Bioanalytical Tools to Evaluate Micropollutants across the Seven Barriers of the Indirect Potable Reuse Scheme

Miroslava Macova[1], Beate Escher[1], Jochen Mueller[1] and Simon Toze[2]

November 2010

Urban Water Security Research Alliance
Technical Report No. 30

Queensland Government
National Research FLAGSHIPS Water for a Healthy Country
CSIRO
Griffith UNIVERSITY
The University Of Queensland AUSTRALIA

Urban Water Security Research Alliance Technical Report ISSN 1836-5566 (Online)
Urban Water Security Research Alliance Technical Report ISSN 1836-5558 (Print)

The Urban Water Security Research Alliance (UWSRA) is a $50 million partnership over five years between the Queensland Government, CSIRO's Water for a Healthy Country Flagship, Griffith University and The University of Queensland. The Alliance has been formed to address South East Queensland's emerging urban water issues with a focus on water security and recycling. The program will bring new research capacity to South East Queensland tailored to tackling existing and anticipated future issues to inform the implementation of the Water Strategy.

For more information about the:

UWSRA - visit http://www.urbanwateralliance.org.au/
Queensland Government - visit http://www.qld.gov.au/
Water for a Healthy Country Flagship - visit www.csiro.au/org/HealthyCountry.html
The University of Queensland - visit http://www.uq.edu.au/
Griffith University - visit http://www.griffith.edu.au/

Enquiries should be addressed to:

The Urban Water Security Research Alliance
PO Box 15087
CITY EAST QLD 4002

Ph: 07-3247 3005; Fax: 07-3405 3556
Email: Sharon.Wakem@qwc.qld.gov.au

Authors: 1 – The University of Queensland, National Research Centre for Environmental Toxicology (Entox);
2 – CSIRO Water for a Healthy Country, Queensland Biosciences Precinct

Macova, M., Escher, B., Mueller, J., Toze, S. (2010). *Bioanalytical Tools to Evaluate Micropollutants across the Seven Barriers of the Indirect Potable Reuse Scheme* Urban Water Security Research Alliance Technical Report No. 30.

Copyright

Disclaimer

Cover Photograph:

Description: Sampling Sites - *Left* - sampling equipment at WWTP, *Right* - sampling site at mid-Brisbane River
Photographer: Leonie Hodgers

ACKNOWLEDGEMENTS

This research was undertaken as part of the South East Queensland Urban Water Security Research Alliance, a scientific collaboration between the Queensland Government, CSIRO, The University of Queensland and Griffith University.

Particular thanks go to Hanne Thoen, Haipu Bi, Ben Mewburn and Kristie Lee Chue (Entox) for experimental assistance, Leonie Hodgers and Peter Sillivan (CSIRO) for collecting and extracting the samples.

The authors acknowledge Brisbane Water* for access to Oxley Creek Wastewater Treatment Plant, WaterSecure and Veolia Water Australia for access to the advanced water treatment plants and PRW pipeline, Seqwater for samples form Wivenhoe Dam, mid-Brisbane River and the Mt Crosby Water Treatment Plant, Ipswich Water* for access to their water distribution system, Moreton Bay Water for access to the South Caboolture Water Reclamation Plant, and Gold Coast Water for access and assistance in samples form the Gold Coast water distribution system, Julien Reungoat (AWMC, UQ) and Michael Bartkow (Seqwater) for their help during sampling.

We would like to also thank Michael Denison (University of California Davis, USA) for providing the H4G1.1c2 cells, Georg Reifferscheid (German Federal Institute of Hydrology, Germany) for providing the bacteria *Salmonella typhimurium* TA1535/pSK1002, and Ana Soto (Tufts University, USA) for providing the MCF7-BOS cells.

Additional material used in the Microtox study is part of the follow-up project "Bioassay and Risk communication". We would like to acknowledge also Seqwater for financial support of the *umuC* TEQ study.

* Brisbane Water and Ipswich Water are now part of Queensland Urban Utilities.

FOREWORD

Water is fundamental to our quality of life, to economic growth and to the environment. With its booming economy and growing population, Australia's South East Queensland (SEQ) region faces increasing pressure on its water resources. These pressures are compounded by the impact of climate variability and accelerating climate change.

The Urban Water Security Research Alliance, through targeted, multidisciplinary research initiatives, has been formed to address the region's emerging urban water issues.

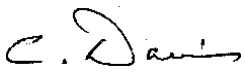
As the largest regionally focused urban water research program in Australia, the Alliance is focused on water security and recycling, but will align research where appropriate with other water research programs such as those of other SEQ water agencies, CSIRO's Water for a Healthy Country National Research Flagship, Water Quality Research Australia, eWater CRC and the Water Services Association of Australia (WSAA).

The Alliance is a partnership between the Queensland Government, CSIRO's Water for a Healthy Country National Research Flagship, The University of Queensland and Griffith University. It brings new research capacity to SEQ, tailored to tackling existing and anticipated future risks, assumptions and uncertainties facing water supply strategy. It is a $50 million partnership over five years.

Alliance research is examining fundamental issues necessary to deliver the region's water needs, including:

- ensuring the reliability and safety of recycled water systems.
- advising on infrastructure and technology for the recycling of wastewater and stormwater.
- building scientific knowledge into the management of health and safety risks in the water supply system.
- increasing community confidence in the future of water supply.

This report is part of a series summarising the output from the Urban Water Security Research Alliance. All reports and additional information about the Alliance can be found at http://www.urbanwateralliance.org.au/about.html.



Chris Davis
Chair, Urban Water Security Research Alliance

CONTENTS

LIST OF FIGURES

LIST OF TABLES

EXECUTIVE SUMMARY

The aim of this project was to evaluate the use of bioanalytical tools for monitoring micropollutants across the seven treatment barriers of the Western Corridor indirect potable reuse scheme in South East Queensland and to assess the efficacy of different treatment barriers. Bioanalytical results provide complementary information to chemical analysis on the sum of micropollutants present during water treatment. Six endpoints targeting the groups of chemicals of particular relevance for human and environmental health were included in the evaluation: genotoxicity, endocrine activity, neurotoxicity, phytotoxicity, dioxin-like activity and non-specific cell toxicity.

The toxicity of samples was reduced across the seven treatment barriers in all six selected bioassays. Each bioassay showed a differentiated picture representative of a different group of chemicals and their mixture effect. The toxicity of the samples across the seven barriers ranged in the same order of magnitude as seen during previous studies in wastewater and advanced water treatment plants and reservoirs. Barrier 2 treatment (wastewater treatment plant) reduced the responses in all selected bioassays. Further decrease in toxic effects of the water was observed after microfiltration (Barrier 3) and reverse osmosis treatment (Barrier 4).

Baseline toxicity of the samples, expressed as baseline toxicity equivalent concentration (baseline - TEQ), was decreased by 94% after treatment in Oxley Creek WWTP (Barrier 2). Microfiltration (Barrier 3) did not significantly alter the baseline toxicity. Further reduction in the baseline toxicity was observed after reverse osmosis and advanced oxidation at Bundamba AWTP (Barriers 4-5) to 2% and 0.5% of the original activity, respectively. Apart from a Mt. Crosby outlet sample, the baseline toxicity of samples collected after Barrier 5 was not significantly different from the blank. The observed increased baseline toxicity of the Mt. Crosby outlet sample was reproducible in a second sampling campaign but the level is of no concern as it was below effect levels modelled for this endpoint, under the assumption that all chemical concentrations are present below their drinking water guideline values.

The estrogenic effect of the samples, expressed as estradiol equivalent concentration (EEQ), decreased by 90% after Barrier 2 - from 3.2 ng/L in Oxley Creek WWTP influent to 0.3 ng/L in the effluent. Estrogenic effect of all samples collected after Barrier 3 (microfiltration at Bundamba AWTP) was reduced to below the detection limit of the assay (<0.01 ng/L).

Results of the bioassay targeting organophosphates and carbamate insecticides, expressed as parathion equivalent concentration (PTEQ), also showed significant decrease in the toxicity post Barrier 2, with further decrease after Barrier 4 to 0.1 µg/L of PTEQ. Low PTEQs not exceeding 0.7 µg/L were observed across Barriers 6 and 7.

A similar decrease of the toxicity across the seven barriers was observed in the remaining three assays targeting triazine and phenylurea herbicides, compounds binding to the aryl hydrocarbon receptor and compounds that cause a genotoxic effect. In all cases, the micropollutant burden was significantly decreased post Barrier 2 (but to a different extent) and further reduced after reverse osmosis treatment (Barrier 4) to levels not significantly different from the blank, or below assay detection limits.

Detection limits of the bioassays, comparable to or lower than the quantification limits of the routine chemical analysis, allowed monitoring of the presence and removal of micropollutants post Barrier 2. 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 provide valuable complementary information to identify potential issues or to predict exposure/risks of micropollutants to humans or the environment. In the next two years of the UWSRA project "Bioassays and Risk Communication", we plan to further validate and expand the test battery and put a particular focus on developing an effective communication strategy.

1. INTRODUCTION

The Western Corridor Recycled Water Scheme in South East Queensland (SEQ) is the largest indirect potable water recycling scheme in Australia and one of the largest in the southern hemisphere. The scheme consists of seven treatment barriers: 1 – source control; 2 – wastewater treatment plant (WWTP); 3 – microfiltration; 4 – reverse osmosis; 5 – advance oxidation (combining hydrogen peroxide and UV irradiation); 6 – natural environment; and 7 – 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 in three advanced water treatment plants (AWTP). The resulting purified recycled water (PRW) is then able to be piped to Lake Wivenhoe, the largest of the freshwater reservoirs in SEQ. This reservoir supplies more than 60% of the freshwater resources for the city of Brisbane. The production of the PRW is based on international experiences of other potable 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 train 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 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 meets potable standards, but is presently only used for industrial purposes and has not been reintroduced to the Wivenhoe Dam yet. Supplementation of drinking water storage dams is envisaged only once the combined level of water in the dams 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. Many organic and inorganic micropollutants are continuously monitored. 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 and optimised 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 (Macova et al., 2010).

The use of bioanalytical techniques in this project links directly to other projects, including the Enhanced Treatment project of the Urban Water Security Research Alliance, the baseline monitoring project in Wivenhoe Dam (Seqwater), as well as work that is planned to be carried out on Advanced Water Treatment through the Research Program "Chair in Water Recycling" (Veolia Water Australia).

Objective of the Project

- Validate the applicability of the selected bioassays for the monitoring of micropollutants across all seven treatment barriers.
- Interpretation of the bioanalytical results to ensure that the limitations and strengths of bioanalytical tools are understood.
- Address QC/QA issues and assess the usefulness of established bioanalytical techniques for source water evaluation.
- Assess the effectiveness of Barrier 2 in removing micropollutants and, where possible, relate the detected toxicity to chemical data.
- Compare the toxicity post Barrier 2 with the toxicity in samples collected during previous studies from the Advanced Water Treatment project, later barriers in the PRW scheme and baseline studies from the dam.

2. DESIGN OF THE BIOANALYTICAL TEST BATTERY

Baseline toxicity, representing the integrative effect of all micropollutants in the sample, was assessed with the bioluminescence inhibition test using the marine bacterium *Vibrio fischeri*. Groups of chemicals with specific modes of toxic action were targeted with five additional bioassays: E-SCREEN (which targets estrogens and estrogenic industrial chemicals); AhR-CAFLUX (which targets polychlorinated dibenzodioxins/furans, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs)); genotoxicity (chlorinated by-products, aromatic amines, PAHs); neurotoxicity (organophosphates and carbamate insecticides); and phytotoxicity (triazine and phenylurea herbicides) (Table 1).

Table 1: Overview of the Bioanalytical Test Battery (adapted from Macova et al., 2010).

