Australian Water Recycling
Centre of Excellence

Project Report

Bioanalytical tools in recycled water quality assessment in Australia: historical context, application and communication

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Bioanalytical tools in recycled water quality assessment in Australia: historical context, application and communication (Sub-Stream 1.2)

Authors

Heather Chapman and Frederic Leusch, Griffith University

Sub-Stream Project Leader

Frederic Leusch
Smart Water Research Centre
School of Environment
Griffith University
Southport QLD 42222 AUSTRALIA
Telephone: +61 7 5552 7832

Contact: Frederic Leusch f.leusch@griffith.edu.au

About the Australian Water Recycling Centre of Excellence

The mission of the Australian Water Recycling Centre of Excellence is to enhance management and use of water recycling through industry partnerships, build capacity and capability within the recycled water industry, and promote water recycling as a socially, environmentally and economically sustainable option for future water security.

The Australian Government has provided $20 million to the Centre through its National Urban Water and Desalination Plan to support applied research and development projects which meet water recycling challenges for Australia's irrigation, urban development, food processing, heavy industry and water utility sectors. This funding has levered an additional $40 million investment from more than 80 private and public organisations, in Australia and overseas.

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Executive summary

This document reviews the historical context, application, and communication of bioanalytical tools for recycled water quality assessment in Australia, and presents this information in four chapters.

Chapter 1 -

An Australia wide study in and again in 1978 concluded that "representative studies of the economics of 'reclaimed water' be undertaken as water deficits would become a problem in Victoria by 2000". These studies had little impact for approximately the next 25 years. Water recycling for irrigation was occurring and a small number of projects initiated in the 1990's began to recognize waste water as a resource rather than a disposal problem. In 1991, the Ecologically Sustainable Development Report noted the growing demand for water for conservation, recreation, irrigation, industry and domestic use. There was concern regarding the capacity of existing supplies to meet future demands.

This report examines events, issues and responses in SE Qld to emerging topic of chemicals in recycled water, intended to augment drinking water supplies and the drivers behind these actions. Since the issue of IPR in Australia came into focus in the late 1990's and mid-2000s, a number of studies have been conducted on the science relating to endocrine disruptors in recycled water, chemical mixtures and the so called 'unknown unknowns'. This was the term adopted to describe contaminants that could be in water that we do not know about using the terminology used by Donald Rumsfeld, the United States Secretary of Defence in 2002.

"There are things we know that we know. There are known unknowns. That is to say there are things that we now know we don't know. But there are also ***unknown unknowns****. There are things we do not know we don't know."*

This report also contains a review of the use of bioanalytical method development for water quality assessment – and how that can contribute to understanding and acceptance of recycled water using a holistic approach to management of chemical hazards. The information is mostly from Australia, but has relevance to chemicals and water recycling any-where in the world. This chapter will focus on studies that responded directly to the challenges of technical issues in chemical safety in drinking water sourced all or in part from recycled water. Part 3 of this report reviews research on risk communication including barriers to implementation of water recycling schemes and the effect of technology transfer workshops on shifts in perception and attitudes within water industry professionals including researchers, policy makers, regulators and water providers.

Chapter 2 -

Water recycling holds great promise for a sustainable water future, but reclamation of water from compromised sources such as treated sewage requires a shift in our risk assessment and monitoring paradigms. Indeed, the large number of chemicals potentially present in complex matrices such as wastewater means that it is no longer feasible nor sufficient to monitor a small number of chemicals one by one. *In vitro* bioassays (a category of bioanalytical tools) not only provide the ability to cast a much wider net to detect chemicals potentially present in waters, but also integrate concentration and toxic potency of individual chemicals to provide a risk-scaled summative measure of toxic chemicals in a sample.

Bioanalytical tools have been used in recycled water quality assessment since the early days of water recycling, mostly to measure mutagenicity using the Ames test. Bioanalytical methods have been applied to a number of schemes, both large and small, not only during validation and verification monitoring, but also to compare different treatment configurations at the pilot scale and fine-tune advanced water treatment trains. Over the past 10 years, the scope, reliability and capability of bioanalytical tools has greatly expanded, and this application of increasingly complex batteries of *in vitro* assays to recycled water quality assessment. It needs to be absolutely clear however that bioassay methods only detect the potential for harm, and do not correlate fully with whole organism effects

Bioanalytical tools have confirmed that advanced technologies are capable of producing water of very high quality, comparable and often exceeding current drinking water quality. Treated sewage produces high biological responses in bioanalytical tools, but advanced water treatment significantly reduces biological activity, often to below detection limits. Low mutagenic and genotoxic responses have often been detected, but these are clearly attributable to disinfection by-products, and are equally present (at similar or higher concentrations) in current drinking water.

The studies presented in this review clearly show that bioanalytical tools have a valuable place in risk assessment of reclaimed water. One issue that is limiting greater application of bioanalytical methods

is the lack of bioassay-based guidelines that compare bioanalytical results to human and environmental health. While there have been several proposals by researchers in this area, these still needs to be evaluated by health regulators.
It is important to keep in mind that adoption of bioanalytical tools for recycled water monitoring will most likely not lead to lower monitoring costs. However, recent developments in high-throughput testing are likely to lead to a reduction in the per sample cost of *in vitro* testing, and application of intelligent testing strategies combining screening with bioanalytical tools and suitable surrogate and indicator chemicals would most likely to lead to a reduction of total analytical costs.
Based on the information currently available, the following endpoints appear particularly well suited for recycled water quality assessment: estrogenic and glucocorticoid activity, mutagenicity and genotoxicity, adaptive stress response assays (particularly oxidative stress) and xenobiotic metabolism assays (particularly AhR and PXR pathways). Bacterial toxicity assays may also be ideally suited as performance indicator bioassays, especially when applied online. This list should not be seen as a comprehensive and final list, and future research may well identify other mode of toxic action that is relevant to drinking water. The logical next stage of evolution of *in vitro* bioassays will be application to online monitoring.

Chapter 3 -
This report briefly reviews the role of communication relating to the use of bioanalytical tools in water quality assessment. This begins by a brief introduction to theory regarding to science in policy making and regulation and refers to reports where this is considered in more detail. It is recognised uptake and use of new knowledge is an active process and an essential outcome of water quality research that. In this section three case studies on risk/science communication are presented, each of which aimed to understand and improve communication aspects regarding water recycling and specifically bioanalytical tools quality assessment in quality assessment of recycled water. The goal of each of the projects developed over time, all based on prior learnings as follows.
Case study 1 Risk communication from science to policy and regulation and implementation of recycled water in Australia

- The primary aim of this project was therefore to understand barriers to communication between scientists, policy makers and regulator and managers of recycled water.
- A range of barriers to communication were identified including clear regulatory guidance, clear process for communication to the public, policy and regulation. A clear message was also that confusing and inconsistent use of language was a barrier to communicating about recycled water both within the industry and when communicating with the public.

Case study 2 "Evaluating a science communication workshop as an educational tool"

- The primary aim of this project was to evaluate the transfer of knowledge on the application and interpretation of bioanalytical tools for industry, policy and regulation in recycled water quality monitoring programs through a workshop using semi-structured interviews.
- This research indicated a clear shift in attitudes and acceptance of bioanalytical methods following attendance at a technology transfer workshop. There acknowledgement that there is more work to be done on data interpretation and communication but that the methods were under-utilised and can make a substantial contribution to water assessment and be used for purposes that traditional analyses have problems doing such as measurement of the effect of mixtures, 'unknown unknowns' and mode of toxicity.

