Is there a needle in the haystack?

Combining an *in vitro* bioassay battery with targeted chemical analysis to detect "unknown" organic contaminants in recycled water

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Introduction

A rising population and drier climate in Australia are leading to chronic water shortages in capital cities, prompting exploration of alternate water sources and reuse of available waters. There is a need to thoroughly characterize the human and ecological risks associated with these new water sources, particularly water reclaimed from wastewater for potable use. However analysis of these complex mixtures of trace chemicals presents challenges for standard chemical analysis methods, which require foreknowledge of the likely contaminants. Bioanalytical methods such as *in vitro* bioassays are ideal screening tools that can detect a wide range of contaminants based on their biological effect rather than their chemical structures, which means that no expectation bias is introduced in the analysis. In combination with chemical analysis, "unknown" biologically-active contaminants can be detected and sometimes identified. This project will apply a combination of *in vitro* bioassay and chemical methods to screen water produced from several Australian water recycling schemes for potentially harmful chemicals.

Site selection and sampling

Nine water reclamation plants in 6 Australian states/territories were sampled. These plants were selected to provide a variety of treatment technologies (from pond- to membrane-based systems) in a range of climatic conditions. Grab samples (2×2L) were taken of the source (usually treated sewage) and the final recycled water in methanol-rinsed glass bottles. Metropolitan tap water, bottled and ultrapure water samples were also taken as negative control. All samples were kept on ice until brought back to the laboratory. Samples were processed on the same day by passage through two 6cc solid-phase extraction cartridges in series, first an Oasis HLB (Waters Corp) and then a Supelclean coconut charcoal cartridge (Sigma-Aldrich). Once dried, the cartridges were eluted with 100% methanol, the extracts blown down to dryness under gentle nitrogen stream, and reconstituted to 1mL. The same aliquots were used for chemical and bioassay analysis.

Chemical analysis

A list of 39 priority chemicals was narrowed down from an initial list of 342 chemicals from a variety of sources (including scientific literature, Australian guidelines and other reports) based on criteria such as the availability of chemical analysis methods, predicted biological activity, actual and perceived toxicity, presence on industrial inventories and likelihood of occurrence in recycled water sources. The priority list includes chlorinated and brominated disinfection by-products, natural hormones (*e.g.* estrogens, androgens), industrial compounds (*e.g.* bisphenol A, nonylphenol), a personal care product (DEET), pesticides (*e.g.* atrazine, diuron, pentachlorophenol), pharmaceuticals (*e.g.* caffeine, carbamazepine, ethynylestradiol) and a veterinary drug (trenbolone) (Figure 1). These priority chemicals will be analysed in the SPE extracts by a combination of high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) techniques. Analytical quantification will be undertaken with isotope dilution to the SPE extracts in order to control for any matrix-effects variability.

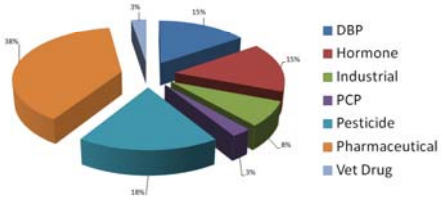

Fig 1. Priority chemical classes

Bioanalytical tools

Following a review of potential human health effects from drinking water exposure to toxic chemicals and the current state-of-the-science of bioanalytical methods, 12 *in vitro* bioassays were selected for this project. The selected assays provide measures of primary non-specific (basal cytotoxicity), specific (endocrine effects, hepatoxicity, and limited measures of immunotoxicity and neurotoxicity) and reactive toxicity (mutagenicity and genotoxicity) (Table 1).

The priority chemicals and field samples will be tested in all bioassays by adding an aliquot of the compound or SPE extracts, respectively, to the incubation media, ensuring the final carrier solvent concentration does not result in toxicity (usually ≤ 0.1%). The response in each of the bioassays will then be compared to a positive standard dose-response curve and expressed relative to that standard, as a toxic equivalent. Where possible, this will allow determination of the toxic equivalency factor (TEF) for the priority chemicals and of a toxic equivalent concentration (TEQ) in the field samples.

Two important potential health outcomes from exposure to toxicants from drinking water were not included in our battery: developmental and reproductive toxicity. Development and reproduction are meta-cellular events and it is currently not possible to adequately predict toxicity to these events in humans using *in vitro* models.

