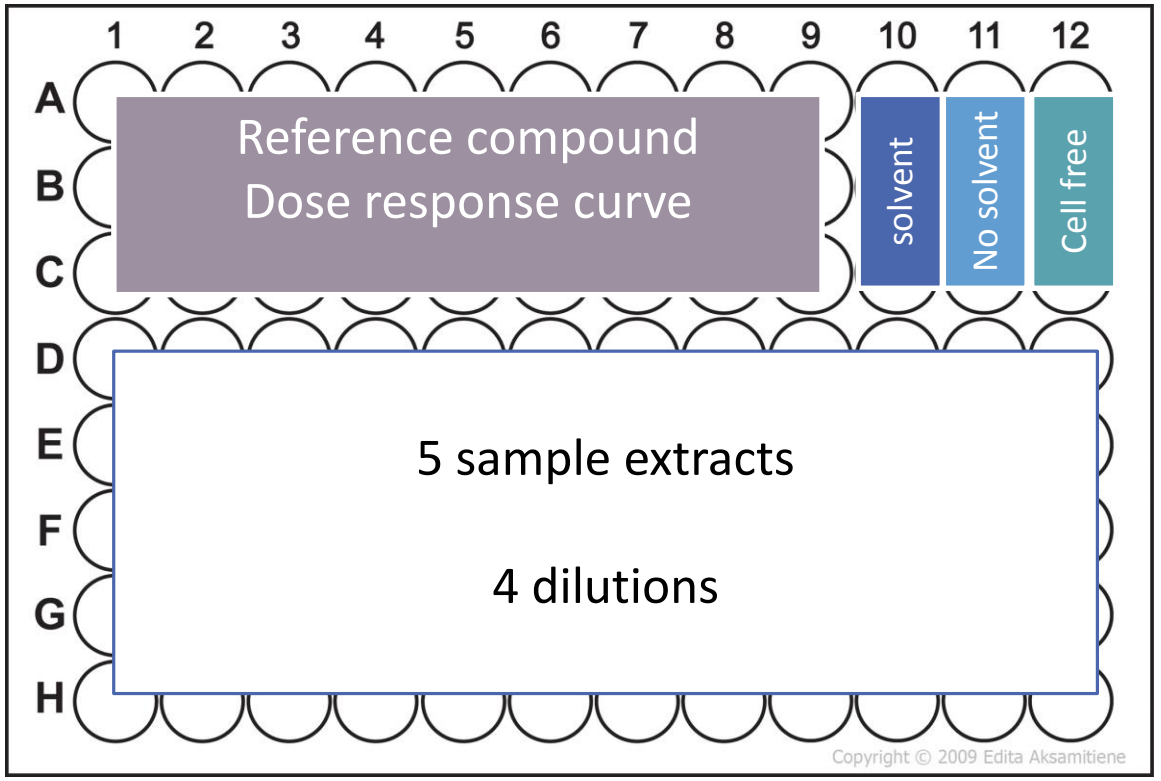
➢ EC50 should agree with historical/specified value, e.g. to within 30%