Case study 3 "Evaluation of an online survey to assess the effectiveness of technology transfer workshops on acceptance of bioanalytical methods"

- The primary aim of this project was to use an online survey to assess the effectiveness of technology transfer workshops on acceptance of bioanalytical methods.
- The online survey was successful in obtaining quality feedback on the workshops that were presented. The workshops clearly achieved the goal of increasing the perceived value and acceptance and acceptance of bioassay methods for measuring water quality but there remains a lack of clarity about the data interpretation and extrapolation to human health and the cost effectiveness of incorporation of the methods into routine monitoring.

Chapter 4 -
Chapter 4 provides a brief overview of the lessons learned from the research that has been carried out thus far on application and communication of bioanalytical tools for recycled water quality assessment. It also provides short answers to frequently asked questions (FAQ) in this context.

Abbreviations

4NQOEQ	4-Nitroquinolone-oxide Equivalent Concentration (genotoxic activity)
AChE	Acetylcholinesterase
ACT	Australian Capital Territory
ADI	Acceptable Daily Intake
AGWR	Australian Guidelines for Water Recycling
AhR	Aryl hydrocarbon Receptor, a protein involved in detoxification of foreign compounds (particularly dioxin-like compounds) in the body
AO	Advanced Oxidation
ARC	Australian Research Council
AWTP	Advanced Water Treatment Plant
BAC	Biological Activated Carbon
BaPEQ	Benzo[a]pyrene Equivalent Concentration (genotoxic or metabolic activity)
BEQ	Bioanalytical Equivalent
CADS	Citizens Against Drinking Sewage
CAFLUX	Chemically Activated Fluorescent Expression
CALUX	Chemically Activated Luminescent Expression
CAR	Constitutive Androstane Receptor, a protein involved in detoxification of foreign compounds in the body
ChlorpyEQ	Chlorpyrifos Equivalent Concentration (neurotoxic activity)
COAG	Council of Australian Governments
CPA	Cytokine Production Assay
CRC	Cooperative Research Centre
CRCWQT	Cooperative Research Centre for Water Quality and Treatment (now Water Research Australia)
DEMEAU	A European Union-funded project on "demonstration of promising technologies to address emerging pollutants in water and waste water"
DEQ	Diuron Equivalent Concentration (phytotoxic activity)
DEXAEQ	Dexamethasone Equivalent Concentration (glucocorticoid or immune activity)
DHTEQ	Dihydrotestosterone Equivalent (androgenic activity)
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DOI	Digital Object Identifier
DTA	Direct Toxicity Assessment
EBT	Effects-Based Trigger
ECVAM	European Centre for Validation of Alternative Methods
EDC	Endocrine Disrupting Compound
EEQ	Estradiol Equivalent Concentration (estrogenic activity)
EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Environmental Protection Agency
EPHC	Environment Protection and Heritage Council
ERBA	Estrogen Receptor Binding Assay
FCMN	Flow Cytometry Micronucleus
GAC	Granular Activated Carbon
GTU	Genotoxic Unit (genotoxic activity)
GU	Griffith University
GWRC	Global Water Research Coalition
HGPRT	Hypoxanthine-Guanine Phosphoribosyltransferase
IPR	Indirect Potable Reuse
ISO	International Organisation for Standardisation
ITS	Integrated Testing Strategy (also called Intelligent Testing Strategy)
IWA	International Water Association
LDH	Lactate Dehydrogenase (used as a measure of cytoxocity)
LLE	Liquid-Liquid Extraction
MF	Microfiltration
MFO	Mixed-Function Oxidase, a family of oxidase enzymes
NHMRC	National Health and Medical Research Council

NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NRC	National Research Council (USA)
NRMMC	Natural Resource Management Ministerial Council
NRU	Neutral Red Uptake (used as a measure of cytotoxicity)
NSW	New South Wales
NWC	National Water Commission of Australia
NWI	National Water Initiative
NWQMS	National Water Quality Management Strategy
O_3	Ozonation
OECD	Organisation for Economic Cooperation and Development
PAC	Powdered Activated Carbon
PAM	Pulse Amplitude Modulated (a method used to quantify photosynthesis inhibition)
PCR	Polymerase Chain Reaction
PPAR	Peroxisome Proliferator-Activated Receptor
PTEQ	Parathion Equivalent Concentration (neurotoxic activity)
PXR	Pregnane X Receptor, a protein involved in detoxification of foreign compounds in the body
QLD	Queensland
RAR	Retinoic Acid Receptor
RNS	Raising National Standards
RO	Reverse Osmosis
SAT	Soil Aquifer Transfer
SEQ	SouthEast Queensland
SPE	Solid Phase Extraction
TCDDEQ	2,3,7,8-Tetrachlorodibenzo-dioxin Equivalent Concentration (AhR activity)
TEQ	Baseline Toxic Equivalent (Microtox assay); or Testosterone Equivalent Concentration (androgenic activity).
TIE	Toxicity Identification and Evaluation
TMXEQ	Tamoxifen Equivalent Concentration (anti-estrogenic activity)
TU	Toxic Unit (cytotoxic activity)
UF	Ultrafiltration
UNSW	University of New South Wales
UV	Ultraviolet
UWSRA	Urban Water Security Research Alliance
VIC	Victoria
WERF	Water Environment Research Foundation
WHO	World Health Organisation
WQRA	Water Quality Research Australia (now Water Research Australia)
WRF	Water Research Foundation
WRP	Water Reclamation Plant
WWTP	Wastewater Treatment Plant
YAS	Yeast Androgen Screen
YDS	Yeast Dioxin Screen
YES	Yeast Estrogen Screen

Note: Throughout this document, "in vitro bioassays" and "bioanalytical tools" are used interchangeably.

How this review is organised

This review reports on a decade of research in response to concerns raised in the 1990s regarding the capacity of existing drinking water supplies to meet future demands for Victoria and subsequently in other regions of Australia. The review is organised in four chapters and includes the following:

Chapter 1 of this report reviews the recent history of issues associated with emerging chemicals in IPR schemes in SE Qld and the initial research responses to those issues, specifically with regards to the use of bioanalytical tools for water quality monitoring.

Chapter 2 details the application of bioanalytical tools in water quality assessment. This includes both a national and global application of bioanalytical tools for water quality assessment.

Chapter 3 presents three case studies where risk and science communication was specifically investigated. The studies described have identified some of the key barriers to communication about water recycling (Case study 1) and more specifically barriers to communication regarding the use of bioanalytical tools in the assessment of the quality of recycled water (Case studies 2&3).

Chapter 4 brings together some of the key messages from the research and provides some short answers to frequently asked questions regarding the application of bioanalytical tools in water quality assessment.