Assay	Targeted Chemicals	Reference Compound	Result Expression	Literature Reference
Baseline Toxicity – Bioluminescence inhibition in *Vibrio fischeri*	All chemicals	Virtual baseline toxicant; Phenol [a]	Baseline toxicity equivalent concentrations (baseline-TEQ)	ISO, 1998; Johnson et al., 2005; Farré et al., 2006; Escher et al., 2008a
Neurotoxicity – AChE	Organophosphates and carbamate insecticides	Parathion	Parathion equivalent concentrations (PTEQ)	Ellman et al., 1961; Hamers et al., 2000; DIN, 1995;
Phytotoxicity – Max-I-PAM	Triazine and phenylurea herbicides	Diuron	Diuron equivalent concentrations (DEQ)	Schreiber et al, 2003; Schreiber et al., 2007
Estrogenicity – E-SCREEN	Estrogens, estrogenic industrial chemicals	17ß-estradiol (E_2)	Estradiol equivalent concentrations (EEQ);	Soto et al., 1995 ; Körner 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; ISO, 2000

[a] only used as positive control, not as reference compound.

2.1. Baseline Toxicity: Bioluminescence Inhibition in *Vibrio fischeri*

This non-specific toxicity assay is widely recognised in the field of ecotoxicology as the standard assay to measure acute cytotoxicity (Johnson, 2005; Farré et al., 2006). This assay measures the relative decrease in light output from the naturally bioluminescent marine bacteria, *Vibrio fischeri*, following its exposure to a toxicant. Damage occurring at any level of cellular organisation, including the disruption of membranes, the electron transport chain, enzymes and cytoplasm composition, can result in decreased light output. Hence, the assay reflects the general "energy status" of the bacteria and can indicate the toxic potency of a broad spectrum of compounds with different modes of action. In general, the mixture concept of concentration addition (Altenburger, 2004) holds for chemicals with the same mode of action, but it has been demonstrated that it is also applicable for more than thirty compounds (Warne and Hawker, 1995) and for an unresolved mixture in WWTP effluent (Escher et al., 2008ab). Therefore, we consider this assay to represent baseline toxicity and to respond non-specifically to all compounds present in the sample. As such, it is integrative, while the other selected bioassays respond specifically to a defined mode of toxic action. Note that baseline toxicity is the underlying toxicity of every compound and one needs to take precautions at higher effect levels of the specific bioassays that the specific effect is not influenced by non-specific baseline toxicity. The effect is expressed as baseline toxicity equivalent concentration (baseline – TEQ) (Table 1).

2.2. Neurotoxicity: Acetylcholinesterase Inhibition Assay

Organophosphate pesticides are the most widely used group of insecticides in Australia. This group of chemicals acts by specific binding of the pesticides to the enzyme acetylcholinesterase (AChE), blocking AChE and thereby inhibiting the hydrolysis of the neurotransmitter acetylcholine (ACh). We chose this endpoint as a relevant representative of neurotoxicity although there are other mechanisms for this mode of action. AChE inhibition can be used to quantitatively determine relative concentrations of acetylcholine inhibitors using an enzymatic assay. The AChE assay was developed by Ellmann et al. (1961) and modified to 96-well plate format by Hamers et al. (2000). Here, we used commercially available AChE enzyme from *E. electricus* and the synthetic substrate acetylthiocholine iodide. The acetylcholinesterase enzyme hydrolyses the substrate to yield acetate and thiocholine. The chromogenic reagent dithio-bis(2-nitrobenzoic acid) (DTNB) then reacts with free thiol groups producing the yellow 4-nitrothiolate which can be measured colorimetrically. The effect in this assay is expressed as parathion equivalent concentration (PTEQ). Parathion is a well-known and potent inhibitor of AChE. The reported PTEQ indicates the concentration of parathion that would exhibit the same effect as the given water sample (Table 1).

2.3. Phytotoxicity: PS II Inhibition I-PAM Assay

Phytotoxicity was quantified via inhibition of photosystem II (PS II), a key target mode of action of herbicides. PS II herbicides disrupt the electron flow and excitation energy is re-emitted as fluorescence rather than driving photochemical processes. A fluorescence-based photosynthetic yield analysis technique was applied for phytotoxicity assessment. The PS II inhibition I-PAM assay is based on the pulse-amplitude modulated fluorometry techniques and has evolved from the ToxY-PAM assay (Schreiber et al., 2003). It has developed as a rapid tool for the detection of chemicals such as phenylurea and triazine herbicides, which bind to and inhibit PS II (Schreiber et al., 2007). The effect of a water sample in this assay is expressed as diuron equivalent concentration (DEQ) (Table 1), i.e. the effect that an equivalent concentration of diuron would elicit.

2.4. Estrogenic Activity: E-SCREEN Assay

The E-SCREEN assay was developed by Soto et al. (1995) and modified by Körner et al. (1999). It responds specifically to estrogenic and xeno-estrogenic compounds that can mimic the activity of the female sex hormone estradiol. Similar to estradiol, pseudo-estrogens induce the proliferation of human breast cancer cells. The results of this bioassay are reported as estradiol equivalent concentrations (EEQ) (Table 1).

2.5. Arylhydrocarbon Receptor Response: AhR-CAFLUX Assay

The AhR-CAFLUX (Chemically Activated FLUorescent gene eXpression) assay was developed by the Denison group (Nagy et al., 2002; Zhao and Denison, 2004). It responds specifically to compounds that bind to the arylhydrocarbon receptor (AhR), such as dioxin-like compounds. The AhR-CAFLUX assay makes use of a rat hepatoma cell line (H4IIE) stably transfected with a vector containing the gene of an enhanced green fluorescent protein (pGreen1.1) under the control of the dioxin-responsive elements (DREs) (Zhao and Denison, 2004). Upon binding, the AhR-ligand complex is activated and translocated to the nucleus where it specifically binds to dioxin-responsive elements and induces or inhibits the transcription of genes under control of DREs. The AhR-CAFLUX assay is performed twice. Once with the same extract as all other bioassays and then again after sulphuric acid silica gel clean-up of the extract. The former gives a response from all compounds that can induce the AhR, while the latter only responds to compounds such as dioxin-like chemicals that are not removed (oxidised and adsorbed by the silica gel) during this additional purification step. In this study, only the assay without additional clean up was performed. Results are reported as TCDD equivalent concentration (TCDDEQ) (Table 1).

2.6. Genotoxicity: *UmuC* Assay

The *umuC* assay was developed by Oda et al. (1985) and responds specifically to genotoxic compounds. DNA damage induces expression of the *umuC* gene, which belongs to the bacterial SOS regulatory network consisting of many genes with increased expression in the case of DNA damage. Again, there is a suite of different bioanalytical tools available to assess DNA damage and other reactive mechanisms of toxicity but we chose the *umuC* assay because of its wide use for water quality assessment, simplicity and relevance of the SOS response. In the *umuC* assay, a strain of *Salmonella typhimurium* bacteria (TA1535) is used that is stably transfected with the plasmid *pSK1002* carrying a *lacZ* gene under the control of the *umu* regulatory region. The *lacZ* reporter gene encodes for proteins with ß-galactosidase activity that can metabolise a colourless substrate o-nitrophenyl-galactopyranoside (ONPG) into a yellow-coloured product (o-nitrophenol). Thus, the induced enzyme activity is quantified by an increase in absorption at 420 nm, which is a direct measure for the exposure level of bacteria to genotoxic compounds in the medium.

3. MATERIAL AND METHODS

3.1. Samples and Sites

Grab samples were collected at 21 sites across the seven barriers of the indirect potable reuse scheme: Oxley Creek waste water treatment plant (WWTP) (Barrier 1-2), microfiltration, reverse osmosis and advanced oxidation at Bundamba advanced water treatment plant (AWTP), Bundamba off-take, Lowood and Caboonbah Pipeline (Barrier 3-5), Power Station lake, Wivenhoe dam and mid-Brisbane river representing natural environment (Barrier 6), Mt. Crosby drinking water treatment plant (DWTP) and the drinking water distribution system (Barrier 7) (Figure 1). Sampling was complemented by: three additional samples collected at Caboolture WWTP and Caboolture enhanced water treatment plant (EWTP) fed with the Caboolture WWTP effluent, to compare the treatment/removal efficiency; two samples collected at Hinze dam and Gold Coast water distribution system; and by two types of bottled water to compare the quality of purified recycled water (see Appendix II, Table 2).

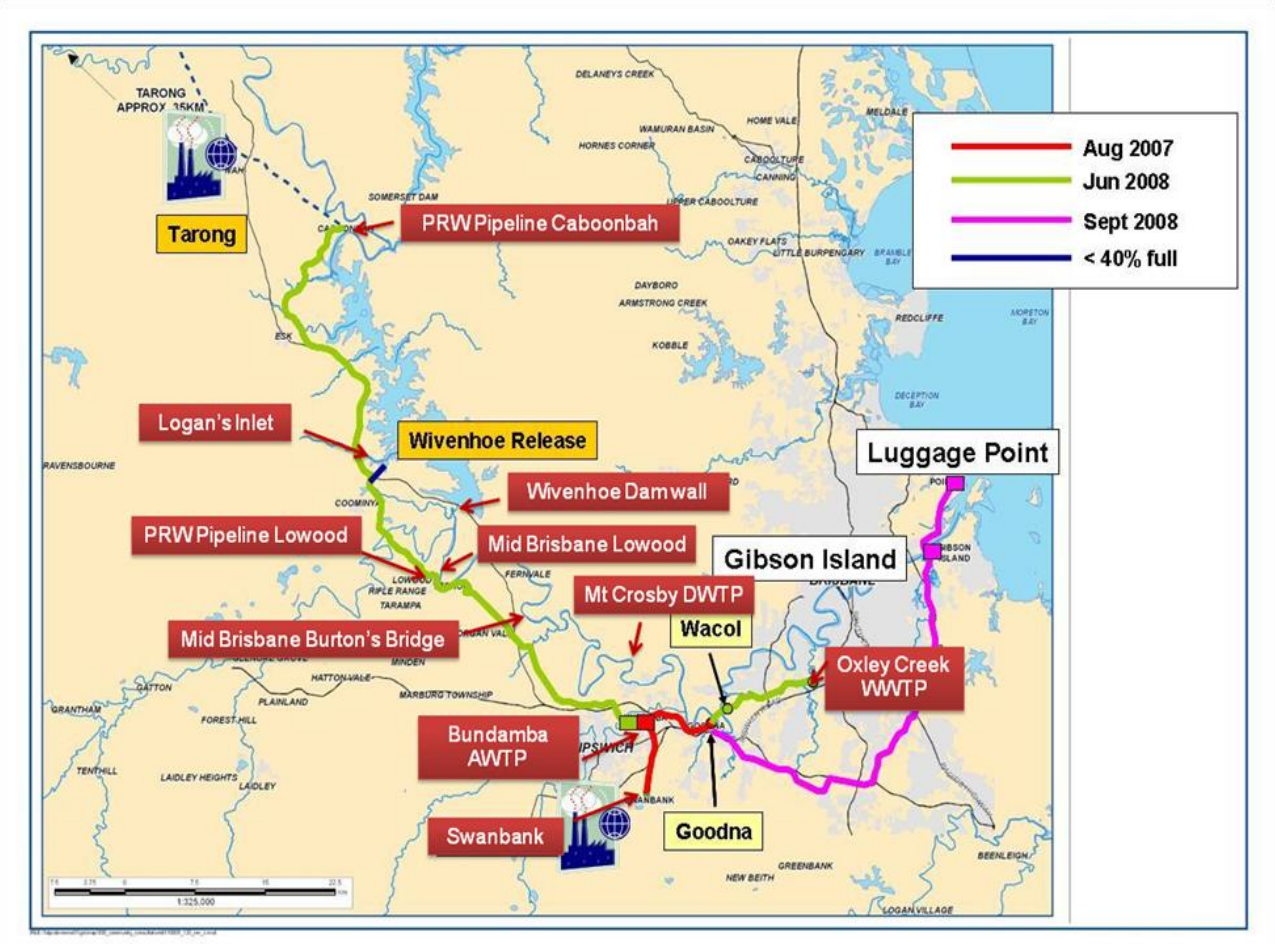


Figure 1: Map of the Indirect Potable Reuse Scheme with Sampling Sites.

Source: Map of the indirect potable reuse scheme kindly provided by Yvan Poussade, Veolia Water Australia.

Note: 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 Wivenhoe Dam at this time.

