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In vitro bioassays to evaluate complex chemical mixtures in recycled water

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Abstract

With burgeoning population and diminishing availability of freshwater resources, the world continues to expand the use of alternative water resources for drinking, and the quality of these sources has been a great concern for the public as well as public health professionals. *In vitro* bioassays are increasingly being used to enable rapid, relatively inexpensive toxicity screening that can be used in conjunction with analytical chemistry data to evaluate water quality and the effectiveness of water treatment. In this study, a comprehensive bioassay battery consisting of 36 bioassays covering 18 biological endpoints was applied to screen the bioactivity of waters of varying qualities with parallel treatments. Samples include wastewater effluent, ultraviolet light (UV) and/or ozone advanced oxidation processed (AOP) recycled water, and infiltrated recycled groundwater. Based on assay sensitivity and detection frequency in the samples, several endpoints were highlighted in the battery, including assays for genotoxicity, mutagenicity, estrogenic activity, glucocorticoid activity, aryl hydrocarbon receptor activity, oxidative stress response, and cytotoxicity. Attenuation of bioactivity was found to be dependent on the treatment process and

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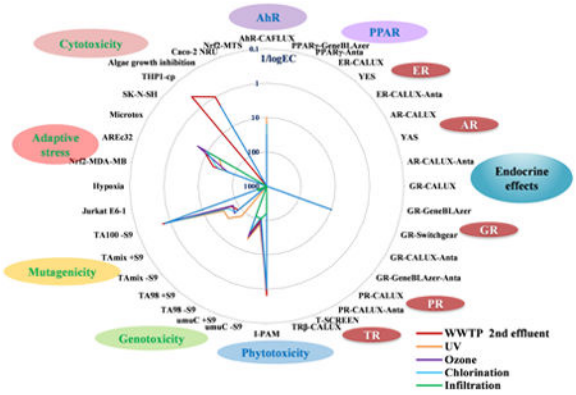
Supplementary Data: Data process, detection summary for all the samples in the bioassay battery, and comparison of all EC values between two WWTP effluents.

bioassay endpoint. For instance, ozone technology significantly removed oxidative stress activity, while UV based technologies were most efficient for the attenuation of glucocorticoid activity. Chlorination partially attenuated genotoxicity and greatly decreased herbicidal activity, while groundwater infiltration efficiently attenuated most of the evaluated bioactivity with the exception of genotoxicity. In some cases, bioactivity (*e.g.*, mutagenicity, genotoxicity, and arylhydrocarbon receptor) increased following water treatment, indicating that transformation products of water treatment may be a concern. Furthermore, several types of bioassays with the same endpoint were compared in this study, which could help guide the selection of optimized methods in future studies. Overall, this research indicates that a battery of bioassays can be used to support decision-making on the application of advanced water treatment processes for removal of bioactivity.

Graphical abstract



Keywords

Bioassay; *in vitro*; water treatment; advanced oxidation process; toxicity; recycled water

1. Introduction

Global concerns have been raised regarding the impact of complex mixtures of pollutants in water on environmental safety and public health (Schwarzenbach et al., 2006). Despite the rapid development of environmental chemistry analysis methods such as modern mass spectrometry analyses, it remains infeasible to track every pollutant potentially present in water because of limitations in analytical capacity, cost, and time. Many environmental chemicals are not readily identified because of their trace concentration, unknown structure/reaction pathway, or lack of sample preparation and detection methods. Moreover, conventional chemical monitoring cannot provide information about health impacts or account for cumulative effects from complex mixtures of environmental contaminants. Complementary techniques such as *in vitro* bioassays can provide a high throughput measurement of the potential toxicity of individual chemicals or chemical mixtures and can also indicate the presence of unknown toxic chemicals and have therefore been increasingly applied to water quality assessment over the past two decades (Escher and Leusch, 2012; Liu and Zhang, 2014; Pan et al., 2014; Yang and Zhang, 2014; Yang et al., 2014). Specifically, bioassays can provide measures of the cumulative effects of chemicals that

exhibit the same mode of toxic action (MOA) and thus concentration-additive effects (Escher and Hermens, 2002). Bioassays can also be used as a complement to chemical analyses in toxicity identification evaluation (TIE) schemes to address complex environmental problems. Such research has been successfully applied in the exploration of several important environmental pollutants, including polyaromatic hydrocarbons (Khim et al., 1999; Qiao et al., 2006) and estrogenic compounds (Furuichi et al., 2004; Snyder et al., 2001).

Due to the exceeding complexity of environmental samples, no single bioassay could represent all potential pathways of bioactivities in these mixtures. Therefore, government agencies such as the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) as well as other professionals have devised programs to push forward the development of bioanalytical technology needed to screen thousands of chemicals for potential toxicity, and to develop cost-effective approaches for prioritizing thousands of chemicals targeted for toxicity evaluation (Collins et al., 2008; Hartung and Daston, 2009; Kavlock and Dix, 2010; NRC, 2007; Shukla et al., 2010; Snyder, 2014). Traditional *in vivo* bioassays have a number of drawbacks such as loss of animal lives, high biological variability, cost, complexity, and long durations, making them particularly poorly suited for routine environmental monitoring. Consequently, there has been a shift toward development of batteries of more rapid, less expensive and relatively simple *in vitro* bioassays for water quality screening (Escher et al., 2014; Leusch et al., 2014).

Furthermore, environmental waters are a “cocktail” of chemicals, containing a potentially vast number of natural or synthetic contaminants that could persist through conventional wastewater treatment and ultimately enter surface waters via effluent discharges. Increasingly, water providers are considering alternative water resources, such as recycled water for potable reuse applications (Asano and Levine, 1996; Leverenz et al., 2011; Solley et al., 2010). Water reuse often utilize advanced oxidation processes (AOPs), including ultraviolet light (UV) with hydrogen peroxide and ozone-based technologies. While AOPs have been proven to effectively break down many organic contaminants in water (Gerrity et al., 2011; Martijn and Kruithof, 2012; Pisarenko et al., 2012; Rosario-Ortiz et al., 2010), several studies have also shown that those technologies typically cannot completely remove contaminants and may also produce transformation products of generally unknown structure and toxicity. Therefore, additional information is still needed about the efficiency for each of these processes for attenuating bioactivity and possible toxic effects of transformation products generated by these oxidative technologies. To evaluate the potential toxicity of mixtures of micropollutants in water, an integrated assessment using a battery of *in vitro* bioassays with various endpoints is necessary. The result of the integrated bioassay battery could also help to identify the presence of groups of chemicals with various MOAs, thus providing information that can improve risk assessment methods and inform future water reclamation and management options. In this study, representative water samples were evaluated, including two wastewater treatment plant (WWTP) effluents, five parallel AOP-treated reclaimed water samples, and two groundwater samples supplied by infiltration of wastewater effluents. These water samples were subjected to a battery of 36 *in vitro* bioassays covering a wide range of MOAs, including xenobiotic metabolism, specific

receptor-mediated effects, reactive toxicity and adaptive stress responses, as well as general cytotoxicity. The efficacy of bioactivity attenuation was compared between different water recycling methods. Furthermore, the performance such as the sensitivity of several different assays with the same endpoint was also compared. The results provide an overview of the mixture effects of micropollutants and their removal during treatment representing the most commonly employed water recycling systems in the USA. The methods developed and optimized also serve as a guide for future water quality screening efforts and for assessment of new water treatment technologies.