Chapter 1. History of events, issues and response to chemicals in recycled water in Queensland

by Heather Chapman

1.1 Introduction

1.1.1 Background on water recycling in Australia

An Australia wide study in commissioned in 1977 (GHD 1977) and another in 1978 (GHD 1978) both cited in Radcliffe (2010), concluded that "representative studies of the economics of 'reclaimed water' be undertaken as water deficits would become a problem in Victoria by 2000". These studies had little impact for approximately the next 25 years (Shuval, 2003 cited in Radcliffe (2004)). Water recycling for irrigation was occurring and a small number of projects initiated in the 1990's began to recognize waste water as a resource rather than a disposal problem. In 1991, the Ecologically Sustainable Development Report noted the growing demand for water for conservation, recreation, irrigation, industry and domestic use. There was concern regarding the capacity of existing supplies to meet future demands. A Productivity Commission enquiry that began in 1991 highlighted the need for wide ranging reform of the water industry to improve its efficiency. This led to policy drivers spearheaded through the Council of Australian Governments (COAG) coming together as the National Water Reform Framework from 1994 however until 2003 the National Water Reform Framework excluded recycled water from its considerations (Radcliffe, 2010). Following the August 2003 COAG meeting, an additional policy driver was announced – The National Water Initiative (NWI). One of the components included the State and local targets with time frames for effluent reuse, stormwater retention and pollution removal, decentralized, small scale sewage treatment and reduced outfall to oceans (Allison et al, 2002). The drought of 2001 – 2003 saw water rationing in most Australian capital cities and brought home to the public that there are limits to accessible sources of drinking water.

1.1.2 Scope of this report

This report examines events, issues and responses in SE Qld to emerging topic of chemicals in recycled water, intended to augment drinking water supplies. Since the issue of IPR in Australia came into focus in the late 1990's and mid-2000s, a number of studies have been conducted on the science relating to endocrine disruptors in recycled water, chemical mixtures and the so called 'unknown unknowns'. This was the term adopted to describe contaminants that could be in water that we do not know about using the terminology used by Donald Rumsfeld, the United States Secretary of Defence in 2002.

"There are things we know that we know. There are known unknowns. That is to say there are things that we now know we don't know. But there are also ***unknown unknowns****. There are things we do not know we don't know."*

In addition to the technical research, attempts have also been made to communicate and use the science to inform industry professionals and risk assessment professionals. It is believed that if the industry itself is well informed they will be better equipped to deal with issues relating to chemicals when they arise. One of the challenges to the implementation of recycled water projects has been a lack of public acceptance and consistent management of recycled water (Ross and Chapman, 2012). Public perceptions have been shown to be influenced by the credibility of the responsible authority and public confidence can be seriously diminished if authorities are perceived as incompetent or biased (Baggett et al, 2006). Therefore it is advantageous that the industry is well informed in order for policy makers, regulators and water managers to make decisions based on the best available science, which it is envisaged will generate trust in the industry.

This report also contains a review of the use of bioanalytical methods for water quality assessment – and how that can contribute to understanding and acceptance of recycled water using a holistic approach to management of chemical hazards. The information is mostly from Australia, but has relevance to chemicals and water recycling any-where in the world. This review will focus on studies

that responded directly to the challenges of chemical safety in drinking water sourced all or in part from recycled water.

1.2 Chronology of events that led to management and policy changes for recycled water

1.2.1 IPR in Australia

Water recycling in SE Queensland was not widespread prior to the 1990s. The standard practice of local authorities at that time was to produce effluent from sewage treatment plants (STPs) to a standard required for discharge to the environment or to land. Around 1993 approximately 88% of effluent was discharged through rivers and estuaries, 1% through ocean outfalls and approximately 10% was discharged to land (Bryan, Gardner and Beavers (1994). In a review by Uhlmann and Head (2011) two main reasons for were suggested.

1. Firstly according to interviews with some professionals working in the field, water scarcity was not in the mindset of SEQ engineers and policy makers at the time. Water was being provided by a series of large dams and local authorities had an adequate supply of water at the time.
2. Secondly, legislation at the time only required a basic level of treatment of effluent before it could be discharged to the environment and therefore it was more cost effective and easier to discharge treated effluent to water ways, rather than reuse the effluent. There was no overarching State legislation facilitating water recycling and no subsidies to local government to encourage the pursuit of water recycling at that time (Uhlmann and Head, 2011).

Change began to occur in the mid-1990s when a number of factors converged to encourage consideration of the use of recycled water including

- A growing population in SE Queensland and elsewhere in Australia, and
- Less predictable rainfall than previously.

Eventually the prolonged drought which commenced in 2001 and agreement between Australia's Commonwealth and the States/Territories governments to progress water reform through the National Water Initiative resulted in new recycling projects in Australian cities (Radcliffe, 2010). A major review of water recycling in Australian was taken by Radcliffe (2004) examining trends (particularly since 1999) in the processing, use and methods of application of recycled water and the policies and regulations governing them. It was suggested that '**drinking water**' should be the term adopted in Australia to describe 'potable water' and that '**water recycling'** be adopted as the preferred term for generic water reclamation and reuse (Radcliffe, 2004). It was also recommended at that time that the National Water Quality Management Strategy should be reviewed and revised based on the risk management framework used for the Australian Drinking Water Guidelines (2004). In the five years following initiation of the review of the NWQMS there was drought in most of Australia potentially due to the impacts of climate change as well as natural climate variability. The resulting demand management and water restrictions that ensued resulted in the Australian community becoming critically aware of the need for water security (Radcliffe, 2010).

1.2.2 IPR in Queensland

1.2.2.1 Caloundra/Maroochy Wastewater Management Strategy

The Caloundra/Maroochy Wastewater Management Strategy was developed in SE Queensland in the 1990s. The strategy proposed to use recycled water to augment drinking water supplies at a local government level. The plan was to reclaim water from the region, treat the water at the Landsborough Water Reclamation Plant and deliver the treated water to Ewen Maddock Dam via a set of constructed wetlands. The treatment plant and the wetlands were constructed however intentional discharge to Ewen Maddock Dam did not occur and approval for overflow from the wetlands was only allowed in extreme wet events. The proposed IPR scheme did not gain Queensland Government approval (in spite of the scheme being encouraged by the State Government in the planning stages) reportedly due to concerns relating to potential for blooms of cyanobacteria due to adding more nutrients to an already mesotrophic water body, plus concern

raised by the local communities regarding the possible presence of chemicals in the treated water that lead to political nervousness.
In 1996 at about the same time as the IPR scheme was proposed, a book titled 'Our Stolen Future' was published (Colborn et al, 1996) and this, combined with easy access to information on the internet appeared to be having an influence on the risk perception of recycled water as part of a drinking water supply. The 'evidence' of endocrine disruption in wildlife was suggested in the book to be sufficient evidence that similar effects could be expected in humans. The issue was cited in the local press at the time as one of the reasons for the Sunshine Coast community opposition to the IPR scheme.
In 1998 a council decision was made not to continue with the IPR scheme due to reluctance of the State Government to issue a licence to release to the dam, and increasing community concern regarding 'hormone disruptors' in the wake of the publication of Our Stolen Future (Colborn et al, 1996). An active group of protesters was formed called Citizens Against Drinking Sewage (CADS) in 1998 in response to the proposed IPR scheme.
Calaqua, part of Caloundra City Council, never-the-less commissioned a 2 year investigation by the School of Public Health at Griffith University into the issue of chemical hazards and in particular the issue of endocrine disruption (community concerns). The research found that there was little evidence for the potential for harm from endocrine disruptor in recycled from Landsborough (Leusch et al, 2005). The view on endocrine disruption and human health risks was also investigated by a comprehensive review of the topic conducted by the World Health Organisation (WHO) titled 'Global assessment of the state-of-the-science of endocrine disruptors' (WHO/IPCS, 2002). One of the conclusions was *"Analysis of the human data by itself, while generating concerns, has so far failed to provide firm evidence of direct causal associations between low-level (i.e., levels measured in the general population) exposure to chemicals with EDCs and adverse health outcomes.*"