Based on the previous studies, sample volume ranged from 0.5 L – 4 L depending on the expected toxicity of the samples (Table 2). 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 detection limit of the assay. Samples were kept on ice during transport and until processing. Samples were percolated through the cartridges within 24 hours of collection. Cartridges were sealed and kept at minus 20˚C until elution with the solvent mixture.

All samples were taken and processed in duplicates. In addition, selected samples were spiked with reference chemicals for QA/QC (see QA/QC chapter).

Table 2: 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 offtake)	08.12.2009	4.0
	PRW pipeline (Lowood)	08.12.2009	4.0
	PRW pipeline (Caboonbah)	08.12.2009	4.0
Barrier 6	Power Station lake	08.12.2009	4.0
	Wivenhoe Dam – Logan's Inlet	13.10.2009	4.0
	Wivenhoe Dam - 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*	08.12.2009	4.0
	Bottled water type 2*	08.12.2009	4.0
	Hinze Dam	28.10.2009	4.0
	Gold Coast Water Distribution system	06.11.2009	4.0
QC/QA	Oxley Ck WWTP Post Clarifiers (spiked)	28.10.2009	2.0
	Mid-Brisbane (spiked)	13.10.2009	4.0
	Drinking Water System - mid way on distribution line (spiked)	19.11.2009	4.0
	MilliQ water (spiked positive control)	09.12.2009	4.0
	MilliQ water (not spiked negative control)	09.12.2009	4.0

* purchased in Brisbane supermarket on 29.10.2009.

3.2. Solid Phase Extraction

Immediately after sampling, 1 mL of 0.1% sodium thiosulphate was added to 1 L 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. The cartridges were dried for two to three hours 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.

3.3. Reporting of the Bioanalytical Results: Toxic Equivalent Concentrations

Bioanalytical results were reported in terms of toxic equivalent concentrations (TEQ) (Villeneuve et al., 2000; Escher et al., 2008a; Macova et al., 2010) using a corresponding reference compound representing the group of targeted chemicals in a given assay (see Table 1). The equivalent concentration represents the concentration of the reference compound required to produce the same effect as the mixture of different compounds in the sample.

Equivalent concentrations were calculated from concentration-effect curves of the reference compound and the samples. Both reference compounds and samples generally followed a sigmoidal log-concentration-effect curve (Figure 2).

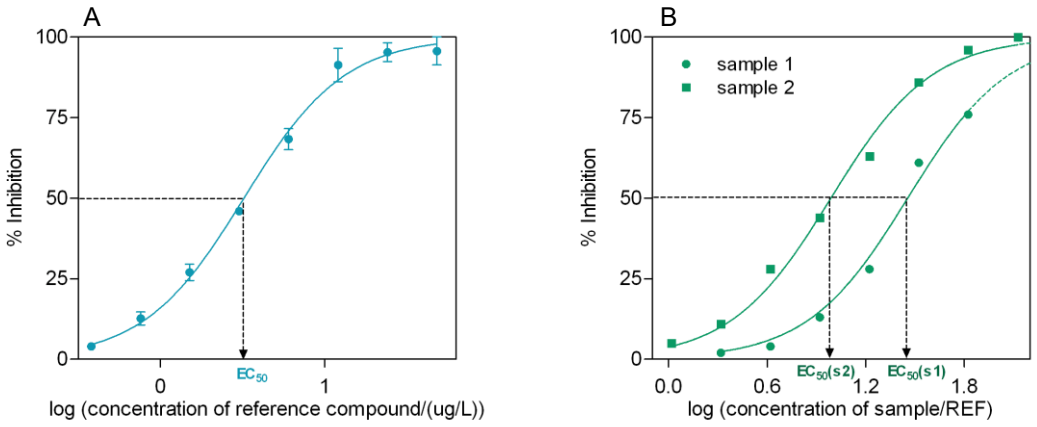


Figure 2: Typical Concentration-Effect Curves of the Reference Compound (A) and Sample Extracts (B).

Solid phase extraction (SPE) extracts are composed of a mixture of unknown substances at unknown concentrations, so the concentration unit of the sample in the concentration effect curve is based on the Relative Enrichment Factor (REF). The REF represents the enrichment or dilution of the original sample in each bioassay and is the combination of the enrichment of the extraction and the dilution in the bioassay (equation 1) (Escher et al., 2006; Muller et al., 2007; Macova et al., 2010). The REF is equivalent to concentration and is expressed in the units [$L_{water\ sample}/L_{bioassay}$].

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

The enrichment factor of the SPE was calculated using equation 2, which represents the enrichment of the extract compared to the source water.

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

Sample volume ranged from 0.5 L to 4 L (Table 2). Final volume of each sample extract was 0.5 mL, therefore the enrichment of the samples was between 1000 and 8000-times.

An aliquot of the enriched sample extracts was then added to the microtiter plate of the respective bioassay and serially diluted by the test medium to obtain a concentration-effect curve. A dilution factor of each bioassay was calculated using equation 3.

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

Toxic equivalent concentrations (TEQ) were then calculated as the ratio of EC_{50} values of the reference compound to the EC_{50} of the sample (equation 4).

$$TEQ = \frac{EC_{50}(\text{reference compound})}{EC_{50}(\text{sample})} \quad (4)$$

Since the EC_{50}(reference compound) is expressed in concentration units such as [µg/L] and the EC_{50}(sample) in concentration units [$L_{\text{water sample}}/L_{\text{bioassay}}$], representing the enrichment or dilution (REF) of the sample required to elicit the 50% effect, the TEQ is expressed in [µg/L], the same units as the corresponding reference compound.

3.4. Bioanalytical Tools

The bioanalytical techniques were performed as previously reported (Escher et al., 2005; Muller et al., 2007; Muller et al., 2008; Macova et al., 2010) according to routine standard operating procedures that have been established at Entox in collaboration with the colleagues from the Swiss Federal Institute of Aquatic Science and Technology. Detailed methodology is described in Appendix I.

3.5. QA/QC

For quality and assurance purposes, all samples were collected in triplicates. Two replicates of each sample were extracted and analysed across the bioanalytical test battery to assess the repeatability of the SPE and the bioassay. The third replicate was percolated through the cartridge and kept in a freezer for a later analysis in a case of inconclusive results.

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. Several QA/QC samples were included to address the matrix effect (Table 2). Based on the preliminary results, the selected samples were spiked with the mixture of diuron, parathion, 17β-estradiol at the concentrations of 3 µg, 17 µg and 0.25 ng per total volume of the sample, respectively (Table 4). The effect of the spiked sample was compared with the effect of the sample where the matrix did not interfere, and the expected effect based on the concentration of the compounds in the spiking mixture.

The detection limit of baseline toxicity assay was defined as three times standard deviation of the negative control response (bacteria *Vibrio fischeri* in the medium). The detection limit of the remaining bioassays was defined as three times standard deviation of the response using the lowest concentration of the reference compound that induced an effect significantly different from the control. Another aspect of the LOD is the maximum enrichment of the sample (REF) we can achieve in the assay; the higher enrichment, the lower detection limit of the assay. Since we collected four different volumes of the samples depending on the expected toxicity (Table 2), there are four different detection limits for each assay (Table 3).

Table 3: Detection Limits of the Assays for Individual Sample Volume.

Assay	Result Expression	Detection Limit 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*	EEQ	0.08 ng/	0.04 ng/	0.02 ng/	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 (+S9) BaPEQ	0.4 µg/L 6.4 µg/L	0.2 µg/L 3.2 µg/L	0.1 µg/L 1.6 µg/L	0.05 µg/L 0.8 µg/L

* If the maximum proliferation of the sample did not reach 50% of the 17β-estradiol, sample were 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).

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

4. RESULTS AND DISCUSSION

4.1. Baseline Toxicity – Bioluminescence Inhibition in *Vibrio fischeri*

The baseline–TEQs were decreased by 94% after treatment in Oxley Creek WWTP (Barrier 2), from 25.57 mg/L in the influent to 1.26 mg/L after activated sludge treatment with no further decrease post clarifiers or UV treatment (Table 3, Figure 3). Microfiltration at Bundamba AWTP (Barrier 3) significantly increased baseline toxicity from 0.91 to 2.66 mg/L. This increase can be caused by removal of particulate matter by microfiltration and release of micropollutants into the dissolved phase, where they become bioavailable. Reverse osmosis and advanced oxidation at Bundamba AWTP (Barrier 4-5) decreased the baseline toxicity to 0.42 and 0.12 mg/L representing 44% and 13% of the original activity in Bundamba AWTP inlet, respectively; both levels not significantly different from the blank.

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 in the influent. Effluent from the conventional Caboolture WWTP was further treated in the Caboolture enhanced water treatment plant (EWTP) with 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 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 (reverse osmosis at Bundamba AWTP) of the indirect potable reuse scheme.

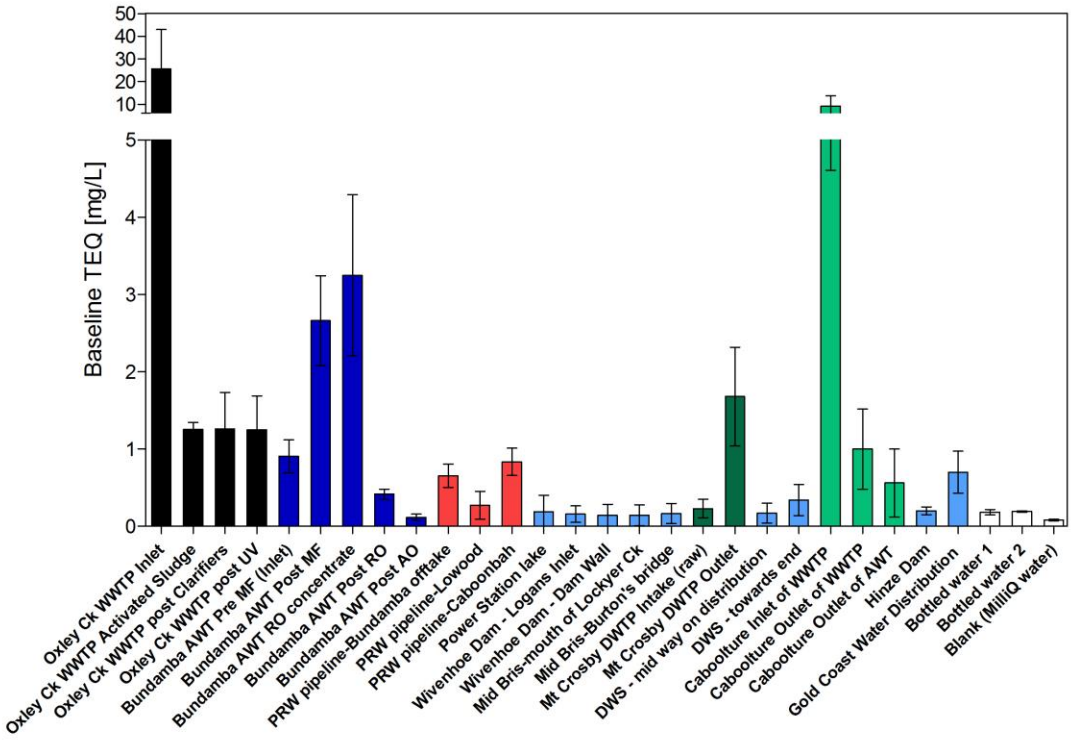


Figure 3: Baseline toxicity equivalent concentrations (baseline-TEQ) of the samples across the seven barriers of the indirect potable reuse scheme and additional samples expressed as average of two replicates. Error bars indicate the standard deviation.

Apart from the Mt. Crosby outlet sample, the baseline toxicity of samples collected after Barrier 5 was not significantly different from the blank (Table 3, Figure 3). The observed increased baseline toxicity of the Mt. Crosby outlet sample was reproducible in a second sampling campaign but the level is of no concern as the levels are below effect levels modelled for this endpoint under the assumption that all chemical concentrations are present below their drinking water guideline values (Vermeirssen et al., 2010; Hawker et al., 2010). In addition, this endpoint also responds to natural organic matter if it is broken down to small molecular weight units, which are also called assimilable organic carbon. Typically, with advanced oxidation processes, assimilable organic carbon is formed but this is a transient process as assimilable organic carbon is easily biodegradable.