2. Experimental Section

2.1. Sample preparation

Nine grab samples (4 L) were collected between February and April 2012 from two WWTPs in Pima County, Arizona, US. Both of the municipal wastewater treatment plants consist mainly of clarifiers, biological treatment and chlorination as shown in Fig. 1. Green Valley WWTP secondary effluent (“GV Sec”) served as the source water for an AOP pilot plant (Xylem Inc., Germany) at a flow rate of 2,000 L/h. Grab samples were collected from the pilot plant at the end of each of the four treatment units, including UV (500 mJ/cm^2; “UV”), ozone (3.0 mg/L, “O_3”), ozone/UV (3.0 mg/L O_3 followed by 500 mJ/cm^2 UV; “O_3/UV”), and UV/H_2O_2 (UV 500 mJ/cm^2, 10 mg/L H_2O_2; “UV/H_2O_2”). The WWTP secondary effluent after chlorination (10 mg/L Cl_2 with 2 h contact time, “Cl_2”) was also collected (Fig. 1). To evaluate the bioactivity of municipal wastewater effluent that is subsequently infiltrated using soil aquifer treatment (SAT), effluent was also collected from the Roger Road Wastewater Reclamation Facility (“RR Eff”), and from two monitoring wells after infiltration (“TW1”, which was located after a few weeks of travel time, and “TW2” which is very close to initial zone of infiltration). A MilliQ water field blank (“FB”) was also collected alongside the samples.

Upon collection, samples containing chlorine (*i.e.*, Cl_2 and RR Eff) were quenched immediately with sodium thiosulfate (50 mg/L). Every water sample was treated with sodium azide (1 g/L) as a preservative to inhibit microbial activity (Vanderford et al., 2011) and then stored at 4 °C until extraction (within one week). Solid phase extraction (SPE) was performed according to a previously published method (Leusch et al., 2014). In brief, after filtration (GF/A, Whatman), 1 L of each sample was passed through two tandem solid phase cartridges, Oasis HLB (500mg/6cc, Waters) followed by coconut charcoal (2g/6cc, Supelco), which were both preconditioned with 10 mL of acetone:hexane (1:1, v/v), 10 mL of methanol and 10 mL of MilliQ water. After rinsing with 10 mL of MilliQ water, the cartridges were dried under vacuum for 2 h and individually eluted with 10 mL of methanol and 10 mL of acetone:hexane (1:1, v/v). Eluates from the two SPE cartridges were then combined and evaporated under a gentle stream of nitrogen. Finally, evaporated eluates were reconstituted to 1 mL with methanol and shared among the collaborating laboratories. An aliquot each sample was further concentrated and the solvent changed to DMSO, depending on each particular assay's requirements.

2.2. Selection of bioassay battery

In total, 36 different bioassays were conducted in this study (Table 1). The bioassay battery encompassed five MOA classes (Escher and Leusch, 2012) and were selected based on fully or partially validated for water quality assessments, or to explore new pathways of potential human health concern. The battery included *in vitro* bioassays based on mammalian, bacteria, and yeast cells. Reporter gene assays were most abundantly used because of their higher throughput, greater sensitivity, and specificity in MOA. Specifically, the peroxisome proliferator-activated receptor (PPARγ), which regulates glucose and lipid metabolism, and arylhydrocarbon receptor (AhR), where the cytochrome P450 monooxygenase (CYP) metabolic enzymes were activated by dioxin-like compounds, were selected as bioassays indicative of toxicokinetic processes (xenobiotic metabolism). Assays specific to estrogenic, androgenic, glucocorticoid, progesterone and thyroid activity were included in both agonistic and antagonistic modes to evaluate potential endocrine activity. Cell-based nuclear reporter gene assays were conducted alongside traditional yeast two-hybrid assay (*i.e.*, ER-CALUX vs YES, AR-CALUX vs YAS) and cell proliferation assay (*i.e.*, TR-CALUX vs T-SCREEN) with the same endpoint as a comparison on their sensitivity and selectivity. Activation and detection of adaptive stress response pathways were assessed through oxygen depletion (hypoxia response), oxidative stress (Nrf2-Keap-ARE32 pathway), and an osmotic stress assay (Jurkat cell line). Mutagenicity and genotoxicity were evaluated using AmesII and umuC assays, respectively. Finally, non-specific cell toxicity (cytotoxicity) was measured by assessing cell growth/survival within various assay endpoints, including neutral red uptake (NRU), other common cell viability test (*i.e.*, MTS), and bacteria bioluminescence inhibition (Microtox). Toxicity at the system level was also assessed to a limited extent by determining cytotoxicity in neuroblastoma cells (SK-N-SH) as an indication of neurotoxicity and cytokine production in a monocyte cell line (THP1) as an indication of immunotoxicity. All experiments were performed as described in (Escher et al, 2014) and in the references given in Table 1.

2.3. Data evaluation

Data processing was described in detail in a recent study (Escher et al., 2014), and more details are also provided in the supplementary data. In brief, the concentrations of samples were expressed as relative enrichment factors (REF), which can be derived by the enrichment factor of the sample extraction multiplied by the dilution factor of the extract in each of the assay.

The bioactivity as %effect was obtained by dividing the sample response by the maximum response at the same experimental day after subtracting background signal (control) (Eq-1). The control signal refers to unexposed cells, or, if samples were dosed in solvents, the solvent control.

$$\%effect = \frac{Signal_{sample} - Signal_{control}}{Signal_{\max} - Signal_{control}} \quad (1)$$

EC_{10} was defined as the sample REF causing 10% effect and it can be calculated from the nonlinear regression fit if a full concentration-effect curve was observed. Alternatively, if

there is no full concentration-effect curve, the EC_{10} was calculated from the slope of the linear range of the sample concentration-effect curve (up to 25% of maximum effect) (Eq-2).

$$EC_{10}=\frac{10\%}{slope} \quad (2)$$

In antagonist mode, the effect was expressed as suppression ratio (SR):

$$\mathrm{SR}=1-\frac{Signal_{sample}-Signal_{control}}{Signal_{agonist}-Signal_{control}} \quad (3)$$

$EC_{SR0.2}$ was defined as the sample REF causing 20% of effect suppression, and similar with EC_{10}, it is calculated directly according to 50% effect concentration ($EC_{SR0.5}$) and the hill slope (s) if a full concentration-effect curve exists (Eq-4), or from the slope of the linear regression (up to 0.3 of suppression) if no full concentration-effect curve can be obtained (Eq-5).