1.2.2.2 South Caboolture Water Reclamation Plant

The shire of Caboolture was a local government, and later part of the Moreton Bay Regional Council, located between the Sunshine Coast and Brisbane and was the one of the first local governments to fully meter water use and to institute water restrictions. However rising water demand due to population growth and insufficient local water forced the council to purchase up to 75% of its water from Brisbane City Council. New dam sites were also being explored and at the same time the EPA was tightening its effluent discharge requirement due to the poor condition of the Caboolture River. Council resolved to solve both water supply and sewage discharge problems with potable water recycling (Uhlmann & Head, 2011). This was a unique project that created a considerable amount of interest and occurred in parallel with and slightly behind the Caloundra/Maroochy strategy, although was not directly influenced by that. Engineers were commissioned to develop a new advanced treatment plant to supply purified water of a potable standard. No community consultation was conducted prior to preparation for the plant. Following the announcement of the proposal began a six month period of consultation that consisted of a leaflet, two full day community workshops, a telephone hotline and fact sheets. This did little to win community support with groups forming to oppose the proposal including CADS (Citizens Against Drinking Sewage). There was a strong reaction against potable reuse by one councillor and a small but vocal group of protesters. Subsequent marketing did little to change opinions and the council decided to only use the water for non-potable uses.
The South Caboolture Water Reclamation Plant (WRP) is one of the largest advanced wastewater treatment plants (AWTPs) in SEQ and was officially opened in March 1999. However, it has been substantially under-utilised in terms of wastewater reuse. Operation of this plant has traditionally been characterised by high throughput (average 7.5 ML/d) but low actual use (~ 2 ML/d) of the product water. The primary role for the AWTP has been to remove additional nutrients from secondary treated effluent prior to discharge to the Caboolture River. Whilst the plant provides water for non-potable applications, it was designed to meet drinking water standards (Ruengoat et al, 2012).
Although the treatment plant was eventually built to improve the quality of water discharged to the environment based on comparison with proposed or operating re-use schemes overseas, treated water from the plant would ultimately be suitable for potable reuse. It was considered that once the quality of the water had been demonstrated, the water would be mixed into the weir from where the town drinking water supply was taken (Radcliffe, 2004).

1.2.2.3 Gold Coast Water Future and Pimpama-Coomera Waterfuture Masterplan project

Gold Coast Water Future project starting in 2004 with the goal to investigate 'every possible source of water' (Gold Coast Water, 2006). Drivers included interest in water sustainability along with the need

for security of supply. The strategy was revised in 2006 when Hinze Dam was down to 20% capacity and included the need for climate-independent solutions including indirect potable reuse. Extensive community consultation over a 2 year period had shown only mixed support for indirect potable reuse (Gold Coast Water, 2006). It is unclear if there were concerns over chemicals in water at that time. As the Pimpama-Coomera Waterfuture Master Plan (2004) included a range of water sources including the use of 'Class A+' for dual reticulation (third pipe) and not drinking water, the same level of emotion relating to chemicals may not have emerged. Support existed for the third pipe at that time.

1.2.2.4 IPR - Water Futures, Toowoomba

Toowoomba, located in Queensland on the crest of the Great Dividing Range is a city of approximately 95,000 people in 2006. Toowoomba's water comes from three storages – Lake Cooby, Lake Perserverence and Lake Cressbrook. Toowoomba's population and industrial development has been increasing over the last two decades along with increasing demand for water. Water restrictions were first put in place in 2003. In 2005/2006 the Toowoomba City Council committed to a Water Demand Initiative to reduce demand as the total need exceeded the supply at that time in spite of water use restrictions being in place.

In 2005 the Toowoomba developed a proposal "Water Futures Initiative" for the recycling of wastewater for indirect potable reuse, urban use and agricultural irrigation. The Water Futures Initiative was launched by the Federal Member for Groom (including Toowoomba), the Honourable Ian MacFarlane, the then Premier of Queensland, the Honourable Peter Beattie and all three local Members of State Parliament (Hurlimann and Dolincar 2010).

Council, supported by all local members of State and Commonwealth Parliaments, lodged an application to the National Water Commission for funding towards the project. The Australian Government agreed to contribute $22.9 million from the Water Smart Australia programme subject to community support in a poll (Turnbull, 2006). The reason for requiring the poll is unclear but it may have been due to increasing opposition to the scheme developing in Toowoomba.

In reaction to the Water Futures Initiative in 2005, the CADs Toowoomba group formed and held their first public meeting on 25th Aug 2005 and by February 2006 10,000 people had signed the CADS petition (Hurlimann and Dolincar 2010). A key platform for opposition of the scheme was contaminants that could be present in the water. Some of those identified included phthalates (plasticisers), human hormones, RU486 abortion pills, prions and hospital waste including drugs and radiological substances. There was also concern regarding 'unknown unknowns' referring to chemicals that we do not know are there and therefore do not monitor for. This expanded the list from those used by the CADs in the campaign against the Caloundra/Maroochy scheme that focussed mostly on hormones (endocrine disruptors).

The council did not approve of the referendum but because the funding for the scheme was conditional on this there was no choice but to proceed. Toowoomba City Council began a 10 week information campaign and was in the position where they needed to condense what was to have been a three year community program into what was essentially a three month political campaign. By the time Council started informing the public the CADs had been communicating with the community for half a year. On the 29th July 2006 the referendum was held and the majority (61.55%) were against the scheme and the Water Futures Project was abandoned.

1.2.2.5 IPR - South East Qld, Western Corridor Scheme

Soon after the failed poll on indirect potable reuse (IPR) in Toowoomba the South East Queensland Water Recycling Strategy was established. In January 2007, Peter Beattie, the then Premier of Queensland, publically announced a decision not to let the public vote on whether or not to proceed with a large scales recycled water scheme for the States capital of Brisbane. This was contrary to his prior commitment to a referendum. His position was that there was no other option than to put in place ways to augment water with reclaimed water. The project began construction and was completed at the end of 2008. In response the CADs again began a campaign and distributed a booklet titled "Think before you agree to drink – Is sewage a source of drinking water" to 500,000 Brisbane residents early in 2007 (http://www.valscan.com.au/tbyatd.pdf). In responding to this booklet in a blog maintained by Dr Stuart Khan a respected scientist from the University of New South Wales, Stuart commented – "In general, I think Manners and Dowson have done a nice job of highlighting the important issue of endocrine disrupting chemicals (EDCs) in the environment. Some of the information presented should indeed cause people to think more carefully about the presence of chemicals in our environment generally. However, my major concern is that almost all of the information presented is done so without explanation of its original context. Context can be important for accurate interpretation of many findings and statements. The inclusion of such context-devoid

statements in this booklet may leave readers with the impression that the context had something to do with advanced treated recycled water. This is unfortunate, since in many cases it did not, and is thus somewhat misleading." (Khan, 2007).
The Western Corridor Scheme encompasses a desalination plant at Tugan on the Gold Coast, and three advanced water treatment plants at Bundamba, Gibson Island and Luggage Point. The drinking water quality produced was intended to be pumped to Brisbane's principal water storage, Wivenhoe Dam. The proposal to proceed was to have been preceded by a plebiscite in March 2007 but the Queensland Government dispensed with this and made the decision to proceed at a time when South-East Queensland was under severe drought stress. The then Premier Anna Bligh announced that treated wastewater would only be added to Wivenhoe Dam once the dam capacity was down to 40%. Subsequent rainfall meant that the dams were refilled and the water has not been added to the dam.

