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Bioanalytical tools for the evaluation of organic micropollutants during sewage treatment, water recycling and drinking water generation

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ABSTRACT

A bioanalytical test battery was used for monitoring organic micropollutants across an indirect potable reuse scheme testing sites across the complete water cycle from sewage to drinking water to assess the efficacy of different treatment barriers. The indirect potable reuse scheme consists of seven treatment barriers: (1) source control, (2) wastewater treatment plant, (3) microfiltration, (4) reverse osmosis, (5) advanced oxidation, (6) natural environment in a reservoir and (7) drinking water treatment plant. Bioanalytical results provide complementary information to chemical analysis on the sum of micropollutants acting together in mixtures. Six endpoints targeting the groups of chemicals with modes of toxic action of particular relevance for human and environmental health were included in the evaluation: genotoxicity, estrogenicity (endocrine disruption), neurotoxicity, phytotoxicity, dioxin-like activity and non-specific cell toxicity. The toxicity of water samples was expressed as toxic equivalent concentrations (TEQ), a measure that translates the effect of the mixtures of unknown and potentially unidentified chemicals in a water sample to the effect that a known reference compound would cause. For each bioassay a different representative reference compound was selected. In this study, the TEQ concept was applied for the first time to the *umuC* test indicative of genotoxicity using 4-nitroquinoline as the reference compound for direct genotoxicity and benzo[a]pyrene for genotoxicity after metabolic activation.

The TEQ were observed to decrease across the seven treatment barriers in all six selected bioassays. Each bioassay showed a differentiated picture representative for a different group of chemicals and their mixture effect. The TEQ of the samples across the seven barriers were in the same order of magnitude as seen during previous individual studies in wastewater and advanced water treatment plants and reservoirs. For the first time a benchmarking was performed that allows direct comparison of different treatment technologies and covers several orders of magnitude of TEQ from highly contaminated sewage to drinking water with TEQ close or below the limit of detection. Detection limits of the bioassays were decreased in comparison to earlier studies by optimizing sample preparation and test protocols, and were comparable to or lower than the quantification limits of the routine chemical analysis, which allowed monitoring of the presence and

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removal of micropollutants post Barrier 2 and in drinking water. The results obtained by bioanalytical tools were reproducible, robust and consistent with previous studies assessing the effectiveness of the wastewater and advanced water treatment plants. The results of this study indicate that bioanalytical results expressed as TEQ are useful to assess removal efficiency of micropollutants throughout all treatment steps of water recycling.

1. Introduction

The indirect potable reuse scheme (IPR, http://www.westerncorridor.com.au) investigated in this study is the largest potable water recycling scheme in Australia and one of the largest in the Southern Hemisphere (Freeman et al., 2008; Traves et al., 2008). The scheme consists of seven treatment barriers: 1 – source control; 2 – wastewater treatment plant (WWTP); 3 – microfiltration; 4 – reverse osmosis; 5 – advanced oxidation (combining hydrogen peroxide and UV irradiation); 6 – natural environment; and 7 – drinking water treatment plant. It takes treated wastewater from five of the largest wastewater treatment plants in the greater Brisbane area and treats this water to potable standards via three advanced water treatment plants. The resulting purified recycled water (PRW) can then be piped to Lake Wivenhoe, the largest of the freshwater reservoirs in South East Queensland (SEQ). This reservoir supplies greater than 60% of the freshwater resources for the city of Brisbane. The production of the PRW is based on international experiences of other recycling plants such as Water Factory 21 in California, Singapore's NEWater, and the Torreele project in Belgium. All of these schemes use a similar treatment process of water treatment plants followed by membrane and reverse osmosis filtration and at least UV disinfection. The Torreele and Water Factory 21 schemes then add the purified recycled water to a local aquifer prior to recovery and addition to the drinking water system. Singapore's NEWater is the same as the indirect potable reuse scheme in SEQ in that the purified water is added to a reservoir.

The water produced in the studied PRW scheme meets potable standards, but is presently only used for industrial purposes and has not yet been introduced to Lake Wivenhoe. Supplementation of drinking water storage reservoirs is envisaged only after the combined level of water in the three major SEQ reservoirs falls below 40%. Water at all stages of the treatment process is subject to quality monitoring to assess the efficacy of the treatment barriers and to ensure the water meets health and safety requirements. A number of organic and inorganic micropollutants have been monitored during the last two years in PRW (Queensland Water Commission, 2009; WaterSecure, 2010; Hawker et al., 2011).

Toxicity testing may provide complementary information to chemical analysis on the sum of micropollutants present during water treatment. Therefore, a bioanalytical "mode of action" test battery, developed or optimized at Entox in collaboration with colleagues from the Swiss Federal Institute of Aquatic Science and Technology, has been included in water recycling projects to support water quality assessment. Bioanalytical techniques have been selected to target the groups of chemicals of particular relevance for human and environmental health including genotoxicity, endocrine activity, neurotoxicity, dioxin-like activity and non-specific cell toxicity (Escher et al., 2008, 2009; Macova et al., 2010). For better comparability, the results in all toxicity tests were expressed as toxic equivalent concentrations that give an account of the concentration of a reference chemical that would elicit the same effect as the sample does (Villeneuve et al., 2000). The TEQ concept was previously established for five of the bioassays used (Escher et al., 2008; Macova et al., 2010) and was newly developed for the umuC assay for genotoxicity (International Organization for Standardization, 2000) in the present study.

The goal of this study was to evaluate the applicability of this bioanalytical test battery for monitoring the micropollutants across all seven barriers of the indirect potable reuse scheme and to obtain a benchmark of water quality that may serve in the future for classification of water samples from emerging technologies and for alternative source water such as stormwater and bore water. To achieve these goals, the existing and validated bioassay test battery was further optimized to achieve lower detection limits and a testing strategy was developed to allow the assessment of samples with a wide range of chemical contamination level.