4.2. Estrogenic Activity – E-SCREEN Assay

The estrogenic effect of the samples, expressed as estradiol equivalent concentration (EEQ), decreased by 86%, after Barrier 2 from 3.2 ng/L in Oxley Creek WWTP Influent to 0.44 ng/L by activated sludge treatment (Table 3, Figure 4). EEQ was further reduced after the clarifiers but UV treatment did not alter the EEQ. Microfiltration at 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 detection limit 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. Estrogenic effect of the samples collected at Hinze dam, the Gold Coast Water distribution system and bottled water was also below the detection limit of the assay (<0.01 ng/L for 4 L water samples).

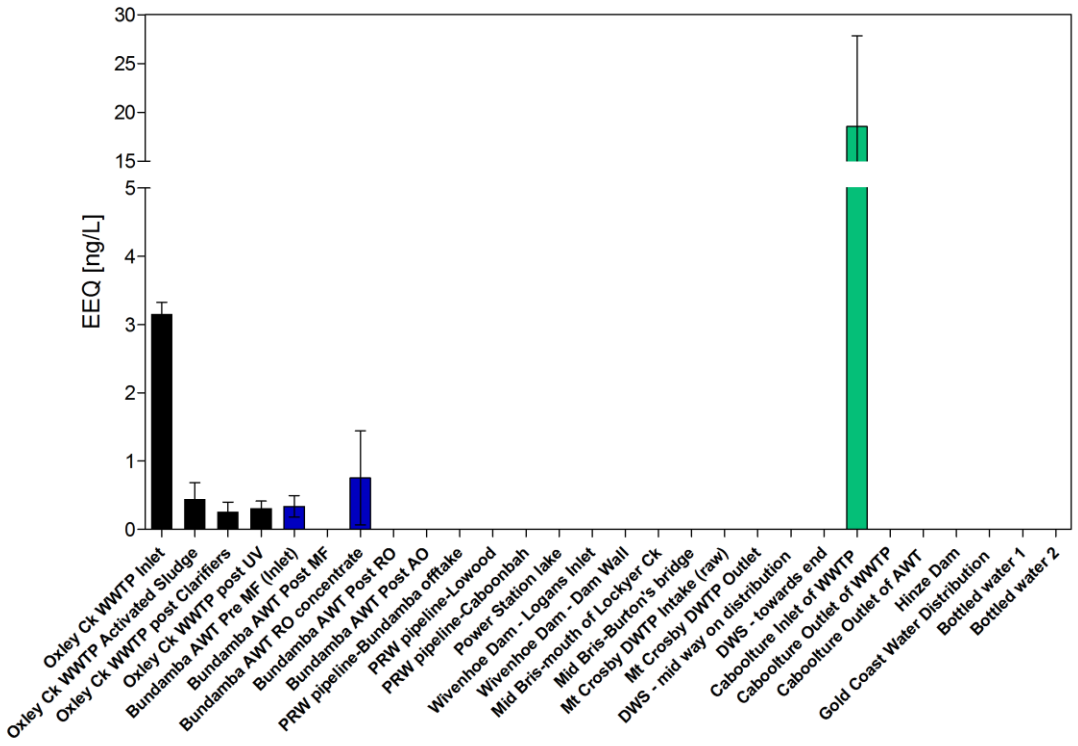


Figure 4: Estradiol equivalent concentrations (EEQ) of the samples across the seven barriers of the indirect potable reuse scheme and additional samples expressed as average of two replicates. Error bars indicate the standard deviation. Missing bars represent data below detection limit < 0.01 ng/L.

Estrogenicity of the influent to Oxley Creek WWTP was lower than typically seen in the raw sewage samples. EEQ in the raw sewage sample from Caboolture WWTP in the previous study ranged from 68 to 91 ng/L in three different samples collected in the course of one month (unpublished results). This is comparable with EEQ reported in the raw sewage in the Brisbane area using the same bioassay, with up to 74 ng/L EEQ (GWRC 2008). EEQ in the raw sewage reported in other studies ranged from <4 - 185 ng/L in Australia and New Zealand (estrogen receptor binding assay, (Leusch et al., 2006)), up to 120 ng/L in the Netherlands (ER-CALUX, (Murk et al., 2002)) and from 58 to 70 ng/L in Germany (E-SCREEN, (Körner et al., 2000)).

EEQ of the additional samples collected at Caboolture WWTP was 18.5 ng/L, which is again in the lower range of what is typically seen in raw sewage. Surprisingly, EEQ was reduced to below the detection limit of the assay (0.02 ng/L for 2 L samples) 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 0.02 ng/L by activated carbon treatment, key steps of the enhanced treatment chain in the removal of the estrogenic effect (Macova et al., 2010). Removal efficiency of ozonation on the estrogenic activity was previously reported in the literature (Lee et al., 2008; Escher et al., 2009b).

4.3. 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 3, Figure 5).

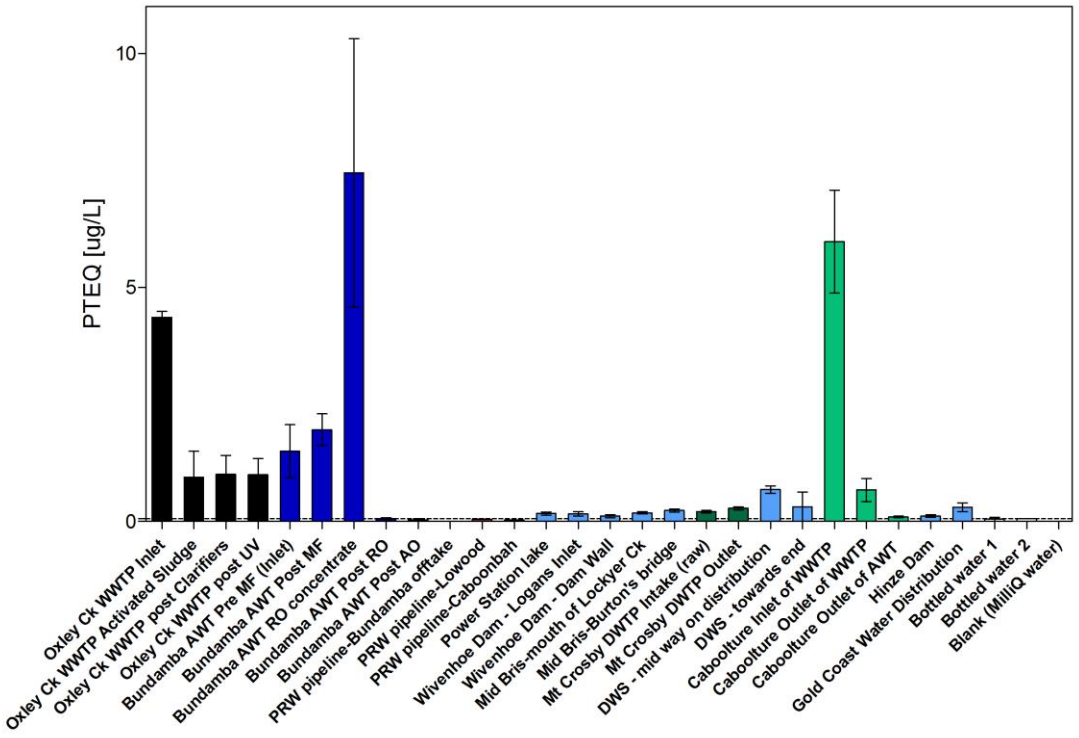


Figure 5: Parathion equivalent concentrations (PTEQ) of the samples across the seven barriers of the indirect potable reuse scheme and additional samples expressed as average of two replicates. Error bars indicate the standard deviation. Missing bars represent data below detection limit < 0.06 µg/L.

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

PTEQ of the samples collected across Barrier 6 and 7 ranged between 0.11 and 0.31 µg/L, with slight increase in the drinking water system (mid-way along the distribution line) not exceeding 0.7 µg/L. 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 detection limit of the assay (<0.06 µg/L for 4 L water samples).

Similar to 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 3). Further decrease to 0.1 µg/L was observed after treatment throughout the treatment chain of Caboolture EWTP. Results are in agreement with a previous study at Caboolture EWTP, where PTEQ was significantly decreased by the enhanced treatment chain to 0.36 µg/L (Macova et al., 2010).

4.4. 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 post Barrier 2 from 2.15 µg/L to 1.31 µg/L of DEQ (Table 3, Figure 6).

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., 2009a). DEQ was not altered by microfiltration (Barrier 3) but was significantly reduced by reverse osmosis (Barrier 4) from 0.2 to 0.04 µg/L. DEQ of the samples collected after Barrier 5 was not significantly different from the blank (0.04 µg/L).

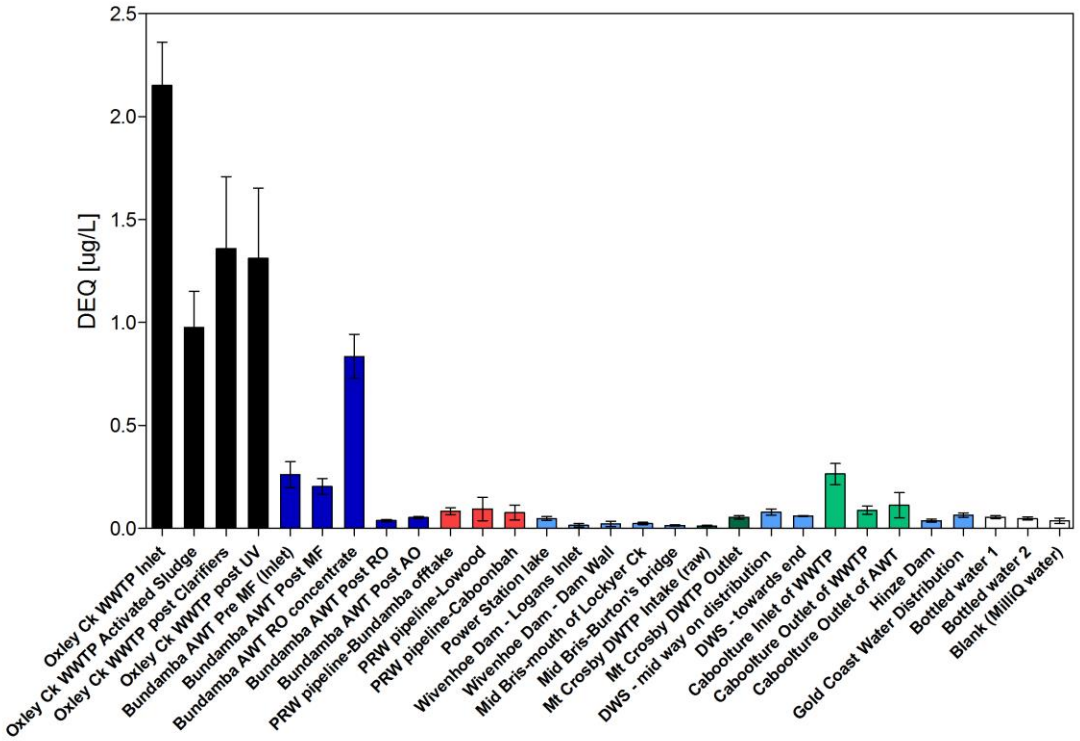


Figure 6: Diuron equivalent concentrations (DEQ) of the samples across the seven barriers of the indirect potable reuse scheme and additional samples expressed as average of two replicates. Bars indicate the standard deviation.

DEQ was also reduced by treatment in Caboolture WWTP from 0.26 to 0.09 µg/L. No further reduction in DEQ was observed after enhanced treatment (Table 3). Results are not in agreement with a previous study at Caboolture EWTP where the enhanced treatment (particularly pre–ozonation) reduced the DEQ effect to a level below the detection limit of the assay (Macova et al., 2010). The MilliQ blank also showed an effect slightly higher than the detection limit of the bioassay with 0.04 µg/L.

4.5. Arylhydrocarbon Receptor Response – AhR-CAFLUX Assay

Similar to previous bioanalysis, 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 3, Figure 7). 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 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 the previous study at 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.

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

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 binding to the arylhydrocarbon receptor present in the sample not only the persistent compounds. The less persistent group includes for example polycyclic aromatic hydrocarbons.