1.2.3 Timeline

Figure 1-1 represents a time line of events that lead to a response to the drought of the 2000s and rapid escalation of consideration of indirect potable reuse (IPR). There were a number of policy responses during this time including water recycling guidelines as discussed in Section 1.2.5. Over the same time frame gaps in the knowledge relating to chemicals in water that could (in part) be addressed through the use of bioanalytical tools arose and was the impetus for a number of research projects as discussed in Section 1.3.

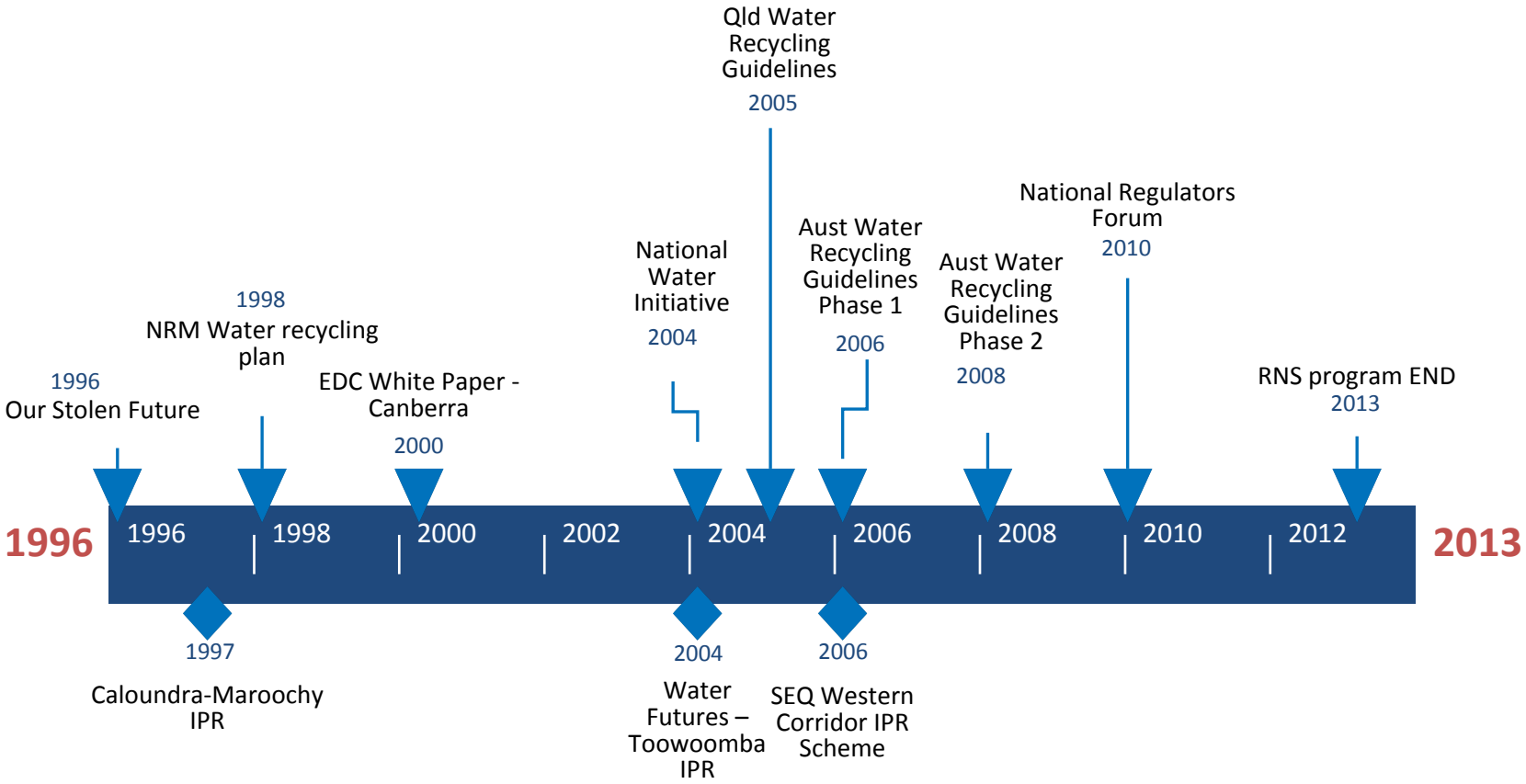

Figure 1-1. Issues and drivers for indirect potable reuse (IPR) that resulted in conduct of the research

1.2.4 Chemicals in recycled water

The presence of biologically-active chemicals in water is of interest because of potential adverse effects on wildlife and humans. This includes but extends beyond endocrine disruptors and includes all of the so called 'emerging contaminants' including a range of chemicals from pharmaceuticals, personal care products, house hold products etc. In the development of guidelines for drinking water it is necessary to extrapolate from animal studies (usually mice or rats), or other wildlife such as fish, and then to apply safety factors to the data to determine guideline values for humans or trigger values for ecosystems. It can take years or decades to develop the necessary information and so the new methods are a valuable source of additional knowledge that is useful in the short term. There is concern expressed regarding the impacts of chemical mixtures and chemicals that are not predicted to present, of treatment technology failure. Bioanalytical tools provide some solutions to these issues but the science is still evolving and yet to gain acceptance by regulators and water providers. Chemicals in water have traditionally been regulated or managed on a chemical by chemical basis, by analysis of a suite of chemicals and comparison to guidelines. Chemical analysis alone is problematic due to the large number of compounds that can be in the water at ultra-low concentrations. Chemical analyses also have to be constantly updated to detect new chemicals on the market, existing chemicals with new uses, or illegal use or disposal of chemicals that may enter water sources. Chemical measurements also only account for those we know to test for and therefore new or unexpected chemicals may be missed altogether. With the increasing number of new chemicals developed and eventually released into water, it is becoming increasingly difficult to measure all possible contaminants using standard chemical techniques (such as gas or liquid chromatography - mass spectrometry). "As any analytical chemist knows, what you see depends on what you look for" (Lynn Roberts, Johns Hopkins University). Also important is that chemicals do not occur in isolation in reclaimed source water and present mostly as a complex mixture. In our efforts to ensure users are safe from pathogenic organisms, high levels of disinfection are used. This can result in a new hazard from treatment by created transformation products such as disinfection by-products (DBPs). DBPs are known and/or suspected to be a hazard and therefore need to be fully understood and managed to protect public health.

1.2.5 The guidelines

The reform of the Australian water industry was initially agreed to at a Council of Australian Governments (COAG) meeting in 1994. Land titles were separated from right to water titles with both becoming separately tradable. Water resource management became separated from the water supply function which was transferred to commercial corporatized entities, albeit mostly still government owned. In 2004 – 2006 the Commonwealth and all States and Territories progressively signed a 108 clause "Intergovernmental Agreement on the National Water Initiative'. Preparation of an Implementation Plan by each government was a requirement of the NWI and the National Water Commission was established in 2005 (Radcliffe, 2010) to assist with the implementation of that Agreement and to undertake two-yearly evaluations of progress.

In 2006, the first phase of the new Australian guidelines for water recycling was published. Titled *Australian Guidelines for Water Recycling: Managing Health and Environmental Risks*, the document was released under the National Water Quality Management Strategy. The guidelines document provides a mechanism for the expanded use of recycled water in a nationally consistent manner, and covered particular sources of water and also different water uses. The guidelines are based on the National Water Quality Management Framework, a risk prevention approach involving anticipation of potential problems and preventing them from arising. An important feature of these guidelines is that they use a risk management framework to protect the health the public as used for example in the Australian Drinking Water Guidelines (2004) and retained in 2011 revision of that document.