2. Materials and methods

2.1. Samples and sites

Grab samples were collected at 21 sites across the seven barriers of the indirect potable reuse scheme (Table 1, for a map see http://www.westerncorridor.com.au/resources/factsheets, select South East Queensland Water Grid): raw wastewater and tractor effluent at the Oxley Creek wastewater treatment plant (WWTP) (Barrier 1–2), product water from microfiltration, reverse osmosis and advanced oxidation at the Bundamba advanced water treatment plant (AWTP), PRW from the Bundamba off-take, Lowood and Caboonbah Pipeline (Barrier 3–5), water from the Swanbank Power Station lake, Lake Wivenhoe and mid-Brisbane river representing the natural environment (Barrier 6), as well as samples from the inlet and outlet of the Mt. Crosby drinking water treatment plant (DWTP) and the drinking water distribution system (DWS) (Barrier 7). While PRW has not been introduced into the drinking water reservoir Lake Wivenhoe, the Power Station lake is a reservoir that receives PRW. Sampling was complemented by three additional samples collected at Caboolture WWTP and Caboolture enhanced

Table 1 – Sample description.

Barrier	Sample location	Sampling date	Sample volume (L)
Barrier 1–2	Oxley Ck WWTP Inlet	28.10.2009	0.5
	Oxley Ck WWTP Activated Sludge	28.10.2009	1.0
	Oxley Ck WWTP post Clarifiers	28.10.2009	2.0
	Oxley Ck WWTP post UV	28.10.2009	2.0
Barrier 3–5	Bundamba AWTP pre MF (Inlet)	29.10.2009	2.0
	Bundamba AWTP post MF	29.10.2009	4.0
	Bundamba AWTP post RO	29.10.2009	4.0
	Bundamba AWTP post AO	29.10.2009	4.0
	Bundamba AWTP RO concentrate	29.10.2009	1.0
	PRW pipeline (Bundamba off-take)	08.12.2009	4.0
	PRW pipeline (Lowood)	08.12.2009	4.0
	PRW pipeline (Caboonbah)	08.12.2009	4.0
Barrier 6[a]	Power Station lake	08.12.2009	4.0
	Lake Wivenhoe – Logan's Inlet	13.10.2009	4.0
	Lake Wivenhoe – Dam Wall	13.10.2009	4.0
	Mid-Brisbane – Lowood	13.10.2009	4.0
	Mid-Brisbane – Burton's Bridge	13.10.2009	4.0
Barrier 7	Mt Crosby DWTP Intake (raw)	13.10.2009	4.0
	Mt Crosby DWTP Outlet	13.10.2009	4.0
	Drinking Water System – mid way on distribution line	19.11.2009	4.0
	Drinking Water System – towards end of distribution line	19.11.2009	4.0
Others	South Caboolture WWTP Influent (raw)	22.10.2009	0.5
	South Caboolture EWTP Influent (Effluent from WWTP)	22.10.2009	2.0
	South Caboolture EWTP Effluent	22.10.2009	4.0
	Bottled Water type 1[b]	08.12.2009	4.0
	Bottled water type 2[b]	08.12.2009	4.0
	Hinze Dam (Lake Advancetown)	28.10.2009	4.0
	Gold Coast Water Distribution system	06.11.2009	4.0

a There is a discontinuation between the AWTPs and the natural environment. The resulting PRW is currently used for industrial purposes and has not been reintroduced to the Lake Wivenhoe at this time but is supplementing the Power Station lake.
b Purchased in a Brisbane supermarket on 29.10.2009.

water treatment plant (EWTP) fed with the Caboolture WWTP effluent, to compare the treatment/removal efficiency of the membrane processes with ozonation followed by biologically activated carbon. Two samples were collected at the Gold Coast Hinze Dam (Lake Advancetown) and water distribution system to compare drinking water sourced from different catchments. Two types of bottled water were purchased at a local supermarket to compare with the quality of the purified recycled water (Table 1).

Based on previous studies (Macova et al., 2010), collected sample volumes ranged from 0.5 L–4 L depending on the expected toxicity of the samples (Table 1). Different sampling volume allows us to test the sample extracts across the bioanalytical test battery without pre-dilution of the extract and to achieve low limits of detection in the assay. Samples were kept on ice during transport and until processing. Samples were extracted by solid phase extraction within 24 h of collection.

2.2. *Solid phase extraction*

Immediately after sampling, 1 mL of 0.1% sodium thiosulphate was added per 1 L of sample to neutralise the presence of chlorine and concentrated HCl (36%) was added to a final concentration of 5 mM for preservation. It was demonstrated in earlier work that a pharmaceutical cocktail in a wastewater matrix had highest recoveries for HLB at pH 3 (Escher et al., 2005).

Samples were extracted using 1 g OASIS® HLB solid phase material in 20 mL cartridges (Waters, Australia) following filtration with a glass fibre filter. After conditioning the cartridges with 10 mL methanol and 20 mL of 5 mM HCl in MilliQ water, a known volume of sample was percolated under vacuum. Cartridges were sealed and kept at −20 °C until elution with the solvent mixture.

Immediately before elution, the cartridges were dried for 2–3 h under vacuum and were eluted with 10 mL methanol and 10 mL hexane:acetone (1:1). All eluates were evaporated to approximately 1 mL under purified nitrogen gas and were solvent exchanged to methanol at a final volume of 500 μL.