$$\log EC_{SR0.2}=\log EC_{SR0.5}+\frac{1}{s}\log(\frac{1}{4}) \quad (4)$$

$$EC_{SR0.2}=\frac{0.2}{slope} \quad (5)$$

The induction ratio (IR) was used as a measure of effect in those bioassays where no maximum response could be obtained (*i.e.*, umuC, Nrf2-MDA-MB and AREc32). The IR is the ratio of the measured signal, *e.g.*, absorbance, relative fluorescence unit (RLU), or relative light unit (RFU), to its control value (Eq-6), and the $EC_{IR1.5}$ (Eq-7) is the sample REF causing an induction ratio of 1.5, which was derived from the linear concentration-effect curve up to an IR of 5, *i.e.*, the linear range of the concentration-effect curve (Escher et al, 2014).

$$IR=\frac{Signal_{sample}}{Signal_{control}} \quad (6)$$

$$EC_{IR1.5}=\frac{0.5}{slope} \quad (7)$$

Detailed data transformation is also provided in the supplementary data.

In this study, the $EC_{IR1.5}$ evaluation was used in the mutagenicity assay, genotoxicity assay, and oxidative stress response assay. For AmesII mutagenicity test, the numbers of revertant were used for calculation thus $EC_{RR1.5}$ was utilized as the expression, where RR is the revertant ratio.

Bioanalytical equivalent concentrations (BEQ) were calculated from the EC_{10} or $EC_{IR1.5}$ of the assay-specific reference compound divided by the EC values from the sample (in unit of REF) using the following equation (Eq-8):

$$BEQ=\frac{EC(reference)}{EC(sample)} \quad (8)$$

Known highly potent bioactive compounds were selected as positive controls as well as reference compounds in each assay. For Caco-2 and SK-N-SH cytotoxicity test, the carrier solvents (MeOH for Caco-2 and DMSO for SK-N-SH) were used as the positive control. The cell viability test of Nrf2-MDA-MB (Nrf2-MTS) was conducted alongside the assay, and thus the same reference compound as the assay was used which was similar to a previous process (Escher et al., 2012).

The efficiency of treatment was then evaluated according to the sample BEQ, which can simply derived from the EC values before and after treatment (Eq-9):

$$BEQ\ reduction(\%)=(1-\frac{BEQ(after\ treatment)}{BEQ(before\ treatment)})\times 100=(1-\frac{EC(before\ treatment)}{EC(after\ treatment)})\times 100 \quad (9)$$

If EC(after treatment) was higher than EC(before treatment), the sample bioactivity decreased during treatment, and the BEQ reduction is a positive value (*i.e.*, removal of bioactive chemicals); in contrast, if the value was negative, BEQ increased, EC decreased, and more toxic transformation byproducts may have been formed after treatment.

3. Results and Discussion

3.1. Performance of the bioassay battery

The detailed assay evaluation and detection results are provided in Table 1 as well as Table S1 in the supplementary data. In most assays in the battery, the effect concentrations (EC_{10}, $EC_{SR0.2}$, $EC_{IR1.5}$ or $EC_{RR1.5}$) of the reference compounds were in nanomolar concentrations or less, which indicate good sensitivity and make them plausible to be applied in environmental water samples where most compounds occur at trace levels. The EC_{50} concentrations of reference compounds were comparable with previous reported data for all bioassays, suggesting the robustness of *in vitro* assays (Table 1). For the same endpoint, the sensitivity was dependent on specific cell lines. For example, the sensitivity of GR-CALUX assay was comparable with the GR-Switchgear and GR-GeneBLAzer assays. However, the sensitivity of the human cell line nuclear receptor assay was much higher than yeast based assay (ER-CALUX vs YES, AR-CALUX vs YAS), which was also consistent with a previous study (Leusch et al., 2010). The result suggests human cell-based assays may be a more preferable choice in the future where sensitivity is critical, such as in drinking and recycled water monitoring. For oxidative stress, the Nrf2-MDA-MB assay ($EC_{IR1.5}$: 33 μM) was one order of magnitude less sensitive than the AREc32 ($EC_{IR1.5}$: 1.7 μM). Also, the sensitivity of T-SCREEN and TRβ-CALUX varied by one to two orders of magnitude. Overall, this study showed that the sensitivity between different bioassays varied largely and

the result of this inter-assay comparison could guide future studies in bioassay selection. Different assays may be suitable under different conditions and drivers depending on their characteristics. For example, yeast-based assays, while less sensitive, are significantly easier and cheaper to run than mammalian-based assays (Leusch et al., 2010), and may be more suitable to high throughput testing of secondary treated wastewater, while the higher sensitivity of mammalian-based assays make them preferable for monitoring high quality water samples, such as drinking or recycled water. However, it should be noted that different assays were conducted in different laboratories, which inherently includes some inter-laboratory variability. It also should be noted that the sample activity is determined not only by the assay's intrinsic sensitivity, but also by the concentration of chemicals in a given sample and how much they were enriched by sample pretreatment (such as SPE). When testing highly treated water samples, efforts should also be made to achieve the highest sample enrichment possible (*i.e.*, highest REF), while ensuring that there is no cytotoxicity from the solvent and the sample. Depending on cell type, the maximum allowable solvent exposed to the cells is generally 0.1% of methanol or 5% of DMSO.

3.2. Effects detected in water samples

Of the 36 bioassays applied, 16 showed response in at least one of the nine water samples tested (Table 1 and Table S1). No significant responses were detected in laboratory or field blanks (Table S1). Eight different endpoints including AhR receptor (AhR-CAFLUX), glucocorticoid effects (GR-Switchgear), algae photosynthesis inhibition (I-PAM), genotoxicity (umuC), mutagenicity (TAmix, TA100, and TA98), oxidative stress (AREc32), and bacteria/algae cytotoxicity (Microtox and algae growth inhibition) showed responses in both of the effluents from municipal wastewater treatment plants (Fig. S1). These results are consistent with a previous study of three Australian WWTPs (Escher et al., 2014; Tang et al., 2014). After different water treatment processes, biological activity was still present in at least 5 out of 7 samples except GR activity (2 out of 7), suggesting current treatment cannot completely remove the chemicals responsible for these biological effects. Detailed EC values and the BEQ reduction in different treatments are compiled in Table 2.