Phase 1 of the Australian Guidelines for Water Recycling: Managing Health and Environmental Risks focussed on the use of recycled water for uses such agriculture, fire control, municipal, residential and commercial property and industry. Augmentation of drinking water supplies was not on the National agenda at that time. The National guidelines were required because pressure was increasing in many cities and regional areas due to widespread drought and movement of population to large centres near capital cities. As the drought continued there was a need to develop further guidelines. Phase 2 – Module 1 "Augmentation of drinking water supplies" was published in 2008. For the first time in relation to drinking water, a section on the use of bioassays to assess biological activity for verification monitoring (Section 4.5.1, AGWR Phase 2, Module 1, 2008) was proposed. The relevant section of the guidelines is reproduced below.

Biological screening assays (reproduced from Section 4.5.1, AGWR Phase 2, Module 1)

Traditional assays for chemicals do not deal with the issues of complex mixtures or biological activity. Both of these issues have been raised for drinking water in general, and for drinking water augmentation schemes in particular.

*Biological activity is most commonly raised as an issue for chemicals, including natural human hormones that might cause endocrine disruption. Fish exposed to treated sewage have exhibited reproductive abnormalities (Jobling and Tyler 2003). As discussed above (*Section 4.2.2 of the Phase 2 guidelines*), it is difficult to extrapolate from these observations to possible effects on human health from much lower levels of such chemicals in highly treated recycled water. Nevertheless, it may be useful to include biological screening assays in verification monitoring programs. Biological assays can also provide an indication of potential impacts of complex mixtures contained in recycled water.*

International experience has shown that biological monitoring of recycled water after complete treatment does not detect any biological activity (NRC 1998, Khan and Roser 2007). Product water should be tested, but it is also informative to test source water and water after initial treatment steps (e.g. after secondary treatment), to verify the effectiveness of treatment processes.

In vitro tests have been used to measure chemical quality of Australian sewage (Leusch et al 2005 and 2006, Muller et al 2007), and a similar approach could be used to monitor the quality of source waters, and of partially and completely treated recycled water. Detection of biological activity should lead to further investigations into the cause of that activity. Biological tests can be used as a screening and prioritisation tool for subsequent chemical analysis.

A range of bioassays can be applied to test for end points such as genotoxicity, mutagenicity, tumour induction, whole-animal toxicity, estrogenicity and androgenicity (Leusch et al 2006, Chapman 2007, Khan and Roser 2007, WERF 2007). Biological screening can include both in vivo and in vitro assays.

Selection of tests will be influenced by a range of factors, including the end point of interest and availability and accessibility to laboratories able to undertake testing. Due to ethical considerations and speed of completion, in vitro tests should take priority. Researchers are evaluating and comparing the efficacy and sensitivity of in vitro tests, and the findings will influence test selection (CRCWQT 2007).

In vivo assays

In vivo tests can include assessments for a range of end points, including whole-animal toxicity, carcinogenicity, androgenicity or estrogenicity. Whole-animal tests often use mice and rats, and guideline values for many chemicals have been generated from this type of testing. However, there are ethical issues that have to be addressed before this type of testing can be applied, and applicability of the finding to humans can vary. Testing using mice and rats can take anything from several months to years. In Singapore, for example, a mice-feeding study over two years was undertaken in association with NEWater. One alternative is to use fish, which can be exposed to recycled water continuously, and are relative inexpensive to maintain. Disadvantages of fish are that (NRC 1998):

- *fish and humans differ significantly in biological terms*
- *being completely immersed in water, the sensitivity of fish gills, in particular, may result in overestimation of acute toxicity*
- *pharmacokinetics and metabolism of chemicals in fish may differ significantly from mammals.*

In vitro assays

In comparison to whole-animal assays, in vitro testing — performed at the molecular or cellular level — can provide results within hours or days. Examples of molecular end points include binding to specific biological receptors or induction of particular biomolecular pathways, whereas cellular events

could be cell death, maturation or growth. In vitro assays can be based on human cells, thus eliminating the interspecies predicament of in vivo testing (Barratt et al 1995). In vitro tests can also detect biological effects at much lower, environmentally relevant concentrations, which are often below detection limits of chemical analysis and in vivo testing (Asano and Cotruvo 2004). Limitations to in vitro bioassays include a lack of metabolism and transport mechanisms — factors that may modulate toxicity in whole organisms (NRC 1998). Nevertheless, in vitro bioassays can be useful adjuncts to traditional analyses for individual parameters, and there has been progress in standardising in vitro tests; for example, through programs such as those operated by the European Centre for the Validation of Alternative Methods and the US National Toxicology Program Interagency Centre for the Evaluation of Alternative Toxicological Methods.

For more information on the application of these types of tests refer to '*Part 2 Application of bioanalytical tools during validation and/or verification of recycled water schemes*' of this report.

1.3 Research response

1.3.1 Endocrine disruption

The issues identified initially on the Sunshine Coast regarding endocrine disruptors and risks from recycled water were the impetus for a number of research initiatives relating to trace chemicals in water that are ongoing today. This began with direct funding from CalAqua to the School of Public Health at Griffith University. A two year intensive study found quite a body of evidence for impacts to wildlife from endocrine disruptors (mostly from estrogens) but little evidence for impacts to human health. It became evident at the end of that initial study that risk analysis based on chemical data was limited with respect to the end point of endocrine disruption and that the research needed to extend to the use of bioanalytical tools that measure the presence of chemicals by their biological activity, rather than individual chemical concentrations. One of the reasons was that the putative endocrine disruptors were thought to be biologically active below the limit of quantification at that time (~2000). The report on that work was never made public due to the controversial nature of the Caloundra/Maroochy Wastewater Management Strategy which was not progressed for political reasons at that time. Consequently, this information was not published or in the public arena. Research subsequent to this was subsequently funded by a Commonwealth funded ARC SPIRT (now known as Linkage) grant with Calaqua as an industry partner. This project "Behaviour and effects of drugs, hormones and other endocrine disrupting chemicals – significance in the Australian aquatic environment" began the local work of investigating the combined use of bioanalytical tools (*in vitro* bioassays) to characterise the risks posed by estrogens on human health and aquatic ecosystems. Further details of the various projects and the science are given below in Section 3 of Part 1 of this document. Prior to this evidence of endocrine disruption in fish had been identified by Batty and Lim (1999). The evidence of impacts in wildlife, became the source in the belief (with little substantiation at that time) that if it can happen to fish, it can happen to humans. This was driven in part by the publication of Our Stolen Future (Colborn et al, 1996).

1.3.2 Application of bioanalytical tools in risk assessment

Short term *bioanalytical* tests (such as *in vitro* testing) can aid predicting the potential for (but not proof of) whole animal effects. This can be due to exposure to biologically active compounds present at very low concentrations, often below analytical detection limits or below individual exposure limits where these exist. These methods, previously developed for the pharmaceutical industry, provide a much-needed new approach to contaminant testing in water. Bioanalytical methods can detect multiple contaminants in one test because they do not measure chemicals by their structure but by their net effect on biological systems (molecules or cells).