2.3. *Bioanalytical tools*

An overview on the bioanalytical test battery comprising six endpoints is given in Table 2. The phytotoxicity assay with the green algae Pseudokirchneriella subcapitata was performed as described by (Escher et al., 2008). The detailed methodology of the remaining bioanalytical techniques was described in (Macova et al., 2010).

Bioanalytical results were reported in terms of toxic equivalent concentrations (TEQ) (Villeneuve et al., 2000; Escher et al., 2008; Macova et al., 2010) using a corresponding reference compound representing the group of targeted chemicals in a given assay (Table 2). In previous work, we had not used the TEQ concept for the *umuC* assay for genotoxicity. Here we tested a series of potential reference compounds (see

Table 2 – Overview of the bioanalytical test battery (adapted from Macova et al. (2010)).

Assay	Targeted chemicals	Reference compound	Result expression	Literature reference
Bioluminescence inhibition in *Vibrio fischeri*	All chemicals	Virtual baseline toxicant; Phenol[a]	Baseline toxicity equivalent concentrations (baseline-TEQ)	(International Organization for Standardization, 1998; Johnson, 2005; Farré et al., 2006)
Neurotoxicity – AChE	Organophosphates and carbamate insecticides	Parathion	Parathion equivalent concentrations (PTEQ)	(Ellman et al., 1961; Hamers et al., 2000)
Phytotoxicity – Max-I-PAM	Triazine and phenylurea herbicides	Diuron	Diuron equivalent concentrations (DEQ)	(Schreiber et al., 2002; Schreiber et al., 2007)
Estrogenicity – E-SCREEN	Estrogens, estrogenic industrial chemicals	17β-Estradiol (E_2)	Estradiol equivalent concentrations (EEQ);	(Soto et al., 1995; Korner et al., 1999)
Ah-Receptor – AhR-CAFLUX	Polychlorinated dibenzodioxins/furans and biphenyls, polycyclic aromatic hydrocarbons	2,3,7,8-Tetrachlorodibenzo dioxin (TCDD)	TCDD equivalent concentrations (TCDDEQ)	(Nagy et al., 2002; Zhao and Denison, 2004)
Genotoxicity – *UmuC*	Chlorinated byproducts, aromatic amines, polycyclic aromatic hydrocarbons	(–S9) 4-nitroquinoline -N-oxide (4NQO) (+S9) Benzo[a]pyrene (BaP)	4NQO and BaP equivalent concentrations (4NQOEQ and BaPEQ)	(Oda et al., 1985; Reifferscheid et al., 1991; International Organization for Standardization, 2000)

a Phenol was only used as positive control, not as reference compound.

Supplementary Information) and validated 4NQO as the reference compound without prior metabolic activation by a rat liver S9 enzyme extract and benzo[a]pyrene as the reference compound for genotoxicity after metabolic activation.

2.4. QA/QC

For quality and assurance purposes, all samples were collected in duplicates and were extracted and analysed across the bioanalytical test battery to assess the repeatability of the SPE and the bioassay. Both replicates of the sample extract were tested in duplicates or triplicates per run depending on the assay, with the standard error typically between 10 and 15%. To assess the day-to-day variation of the assays, a second replicate of the sample extract was analysed in bioassays on a different day than the first replicate. Final TEQs were expressed as the average ± standard deviation of two independent replicates reflecting day-to-day variation of the assays. Another QA/QC parameter of the bioanalytical results was the long term record of the EC_{50} values of the reference compounds. If the EC_{50} value of the reference compound of a given run varied more than three times the standard deviation of the long term average, the results were not included and the run was repeated. To assess any effect associated with the extraction process or with the solvent, MilliQ water was processed the same way as the samples and assessed in all bioassays as a procedural blank.

There are two aspects that influence the limit of detection: the variability of the response in a given assay assessed by the concentration-effect curve of the reference compound and the maximum enrichment of the sample that could be achieved in the assay. The detection limits of all assays were defined as three times standard deviation of the control response. For example, if the average of effect of the control was 2.3 ± 3.1%, then the LOD was assigned to the concentration of sample that produced 3 × 3.1% = 9.3% effect. For the bacteria *Vibrio fischeri* the control diluted in the medium was used to derive this standard deviation, for the other five bioassays the response using the lowest concentration of the reference compound that induced an effect significantly different from the control was used. Typically, the thus derived standard deviation was in the range of an effect level of 8–10% effect. Since four different volumes of the samples were extracted depending on the expected toxicity (0.5, 1, 2 and 4 L), there are four different LODs for each assay summarised in Table 3.

Analysis of variance (ANOVA, GraphPad Prism, San Diego, CA, USA) was used to analyse statistical differences among the average TEQs of the samples.

3. Results and discussion

3.1. *Baseline toxicity – bioluminescence inhibition in* Vibrio fischeri

The baseline–TEQs were observed to decrease by 94% after treatment in the Oxley Creek WWTP (Barrier 2), from 25.6 mg/L in the influent to 1.26 mg/L after activated sludge treatment with no further decrease post clarifiers or UV treatment (Table 4, Fig. 1). Microfiltration at Bundamba AWTP (Barrier 3) significantly increased baseline toxicity from 0.91 to 2.66 mg/L. This increase may be caused by chloramination that is preceeding microfiltration or by removal of particulate matter by microfiltration and release of micropollutants into the dissolved phase, in which they are bioavailable. Reverse osmosis and advanced oxidation at the Bundamba AWTP (Barrier 4–5) decreased the baseline toxicity to 0.42 and 0.12 mg/L representing 44% and 13%, respectively, of the original activity in the Bundamba AWTP inlet; the latter not being significantly different from the blank (0.08 mg/L, *t*-test, $p = 0.304$).