3.2.1. Xenobiotic metabolism—The PPARγ-GeneBLAzer assay showed no response in either agonist or antagonist mode, while the AhR-CAFLUX assay showed positive response in all samples except infiltrated groundwater samples (TW1 and TW2) with EC_{10} values ranging from 6.1 to 51 REF (Table 2 and Table S1). RR Eff had the highest activity (EC_{10} of 6.1 REF) among all of the test samples. In contrast, TW1 and TW2 did not yield any positive responses at an REF up to 100, indicating infiltration processes were able to significantly reduce the AhR activity (>94%). Similarly, EC values between GV Sec and the AOP treated samples suggested that UV, oxidation and chlorination had limited effect on AhR removal (<39%). TCDD equivalent in the two effluents was 6.8 and 34 pg/L and ranged from 4.1 to 21 pg/L in subsequent AOP-treated samples (Table S1), which demonstrates limited attenuation through the treatment processes. The AhR activity in this research was slightly lower than the activity reported in municipal wastewater effluent in Australia (TCDD-EQ 0.59-0.98 ng/L) (Macova et al., 2010); however, the EC_{10} concentrations were comparable with a recent Australia study (Escher et al., 2014). The BEQs were comparable with Chinese WWTP effluents (<14 pg/L TCDD-EQ) (Ma et al.,

2005). A slight increase in activity was observed after chlorination and UV treatment, which likely was caused by transformation products formed during treatment. For instance, it has been reported that the commonly used antimicrobial triclosan can form dioxin-like structures during chlorination (Buth et al., 2011). The Ah receptor is not only activated by known polycyclic aromatic hydrocarbon (PAHs) and polychlorinated biphenyls (PCBs), but also a big range of structurally divergent chemicals including numerous waterborne chemicals (Denison and Nagy, 2003; Martin et al., 2010), which have potential for carcinogenicity (Nagy et al., 2002). The results in this study show that future assessment for AhR activity change during water treatment is warranted.

3.2.2. Specific modes of action—Of the five endocrine nuclear receptors evaluated, only the estrogen and the glucocorticoid receptors were activated by some of the water samples in some of the assays. Only one sample (RR Eff) was found to be estrogenic using the YES assay, with an EC_{10} value of 82 REF. This translates into an EE2-EQ concentration of 1.7 ng/L. In comparison, the ER-CALUX assay did not detect estrogenicity in any of the samples (including that particular sample), which would suggest an EEQ of <0.2 ng/L. This is below a recently derived safe bioassay threshold concentration of 0.2 ng/L (Jarošová et al., 2014; Jarosova et al., 2014), suggesting that all water samples are unlikely to induce negative ecological effects if discharged. It is unclear why the YES result for the RR Eff sample is so high, although previous studies have shown the yeast assays to be particularly sensitive to alkylphenols (Leusch et al., 2010), which are commonly found in wastewater samples (Soares et al., 2008). It is therefore possible that the higher response in the YES assay is due to the presence of alkylphenols in these wastewater effluent samples.

The ER-CALUX samples were tested at a maximum REF of 1 due to conservative consideration of solvent toxicity in the assay (which limited how much sample could be added). This comparatively low REF somewhat limited the overall sensitivity of the method and future work should identify if higher solvent concentration can be used in the CALUX assay, which would improve the overall method detection limit.

It should be mentioned that this study also applied the E-SCREEN assay, and it reported positive responses in 4 out of the 9 samples evaluated. Due to high variability, the results had to be excluded from this paper as they failed quality control. It is interesting to note, however, that the E-SCREEN results showed the same tendencies as the ER-CALUX results. Estrogenicity has been studied in aquatic system for the past two decades, and it has been reported in WWTP effluents worldwide. Despite the low frequency of detection in this study, it remains a highly relevant endpoint and should always be considered in water quality assessment.

Glucocorticoid activity was detected by only one of the three GR cellular assays (GR-Switchgear). Considering their similar sensitivity for the reference compound dexamethasone (Table 1), it is most likely that the difference in detection is due to the comparatively low REF applied for operational reasons in GR-CALUX and GR-GeneBLAzer compared with GR-Switchgear (Table S1). In the GR-Switchgear assay, four out of nine tested samples showed glucocorticoid-activity including GV Sec, O_3, Cl_2 and RR Eff, with the EC_{10} ranging from 8.1 to 12 REF, and dexamethasone equivalent (Dex-

EQ) concentrations ranging from 16-24 ng/L. UV-based technology (including O_3/UV and UV/H_2O_2) and infiltration appeared to be the most efficacious treatment for GR activity attenuation with no activity detected after these treatments. Several glucocorticoid compounds have been detected in municipal wastewater effluents in China (Chang et al., 2007) and the Netherlands (Schriks et al., 2010; Van der Linden et al., 2008), with concentrations of 0.13-1.9 ng/L and 14 ng/L, respectively. Glucocorticoid activity was also reported within hospital wastewater, industry wastewater, WWTP effluent and surface waters (Escher et al., 2014; Schriks et al., 2010; Stavreva et al., 2012) with Dex-EQ reported as 38 ng/L in WWTP effluent through the GR-CALUX assay (Schriks et al., 2010). There are still far fewer studies on the occurrence and impact of glucocorticoid-active substances in the environment compare with estrogenic effects.

Other hormone pathways including androgen, progesterone, and thyroid effects were not observed in any of the water samples, which might be partly due to the low REF used (maximum REF of 1). In contrast, several recent studies have observed a strong anti-androgen effect of the effluent from WWTPs and several possible targeted compounds have been identified using effect-directed analysis (Rostkowski et al., 2011), and progesterone activity has been reported in Australian treated sewage (Leusch et al., 2014). To observe these effects, changes to the sample dosing in the bioassay protocols may be needed to concentrate the samples further, and further sample cleanup prior to dosing might also be necessary to avoid masking by cytotoxicity.

In all of the test samples, the EC_{10} of algae photosynthesis inhibition effect (I-PAM) was between 0.4 (UV/H_2O_2) and 170 REF (TW2), with the diuron-EQ ranging from 0.003 μg/L (TW2) to 1.3 μg/L (UV/H_2O_2). No treatment method except infiltration in this study significantly reduced I-PAM activity. Herbicides are already well-known to impact algae photosynthesis (Muller et al., 2008), and many of them are listed in the US EPA “list of contaminants and their maximum contaminant level” for drinking water regulations (USEPA, 2009). Mixture experiments have also indicated that in water samples the herbicides typically dominate the algal photosynthesis inhibition even if non-specifically acting organic compounds can also trigger those effects (Tang and Escher, 2014). These data show that I-PAM activity can be a useful tool for system phytotoxicity.

3.2.3. Reactive modes of action—Both the mutagenicity and genotoxicity assays rely upon *Salmonella typhimurium* bacteria strains; however, the umuC assay is related to DNA damage repair (Oda et al., 1985) and the Ames test to mutations (Ames et al., 1975). Three different strains were used with the AmesII assay, including TA98 (frameshift mutation), TA100 (base-pair substitution), and TAmix (a 6-strain mixture from TA7001 to TA7006, indicating different location of base-pair substitution). The bacteria strain TA1535 [pSK1002] was used for the umu/SOS reaction. In addition, rat liver metabolic enzyme S9 fractions were applied for umuC, TA98, and TA mixture assays to compare the sample toxicity before and after metabolic activation of organic contaminants present within water extracts.