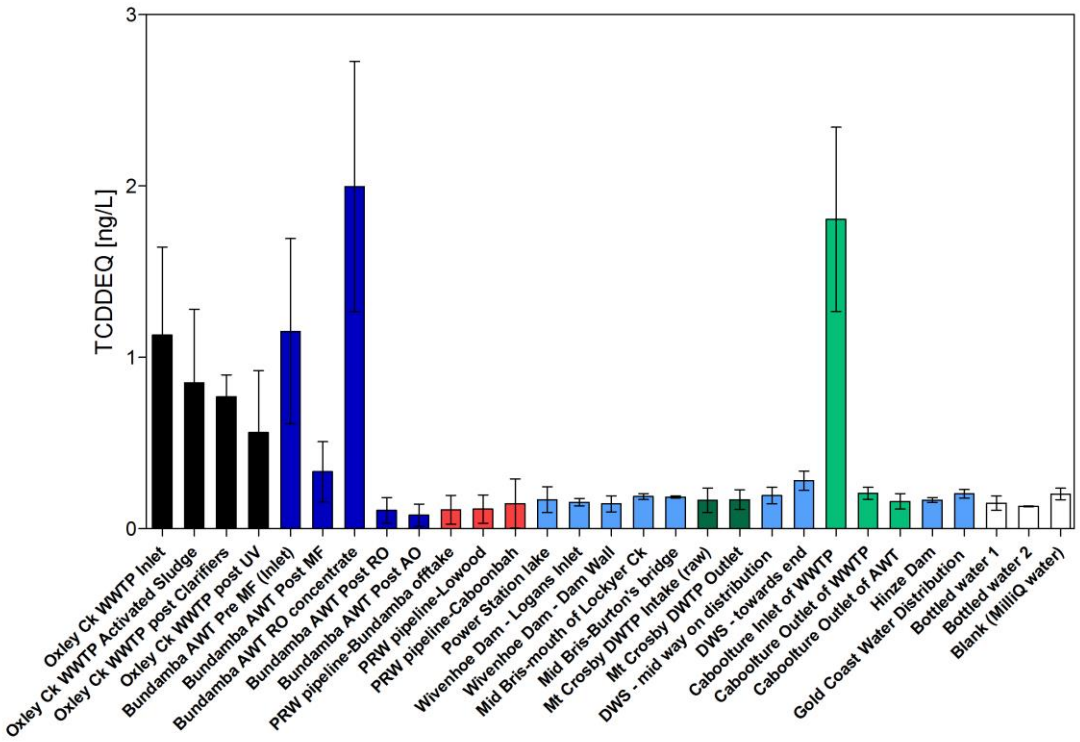


Figure 7: TCDD equivalent concentrations (TCDDEQ) of the samples across the seven barriers of the indirect potable reuse scheme and additional samples expressed as average of two replicates. Error bars indicate the standard deviation.

4.6. Genotoxicity – *UmuC* Assay

To enable detection of progenotoxins that require metabolic activation to become genotoxic, samples were tested both with and without 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. Results of the *umuC* assay are 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). Unlike the 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 EN International Standard Organisation (ISO) guideline as the threshold of genotoxic effect (ISO, 2000), providing the sample was not cytotoxic (growth < 0.5). (Table 3, Figure 8).

Similar to all other bioanalyses, genotoxic effect was significantly decreased post Barrier 2. Activated sludge at Oxley Creek WWTP reduced the genotoxic effects both without and with metabolic activation by 90% and 87%, respectively (Table 3). No further decrease was observed post clarifiers and after UV treatment. Barrier 3 treatment (microfiltration) at 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.

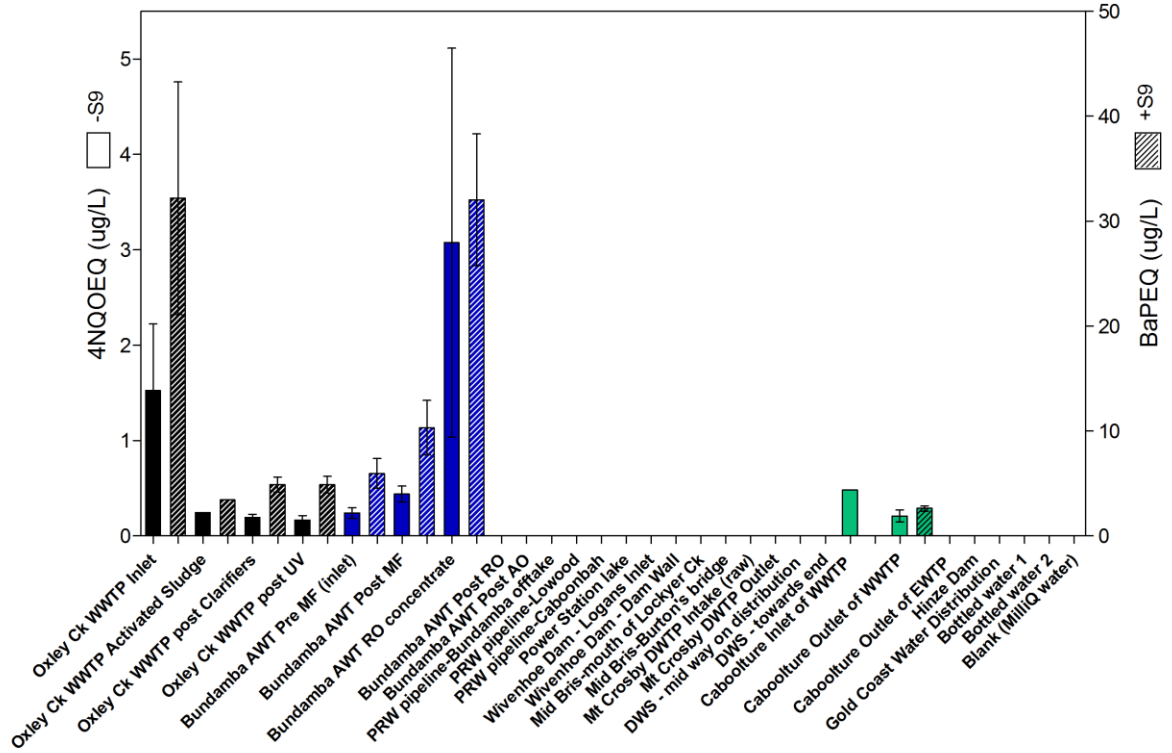


Figure 8: Genotoxic effect of the samples across the seven barriers of the indirect potable reuse scheme and additional samples expressed as 4-nitroquinoline-oxide equivalent concentration (4NQOEQ) (assay without metabolic activation –S9) and benzo[a]pyrene equivalent concentration (BaPEQ) (assay with metabolic activation +S9). Data represent the average of two replicates. Error bars indicate the standard deviation. Missing bars represent data below detection limit: 0.05 µg/L of 4NQOEQ and 0.8 µg/L of BaPEQ.

A surprisingly low genotoxic effect was observed in the inlet of the Caboolture WWTP in comparison with a previous study (2008, unpublished data) and in comparison to 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 Caboolture WWTP, to a level below the detection limit of the assay at the outlet of the EWTP (< 0.05 µg/L of 4NQOEQ and < 0.8 µg/L of BaPEQ).

Table 4: Summary of the Bioanalytical Results Expressed as the Average ± sd of Two Replicates.

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
Oxley Ck WWTP Inlet	25.57	17.40	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
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	
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	
Wivenhoe Dam – 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	
Wivenhoe Dam - 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 - mouth of Lockyer Creek	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	
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	
Drinking Water System - 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	
Drinking Water System - 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	
South Caboolture WWTP Influent (raw)	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	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 (not spiked 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.

4.7. QA/QC Samples

If there are additive interactions of chemicals in a mixture, then their toxic equivalent concentrations will sum up. However, if there are synergistic or antagonistic interactions of chemicals in mixtures, or the matrix interferes with the solid-phase extraction or the bioassay response, then a deviation from additivity of TEQs would be observed. We tested the potential of the samples to cause deviation from concentration addition by spiking a solution of a mixture of reference compounds to three selected samples and MilliQ water.

The MilliQ water sample and parallel samples collected at 3 sites (Oxley Creek WWTP post clarifiers, Mid-Brisbane at Burton's Bridge and the Drinking Water System mid-way along the distribution line (DWS mid-way)) were spiked with the mixture of the reference compounds diuron, parathion and 17β-estradiol. The amount of diuron, parathion and 17β-estradiol in the spiked samples was 3 µg, 17 µg and 0.25 ng per total volume of the sample, respectively (Table 4). The concentration of the reference compounds per litre of the sample was calculated based on the total volume of the sample collected. Since the volume of the sample collected at Oxley Creek WWTP post clarifiers was 2 L, the concentration of the diuron, parathion and 17β-estradiol per litre of the sample was 1.5 µg/L, 8.6 µg/L and 0.13 ng/L, respectively. The concentration of the diuron, parathion and 17β-estradiol in the remaining three samples (including MilliQ water) where we collected 4 L was 0.75 µg/L, 4.3 µg/L and 0.065 ng/L, respectively.

The effects of the spiked samples in selected bioassays were compared with the predicted effect and with the effect in the sample where the matrix did not interfere (MilliQ water).

Table 5: Reference Compound Mixture Details.

Reference Compound	Concentration in the Spiking Mixture Stock (MeOH)	Concentration in Total Volume of Sample	Concentration in 2 L Sample (Oxley Creek WWTP Post Clarifiers)	Concentration in 4 L Samples[a]
diuron	60 µg/mL	3 µg/sample	1.5 µg/L	0.75 µg/L
parathion	0.34 mg/mL	17 µg/sample	8.6 µg/L	4.3 µg/L
17β-estradiol	5 ng/mL	0.25 ng/sample	0.13 ng/L	0.065 ng/L

[a] Mid-Brisbane at Burton's Bridge, Drinking Water System – mid-way on the distribution line and MilliQ water samples

4.7.1. Phytotoxicity – PSII Inhibition I-PAM Assay

The predicted effect of the spiked sample in the I-PAM assay was calculated based on the concentration of the diuron in the spiking mixture. The effect of the spiking mixture in the I-PAM assay was comparable with the effect of the diuron based on the EC_{50} interpolated from concentration–effect curves (Figure 9A). This indicates that none of the other components in the reference compound mixture interfered with the IPAM assay.

The diuron equivalent concentration (DEQ) of the native sample collected at Oxley Creek WWTP post clarifiers was 1.36 µg/L in comparison with the 3.05 µg/L of the DEQ in the spiked sample (Figure 9B). The theoretical contribution of the spike represented 1.5 µg/L, assuming that there was no cross reactivity of the reference compounds at the concentration tested and only diuron had an effect in the I-PAM assay. Therefore, the predicted effect of the spiked sample in the I-PAM assay would be 2.86 µg/L (sum of the bar "unspiked sample (exp.)", plus "theoretical contribution of spike (calc.)" in Figure 9), which was comparable with the 3.05 µg/L of DEQ measured in the I-PAM assay.

The measured DEQ of the spiked sample collected in Mid-Brisbane at Burton's bridge sample and at DWS mid-way was lower than the predicted DEQ, representing 61% and 45% respectively of the calculated sum DEQ of sample plus spiked concentration. A comparable, low effect was observed in the MilliQ water sample where the measured DEQ represented 58% of the predicted effect, suggesting lower recovery of the PSII herbicides in the SPE extraction.