Validate assay response

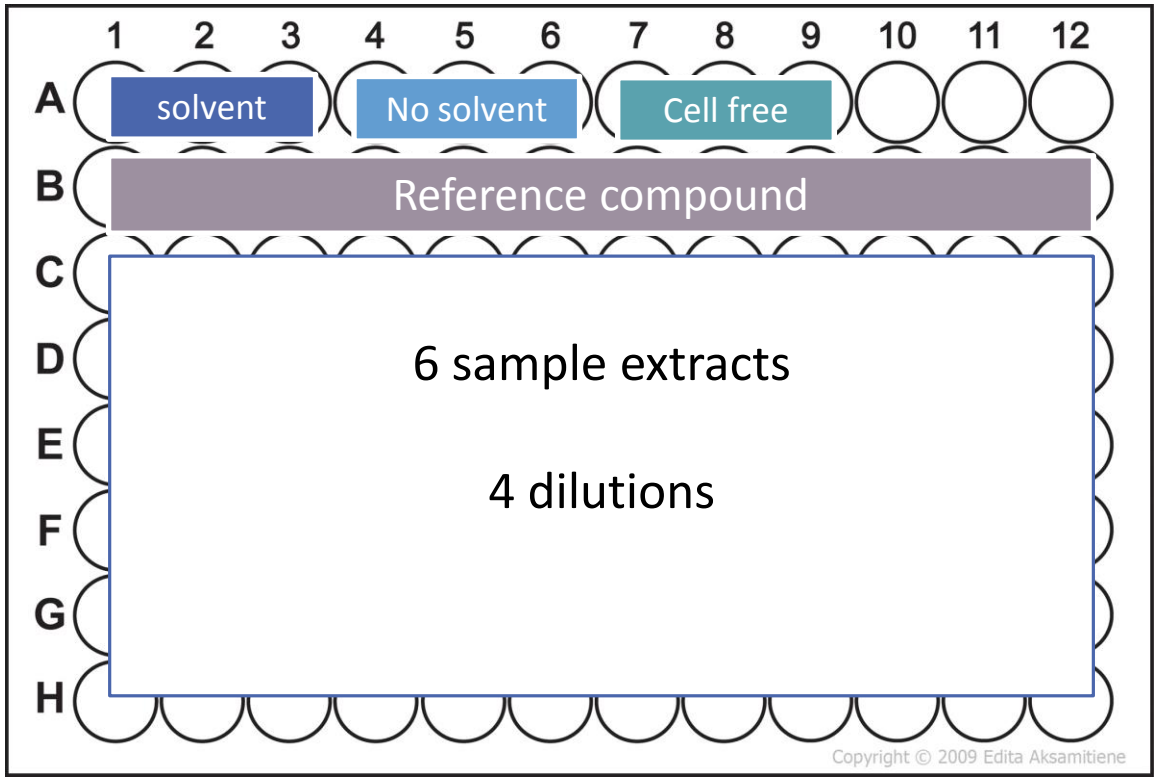
- Include spiked sample

➢ Response should be within the expected range of positive assay response

Cell Assay Protocol (96-well plate format)