Use of the bioassay data can also be a powerful tool the interpretation and translation of technical information in risk assessment. Most regulation of chemicals globally is based on individual chemicals. While this is accepted methodology it is limited in risk assessment as it does not normally allow for chemical interactions, and the risk that may be posed by chemical we do not know are present, and we therefore do not look for. That is, the unknown unknowns. There has been a substantial effort in the last two decades relating to emerging chemicals in water. Some substantial reviews have been written and it is not intended to cover that material again. Escher et al (2012) and Poulsen et al (2011) can be accessed for further background.

1.4 Research responses (technical) to the issue of chemicals in recycled water

The purpose of this summary here is to document research activity that occurred locally in direct response to the issues that arose due to drought in Australia, particularly in Queensland in the period from 2000. The research in the first few years addressed technical issues relating to monitoring and risk from exposure to chemicals in water, specifically endocrine disruptors. Table 1-1 lists a number of key projects that were initiated in response to the issues that were raised by the media, politician, water industry practitioner and the general community.

This research was occurring at the same time as, and in response to policy and regulatory change that was being implemented and some of the new knowledge was incorporated into that process. The National Water Commission/WQRA project (Chapman et al, 2011) was the first of these projects to specifically research the risk and communication issues.

In spite of little evidence of the potential for harm due to exposure to chemicals in treated recycled water, there remained concerns relating to this. Due to the world wide exposure to these issues in the media and by misinformed politicians there was a continuation of concern. Decision making regarding recycled water had become political and risk averse in spite of evidence that the risks were low.

Table 1-1. Research projects responding to development of bioanalytical tools

Completion date	Project title	Aims/objectives	Major findings	Project/reference
2000	Toxicant evaluation for Ewen Maddock Dam, Landsborough Ornamental Wetlands and the Landsborough Water Reclamation plant.	Review the scientific literature on endocrine disruptors relative to wildlife and humans, conduct baseline study of chemicals in water and sediments in Ewen Maddock Dam and conduct a hazard assessment of endocrine disruptor effects at realistic concentrations.	Analysis of water, sediments and fish showed no significant chemical contamination of the dam or wetlands. Few chemicals were detected in the final water (advanced treatment with sandfilter, ozone, BAC and UV). Low risk was indicated due to low concentrations of putative EDCs present, low potency of those that were present, resulting in both low exposure and low erstrogenic potency.	Chapman H, Connell D, Krishnamohan M, Zalucki J, Wesche S and G Palmer (Dec 2000) Confidential report to CalAqua, Caloundra City Council, Caloundra, QLD 4551. Unpublished report (not available).
2003	Behavioral and effects of drugs, hormones and other endocrine disrupting chemicals in the Australia aquatic environment	The controversial topic of endocrine disrupting chemicals is of international and Australian signigicance with the need for sustainable management of water resources increasing. This research aims to use novel measurement techniques (bioanalytical tools) to carry out risk assessment of EDC chemicals based on biological response rather that chemical measurement.	The research found that there was a low risk due to exposure to chemicals with endocrine disrupting chemicals from the Landsborough Water Reclamation Plant, the constructed wetlands and Ewen Maddock Dam. In spite of this the IPR scheme was abondoned due to political nervousness and policy constraints. The project begain the research partnership with Louis Tremblay and other international researchers for subsequent projects including two PhD scholars (Fred Leusch and Benjamin Tan)	Connell D, Yu J, Tremblay L and HF Chapman (2001 - 2003) ARC SPIRT GRANT. Industry partner - CalAqua, Caloundra City Council. (Leusch et al, 2005; Chapman, 2003)
2003	Endocrine disruptors in the Context of Australian Drinking Water CRCWQT Occasional paper # 7.	This review was commissioned by the CRC for Water Quality and Treatment to provide an overview of the effects of endocrine disrupting chemicals (EDCs) and their potential to contaminate drinking water in Australia. The particular issue for the water industry is the protection of safe drinking water from sources of varying quality and the implications for public health.	This review concluded that while the concentrations of EDCs in domestic wastewater may cause changes to aquatic fauna in the discharge plume, the concentrations were orders of magnitude less than those likely to cause health effects in humans. On the basis of a larg amount of research being conducted at that time in Europe and the USA, it was not recommended that Australia embark on similar programs at that time. The World Health Organisation at that time concluded that adverse effects on human health from low level exposure was unlikely (WHO/IPCS 2002)	Falconer, Ian R, Chapman, Heather F, Moore, Michael R and Geetha Ranmuthugala (2006) Endocrine Disrupting Compounds: A review of their challenge to sustainable and safe water supply and water re-use. *Journal of Environmental Toxicology.* 21:181-191
2004	Estrogenic and androgenic potential of municipal sewage in Australia and New Zealand. Frederic Leusch, Doctoral Thesis, Lincoln University, New Zealand.	The main objective of this research was to examine the estrogenic and androgenic activity in treated sewage to determine the risk associated with treated sewage discharges in Australia and New Zealand using *in vitro* and *in vivo* bioassay methods using waters from the Landsborough Water Reclamation Plant and a constructed wetland receiving sewage effluent in SE Queensland.	Mosquitofish in a constructed wetland receiving sewage effluent exhibited morphological changes consistent with exposure to androgens although this was not measureable downstream. Based on this result it is unlikely mosquito fish would be impacted by endocrine disruptors in this location.	Leusch et al (2005; 2006a,b,c)
2006	Chemical and biological analyses of selected endocrine disruptors in wastewater treatment plants in South East Queensland, Australia. Benjamin Tan, Doctoral Thesis, Griffith University, Brisbane, Australia	The main aim of this study was to utilise chemical analyses to assess concentrations of slected endocrine disruptors to measure the potential estrogenic effects of EDCs in water discharged from waste water treatment plants (WWTP) in South East Queensland, Australia. At this time there were few reported studies on estrogenic effects of EDCs released from WWTP into receiving environments in Australia.	Chemical results were compared to biological analysis and found to be complementary. The results demonstrated that the EDCs discharged from the monitored wastewater treatment plants would be expected to have a low impact on the receiving environment.	Tan et al (2007a,b; 2008)