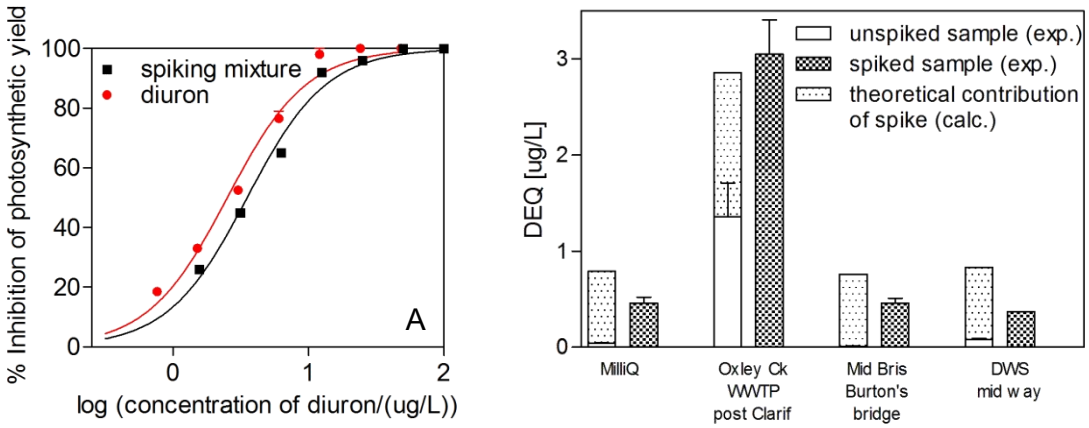


Figure 9: (A) Concentration-effect curves of the reference compound diuron and the spiking mixture (diuron, 17β-estradiol and parathion) in I-PAM assay; and (B) the effect of the three selected samples and MilliQ water in I-PAM assay expressed as diuron equivalent concentration (DEQ). DEQ of the native (not spiked) and spiked samples were expressed as average ± standard deviation of two replicates. Theoretical contribution of the spike was calculated based on the concentrations of the diuron in the spiking mixture.

4.7.2. Neurotoxicity – Acetylcholinesterase Inhibition Assay

Similar to I-PAM assay, the effect of the mixture was comparable with the effect of the reference compound parathion (Figure 10A), indicating that the other reference compounds did not interfere with the activity of parathion.

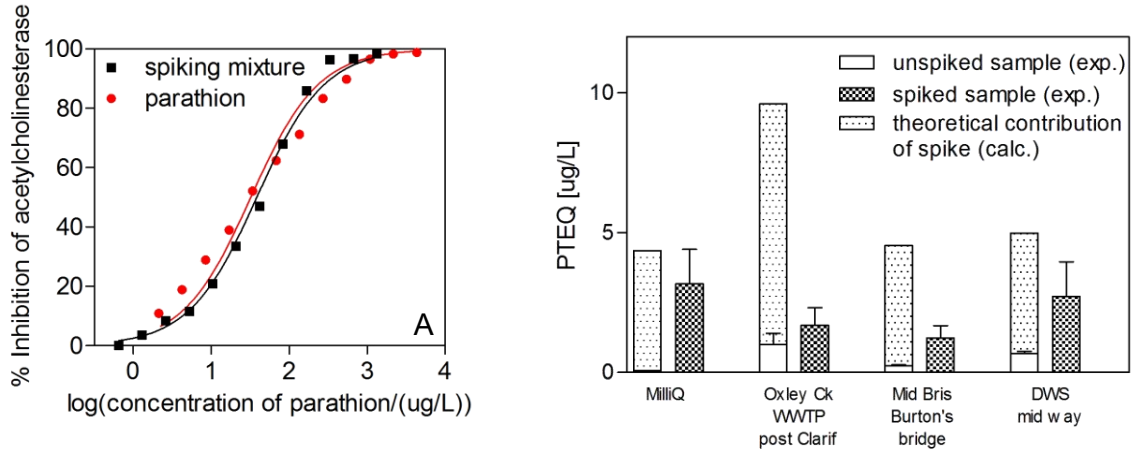


Figure 10: (A) Concentration-effect curves of the reference compound parathion and the spiking mixture (diuron, 17β-estradiol and parathion) AChE assay. (B) The effect of the three selected samples and MilliQ water in AChE assay expressed as parathion equivalent concentration (PTEQ). PTEQ of the unspiked and spiked samples were expressed as average ± standard deviation of two replicates. Theoretical contribution of the spike was calculated based on the concentrations of the parathion in the spiking mixture.

The measured effect of the spiked sample collected at Oxley Creek WWTP post clarifiers exhibited only 18% of the predicted effect, calculated as the PTEQ in the original sample (1 µg/L) plus the theoretical contribution of the spike (8.6 µg/L of parathion) (Figure 10B). A similar reduction of PTEQ was observed in the sample collected at Mid-Brisbane at Burton's bridge, where the experimental PTEQ value represented 27% of the predicted PTEQ (unspiked sample plus theoretical contribution of the spike). However, the predicted effect in the sample collected at DWS mid-way and in the MilliQ water was comparable with the measured PTEQ in the AChE assay (Figure 10B).

These results suggested that the recovery in SPE extraction is not a problem, but enzymes might be partially destroyed in highly contaminated samples and the matrix effects might lead to suppression by quenching the effect of the organophosphates present in the sample. This non-specific effect is being further investigated in the recently started UWSRA "Bioassays and Risk Communication" project, with a series of mixture experiments to assess the contribution of the non-specifically acting compounds, particularly in highly contaminated samples such as raw sewage, secondary treated sewage or reverse osmosis concentrate. The main difference between the AChE assay and all other assays in the test battery used here is that, for AChE, an isolated enzyme is used. Therefore, it is not possible to differentiate between non-specific disturbance of enzyme activity and specific inhibitor binding. In contrast, all other assays are cell based and the cytotoxicity is always assessed in parallel to give a warning in case cytotoxicity exceeds specific effects. There remains a grey zone, when cytotoxicity is not observed but non-specific effects can modify the investigated specific endpoint, but this zone is likely to cover a much smaller concentration range that in the case of the isolated enzyme, where no cytotoxicity control exists.

4.7.3. Estrogenic Activity – E-SCREEN Assay

In contrast to the previous two assays, the effect of the spiking mixture in the E-SCREEN assay was significantly lower than the effect of the reference compound 17β-estradiol (Figure 11A).

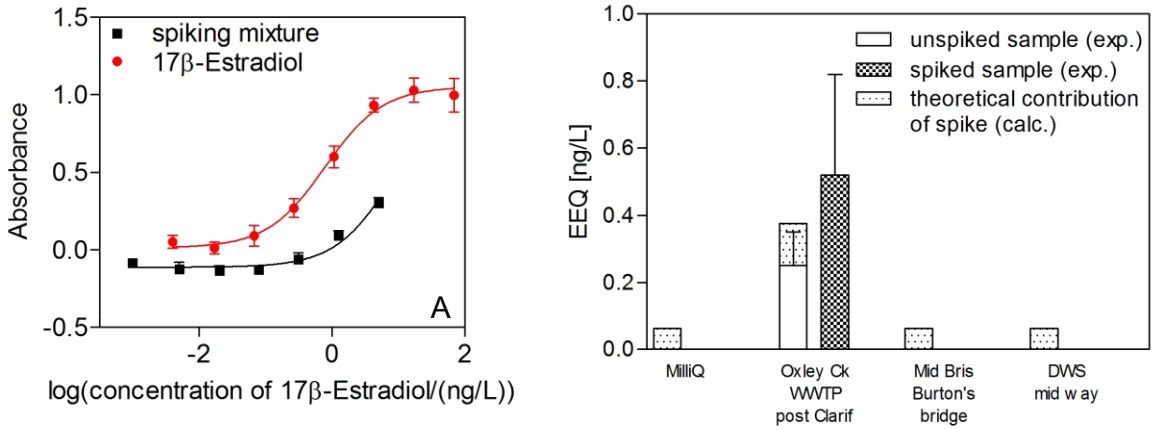


Figure 11: (A) Concentration-effect curves of the reference compound 17β-estradiol and the spiking mixture (diuron, 17β-estradiol and parathion) in E-SCREEN assay. (B) The effect of the three selected samples and MilliQ water in E-SCREEN assay expressed as estradiol equivalent concentration (EEQ). EEQ of the unspiked and spiked samples were expressed as average ± standard deviation of two replicates. Theoretical contribution of the spike was calculated based on the concentrations of the 17β-estradiol in the spiking mixture.

Low relative potency of the spiking mixture in comparison with the 17β-estradiol might explain the low estrogenic effect < 0.01 ng/L detected in the spiked samples collected at Mid-Brisbane at Burton's bridge, DWS mid-way and in the MilliQ water (Figure 11B). However, the low stability of the 17β-estradiol or recovery issue cannot be excluded. The EEQ levels at Oxley Creek WWTP post clarifiers were inconclusive due to high standard deviation of the spiked sample. These results need further investigation and the experiment will be repeated after modification of the spiking mixture using a xenoestrogen like nonylphenol which is actually present in waste water while 17β-estradiol is typically not found in appreciable concentration in secondary treated effluent as it is readily biodegradable.

No effect of the spiked samples was observed in Microtox, AhR-CAFLUX or *umuC* assay, suggesting that the reference compounds in the spiking mixture at the concentration tested did not cross-react in these three assays (data not shown).

Despite the limitations, this pilot study focused on the potential matrix effects of the samples provided valuable information about the selected bioanalysis and initiated a series of experiments to further validate the bioanalytical tools and the interpretation of the results.

5. CONCLUSIONS

- This report describes the first study that covers the monitoring of all steps in the seven treatment barriers of the Western Corridor Recycled Water Scheme.
- The toxicity of samples was reduced across the seven treatment barriers in all six selected bioassays (Figure 12). 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 of a different group of chemicals and their mixture effect. The effects after Barriers 6 and 7 and in drinking water were very low for many endpoints, typically falling below the detection limit.

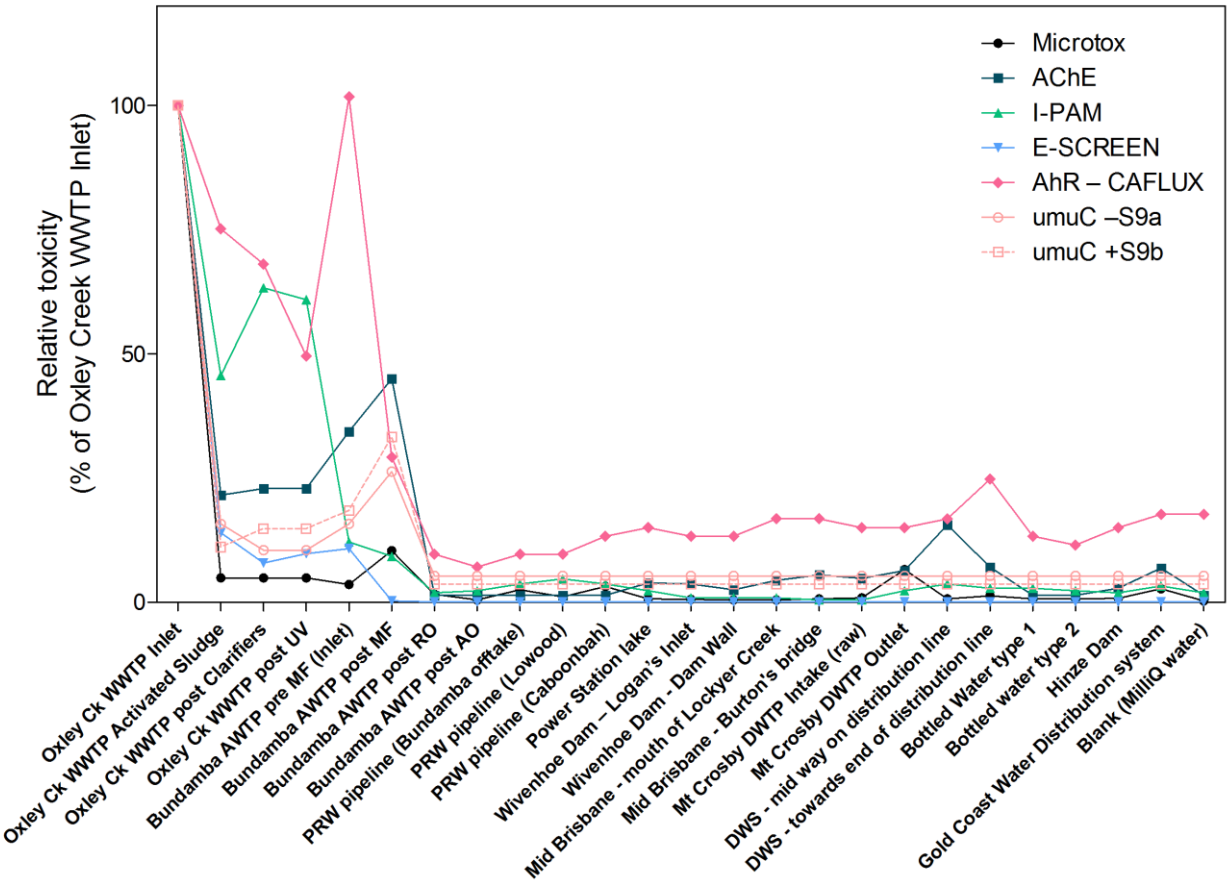


Figure 12 Reduction of toxicity across treatment barriers in all six bioassays. (Note: Data represent the average of two replicates, DWS – Drinking Water System).