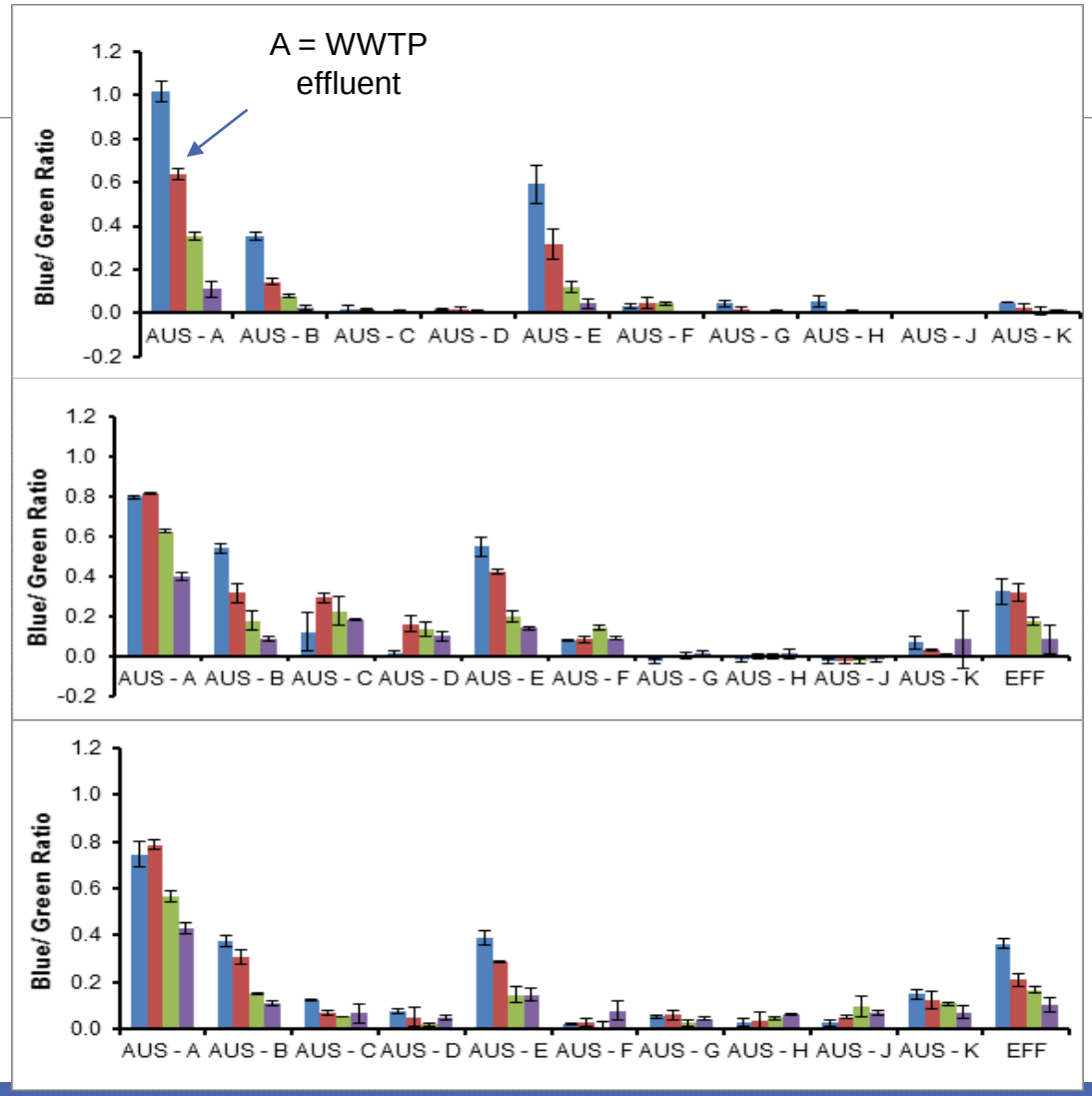
Standard plate



Additional plates

WRF Results

- Example with ERa cell assay
- Good agreement between CA team participating laboratories



Future Goals

Time/Cost Improvements:

- Customized kit with specific cell density, number of aliquots per plate, etc.
- Scale up to higher density plates to run samples more cost effectively
- Automation of protocol
- Multiplex endpoints for a given cell line

DATA INTERPRETATION & GUIDANCE

- **Translate bioassay results into quantifiable threshold**
  - total equivalent concentrations or quotients (TEQs)
- **Investigate relationship to priority CEC concentrations & health based trigger levels**
  - compile reference doses or “TTCs” for known/measured CECs
- **Develop tiered framework that best utilizes bioassay results**
  - first tier screening tool
  - bioassay threshold exceedances that trigger appropriate response
- **Conduct workshop for stakeholders**
  - appropriate role, implementation and use of bioassay results

DATA ANALYSIS

- **Step 1. Confirm bioassay results are valid (QA/QC checks)**

  Calculate EC_x (reference chemical) and compare to historical values

  If within specification, go to next step. If outside, take corrective action

  Assess blank contribution

  If within specification, go to next step. If outside, take corrective action

- **Step 2. Determine behavior of sample results**
  - Test for difference in fold response among sample dilution series
  - If dose-response exists, calculate EC_{10} and/or EC_{50}
  - If no dose-response, compare mean to blank
    - If no difference, report as "ND" (e.g. max REF * 2)

- **Step 3. Compute bioassay equivalents (BEQs)**
  - represent in units of ng/L based on reference chemical

    BEQ = EC_x (reference chemical) / EC_x (sample)

MONITORING THRESHOLDS

- **Step 1. Consult with regulators to identify current guidelines**
  - Fed, state MCLs for target analytes or analogs thereof
  - State, regional investigative benchmarks (e.g. notification levels)
  - International published thresholds based on human health effects

- **Step 2. Assess linkage of bioassay and higher order effects**
  - Compile relative potency factors (PFs) as $EC_{x,\text{in vitro}}$ / $EC_{x,\text{ in vivo}}$
  - Rank or weight PFs based on relevance/rigor of study
    - (epi > individual > organ > molecular)

- **Step 3. Apply margin of safety based on monitoring goals and uncertainty**
  - Action Level (AL) = PNEC or NOEC / (PF * SF)