Table 1. Bioassay battery

Mode of toxicity *	Endpoint	Mechanism	Bioassay
Non-specific	Cytotoxicity	Basal cytotoxicity to gastro-intestinal cells	Caco2-NRU [1]
	Cytotoxicity to liver cells	Basal cytotoxicity to liver cells	C3A-cytotox
Specific	Endocrine effect: estrogenic	ER-mediated transcriptional activation	ER-CALUX [2] / E-SCREEN [3]
	Endocrine effect: androgenic	AR-mediated transcriptional activation	AR-CALUX [2]
	Endocrine effect: glucocorticoid	GR-mediated transcriptional activation	GR-CALUX [2]
	Endocrine effect: progesteronic	PR-mediated transcriptional activation	PR-CALUX [2]
	Endocrine effect: thyroid receptor	TRb-mediated transcriptional activation	TRb-CALUX [2]
	Hepatotoxicity	CYP450 induction in liver cells	C3A-CYP450 induction
	(Imunotoxicity)	Immunomodulation of cytokine production by monocytes	THP1 cytokine production assay [4]
	(Neurotoxicity)	Inhibition of acetylcholinesterase	AChE assay [5]
Reactive	Mutagenicity	Mutagenic potential	Ames test [6]
	Genotoxicity	Micronucleus formation	WIL2NS FCMN [7]

* Classification is based on [8]

Analysis plan and the effects fingerprint

The priority chemicals will be tested in the full bioassay battery to establish an "effect fingerprint" (Table 2). This fingerprint will be used to direct chemical analysis during real sample analysis. Samples collected at several water reclamation plants in Australia will be tested in the full battery of bioassays. Those inducing biological responses *in vitro* will be tested using targeted chemical analysis, directed by the effects fingerprints (Figure 2).

Table 2. Tentative fingerprint of priority chemicals

CASRN	Chemical Name	Cyto	Ames	MN	Hepato	ER-CALUX	AR-CALUX	GR-CALUX	PR-CALUX	TRb-CALUX	AChE	THP-1 CPA
80-05-7	Bisphenol A (BPA)											
1080-34-5	Triclosan											
104-40-5	4-Nonylphenol											
1912-24-9	Atrazine											
50-28-2	17b-Estradiol (E2)											
140-66-9	4-tert-octylphenol											
67-66-3	Chloroform											
57-83-0	Norethindrone/ethynyl											
123-46-6	sulfamethoxazole (Contrim*)											
122-34-9	Simazine											
57-63-6	17a-Ethynylestradiol (EE2)											
53-19-7	Estrone (E1)											
62-75-9	NDMA (N-Nitrosodimethylamine)											
59-05-2	Methotrexate											
1582-09-8	Trifluralin											
330-54-1	Diuron/gem (Valium*)											
124-48-1	Dibromochloromethane (Chlorodibromomethane)											
75-27-4	Bromodichloromethane											
330-54-1	Diuron											
134-62-3	DEET (Diethyltoluamide)											
65161-33-8	17b-Trenbolone											
15307-86-5	Diclofenac (Cataflam*, Voltaren*)											
498-46-4	Carbamazepine											
58-22-0	Testosterone											
15652-30-0	5am(Bboid (Lopid*)											
75-25-2	Bromoform											
57-91-0	17a-Estradiol (aE2)											
50-27-1	Estriol (E3)											
103-90-2	Paracetamol											
69-72-7	Salicylic acid											
51-86-1	Indomethacin											
58-08-2	Caffeine											
521-18-6	5a-Dihydro-testosterone (5a-DHT, stanolone)											
5588-90-8	Aromochloroacetic acid											
23122-68-7	Nonoid (Tenormin*)											
330-43-0	Diazinon											
2921-88-2	Chlorpyrifos											
72-33-3	Mestranol											
797-63-7	Levonorgestrel											

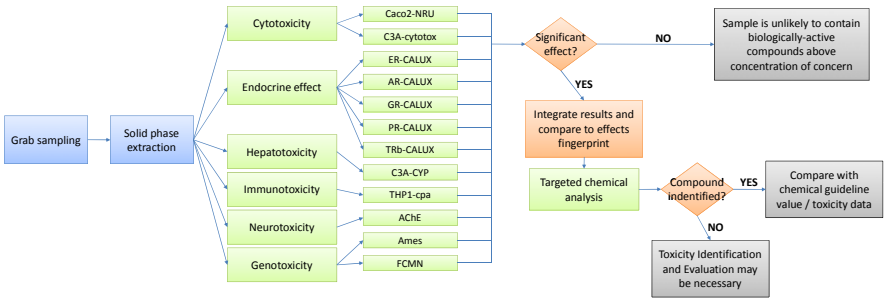

Fig 2. Tentative analysis plan

Anticipated outcomes

1. Enhance current knowledge regarding the performance of a suite of available *in vitro* bioassays for detecting trace chemicals that may be of human toxicological significance in potable water samples.
2. Provide guidelines for selection and deployment of robust set of bioassays, which can then help inform development of HACCP risk management for water recycling and support monitoring programs.
3. Incorporate this new knowledge as an additional tool for risk assessment, management and communication of recycled water projects.
4. Assist further development and implementation of the Australian Guidelines for Water Recycling by identifying potentially unexpected and/or unregulated chemicals and provide a first step towards the development of bioassay-based guidelines.

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