No positive results were observed in most of the test samples except RR Eff and UV for TA98 assay with and without the addition of S9 fraction (Table 2). The $EC_{RR1.5}$ in RR Eff

was 30 with TA98 without S9 and decreased to 3.5 with S9 activation, suggesting some of the compounds have higher toxicity after metabolic activation. Mutagenic responses were prevalently found in both of TA100 and TAmix strains, which target different location of base-pair substitution. The $EC_{RR1.5}$ of TA100 was much lower than TAmix, suggesting that TA100 has a higher sensitivity than TAmix in detecting mutagenicity for environmental samples, and this is possibly due to a diluted strain density of TAmix compared with pure strain TA100. With TA100 without S9, all test samples except TW1 showed mutagenicity at very low REF from 0.5 to 4.8, while in TAmix the $EC_{RR1.5}$ ranged from 8.5 to 83 REF. The results suggest that TA100 may be a useful strain for base-pair substitution detection in the future mutagenicity test. The results in TA100 and TAmix also suggested that providing enough retention time in SAT would help for mutagenicity removal, when we were comparing the results in TW2 which was more close to the infiltration start point with TW1, where water had gone through four recharge basins (Table 2).

All tested samples showed umuC response with the $EC_{IR1.5}$ value ranged from 42-120 and 13-130 REF in the absence or presence of S9, respectively. The role of *in vitro* S9 metabolic activation appeared to be sample/site dependent in the mutagenicity and genotoxicity test. An increase of genotoxicity was generally observed in all samples originating from GV with the addition of S9; while for RR Eff and its downstream infiltrated TW1 and TW2, samples showed more genotoxicity without the S9 activation (Fig. 2b). While in TAmix mutagenicity test, fewer effects were observed for S9 bioactivation (Fig. 2a). These differences point to a different chemical composition of water samples as well as the different targeting chemicals in these two assays which requires future chemical verification.

The result also showed that few treatment methods except infiltration could effectively remove mutagenicity and genotoxicity. For example, the genotoxic equivalent concentration in two effluents reached 120 and 240 ng/L 4-NQO-EQ in GV Sec and RR Eff, respectively, in the umuC without S9 test (Table S1). High 4-NQO-EQ resulted after AOP treatment (130-290 ng/L) suggest that AOPs using ozone and UV were ineffective in reducing genotoxicity and rather produced reactive intermediates. A similar result was also observed in the mutagenicity test (Table 2 and Table S1). In all cases, infiltration appeared to be able to partially attenuate the effects especially in all of the mutagenicity tests (44%- >97%) and in the umuC with the absence of S9 (52-55%) (Table 2). Both of the tests should be conducted in drinking water system in the future, particularly when considering that both mutagenicity and genotoxicity can induce DNA damage and ultimately lead to the formation of cancerous tumors.

3.2.4. Induction of adaptive stress response pathways—Both osmotic stress and hypoxia induction were negative in all of the samples. In the oxidative stress response, the AREc32 assay showed positive response in 7 out of the 9 samples except the two infiltrated samples; while no activity was found in Nrf2-MDA-MB cell line, which was probably due to the relatively low applied REF and sensitivity in the Nrf2 assay (Table 1 and Table S1).

The $EC_{IR1.5}$ in AREc32 assay ranged from 12 (RR Eff) to 67 (O_3/UV) REF, and the EC of the treated samples were all higher than that of the untreated water GV Sec (Table 2), suggesting that the treatment process partially removed the chemicals that cause oxidative

stress in water. The tBHQ-EQ of two effluents were 13 and 23 μg/L for GV Sec and RR Eff, respectively, which were in a similar range of the value reported in Australia (tBHQ-EQ 41-52 μg/L) for similar treatment processes (Escher et al., 2012). The Nrf2-Keap-ARE pathway plays a central role in the protection of cells against oxidative and xenobiotic damage and a very wide range of chemicals have been reported to induce this pathway. Thus, AREc32 may represent a promising indicator of water treatment efficacy, although the health significance of the oxidative stress concentrations detected in these water samples is currently unclear.

3.2.5. General cytotoxicity and models for system response—Cytotoxicity/cell viability serves as a sum parameter for overall toxicity but was also used to assist the validity of induction assays. In this study, if the cytotoxicity (cell viability inhibition) was larger than 10%, the induction data were not included in the concentration-response assessment. In the literature, some *in vitro* cytotoxicity results were shown to correlate with *in vivo* toxicity tests, for example, EC_{50} values in the Caco2-NRU assay are significantly correlated with rat LD_{50} (Konsoula and Barile, 2005; Payne et al., 2014). Although there are significant uncertainties in extrapolating cytotoxicity results with human health outcomes, the *in vitro* result can still reflect the baseline toxicity of all chemicals in the sample (Escher and Leusch, 2012). No effects were observed in the assay indicative of neurotoxicity. A minimal effect was detected in the immunotoxicity assay with one of the effluent samples (EC_{10} of 0.4 REF in GV Sec; Table S1), but all subsequent treatment removed the effect to below detection limit (>1 REF). In contrast, the bacterial bioluminescence inhibition (Microtox) and algae growth inhibition assays showed detectable responses (Table 2 and Table S1). The relatively low EC values in the Microtox assay confirmed that this assay may be suitable assays for screening the overall cytotoxicity and can be considered in the assessment of environmental water system (Farré et al., 2002; Guzzella et al., 2004). In contrast, algae growth inhibition is strongly influenced by herbicides and this endpoint is therefore likely to not be non-specific although it also responds to baseline toxicants (Tang and Escher, 2014).

3.2.6. Removal efficacy and indicator bioassay—The BEQ reduction/increase during the treatment, which indicates the treatment efficacy, was visualized as a heatmap (Fig. 3). The similarity and correlations of *in vitro* response fingerprints between different water samples as well different assays was characterized by hierarchical clustering. Overall, water treatment efficiently eliminated part of the activity, although this depended both on treatment and individual bioassays. The closest similarity existed between O_3 and O_3/UV, while UV and UV/H_2O_2 are further away in hierarchy distance. Groundwater infiltration appeared to be the most efficient treatment step and removed most of the *in vitro* responses except genotoxicity. Ozone technology was able to significantly remove the oxidative stress assay activity (AREc32); while UV and UV based technology (including UV/H_2O_2 and O_3/UV) appeared to be most efficacious for attenuation of glucocorticoid activity. Conversely, chlorination had fewer effects on most of the assays, but it could partially attenuate genotoxicity and largely decreased algal toxicity. It should also be noted that some of the activity did not change, and in fact even increased after the treatment, such as AhR activity and most of mutagenicity assays after UV treatment (Fig. 3), suggesting limited

removal efficiency and/or production of reactive disinfection by-products generated during the AOP process. This study also suggested that single AOP treatment could not remove all *in vitro* responses; however, a previous study found that AOP combined with other treatments such as active carbon or reverse osmosis could remove most of these effects (Escher et al., 2014; Tang et al., 2014), implying that combination techniques could be applied in future drinking and recycled water treatment.