DECISION MAKING

- **Step 1. Compare bioassay result to action level**

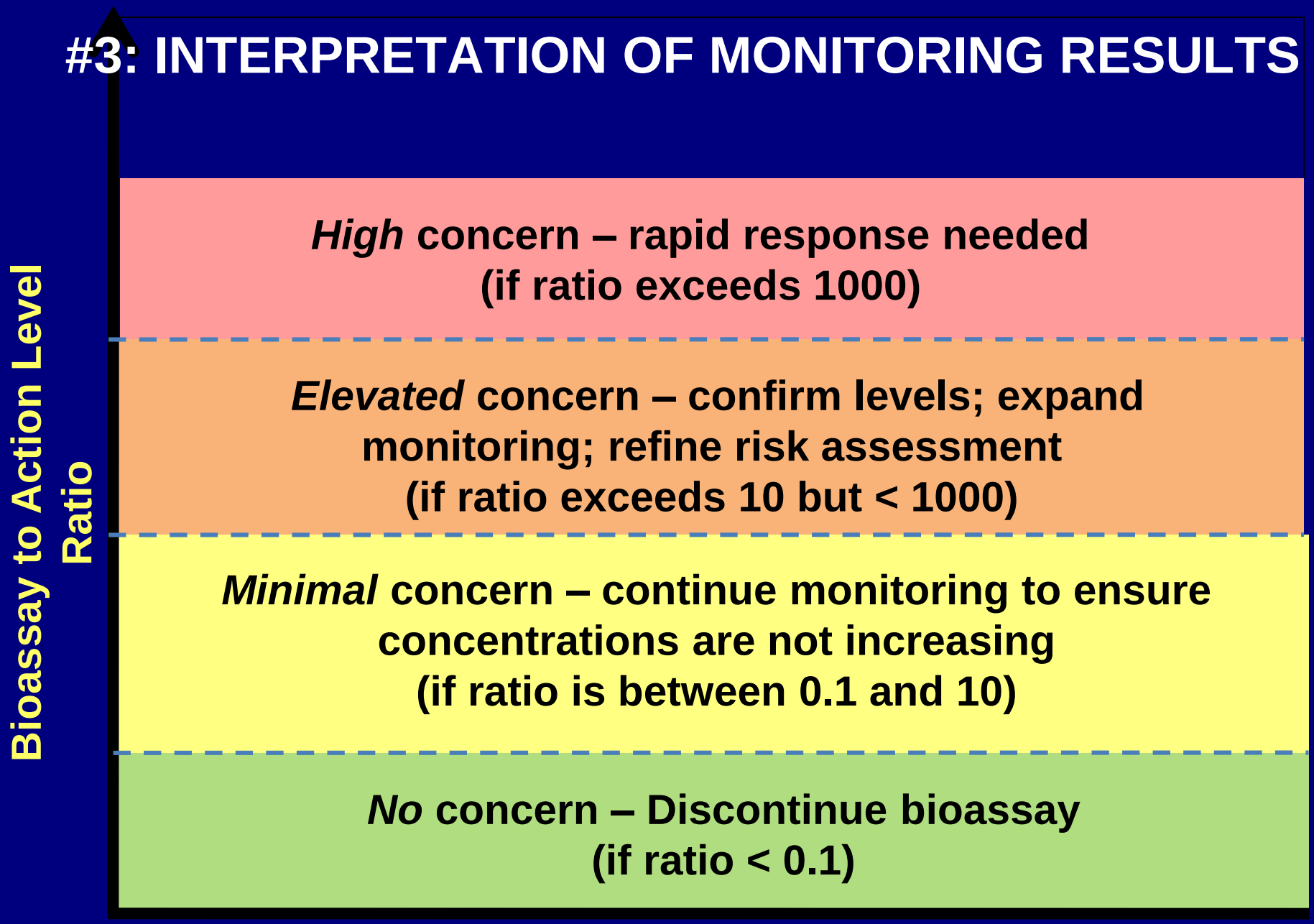
  If BEQ > AL, GO TO STEP 2

  If BEQ < AL, continue with baseline monitoring and GO TO STEP 3

- **Step 2. Define actions commensurate with exceedance**
  - Confirm a single exceedance within specified period of time (e.g. 72h)
  - If confirmed, initiate targeted chemical analysis “directed by bioassay”
  - Increase frequency of monitoring to see if exceedance persists
  - Notify regulatory agency and discuss/implement rigorous solutions

- **Step 3. Review monitoring data on a regular schedule**
  - Off ramp for bioassays that consistent exhibit “safe” response
  - Status quo monitoring for bioassays that show minimal/moderate response
  - Take action to reduce residuals causing consistent bioassay responses at higher levels of concern

#3: INTERPRETATION OF MONITORING RESULTS



Bioassay Wish List

Dan Schlenk
University of California, Riverside

Primary Uses of Bioassays

- Rapid and robust biological response that can be linked through MOA to a higher order adverse outcome
  - BEQ---TEQ----RfD
- Use a biological response that identifies exposure to mixtures of known and unknown stressors.
  - Focus chemical testing;
  - Screening/Tiered process