Baseline toxicity of the sample after Barrier 2 treatment of the indirect potable reuse scheme was comparable with the effluent of the Caboolture WWTP of 1.0 mg/L, representing 11% of the toxicity equivalents in the influent. Effluent from the conventional Caboolture WWTP was further treated in the Caboolture enhanced water treatment plant (EWTP) with

Table 3 – Limits of detection of the assays for individual sample volume.

Assay	Result expression	LOD for different sample volume			
		0.5 L	1 L	2 L	4 L
Baseline Toxicity – Bioluminescence inhibition in *Vibrio fischeri*	Baseline-TEQ	0.1 mg/L	0.05 mg/L	0.02 mg/L	0.01 mg/L
Neurotoxicity – AChE	PTEQ	0.4 μg/L	0.2 μg/L	0.1 μg/L	0.06 μg/L
Phytotoxicity – Max-I-PAM	DEQ	0.05 μg/L	0.02 μg/L	0.01 μg/L	0.005 μg/L
Estrogenicity – E-SCREEN[a]	EEQ	0.08 ng/L	0.04 ng/L	0.02 ng/L	0.01 ng/L
Ah-Receptor – AhR-CAFLUX	TCDDEQ	0.09 ng/L	0.05 ng/L	0.02 ng/L	0.01 ng/L
Genotoxicity – *UmuC*	(−S9) 4NQOEQ	0.4 μg/L	0.2 μg/L	0.1 μg/L	0.05 μg/L
	(+S9) BaPEQ	6.4 μg/L	3.2 μg/L	1.6 μg/L	0.8 μg/L

a If the maximum proliferation of the sample did not reach 50% of the 17β-estradiol, the sample was not classified as estrogenic, therefore EEQ was not quantified and the results were reported as below quantification limit of the assay (Soto et al., 1995).

ozonation and activated carbon treatment (van Leeuwen et al., 2003; Reungoat et al., 2010). Baseline toxicity in the final effluent was decreased to 0.56 mg/L, a level not significantly different from the blank. Results are in agreement with the previous study at the Caboolture EWTP, where the baseline toxicity was reduced throughout the enhanced treatment chain from 2.3 mg/L to 0.52 mg/L in the final effluent (Macova et al., 2010). Baseline toxicity of the Caboolture EWTP final effluent was comparable with the baseline toxicity post Barrier 4 of the IPR, reverse osmosis at Bundamba AWTP.

Apart from the Mt. Crosby outlet sample, the baseline toxicity of samples collected after Barrier 5 was on average elevated by a factor of two as compared to the blank and not significantly different from the bottled water (*t*-test, $p = 0.25$) (Table 4, Fig. 1).

The observed increased baseline toxicity of the Mt. Crosby outlet sample as compared to the inlet of the drinking water plant was reproducible in a second sampling campaign but the level is of no concern relating to potential health impacts because they are below permissible effect levels modelled for this endpoint under the assumption that all chemical concentrations are present at or below their drinking water guideline values using methods detailed in Vermeirssen et al. (2010) and model input parameters from Hawker et al. (2011).

Furthermore this effect decreased significantly in the drinking water supply pipeline and might be related to intermittent production of chlorinated disinfection byproducts or assimilable organic carbon.

3.2. *Estrogenic activity – E-SCREEN assay*

The estrogenic effect of the samples, expressed as estradiol equivalent concentration (EEQ), decreased by 86% during activated sludge treatment after Barrier 2 from 3.2 ng/L in Oxley Creek WWTP influent to 0.44 ng/L in the effluent (Table 4, Fig. 1). EEQ was further reduced after the clarifiers but UV treatment did not alter the EEQ. Microfiltration at the Bundamba AWTP (Barrier 3) reduced estrogenic effect to the level below the detection limit of the assay (<0.01 ng/L). The reverse osmosis concentrate, where micropollutants are enriched by a factor of six to eight times, did show appreciable estrogenic activity, indicating that despite the EEQ being below the LOD after microfiltration, there were still residual estrogenic compounds but they were rejected by reverse osmosis. No further alteration in the estrogenicity was observed in any sample collected after Barrier 3. Results are in agreement with the previous findings where no estrogenic response (EEQ <0.01 ng/L) was observed in any sample collected at Lake Wivenhoe (Seqwater project 2007, unpublished data). The estrogenic effects of the samples collected at Hinze Dam (Lake Advancetown), the Gold Coast Water distribution system and bottled water were also below the LOD of the assay (<0.01 ng/L for 4 L water samples).

Estrogenicity of the influent to the Oxley Creek WWTP was lower than typically seen in the raw sewage samples. EEQ in the raw sewage sample from the Caboolture WWTP in a previous study ranged from 68 to 91 ng/L in three different samples collected in the course of one month (2008, unpublished results). This is comparable with the EEQ reported in the raw sewage in the Brisbane area using the same bioassay and with other bioassays for estrogenicity (reviewed in Macova et al. (2010)).

The EEQ of the additional sample collected at the Caboolture WWTP was 18.5 ng/L, which is again in the lower range of what is typically seen in raw sewage. Surprisingly, the EEQ was reduced to below the LOD of the assay of 0.02 ng/L (for 2 L sample) already by the treatment in the conventional WWTP. In a previous study, this WWTP effluent, which was an influent to the enhanced treatment plant, exhibited EEQ of 6 ng/L (Macova et al., 2010). Estrogenic effect was markedly decreased by ozonation and further reduced to below LOD (<0.02 ng/L) by activated carbon treatment, key steps of the enhanced treatment chain that are effective in the removal of the estrogenic effect (Macova et al., 2010). High removal efficiency of ozonation on the estrogenic activity is consistent with literature (Lee et al., 2008; Escher et al., 2009).