4. Conclusions

In this study, we selected nine representative water samples including two WWTP secondary effluents, five AOP-treated reclaimed water, as well as two infiltrated groundwater, covering a broad range of different water qualities and subsequent treatment methods. All water extracts were screened in a battery of 18 MOA-based endpoints consisting of 36 bioassays. The result of this study showed that toxicity with different endpoints was observed in all the water samples and current technologies could not completely remove the *in vitro* responses. For xenobiotic metabolism induction, AhR seemed to have the highest induction; while for adaptive stress the AREc32 pathway provided the largest number of positive results and thus these bioassays may serve as indicator bioassay. For specific mode of action (*i.e.*, endocrine effects), estrogenic and glucocorticoid activity showed detectable response in some of the water samples. In addition, some endpoints such as AhR activity, genotoxicity, and mutagenicity were increased after treatment, which may indicate a need for further investigation of possible formation of transformation products. Further study should be conducted to identify the primary chemicals responsible for the observed *in vitro* responses. Furthermore, the information this study has provided, in combination with previous studies (Escher et al., 2014; Leusch et al., 2014; Tang et al., 2014), may be used to establish which bioassays are most suited and relevant for water quality assessment, and guides further bioassay deployment in water monitoring. The conclusion of this study could be used to guide future policy on the selection of wastewater and drinking water treatment techniques. Varying degrees of sample enrichment was identified as a significant variable that affects comparison of bioassay results, and further bioassay work should carefully consider sample enrichment factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. 36 bioassays were used to evaluate 18 modes of toxicity.
2. Reclaimed water and various potable reuse treatment processes were screened.
3. Bioactivity attenuation efficacy during different reuse processes is compared.
4. Recommendations for application of bioscreening tools in water quality evaluations are provided.

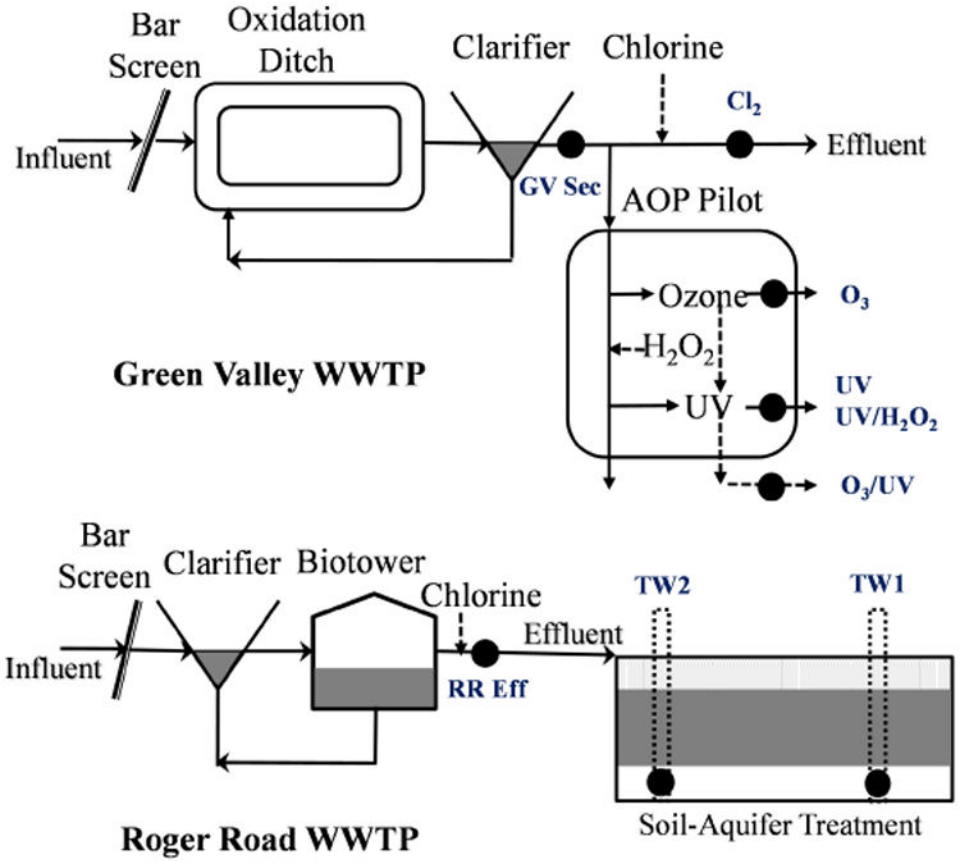

Fig 1.

Treatment process and sampling point at two WWTPs, Tucson, US. (Black dots indicate where the grab samples were collected)

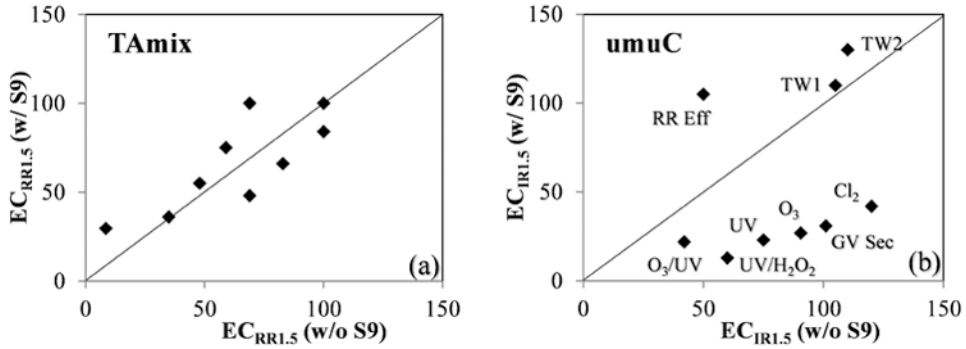

Fig 2.

Comparison of the effect of S9 metabolic activation in mutagenicity and genotoxicity tests. (a) in AmesII TAmix strain. (b) in umuC TA1535 strain.