- Detection limits of the bioassays were comparable to 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 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 toxic equivalent concentrations (TEQ) provide valuable information complementing chemical analytical data to identify potential issues or to predict potential exposure/risks of micropollutants to humans or the environment.

Limitations:

- High enrichment of the samples allowed us to achieve low detection limits in the assays. However, high enrichment might cause matrix effects, increase the effect of the blank, and hence compromise the detection limit of the assay.
- Particular attention has to be paid to estrogenic compounds, which could be degraded in the collected samples within a few days even if stored at 4°C.

6. RECOMMENDATIONS FOR FURTHER RESEARCH / NEXT STEPS

- This project is directly linked to one of the Seqwater projects monitoring the micropollutants at Wivenhoe Dam. A second sampling campaign at Wivenhoe Dam has been conducted and the samples are presently in the stage of bioanalytical testing. The results will be compared to those obtained in this project and will be made available to the public in a respected, peer-reviewed publication.
- Importantly, further work is required to assess the matrix effect of the highly contaminated samples in the AChE assay. Our preliminary experiments indicate that the acetylcholinesterase enzyme might by partially destroyed in highly contaminated samples, such as raw sewage or reverse osmosis concentrate. The potential matrix effect is being currently investigated in the series of mixture experiments.
- The unexpected increased baseline toxicity of the Mt. Crosby outlet sample is currently under investigation. The potential impact of dissolved organic matter on microtox performance will also be considered.
- In the next two years of the UWRSA "Bioassays and Risk Communication" project on bioanalytical tools, we plan to further validate and expand the test battery, with a particular focus on developing an effective communication strategy.
- The results of this project will be presented at the next UWSRA scientific forum. After clearance by stakeholders, results will be submitted to *Water Research* and also presented at "What's in Our Water: The significance of trace organic compounds" (2010) - 3rd Australian Symposium on Ecological Risk Assessment and Management of Endocrine Disrupting Chemicals (EDCs), Pharmaceuticals and Personal Care Products (PPCPs) in the Australasian Environment (Canberra, Nov 2010).

APPENDIX I: Detailed Experimental Methods of the Bioanalytical Test Battery

Baseline Toxicity – Bioluminescence Inhibition in Vibrio fischeri

The bioluminescence inhibition test with *Vibrio fischeri* was conducted according to the standard procedure ((International Standard Organisation, 1998) with modifications for a 96-well microplate (Escher et al., 2008a). Bioassays were performed at laboratory ambient temperature (~23°C) in white 96-well plates using 50 µL/well of reconstituted bacterial reagent (Microtox® reagent, JW Industrial Instruments, Roseville, Australia) incubated for 15 minutes at laboratory temperature in the dark before adding 100 µL of diluted extracts. Each replicate of the sample extract was tested at eight different concentrations, in duplicate, after serial dilution 1:2 in buffer. Each plate also included a negative control (buffer) and a positive control of eight concentrations of the reference compound phenol (in range from 5.21 to 667 mg/L). Bioluminescence was quantified in a BMG Labtech FLUOstar OPTIMA plate reader (BMG LABTECH GmbH, Germany) where luminescence was measured immediately prior to the addition of the sample extracts and then 5, 15 and 30 minutes after dosing. The light output after 30 minutes was used to calculate the EC_{50}.

Toxicity in the bioluminescence inhibition test was expressed in baseline toxicity equivalent concentration (baseline-TEQ, mg/L) using the EC_{50} of a virtual baseline toxicant of 12 mg/L (Escher et al., 2008a).

Neurotoxicity – Acetylcholinesterase Inhibition Assay

The AChE assay was performed according to standardised DIN 38415-1 protocol (Deutsche Norm, 1995). We used commercially available AChE enzyme from *E. electricus* and the synthetic substrate acetylthiocholine iodide. The acetylcholinesterase enzyme hydrolyses the substrate to yield acetate and thiocholine. The chromogenic reagent dithio-bis(2-nitrobenzoic acid) (DTNB) then reacts with free thiol groups producing the yellow 4-nitrothiolate which can be measured colorimetrically. All samples were serially diluted 1:2 in a phosphate buffer in a 96-well plate. Samples were oxidized by N-bromosuccinimide, before the reaction was inhibited with ascorbic acid (0.47 M) for one minute. An *E. electricus* acetylcholinesterase-solution (Sigma, USA) in a concentration of 0.23 U/mL of phosphate buffer was incubated with each sample for 10 minutes before a colour-based product indicator ACT/DTNB (S-acetylthiocholine iodide 60 mM / 5,5'-dithio-bis(-2-nitrobenzoic acid) 10 mM dissolved in $NaHCO_3$ (Sigma, USA), was added. Plates were immediately analysed in a FLUOstar plate reader where absorbance was measured at 420 nm at 30-second intervals. All plates included a negative control of phosphate buffer and a positive control of parathion (Sigma, Germany) in range: 3.95 nM - 8.09 µM.

The percentage of inhibition of acetylcholinesterase in each sample was calculated using the control enzyme rate and the rate of the enzyme-catalysed reaction in the presence of an inhibitor (the sample or standard). Acetylcholinesterase inhibition was expressed as parathion equivalent concentrations PTEQ (µg/L).

Phytotoxicity – PSII inhibition I-PAM Assay

Pulse amplitude modulated (PAM) fluorometry was used to monitor PS II quantum yield (Y(II)) (equation 6) in algal suspensions via repetitive measurements of the chlorophyll fluorescence parameters, basal fluorescence (F') and maximal fluorescence (F'm), following application of a saturation light pulse, which transiently closes all reaction centres (Schreiber et al., 2003).

$$Y(II) = \frac{F'm - F'}{F'm} \qquad (6)$$

Cultures of the freshwater chlorophyte *Chlorella vulgaris* obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO, Hobart, Australia), were grown in MBL media. Young cells show maximal responses and are least affected by saturation pulse application (Escher et al., 2006), hence test cultures were maintained in this exponential growth phase. Consistent cell densities

were maintained for all assays by ensuring basal fluorescence F' was within the range of F' = 0.08 to 0.12 and photosynthetic yield Y(II) within the range 0.50 to 0.55, as described by Muller et al. (2007).

The phytotoxicity assay was performed at laboratory ambient temperature (~23 °C) in black 96-well plates (Greiner) using Maxi-Imaging-PAM (Heinz Walz GmbH, Germany) for the determination of the photosynthesis yield. Each well contained 150 µL of biomaterial and 150 µL of sample or the reference compound diuron serially diluted 1:2 in MBL medium (Muller et al., 2007). Cultures were exposed to test light conditions for at least 10 minutes prior to assay commencement. Phytotoxic response of the algae after two hours of incubation was expressed as diuron equivalent concentrations DEQ.

Estrogenic Activity: E-SCREEN Assay

The MCF-7-BOS breast cancer cell line (obtained from Professor A Soto, Tufts University, Boston, USA) was routinely grown in Dulbecco's Modified Eagle Medium (GIBCO, USA) containing 10% foetal bovine serum (Australian origin, Invitrogen, Australia), 10 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (Thermo Electron, Melbourne, Australia), 0.1 mM nonessential amino acids (GIBCO, USA), 2 mM Glutamax (L-Alanyl-L-Glutamine) (GIBCO, USA), 1% of 10000 U penicillin/10000 µg streptomycin (BioWhittaker, USA) and 1 mM sodium pyruvate (Sigma, USA), in a T25 flask (Iwaki, Japan) at 37°C with 5% CO_2 in a humidified atmosphere (95% relative humidity).

The E-SCREEN assay in this study was performed according to the method described in the literature (Soto et al., 1995; Körner et al., 1999; Leusch et al., 2005) with some modifications to cell seeding number and cell proliferation measurement technique. At the start of each experiment, MCF-7 cells were seeded in 96-well microplates at 5000 cells/well, in 200 µL of phenol-red-free Dulbecco's Modified Eagle Medium (HyClone, USA) containing 10% charcoal-dextran-treated foetal bovine serum (CD-FBS, HyClone, USA), 10 mM HEPES buffer, 0.1 mM non-essential amino acids, 2 mM Glutamax, 1% of 10000 U penicillin and 10000 µg streptomycin, and 1 mM sodium pyruvate. After 24 hours, the medium from each well was aspirated and replaced with 200 µL of phenol-red-free medium containing a serial dilution of sample extracts. Nine dilution volumes were tested with three replicates per sample; six wells per assay without hormones or sample acted as a negative control. 17ß-estradiol (Sigma, Montana, USA) was included as the internal positive control in each assay, in nine final concentrations between 10^{-14} M and 10^{-9} M. After five days of exposure, cell proliferation was measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Wisconsin, USA). The medium was aspirated and replaced with 120 µl of CellTiter reagent mix. The cells were incubated for two hours and then absorbance recorded at 490 nm with a FLUOstar plate reader. The quantity of formazan product as measured by the amount of 490 nm absorbance is, within a certain range, directly proportional to the number of living cells in culture (Promega Corporation, 2005).

The estrogenicity of the samples was expressed as 17ß-estradiol equivalent concentration (EEQ).

Ah-Receptor Response: AhR-CAFLUX Assay

The AhR-CAFLUX assay was performed according to the procedure described by Zhao and Denison (2004) with the following changes. In brief, the H4G1.1c2 cells (provided by Professor M.S. Denison, University of California Davis, USA) were grown in T-75 cm2 flasks in α-MEM (GIBCO) selective medium containing 10% fetal bovine serum (Australian origin, Invitrogen, Australia), 1% of 10000 U penicillin/10000 µg streptomycin (BioWhittaker, USA) and 1 mg/mL geneticin (GIBCO, USA) in a tissue-culture incubator (37˚C, 5% CO2) until they were approximately 80 - 90% confluent. The cells were then trypsinised and counted using a hemocytometer and diluted in selective media to a concentration of 3.0 × 105 cells/mL, seeded into 96-well plates at a volume of 100 µL/well and incubated for 24 hours. After 24 hours, the media was replaced with a non-selective media containing the chemical to be tested in a five-point dilution series. The cells were also dosed with 2,3,7,8-TCDD (Novachem, Collingwood, VIC, Australia) with concentrations ranging from 0.1 nM to 20 µM. The plates were incubated at 33˚C, 5% CO2 for 24 hours and the fluorescent protein expression was read in a FLUOstar plate reader at excitation 485 nm, emission 520 nm, and gain 1500. The H4G1.1c2 cells respond to TCDD and other AhR agonists with the induction of fluorescence in a time, dose, chemical,

and AhR-specific manner. The green fluorescence production is a direct measure for the exposure level of the cells to AhR agonists. These readings were performed every 24 hours for three consecutive days. Positive controls of 2,3,7,8-TCDD standards and ß-napthoflavone, and a negative control of DMSO were also analysed. The AhR response to environmental samples was expressed as the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentration (TCDDEQ).

Genotoxicity - UmuC Assay

The *umuC* assay developed by Oda et al. (1985) was adjusted to a 96-well microplate format by Reifferscheid et al. (1991) and this protocol has been standardised and validated (International Standard Organisation, 2000).

The *umuC* assay was carried out according to the procedure described by ISO 13829:2000 (International Standard Organisation, 2000), using the bacteria *Salmonella typhimurium* TA1535/pSK1002 (provided by Dr. G Reifferscheid, German Federal Institute of Hydrology, Germany). Samples were tested both with and without exogenous metabolic activation using liver supernatant fraction (S9) prepared from rats treated with Aroclor 1254. (Moltox Inc, USA).