3.3. *Neurotoxicity – acetylcholinesterase inhibition assay*

Results of the bioassay targeting organophosphates and carbamate insecticides, expressed as parathion equivalent concentration (PTEQ), showed 78% decrease in the toxicity post Barrier 2, from 4.36 μg/L in the Oxley Creek WWTP inlet to 0.94 μg/L after activated sludge treatment, with no further decrease post clarifiers or UV treatment (Table 4, Fig. 1).

Barrier 3 (microfiltration) did not alter PTEQ, while Barrier 4 (reverse osmosis) reduced PTEQ from 1.96 μg/L to the level below the LOD (<0.06 μg/L). Results are in agreement with a previous study at the Bundamba AWTP where microfiltration did not affect PTEQ (unpublished data).

Table 4 – Summary of the bioanalytical results expressed as the average ± standard deviation (sd) of two replicates.

Treatment Barrier	Sample Location	Bioluminescence Inhibition		AChE		I-PAM		E-SCREEN		AhR – CAFLUX		*umuC* –S9[a]		*umuC* +S9[b]	
		Baseline – TEQ (mg/L)		PTEQ (μg/L)		DEQ (μg/L)		EEQ (ng/L)		TCDDEQ (ng/L)		4NQOEQ (μg/L)		BaPEQ (μg/L)	
		Avg	sd	Avg	sd	Avg	sd	Avg	sd	Avg	sd	Avg	sd	Avg	sd
1–2	Oxley Ck WWTP Inlet	25.6	17.4	4.36	0.12	2.15	0.21	3.15	0.2	1.13	0.51	1.52	0.70	32.19	11.09
	Oxley Ck WWTP Activated Sludge	1.26	0.09	0.94	0.56	0.98	0.18	0.44	0.2	0.85	0.43	0.25		3.42	
	Oxley Ck WWTP Post Clarifiers	1.26	0.47	1.00	0.41	1.36	0.35	0.25	0.1	0.77	0.13	0.19	0.03	4.89	0.72
	Oxley Ck WWTP Post UV	1.25	0.44	1.00	0.34	1.31	0.34	0.31	0.1	0.56	0.36	0.17	0.05	4.87	0.79
3–5	Bundamba AWTP Pre MF (Inlet)	0.91	0.22	1.50	0.57	0.26	0.06	0.34	0.2	1.15	0.54	0.24	0.05	5.95	1.41
	Bundamba AWTP Post MF	2.66	0.58	1.96	0.34	0.20	0.04	<0.01		0.33	0.18	0.44	0.08	10.31	2.60
	Bundamba AWTP post RO	0.42	0.07	<0.06		0.04	0.01	<0.01		0.11	0.08	<0.05		<0.8	
	Bundamba AWTP Post AO	0.12	0.04	<0.06		0.05	0.01	<0.01		0.08	0.06	<0.05		<0.8	
	Bundamba AWTP RO Concentrate	3.25	1.04	7.45	2.87	0.8	0.11	0.75	0.7	2.00	0.73	3.07	2.04	32.04	6.27
	PRW Pipeline (Bundamba off-take)	0.65	0.15	<0.06		0.08	0.02	<0.01		0.11	0.08	<0.05		<0.8	
	PRW Pipeline (Lowood)	0.27	0.18	<0.06		0.1	0.06	<0.01		0.11	0.08	<0.05		<0.8	
	PRW Pipeline (Caboonbah)	0.83	0.18	<0.06		0.08	0.04	<0.01		0.15	0.14	<0.05		<0.8	
6	Power Station Lake	0.19	0.21	0.17	0.03	0.05	0.01	<0.01		0.17	0.08	<0.05		<0.8	
	Lake Wivenhoe – Logan's Inlet	0.16	0.11	0.16	0.04	0.02	0.01	<0.01		0.15	0.02	<0.05		<0.8	
	Lake Wivenhoe - Dam Wall	0.14	0.14	0.11	0.03	0.02	0.01	<0.01		0.15	0.05	<0.05		<0.8	
	Mid-Brisbane – Lowood	0.14	0.13	0.19	0.03	0.02	0.01	<0.01		0.19	0.02	<0.05		<0.8	
	Mid-Brisbane - Burton's Bridge	0.17	0.13	0.24	0.03	0.01	0.003	<0.01		0.19	0.01	<0.05		<0.8	
7	Mt Crosby DWTP Intake (raw)	0.23	0.12	0.21	0.03	0.01	0.003	<0.01		0.17	0.07	<0.05		<0.8	
	Mt Crosby DWTP Outlet	1.68	0.64	0.28	0.03	0.05	0.01	<0.01		0.17	0.06	<0.05		<0.8	
	DWS – Mid Way on Distribution Line	0.17	0.13	0.68	0.08	0.08	0.01	<0.01		0.19	0.05	<0.05		<0.8	
	SEW – Towards End of Distribution Line	0.34	0.20	0.31	0.32	0.06	0.002	<0.01		0.28	0.06	<0.05		<0.8	
Others	South Caboolture WWTP Influent	9.17	4.56	5.98	1.10	0.26	0.05	18.53	9.3	1.80	0.54	0.48		< 6.4	
	South Caboolture EWTP Influent (Effluent from WWTP)	1.00	0.52	0.67	0.25	0.09	0.02	<0.02		0.21	0.04	0.21	0.06	2.61	0.24
	South Caboolture EWTP Effluent	0.56	0.44	0.10	0.02	0.11	0.06	<0.01		0.16	0.04	<0.05		<0.8	
	Bottled Water type 1	0.18	0.03	<0.06		0.06	0.01	<0.01		0.15	0.04	<0.05		<0.8	
	Bottled Water Type 2	0.19	0.01	<0.06		0.05	0.01	<0.01		0.13	0.01	<0.05		<0.8	
	Hinze Dam (Lake Advancetown)	0.20	0.05	0.12	0.03	0.04	0.01	<0.01		0.17	0.01	<0.05		<0.8	
	Gold Coast Water Distribution System	0.70	0.27	0.30	0.09	0.07	0.01	<0.01		0.20	0.02	0.06	0.004	<0.8	
	MilliQ Water (Negative Control)	0.08	0.01	<0.06		0.04	0.01	<0.01		0.20	0.03	<0.05		<0.8	

a (–S9) without exogenous metabolic activation.
b (+S9) with exogenous metabolic activation.