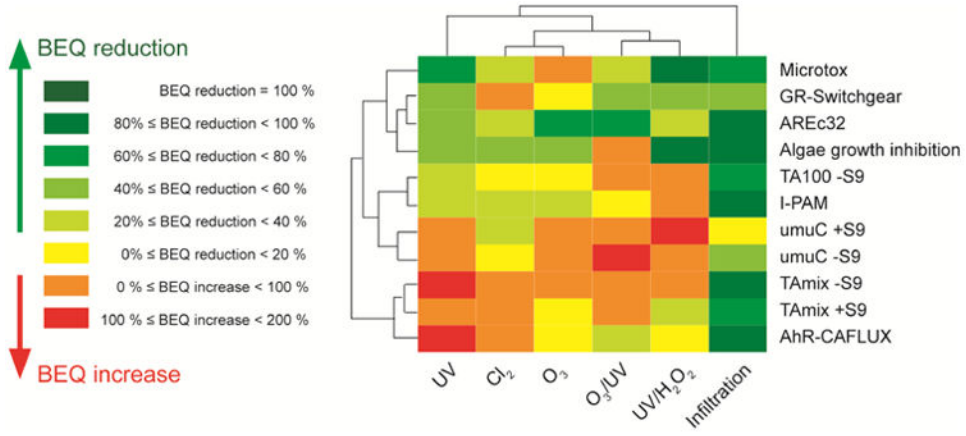

Fig 3.

Summary of test results in 11 primary bioassays (excluding the ones showing no response). Plotted are the bioanalytical equivalent concentration (BEQ) reduction/increase ratio (%) calculated from the sample effect concentration (EC) values before and after treatment. For samples had no activity been detected after treatment, the minimum BEQ reduction value was used using the maximum REF tested.

Table 1

Bioassay evaluation and summary of active water samples.

Endpoint	Bioassay	Method reference	Reference chemical	Assay evaluation by reference chemical	EC_{50} value in literature	Assay response within samples	Number of samples showed positive response in 2 WWTP effluents	Number of samples showed positive response in 7 treated samples
Xenobiotic metabolism								
AhR receptor	AhR CAFLUX	(Jin et al., 2013; Nagy et al., 2002)	TCDD	EC_{10} (0.65 pM); EC_{50} (8.0 pM)	24.8 pM (Macova et al., 2010)	+	2	5
Peroxisome	PPARγ-GeneBLAzer	(US EPA, 2012)	Rosiglitazone	EC_{10} (0.33 nM); EC_{50} (3.9 nM)	3.7 nM (Invitrogen, 2007b)	-	0	0
	PPARγ-GeneBLAzer-Anta	(Huang et al., 2011; US EPA, 2012)	GW9662	$EC_{SR0.2}$ (3.0 nM); $EC_{SR0.5}$ (8.4 nM)	3.5 nM (Invitrogen, 2007b)	-	0	0
Specific modes of action								
Estrogenic effects	ER-CALUX	(Van der Linden et al., 2008)	17β-Estradiol	EC_{10} (0.75 pM); EC_{50} (6.0 pM)	6 pM (Legler et al., 1999), 2 pM (Leusch et al., 2010)	-	0	0
	YES	(Escher et al., 2008b; Routledge and Sumpter, 1996)	Ethinylestradiol	EC_{10} (480 pM); EC_{50} (1700 pM)	800 pM (Conroy et al., 2007), 370 pM (Leusch et al., 2010)	+	1	0
	ER-CALUX-Anta	(Leusch et al., 2014)	Tamoxifen	$EC_{SR0.2}$ (1.5 nM); $EC_{SR0.5}$ (3.7 nM)	n/a	-	0	0
Androgenic effects	AR-CALUX	(Van der Linden et al., 2008)	Dihydrotestosterone	EC_{10} (0.1 nM); EC_{50} (1.0 nM)	0.13 nM (Sonneveld et al., 2005)	-	0	0
	YAS	(Sohoni and Sumpter, 1998; Thomas et al., 2002)	Testosterone	EC_{10} (4.1 nM); EC_{50} (9.6 nM)	3 nM (Conroy et al., 2007)	-	0	0
	AR-CALUX-Anta	(Leusch et al., 2014)	Flutamide	$EC_{SR0.2}$ (1.1 μM); $EC_{SR0.5}$ (3.6 μM)	0.45 μM (van der	-	0	0

Endpoint	Bioassay	Method reference	Reference chemical	Assay evaluation by reference chemical	EC_{50} value in literature	Assay response within samples	Number of samples showed positive response in 2 WWTP effluents	Number of samples showed positive response in 7 treated samples
					Burg et al., 2010 Burg et al., 2010			
Glucocorticoid effects	GR-CALUX	(Van der Linden et al., 2008)	Dexamethasone	EC_{10} (0.8 nM); EC_{50} (3.7 nM)	0.37 nM (Sonneveld et al., 2005)	-	0	0
	GR-GeneBLAzer	(Huang et al., 2011; US EPA, 2012)	Dexamethasone	EC_{10} (0.6 nM); EC_{50} (2.5 nM)	1.8 nM (Invitrogen, 2007a)	-	0	0
	GR-Switchgear	(Switchgear, 2010a)	Dexamethasone	EC_{10} (0.5 nM); EC_{50} (2.6 nM)	n/a	+	2	2
	GR-CALUX-Anta	(Van der Linden et al., 2008)	Mifepristone	$EC_{SR0.2}$ (2.9 nM); $EC_{SR0.5}$ (6.0 nM)	n/a	-	0	0
	GR-GeneBLAzer-Anta	(Escher et al., 2014)	Mifepristone	$EC_{SR0.2}$ (0.1 nM); $EC_{SR0.5}$ (0.4 nM)	0.16 nM (Invitrogen, 2007a)	-	0	0
Progestagenic effects	PR-CALUX	(Van der Linden et al., 2008)	Levonorgestrel	EC_{10} (0.3 nM); EC_{50} (5.7 nM)	n/a	-	0	0
	PR-CALUX-Anta	(Sonneveld et al., 2005)	Mifepristone	$EC_{SR0.2}$ (20 pM); $EC_{SR0.5}$ (78 pM)	n/a	-	0	0
Thyroid effects	T-SCREEN	(Gutleb et al., 2005)	Triiodothyronine	EC_{10} (280 pM); EC_{50} (740 pM)	191 pM (Gutleb et al., 2005)	-	0	0
	TRβ-CALUX	(Leusch et al., 2014)	Triiodothyronine	EC_{10} (8.6 pM); EC_{50} (86 pM)	n/a	-	0	0
Phytotoxicity (photosynthesis inhibition)	I-PAM	(Escher et al., 2008a)	Diuron	EC_{10} (2.3 nM); EC_{50} (6 nM)	68.6 nM (Macova et al., 2010), 12.8 nM (Escher et al., 2008a)	+	2	7
Reactive modes of action								
Genotoxicity	umuC -S9	(ISO13828, 1999; Oda et al., 1985)	4-NQO	$EC_{IR1.5}$ (63.5 nM)	n/a	+	2	7