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doi:10.1093/toxsci/kfs285
Advance Access publication September 28, 2012

In Vitro Perturbations of Targets in Cancer Hallmark Processes Predict Rodent Chemical Carcinogenesis

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TOXICOLOGICAL SCIENCES 135(2), 277–291 2013
doi:10.1093/toxsci/kft164
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EADB: An Estrogenic Activity Database for Assessing Potential Endocrine Activity

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Thomas J. Colatsky,‖ Jason L. Aungst,‖‖ Christina Teng,‖‖‖ Steve C. Harris,* Weigong Ge,* Susie Y. Dai,# Zhenqiang Su,*
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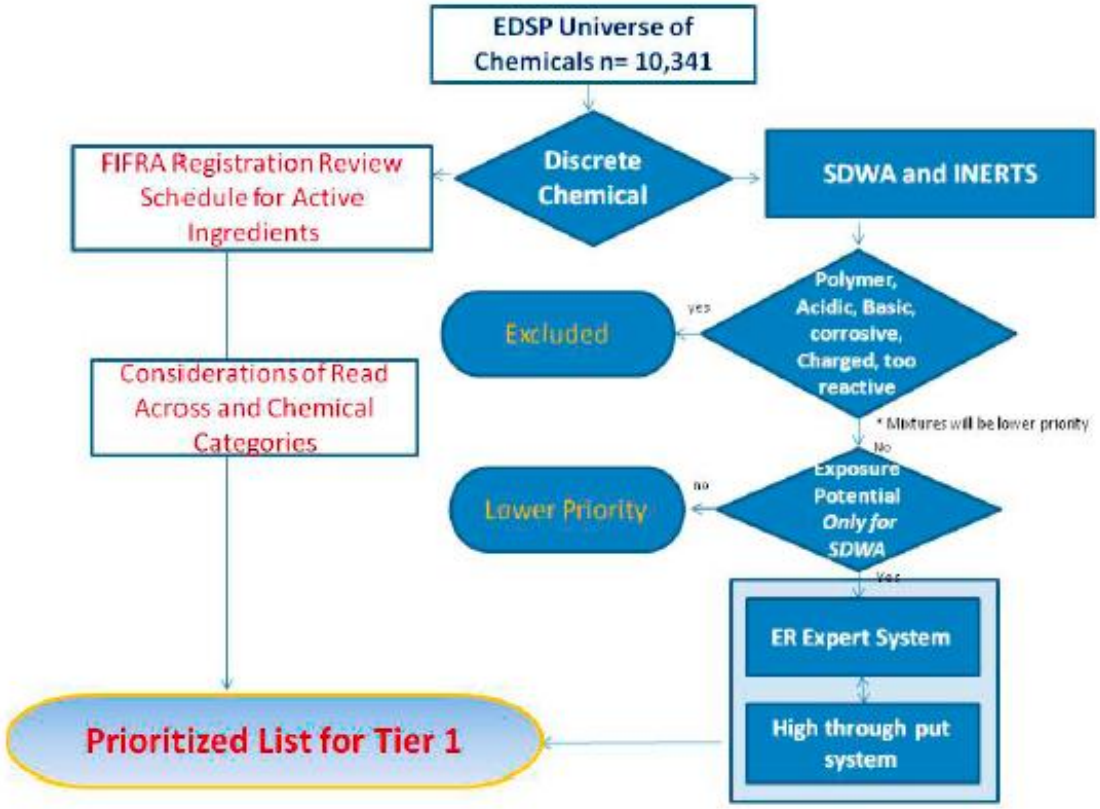


Figure 2.2.1: Proposed EDSP21 Prioritization Scheme

USEPA 2013

EATS Priority?

- Androgens
  - No Tier 1 transactivation assay for AR?
  - Anti-androgens > Androgens
    - Anti-Estrogens?
- Thyroid
  - Limited success with transactivation assays
  - Affinity/Sensitivity?
  - Thyroxine levels in vivo (mammals)
- Steroidogenesis
  - H295R
    - Translates well to steroid hormone concentrations/reproduction

Why EATS?