In brief, the bacteria were grown in Luria-Bertani medium containing 50 µg/mL ampicillin (Sigma, USA), at 37°C for approximately 16 hours with vigorous shaking. The overnight culture was diluted 20-fold in TGA medium (1% bactotryptone, 0.5% NaCl, 0.2% glucose, 50 µg/mL ampicillin) and culturing was continued until the optical density at 600 nm reached about 0.25. 96-well plates were prepared with half of the treatments containing 3% S9 mix. Sample extracts were then added, followed by the bacterial culture. The mixture was incubated at 37°C for two hours with shaking. 30 µL of the mixture was transferred to a new plate with pre-warmed fresh TGA medium for a further incubation of two hours with shaking. The bacterial density at 600 nm was measured and ß-galactosidase activity was assayed using 2-nitrophenyl-ß-D-galactopyranoside (ONPG, 4.5 mg/mL) as a substrate. The activity was determined by the method of Oda et al. (1985). The results are presented as means of triplicate determinations. For QA/QC, positive controls (4-nitroquinoline-N-oxide (4-NQO) and 2-aminoanthracene (2-AA)), negative controls and a solvent control (3% DMSO) were also tested. In the *umuC* assay, a quantification limit was used instead of detection limit and was defined as the maximum REF achieved in this assay depending on the original volume of the sample and the dilution of the extract in the assay.

APPENDIX II: Selected Sampling Sites

Figure 13 Oxley Creek WWTP Influent.

Figure 14 Oxley Creek WWTP post UV.

Figure 15 Mid Brisbane - Burton's Bridge.

Figure 16 Mid Brisbane – Lowood.

Greenacre Dr, ARUNDEL

Sample Run: B-2

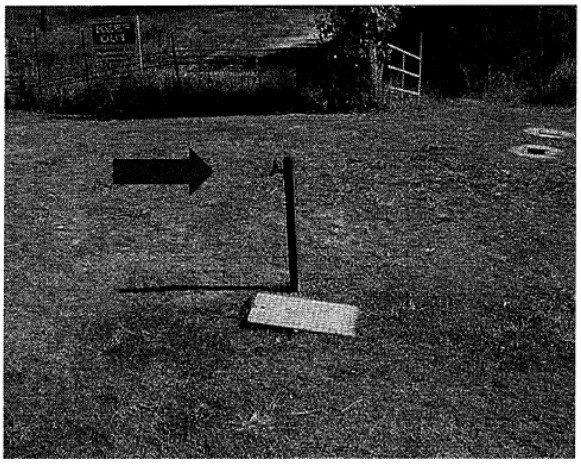
Site ID: B1_SP1

Res Tested: Southport West

Location: Easement at northern end of Greenacre Dr, ARUNDEL
Opposite # 191 Greenacre Drive

UBD 2004 Ref: Map 27 – M2

Notes: N/A



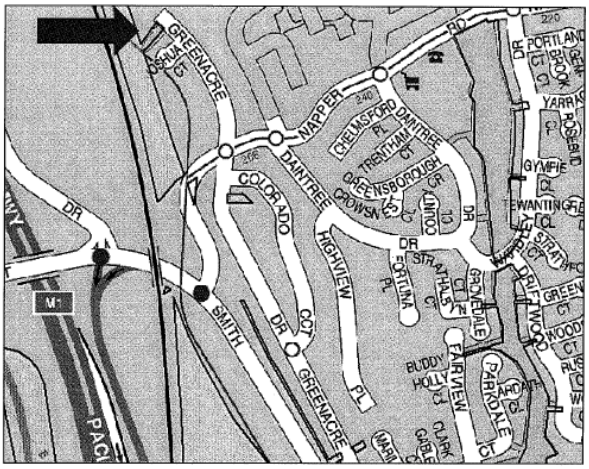


Figure 17 Gold Coast Water Distribution System. Southport West Zone fed from Molendinar (Hinze Dam surface water – no desalination mixed in).

Figure 18 Hinze Dam.

REFERENCES

Altenburger, R., Walter, H., Grote, M., 2004. What contributes to the combined effect of a complex mixture? Environmental Science and Technology 38, 6353-6362.

Deutsche Norm, 1995. Suborganismische Verfahren: Teil 1: Bestimmung von Cholinesterase-hemmenden Organophosphat- und Carbamat-Pestiziden (Cholinesterase-Hemmtest). DIN, N.W.N.i. (ed), DIN 38415-1: 1995-02, Berlin, Germany.

Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7, 88-95.

Escher, B.I., Bramaz, N., Maurer, M., Richter, M., Sutter, D., von Känel, C., Zschokke, M., 2005. Screening test battery for pharmaceuticals in urine and wastewater. Environ. Tox. Chem. 24, 750-758.

Escher, B.I., Bramaz, N., Mueller, J.F., Quayle, P., Rutishauser, S., Vermeirssen, E., 2008a. Toxic equivalent concentrations (TEQs) for baseline toxicity and specific modes of action as a tool to improve evaluation of ecotoxicity tests on environmental samples. J. Env. Monitor. 10, 612-621.

Escher, B.I., Bramaz, N., Quayle, P., Rutishauser, S., Vermeirssen, E., 2008b. Monitoring of the ecotoxicological hazard potential by polar organic micropollutants in sewage treatment plants and surface waters using a mode-of-action based test battery. J. Env. Monitor. 10, 622-631.

Escher, B.I., Bramaz, N., Ort, C., 2009. JEM spotlight: Monitoring the treatment efficiency of a full scale ozonation on a sewage treatment plant with a mode-of-action based test battery. J Environ Monit 11, 1836-1846.

Escher, B.I., Quayle, P., Muller, R., Schreiber, U. and Mueller, J., 2006. Passive sampling of herbicides combined with effect analysis in algae using a novel high-throughput phytotoxicity assay (Maxi-Imaging- PAM). Journal Environmental Monitoring 8, 456-464.

Farré, M., Martinez, E., Hernando, M., Fernandez-Alba, A., Fritz, J.I., Unruh, E., Mihail, O., Sakkas, V., Morbey, A., Albanis, T.A., Brito, F., Hansen, P.D., Barcelo, D., 2006. European ring exercise on water toxicity using different bioluminescence inhibition tests based on *Vibrio fischeri*, in support to the implementation of the water framework directive. Talanta, 323-333.

Hamers, T., Molin, K.R.J., Koeman, J.H. and Murk, A.J., 2000. A small-volume bioassay for quantification of the esterase inhibiting potency of mixtures of organophosphates and carbamate insceticides in rainwater: development and optimization. Toxicological Sciences 58, 60-67.

Hawker, D.W., Cumming, J.L., Neale, P.A., Bartkow, M.E. and Escher, B.I., 2010. A screening level fate model of organic contaminants from advanced water treatment in a potable water supply reservoir. Water Research, *in press*.

International Standard Organisation, 1998. Water Quality–determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test). EN ISO 11348-3, Geneva, Switzerland.

International Standard Organisation, 2000. Water quality - Determination of the genotoxicity of water and waste water using the umu-test, EN ISO 13829 (2000) and 38415-3 (1996), Geneva, Switzerland.

Johnson B.T., 2005. Microtox acute toxicity test. In Blaise C, Ferard J-F, eds, *Small-scale Freshwater Toxicity Investigations* Volume 1: Toxicity Test Methods. Springer.

Körner, W., Bolz, U., Sussmuth, W., Hiller, G., Schuller, W., Hanf, V. and Hagenmaier, H., 2000. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 40(9-11), 1131-1142.

Körner, W., Hanf, V., Schuller, W., Kempter, C., Metzger, J., Hagenmaier, H., 1999. Development of a sensitive E-screen assay for quantitative analysis of estrogenic activity in municipal sewage plant effluents. Sci Total Environ 225, 33-48.

Lee, Y., Escher, B.I., Gunten, U.v., 2008. Efficient removal of estrogenic activity during oxidative treatment of waters containing steroid estrogens. Environmental Science and Technology 42, 6333-6339.

Leusch, F.D., Chapman, H.F., van den Heuvel, M.R., Tan, B.L., Gooneratne, S.R. and Tremblay, L.A., 2006. Bioassay-derived androgenic and estrogenic activity in municipal sewage in Australia and New Zealand. Ecotoxicology and Environmental Safety 65(3), 403-411.

Leusch, F.D.L., Chapman, H.F., Körner, W., Gooneratne, S.R., and Tremblay, L.A., 2005. Efficacy of an advanced sewage treatment plant in southeast Queensland, Australia, to remove estrogenic chemicals. Environmental Science and Technology 39(15), 5781-5786.

Macova, M., Escher, B.I., Reungoat, J., Carswell, S., Chue, K.L., Keller, J., Mueller, J.F., 2010. Monitoring the biological activity of micropollutants during advanced wastewater treatment with ozonation and activated carbon filtration. Water Res 44, 477-492.

Muller, R., Schreiber, U., Escher, B.I., Quayle, P., Bengtson Nash, S., Mueller, J., 2008. Rapid exposure assessment of PSII herbicides in surface water using a novel chlorophyll a fluorescence imaging assay. Science Tot. Environ. 401, 51-59.

Muller, R., Tang, J., Thier, R., Mueller, J., 2007. Combining passive sampling and toxicity testing for evaluation of mixtures of polar organic chemicals in sewage treatment plant effluents. J. Env. Monitor. 9, 104-109.

Murk, A.J., Legler, J., van Lipzig, M.M.H., Meerman, J.H.N., Belfroid, A.C., Spenkelink, A., van der Burg, B., Rijs, G.B.J. and Vethaak, D., 2002. Detection of estrogenic potency in wastewater and surface water with three in vitro bioassays. Environmental Toxicology and Chemistry 21(1), 16-23.

Nagy, S., Sanborn, J., Hammock, B., Denison, M., 2002. Development of a green fluorescent protein-based cell bioassay for the rapid and inexpensive detection and characterization of Ah receptor agonists. Toxicol. Sci. 65, 200-210.

Oda, J., Nakamura, S.I., Oki, I., Kato, T. and Shinagawa, H., 1985. Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens. Mutatation Research 147, 219-229.

Reifferscheid, G., Heil, J., Oda, Y. and Zahn, R.K., 1991. A microplate version of the SOS/umu-test for rapid detection of genotoxins and genotoxic potentials of environmental samples. Mutation Research 253(3), 215-222.

Reungoat, J., Macova, M., Escher, B.I., Carswell, S., Mueller, J.F., Keller, J., 2010. Removal of micropollutants and reduction of biological activity in a full scale reclamation plant using ozonation and activated carbon filtration. Water Res 44, 625-637.

Schreiber, U., Müller, J., Haugg, A., Gademann, R., 2003. New type of dual-channel PAM chlorophyll fluorometer for highly sensitive water toxicity biotests. Photosynth. Res. 74, 317-330.

Schreiber, U., Quayle, P., Schmidt, S., Escher, B.I., Mueller, J., 2007. Methodology and evaluation of a highly sensitive algae toxicity test based on multiwell chlorophyll fluorescence imaging. Biosensors and Bioelectronics 22, 2554-2563.

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N. and Serrano, F.O., 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental Health Perspectives 103 Suppl 7, 113-122.

van Leeuwen, J., Pipe-Martin, C., Lehmann, R.M., 2003. Water Reclamation At South Caboolture, Queensland, Australia. Ozone: Science and Engineering 25, 107-120.

Vermeirssen, E.L.M., Hollender, J., von der Voet, J. and Escher, B.I., 2010. Linking toxicity in algal and bacterial assays with chemical analysis in passive samplers exposed to treated sewage effluent. Environ. Tox. Chem, *in press.*

Villeneuve, D.L., Blankenship, A.L., Giesy, J.P., 2000. Derivation and application of relative potency estimates based on in-vitro bioassays. Environmental Toxicology Chemistry 19, 2835-2843.

Warne, M.S.J., Hawker, D.W., 1995. The Number of Components in a Mixture Determines Whether Synergistic and Antagonistic or Additive Toxicity Predominate - the Funnel Hypothesis. Ecotoxicology and Environmental Safety 31, 23-28.

Zhao, B., Denison, M., 2004. Dvelopment and characterization of a green fluorescence protein-based rat cell bioassay system for detection of Ah receptor ligands. Organohalogen Compounds 66.