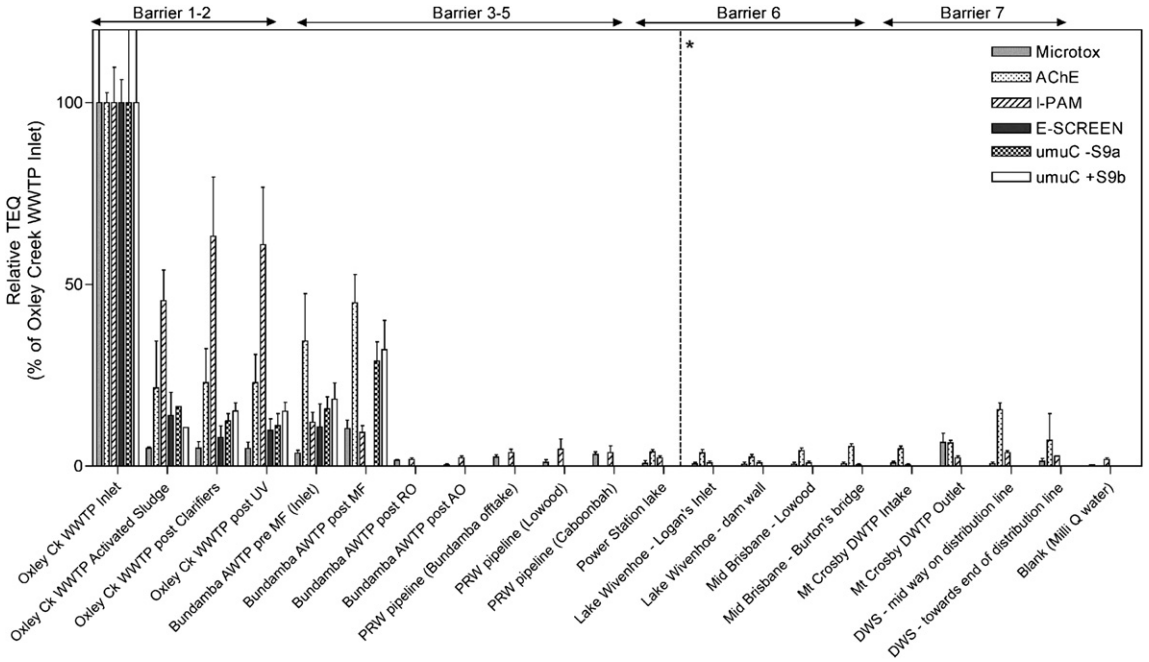

Fig. 1 – Reduction of TEQ across seven treatment barriers in selected bioassays expressed relative to the TEQ of the Oxley Creek WWTP Inlet. Data represent the average of two replicates. Error bars indicate the standard deviation. Missing bars represent data below LOD. *There is a discontinuation between the AWTPs and the natural environment. The resulting purified recycled water is currently used for industrial purposes (represented by Power Station Lake) and has not been reintroduced to the Lake Wivenhoe at the time of this publication.

PTEQ of the samples collected across Barrier 6 and 7 ranged between 0.11 and 0.31 μg/L, with a slight increase in the drinking water system (mid-way along the distribution line) not exceeding 0.7 μg/L but clearly above the blanks. This increase of PTEQ in the drinking water pipeline might be an artefact caused by disinfection with chlorine. Approximately 0.3 mg/L Cl equivalents are used in the drinking water plant for final disinfection but this dose is increased up to 0.8 mg/L in the distribution system (personal communication, Water Grid Manager Technical Committee, 2010). While chlorine should have been quenched prior to extraction of the sample, there might be residual chlorine still present. This should not affect cell-based bioassays but the AChE inhibition assay is done with purified enzyme, which can be denatured or looses activity. Due to the nature of the assay, a non-specific denaturation of the enzyme cannot be differentiated from a specific inhibition. This issue will require further investigation and it is advisable that the AChE inhibition assay be replaced in the future by a cell-based assay indicative of the same endpoint.

PTEQ of the samples collected at Hinze Dam (0.12 μg/L) and the Gold Coast Water distribution system (0.3 μg/L) was comparable with the PTEQ of the samples collected across Barrier 6. PTEQ of both types of bottled water was below the LOD (<0.06 μg/L for 4 L water samples).

Similar to the Oxley Creek WWTP (Barrier 2), PTEQ was decreased by 89% in the Caboolture WWTP from 5.98 μg/L in the inlet to 0.67 μg/L in the WWTP outlet (Table 4). A further decrease to 0.1 μg/L was observed after treatment throughout the treatment chain of the Caboolture EWTP. Results are in agreement with a previous study at the Caboolture EWTP, where PTEQ were significantly decreased during enhanced treatment to 0.36 μg/L (Macova et al., 2010).

3.4. *Phytotoxicity – PSII inhibition I-PAM assay*

A similar decrease of the toxicity across the seven barriers was observed in the I-PAM assay targeting triazine and phenylurea herbicides, expressed as diuron equivalent concentration (DEQ). Phytotoxicity of the samples was reduced from the inlet to post Barrier 2 from 2.15 μg/L to 1.31 μg/L of DEQ (Table 4, Fig. 1).