- EDSP targets
  - Large database and QSAR development Toxcast
- Vetted Protocols/Methods available
  - QA/QC
- Linkages to Adverse Outcomes better quantified

Glucocorticoid and Progesterone

- GR
  - Linkage to Immune/cardiovascular functions, developmental cellular proliferation
    - TEQ?
  - High sensitivity and robust assay that allows TIE analyses (WRF report)
- PR
  - Linkage to Reproductive and Neuroendocrine responses
    - TEQ
  - Environmental interest

Dioxin case study – Key Events Dose Response Framework

Application of National Research Council "Silverbook" Methodology for Dose Response Assessment of 2,3,7,8-Tetrachlorodibenzo(*p*)dioxin.

Authors: Simon T., Stephens M., Yang Y., Manning R.O., Budinsky R.A. and Rowlands J.C.

TEQ RfD for AhR Dysregulation = 30 pg/kg/d

Multiplex?

Nuclear Receptors/transcription factors		Level of activity
AhR	Aryl Hydrocarbon receptor	++++
AP1	Activator protein 1	+
AR	Androgen receptor	+
CAR	Constitutive androstane receptor	+++
ERa	Estrogen receptor alpha	++++
ERb	Estrogen receptor beta	+++
ERRg	Estrogen receptor related gamma	++
FXR	Farnesoid X Receptr	+
GR	Glucocorticoid receptor	++
HNF4a	Hepatocyte Nuclear factor 4 alpha	+
LXR	Liver X receptor	+
NRF2	Nuclear factor erythroid 2-related factor 2	+++

Nuclear Receptors/transcription factors		Level of activity
PPARa	Peroxisome proliferator-activated receptor	++++
PPARd1	Peroxisome proliferator-activated receptor	+
PPARg	Peroxisome proliferator-activated receptor	++
PXR	Pregnane-X-receptor	+
RARa	Retinoic Acid receptor, alpha	++++++
RARb	Retinoic Acid receptor, beta	+++++
RARg	Retinoic Acid receptor, gamma	++++++
RORb	Retinoid related orphan receptor beta	++++
RXRa	Retinoic-X receptor, alpha	+
RXRb	Retinoic-X receptor, beta	+++
VDR	Vitamin D receptor	+

Genotoxicity

- Chemicals of Concern
  - Cr^{VI}, 1,4 Dioxane, NDMA, DBPs (trihalos)
- Ames & uMu (SOS)
  - lack of sensitivity?
    - Exposure of known compounds (NDMA, BaP)
- P53 activities?
  - Adequate D/R
  - Chicken/egg?
  - TEQ?
- TIE?

Wish List Summary

- EDSP/Toxcast
  - ER redundancy
  - Anti-E; Anti-A
  - Thyroid?
  - Steroidogenesis
- Other NR
  - AhR
    - Life Tech Development
  - GR---TIE already performed
  - PR
- Genotoxicity Assays
  - P53

Promising endpoints in the development phase … and promising developments

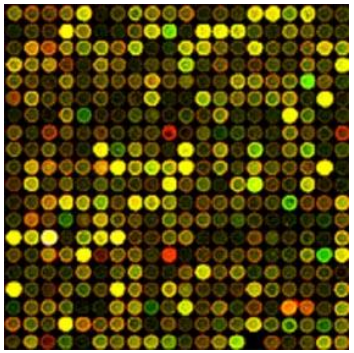
Frederic Leusch

Promising endpoints

- Based on interlab comparison:
  - Pregnane X receptor (PXR)
  - Oxidative stress (ARE-mediated)
- Based on known limitations of *in vitro* methods:
  - High throughput mammalian genotox assay
  - Non-genotoxic carcinogenicity
  - Neurotoxicity
  - Immunotoxicity
  - Developmental
  - Reproductive

Genomic methods
(*e.g.*, RT-PCR, gene arrays)

- Very versatile
- Can help discover new pathways relevant to contaminants in water
- But …
  - Limited throughput
  - Expensive



Metabolic activation

- Metabolic activation is important for:
  - Reactive toxicity
  - Thyroid active compounds (Murk et al)
  - Oxidative stress? Others?
- But often not incorporated in testing strategy
  - Cost: doubles number of analyses required
- Currently available:
  - Rat liver microsomes (S9 fraction)
  - Recombinant human CYP (Corning Supersome)

Moving towards true animal replacement

- Fetal Bovine Serum (FBS):
  - Contains hormones, growth factors, protease inhibitors, proteins, vitamins, amino acids, trace elements, lipids, attachment factors …
  - Significant source of variability, high ethical cost
- Development of serum free media
  - Would have big QAQC and ethical benefits
  - Any TK implications?