Endpoint	Bioassay	Method reference	Reference chemical	Assay evaluation by reference chemical	EC_{50} value in literature	Assay response within samples	Number of samples showed positive response in 2 WWTP effluents	Number of samples showed positive response in 7 treated samples
	umuC +S9	(ISO13828, 1999; Oda et al., 1985)	2-Aminoanthracene	$EC_{IR1.5}$ (0.29 μM)	n/a	+	2	7
Mutagenicity	TA98 -S9	(Heringa et al., 2011; Umbuzeiro et al., 2010)	4-NQO	$EC_{RR1.5}$ (n/a)*	n/a	+	1	0
	TAmix -S9	(Heringa et al., 2011)	4-NQO	$EC_{RR1.5}$ (n/a)*	n/a	+	1	1
	TA98 +S9	(Heringa et al., 2011; Umbuzeiro et al., 2010)	2-Aminoanthracene	$EC_{RR1.5}$ (n/a)*	n/a	+	2	5
	TAmix +S9	(Heringa et al., 2011)	2-Aminoanthracene	$EC_{RR1.5}$ (n/a)*	n/a	+	2	5
	TA100 -S9	(Reifferscheid et al., 2012)	2-Nitrofluorene	$EC_{RR1.5}$ (66 μM)	n/a	+	2	6
Induction of adaptive stress response pathways								
Osmotic stress	Jurkat E6-1	(Escher et al., 2014)	PMA	EC_{10} (3 nM); EC_{50} (20 nM)	n/a	+	1	0
Adaptive stress	Hypoxia-Switchgear	(Switchgear, 2010b)	Desferrioxamine	$EC_{IR1.5}$ (7.5 μM)	n/a	-	0	0
Oxidative stress	Nrf2-MDA-MB	(Wang et al., 2008; Zhang, 2006)	tBHQ	$EC_{IR1.5}$ (33 μM)	n/a	-	0	0
	AREc32	(Escher et al., 2012; Wang et al., 2006)	tBHQ	$EC_{IR1.5}$ (1.7 μM)	1.32 μM (Escher et al., 2012)	+	2	5
General cytotoxicity and modes of system response								
Bacterial cytotoxicity	Microtox	(Tang et al., 2013)	Phenol	n/a	n/a	+	2	7
Neurotoxicity	SK-N-SH	(Manger et al., 2003)	DMSO	EC_{10} (0.11 M); EC_{50} (0.38 M)	n/a	-	0	0
Immunotoxicity	THP1-cp	(Leusch et al., 2014)	Dexamethasone	EC_{10} (2.1 nM); EC_{50} (45 nM)	2.7 nM (Leusch et al., 2014)	+	1	1
Algae growth inhibition	Algae Growth Inhibition	(Escher et al., 2008a)	Diuron	EC_{10} (7 nM); EC_{50} (25 nM)	135 nM (Escher et al., 2008a)	+	2	7

Endpoint	Bioassay	Method reference	Reference chemical	Assay evaluation by reference chemical	EC_{50} value in literature	Assay response within samples	Number of samples showed positive response in 2 WWTP effluents	Number of samples showed positive response in 7 treated samples
Human cellular Cytotoxicity	Caco-2 NRU	(Leusch et al., 2014)	Methanol	EC_{10} (0.4 M); EC_{50} (2.3 M)	n/a	-	0	0
	Nrf2-MTS	(Escher et al., 2012)	tBHQ	EC_{10} (103 μM); EC_{50} (178 μM)	46 μM (Escher et al., 2012)	-	0	0

Abbreviations used: TCDD = 2,3,7,8-TCDD; 4-NQO = 4-Nitroquinoline N-oxide; PMA = Phorbol-12-myristate-13-acetate; tBHQ = tert-Butylhydroquinone; DMSO = Dimethylsulfoxide. n/a = Data not available.

* the data was not available in TA98 and TAmix mutagenicity assay in this study, because only 2 dose points of positive control were applied in each plate due to operatical limit.

Table 2

Summary of sample effect concentration (EC, expressed as relative enrichment factor REF) and BEQ reduction/increase values (%) compared to the influent ("GV Sec" or "RR Eff") in all tested treatments in the subset of bioassays that showed activity in one or more samples (for description of sampling sites see Figure 1).

Bioassay		FB [a]	GV Sec	UV	UV/H_2O_2	O_3	O_3/UV	Cl_2	RR Eff	Infiltration	
										TW1	TW2
AhR CAFLUX	**EC (REF)**	>100	31	10	31	32	51	16	6.1	>100	>100
	BEQ reduction			(198) [b]	0	3	39	(94)		>94 [c]	>94
YES	**EC (REF)**	>200	>200	>200	>200	>200	>200	>200	82	>200	>200
	BEQ reduction									>59	>59
GR-Switchgear	**EC (REF)**	>20	10	>20	>20	12	>20	9.5	8.1	>20	>20
	BEQ reduction			>50	>50	17	50	(6)		>59	>59
I-PAM	**EC (REF)**	>333	0.65	1.0	0.4	1.0	0.7	1.0	2.0	150	170
	BEQ reduction			35	(63)	35	7	35		>99	>99
umuC -S9	**EC (REF)**	>139	100	75	60	90	42	120	50	105	110
	BEQ reduction			(35)	(68)	(12)	(140)	16		52	55
umuC +S9	**EC (REF)**	>139	31	23	13	27	22	42	105	110	130
	BEQ reduction			(35)	(138)	(15)	(41)	26		5	19
TA98 -S9	**EC (REF)**	>100	>100	>100	>100	>100	>100	>100	30	>100	>100
	BEQ reduction									>70	>70
TA98 +S9	**EC (REF)**	>100	>100	72	>100	>100	>100	>100	3.5	>100	>100
	BEQ reduction			>(39)						>97	>97
TAmix -S9	**EC (REF)**	>100	83	35	69	59	48	69	8.5	>100	>100
	BEQ reduction			(137)	(20)	(41)	(73)	(20)		>92	>92
TAmix +S9	**EC (REF)**	>100	66	36	>100	75	55	48	29	>100	84
	BEQ reduction			(83)	>34	12	(20)	(38)		>71	65
TA100 -S9	**EC (REF)**	>20	0.6	0.8	0.5	0.7	0.5	0.7	2.7	>20	4.8
	BEQ reduction			25	(20)	14	(20)	14		>87	44
AREc32	**EC (REF)**	>160	22	43	30	60	67	30	12	>160	>160
	BEQ reduction			49	27	63	67	27		>93	>93

Bioassay		FB [a]	GV Sec	UV	UV/H_2O_2	O_3	O_3/UV	Cl_2	RR Eff	Infiltration	
										TW1	TW2
Microtox	**EC (REF)**	54	7.1	25	47	4.7	9.0	9.7	4.1	9.6	12
	BEQ reduction			71	85	(51)	21	27		57	67
Algae growth inhibition	**EC (REF)**	>333	1.0	2.1	7.0	2.0	0.7	2.0	3.2	>333	>333
	BEQ reduction			52	86	50	(43)	50		>99	>99

[a] FB = Field Blank.

[b] (), negative value, which indicate the BEQ increased after treatment.

[c] >, the BEQ reduction was calculated based on the maximum applied REF where no bioactivity was observed, indicating the minimum BEQ reduction the treatment can achieve.