The relatively high DEQ after Barrier 2 treatment is in agreement with the literature data, showing lower treatment efficiency of biological treatment than ozonation in the removal of herbicides (Escher et al., 2009). DEQ was not altered by microfiltration (Barrier 3) but was five-fold reduced by reverse osmosis (Barrier 4) from 0.2 to 0.04 μg/L. DEQ of the samples collected after Barrier 5 were not significantly different from the blank (0.04 μg/L, *t*-test, $p = 0.61$). Similar results were observed in the previous study at Lake Wivenhoe with the DEQ levels of 0.020 μg/L and 0.025 μg/L at the dam wall and Logan's Inlet respectively (Seqwater project 2007, unpublished data).

DEQ was also reduced by treatment in the Caboolture WWTP from 0.26 to 0.09 μg/L. No further reduction in DEQ was observed after enhanced treatment (Table 4). These results are not in agreement with a previous study at the Caboolture EWTP where the enhanced treatment (particularly pre–ozonation) reduced the DEQ effect to a level below LOD

(Macova et al., 2010) but since herbicide input types and levels are highly variable, which is also evident from the almost tenfold difference in WWTP inlets between Oxley WWT and Caboolture WWTP, the observed difference might reflect rather differences in input than differences in treatment efficiency. The MilliQ blank also showed an effect slightly higher (0.04 μg/L) than the LOD of the bioassay but of no environmental concern.

3.5. *Arylhydrocarbon receptor response – AhR-CAFLUX assay*

In this study, sample extracts were tested in the AhR-CAFLUX assay without acid silica gel clean up, which destroys all but persistent compounds such as polychlorinated dibenzodioxins/furans and biphenyls. Therefore, the TCDDEQ reported in this study represented the sum of all compounds present in the sample that can bind to the arylhydrocarbon receptor, not only the persistent compounds. The less persistent group includes, for example, polycyclic aromatic hydrocarbons.

The response of arylhydrocarbon receptor (AhR) compounds targeted in the AhR-CAFLUX assay and expressed as 2,3,7,8-Tetrachlorodibenzo-dioxin (TCDD) equivalent concentration (TCDDEQ) was reduced across the seven treatment barriers (Table 4). The first decrease was observed post Barrier 2 from an original level of 1.13 ng/L of TCDDEQ to 0.56 ng/L after UV treatment at the Oxley Creek WWTP. TCDDEQ was also decreased throughout the advanced water treatment chain in the Bundamba AWTP, from 1.15 ng/L to 0.33 ng/L after microfiltration (Barrier 3) and further to 0.11 ng/L after reverse osmosis (Barrier 4), a level not significantly different from the blank (0.2 ng/L). In a previous study at the Bundamba WWTP, the TCDDEQ was reduced after reverse osmosis from 1.72 ng/L to 0.12 ng/L (2008, unpublished data). No alterations in the TCDDEQ were observed after Barrier 4 and the effect of all samples was not significantly different from the blank.

A decrease in TCDDEQ was observed also after treatment in the South Caboolture WWTP from 1.8 ng/L in the influent to 0.21 ng/L in the WWTP effluent.

3.6. *Genotoxicity – UmuC assay*

To enable detection of progenotoxins that require metabolic activation to become genotoxic, samples were tested both with and without the inclusion of rat liver supernatant fraction (S9). Response in the *umuC* assay was defined as the induction ratio (IR), the ratio of the sample response to the control, and an effect concentration inducing an IR of 1.5 ($EC_{IR1.5}$) was deduced from linear concentration-response curves. The $EC_{IR1.5}$ in *umuC* assay were expressed as 4-nitroquinoline-oxide (4NQO) equivalent concentration (4NQOEQ) for the assay without metabolic activation (−S9) and benzo[a]pyrene (BaP) equivalent concentration (BaPEQ) for the assay with metabolic activation (+S9) (see Supplementary Information). Similar to other assays, where the TEQs were calculated as the ratio of EC_{50} of the corresponding reference compound to EC_{50} of the sample, the TEQs in *umuC* assay were calculated using the effective concentration $EC_{IR1.5}$ that induces the induction ratio of 1.5 defined by the International Standard Organisation (ISO) guideline as the threshold of genotoxic effect (International Organization for Standardization, 2000), providing the sample was not cytotoxic (growth < 0.5) (Table 4, Fig. 1, and Supplementary information). TEQs were calculated using the average $EC_{IR1.5}$ (4NQO) of 0.008 mg/L and $EC_{IR1.5}$ (BaP) of 0.12 mg/L (see Supplementary Information).

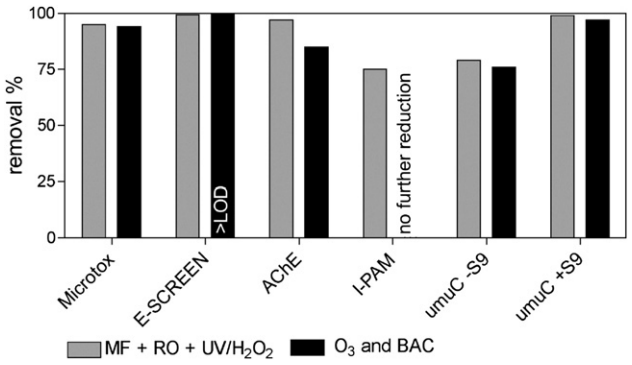

Fig. 2 – Removal of TEQ in the two advanced water treatment chains investigated in this study.