van der Valk et al 2010. Toxicol In Vitro 24: 1053-1063

A change in climate …

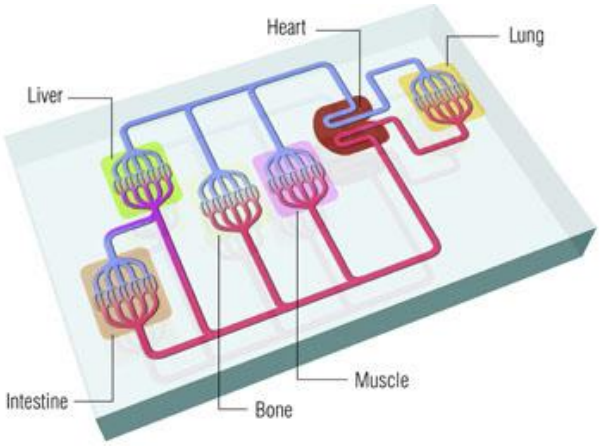
- Growing list of validated *in vitro* methods
  - Driven by ICCVAM and ECVAM (via OECD TG)
- Rapid increase in capacity
  - More than 100 commercial labs can conduct *in vitro* testing (most for drug discovery, but also for env samples)
  - http://www.alttox.org/ttrc/resources/in-vitro-testing.html
- Several projects (*e.g.*, DEMEAU) and publications devoted to development of bioassay guidelines

High throughput screening

- Many assays being adapted to 384-well format
- Electronic pipettes and pipetting robots are more widely available (and cheaper)

A little farther on the horizon …

- Implications of today's discoveries
  - Tox21: discovery of biological pathways induced by exposure to environmental pollutants
- Animal on a chip
  - Microfluidics to replicate organ systems
- 3D tissue and organ printing



Reif et al 2010 Environ Health Perspect 118(12): 1714-1720; Baker 2011 Nature 471: 661-665

MEETING OUTCOME

- **What endpoints are ready to move forward?**
  - Are there superior (commercially available) products that have not yet been tested
- **How should the bioassay results be used (e.g. screening vs. decision?)**
  - Propose a logical flow for use of screening data
  - Which applications?
- **How do we transfer this technology?**
  - Standardization, QA/QC guidelines
  - Lab certification
- **What more can these bioassays be used for?**
  - “hard” decision making
  - Receiving waters

Recommended Studies

- Ensure that water extraction efficiency is universal for all candidate endpoints
- Compare cost of bioanalytical assays vs chemistry
- Need to identify suitable AhR and genotoxicity assays

ER alpha

- Preferred MRL - 1 ng/L human relevance

  (0.1 ng/L ecological relevance

- Max REF - up to 50 depending on water quality
- Existing products: GeneBLAzer EC_{10}=5 ng/L

  BDS ERa-Calux EC_{10}= approx. 1 ng/L

  Possible non-commercial assays e.g. CAFLUX
- Reproducibility: Control charts over time ("Shewart log scale EC_{50}) should be within 2 standard deviations)
- Extraction: 1L using Oasis HLB 6cc recommended

ER data interpretation/ framework

1. Run in vitro assays
2. BEQ > action levels (1 ng/L)
    1. Confirm results
    2. Targeted analysis (e.g. hormomes, alkylphenols, etc.) to account for estrogenicity
    3. If BEQ > CEQ- do effect directed analysis (EDA)/TIE e.g. NTA
    4. If BEQ ~ CEQ: determine relevance to human health

1. CONSIDER BEQ/AL WHEN MOVING TO NEXT STEP

ERa application

- Testing of treatment efficacy
- **Screening**
- Decision making
- CONCLUSION : Do pilot evaluation of bioassay framework before taking next step (is it suitable for decision-making?)

Tech transfer

- General guidelines (performance-based)
  - Cell viability
  - Calibration
  - Required QA/QC
  - Cytotoxicity
  - Certified materials
- Standardized data evaluation (results reporting)
- Laboratory certification (inter-calibration exercises)
- Create & maintain information node
- Workshop

Future

- Additional applications
  - Receiving waters
  - Utility for human health assessment
  - Screening for EPA/TIE
  - Transition from screening to decision making tool
- Additional endpoints
  - GR assay is promising