Similar to all other bioassays, the genotoxic effect was significantly decreased post Barrier 2. Activated sludge at the Oxley Creek WWTP reduced the genotoxic effects both without and with metabolic activation by 90% and 87%, respectively (Table 4). No further decrease was observed post clarifiers and after UV treatment. Barrier 3 treatment (microfiltration) at the Bundamba AWTP did not significantly alter the genotoxic effect of the microfiltration feed. However, the genotoxic effect was markedly reduced by reverse osmosis treatment (Barrier 4) to a level below the detection limits of the assay (<0.05 μg/L of 4NQOEQ and <0.8 μg/L of BaPEQ). No genotoxic effect was observed in any sample collected post Barrier 4.

An unexpectedly low genotoxic effect was observed in the Inlet of the Caboolture WWTP compared to the results of a previous study (2008, unpublished data) and in comparison to genotoxic effect at the Oxley Creek WWTP inlet – 0.48 μg/L of 4NQOEQ and no genotoxic effect in the assay with metabolic activation. The genotoxic effect was reduced throughout the enhanced water treatment chain of the Caboolture EWTP from 0.21 μg/L of 4NQOEQ and 2.61 μg/L of BaPEQ in the outlet of the Caboolture WWTP, to a level below the LOD at the outlet of the EWTP (<0.05 μg/L of 4NQOEQ and <0.8 μg/L of BaPEQ).

4. Conclusions

This paper comprises the first study that monitored mixture toxicity in various bioassays across all steps of a water recycling scheme from sewage to drinking water. This will allow benchmarking of water quality levels in the future, when data from alternative source water, e.g. stormwater or bore water and from alternative treatment processes, e.g. treatment of coal seam gas water, become available.

Fig. 1 represents the relative TEQ in comparison to raw sewage across one (albeit discontinuous) water cycle and subsequent environmental buffers and drinking water

treatment and clearly demonstrates how TEQ were reduced across the seven treatment barriers in all six selected bioassays. In all cases, the micropollutant burden was reduced by one order of magnitude or more, but to a different extent, in Barriers 2 to 5. The six bioassays showed a differentiated picture, each one representative for a different group of chemicals and their mixture effect, and the reduction in the toxicity for certain groups of chemicals could be related to their physicochemical properties and behaviour during the various treatment processes. For example, the fact that herbicides are recalcitrant to biodegradation in wastewater treatment plant but rejected by reverse osmosis and well degraded by ozonation is reflected in the DEQ pattern seen over the treatment chain. Another example refers to the EEQ that are brought down to low level by biodegradation already, so they were below detection limits already after Barrier 3.

The results obtained in this study are also useful to compare different treatment options. Fig. 2 compares the removal of TEQ of the advanced water treatment (Barrier 3–5 with membrane filtration, reverse osmosis and UV/H_2O_2) with an alternative advanced treatment chain composed of ozonation and biological activated carbon filtration (BAC). Both chains performed equally well for the removal of non-specific toxicity and while the membrane technology was slightly superior to the chemical treatment with O_3 followed by BAC, the differences were not large, indicating the comparable efficiency of both treatment technologies. While micropollutant removal is not the only indicator for the selection of a treatment technology, the proposed bioanalytical water quality assessment might prove useful in the future for benchmarking treatment technologies.

The effects in Barrier 6, 7 and in drinking water were very low for many endpoints, typically falling below the detection limit. A similar very low detection was observed in the Power Station lake sample, which represents a reservoir where natural water and indirect purified recycled water (PRW) are mixed. ANOVA of the TEQ in the purified recycled water (Bundamba after AOP), drinking water (two samples in the drinking water distribution line) and bottled water (two samples) revealed no statistically significant differences for baseline-TEQ ($p = 0.75$), DEQ ($p = 0.12$), and TCDDEQ ($p = 0.24$) and the EEQ, BaPEQ and 4NQOEQ were all below detection limit in these samples. Only the PTEQ were slightly increased in the drinking water pipeline as discussed in Section 3.3.

Despite these promising results that indicate that bioassays can be used to assess the fate of mixtures of chemicals across a wide range of water matrices, it is advisable to expand the battery of bioanalytical tools in the future to further endpoints relevant to human health, e.g., oxidative stress, additional genotoxicity endpoints and additional hormonal effects to include a wider variety of chemical groups in the investigation. It has also been demonstrated in a parallel study (Escher et al., 2011) that baseline-TEQ derived from the Microtox are not ideal as they are biased towards compounds of low hydrophobicity. Thus cytotoxicity endpoints based on 24 h (or longer) growth of mammalian cells or even bacteria would be beneficial to complement the bioassay battery as non-specific measure of all chemicals present in a given sample. Further, this study shows only a snapshot in time, given that grab samples were taken, therefore the treatment efficiency given in Fig. 1 is not absolute but will vary in space and time. The novelty of this study is that it connects previous and future studies on individual steps of the water cycle constituting a first benchmark of the entire water cycle and clearly demonstrates that bioanalytical tools are applicable for a wide range of matrices.

Detection limits of the bioassays were comparable or lower than the quantification limits of the routine chemical analysis, and allowed monitoring of the presence and removal of micropollutants post Barrier 2. The results obtained by the bioanalytical tools were found to be reproducible, robust and consistent with findings from previous studies assessing the effectiveness of the wastewater and advanced water treatment plants. The results of this study indicate that bioanalytical results expressed as toxic equivalent concentration (TEQ) provide valuable complementary information on mixture effects of groups of chemicals with a common mode of toxic action that help identify potential issues or to predict potential exposure/risks of micropollutants to humans or the environment and are useful for continuous monitoring of the removal efficiencies of the various treatment processes and natural attenuation.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.watres.2011.05.032.

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