Completion date	Project title	Aims/objectives	Major findings	Project/reference
2007	Chemicals of Concern in Wastewater Treatment Plant Effluent: State of the Science in Australia. CRC Water Quality and Treatment - Occasional Paper No 8.	This CRC occassional paper comprises stand alone research reviews covering some of the chemicals of concern in treated wastewater, namely endocrine disruptors, pharmaceuticals and polyelectrolytes. This review summarised research conducted by independent researcher at the CRC Water Quality and Treatment presenting Australian data within the context of the international literature.	The chapter on endocrine disruptors concluded that the provision of safe and sustainable water supplies will become increasingly challenging in Australia but that more data was required than available from concentional risk assessments. Integration of management practices and monitoring technologies were identified as a need to protection of our water ways and water supplies.	Chapman HF, FDL Leusch and B Tan (2007) Chapter 1 Endocrine disruptors. CRC Water Quality and Treatment - Occassional Paper #8.
2007	Tools to detect estrogenicity in Environmental Waters. Final Report from the Global Water Research Coalition (GWRC) project.	This project evaluated the performance of five *in vitro* bioassays to measure the estrogenicity of water samples including sewage, river, ground and drinkiung water as well as spiked with known estrogenic chemicals. The tests included yeast estrogen screen (YES), ER-CALUX, MELN, T47D-KBluc and E-Screen assays.	The results indicated that ER-Calux and E-screen in this study successfully detected estrogenicity in water samples at very low estrogen equivalents (0.1 - 320 ng/L EEQ). The estrogenity measured using the bioassays was correlated with the comprehensive chemical analyses suggesting either bioassay could be used as in initial screening tool to detect estrogenicity in environmental waters.	GWRC (2007) Tools to detect estrogenicity in Environmental Waters. Final Report from the Global Water Research Coalition (GWRC) project. WERF contract number #03-HHE-4T CRCWQT
2008	Recycled water quality – A guide to determining, monitoring and achieving safe concentrations of chemicals in recycled water.	The purpose of this study was to investigate scientifically justified human-based chemical quality guidelines for recycled water. The information in the report intended to provide the joint steering committee of the NHMRC National Guidelines for Water Recycling - Phase 2 including guidance on the use of recycled water to augment drinking water sources.	The review considered the greatest potential for exposure to be from water reclaimed from STPs with the intent of determining safe exposure concentrations for drinking water. Information produced by this document was using in the development of the guidelines, source control and efficacy of treatment, monitoring and public health surveillance. This included recommendation for the incorpration of an integrated approach (using a range of tools) to managment of safe drinking water augmented with recycled water.	UNIQUEST - Report commissioned by the Environment Heritage Protection Agency and Natural Resources and Natural Resources and Water, QLD and the National Water Commission.
2010	'Development of an Ecotoxicity toolbox to characterise water quality for recycling'.	This project developed an ecotoxicity toolbox to characterise water quality though wastewater treatment processes for a range of toxic responses including in vitro, in vivo and in situ endpoints. Chemical analyses was conducted in paraellel for some chemical groups to correlate standard analytical methods with the bioanalytical approach.	The results showed that there was significant biological activity in raw wasdtewater and presence of chemicals at all plants sampled. The treatment plants achieved significant removal of the chemicals and their activity depending on the treatment processes. RO membranes are particularly effective at removing organic contaminants. There was no identifiable impact on mosquito fish morphology or reproduction. The study demonstrated the usefulness of combining multiple lines of evidence in the assessment of water quality.	Reitsema T, Nice HE, Leusch, FDL, Quayle, P, Chapman, HF, Khan, SJ Trinh, T, Coleman, H., Rawson, C, Gagnon, MM and Blair (2009) 'Development of an Ecotoxicity toolbox to characterise water quality for recycling'. Water Science Technical Series, Report No 36, Dept of Water, WA.
				Leusch FDL, SJ Khan, MM Gagnon, P Quayle, T Trinh, H Coleman, C Rawson, HF Chapman, P Blair, H Nice and T Reitsema (2013) Assessment of wastewater and recycled water quality: A comparison of lines of evidence from *in vitro*, *in vivo* and chemical analyses. *Water Research* (Impact Factor: 4.66). 10/2013; DOI:10.1016/j.watres.2013.10.056

Completion date	Project title	Aims/objectives	Major findings	Project/reference
2010	Bioanalytical tools to evaluate micropollutants across the Seven Barriers of the Indirect Potable Reuse Scheme.	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. 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.	Results from each of the six bioassays showed a significant decrease in biological activity across the seven barriers allowing bench marking of treatment efficacy. The results were reproducable and and consistent with previous studies assessing the effectiveness of wastewater treatment. Results were expressed as toxic equivalents (TEQ) using the same concept as previously used for dioxins. Detection limits are generally lower than for compariable chemical analysis.	Macova M, Escher B, Mueller J and 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 Macova, M., Escher, B.I. , Reungoat, J., Carswell, S., Lee Chue, K., Keller, J. and Mueller, J.F. (2010).Monitoring the biological activity of micropollutants during advanced wastewater treatment with ozonation and activated carbon filtration. *Water Research*, 44 (2), 477-492
2011	A national approach to health risk assessment, risk communication and management of chemical hazards from recycled water. Report to the National Water Commission - Raising National Standards program.	This project was undertaken to extend the range of available bioanalytical tools used in the previous projects. The broad aim was to adopt and validate methods or tools for assessing the potential for health impacts in humans. The project also examined issues regarding communication between scientists, policy officers in government, regulators and the water industry (this is reported on in Section 3 of this report).	In conclusion the project demonstrated that sound scientific evidence and good communication can contribute significantly to water reform in Australia. The recommendations from this project were that multiple barriers should always be deployed in water recycling schemes, *in vitro* bioassays to predict *in vivo* effects should be developed further, the range of endpoints needs ongoing development and that knowledge transfer and uptake into practice requires active communication between all stakeholders.	Chapman HF, Leusch FDL, Prochazka E, Cumming J, Ross V; Humpage A, Froscio S, Laingam S, Khan SJ, Trinh T, McDonald JA (2011) , Waterlines Report 48, National Water Commission, Canberra. http://archive.nwc.gov.au/library/waterlines/48 (accessed 30/1/14) Leusch FDL, SJ Khan, S Laingam, W Prochazka, S Froscio, T Trinh, HF Chapman and Andrew Humpage (2014) Assessment of the application of bioanalytical tools as surrogate measure of chemical contaminants in recycled water. *Water Research* 49:300-315
2011	Bioassays and risk communication: Goal 1 - To strengthen and validate bioanalytical tools for application in water quality assessment (a) Review	The project overall focused on implementing, validating and expanding bioanalytical tools for cost-efficient water quality monitoring. A number of related activities in this project included a review of projects in Australia currently using these techniques.	This review documented historical and current application of bioanalytical tools for water quality assessment within New Zealand and Australia. Basic theory and concepts were detailed before worldwide applications were outlined to set the local resources into a global context.	Poulsen A, HF Chapman, FDL Leusch and B Escher (2011) Application of Bioanalytical Tools for Water Quality Assessment. Urban Water Security Research Alliance - Technical Report # 41. http://www.urbanwateralliance.org.au/publications/UWSRA-tr41.pdf

Completion date	Project title	Aims/objectives	Major findings	Project/reference
	Bioassays and risk communication: Goal 1 - To strengthen and validate bioanalytical tools for application in water quality assessment (b) Book.	The objective of this book was to summarise the scientific background underlying the application of bioanalytical tools in water quality assessment for a non-specialist audience and to review the state-of-the-science.	Chapters 1-3 provided background information of the field, an introduction to risk assessment, standards and guidelines and the scientific basis for bioanalytical tools. Chapters 4-8 takes the reader to cellular mechanistic level, explains toxicity pathways and provides an overview of mixture toxicity. Chapters 9-12 describes the application of the tools and an outlook on future developments.	Escher, B., Leusch, FDL, Chapman HF and Poulsen (2012) *Bioanalytical Tools for Water Quality Assessment.* IWA Publishing, Dec 15th 2011
2012	Bioassays and risk communication: Goal 2. Communication and adoption.	The previous projects, particularly those involving endocrine disruption, highlighted that concerns regarding chemicals in recycled water was a societal problem as well as a technical one. It was thought that part of the reason could be a lack of understanding of the science by the water industry. This project aimed to assess the effectiveness of a science communication workshop as an educational tool for the uptake and application of bioanalytical tools for water quality assessment.	All attendees at the workshop felt that attendance at the workshop was beneficial in improving their knowledge of bioanalytical tools. The research also showed that barriers to communication within the water sector included inconsistent use of water recycling terminology, content-free approaches to policy, staffing changes, and policy makers, industry and regulators not being 'on the same page'. The research demonstrated that the science communication workshop was an effective means of informing industry professionals about the application of bioassays and ecouraging their uptake of the tools.	Ross, Victoria and Heather Chapman (2012). Evaluating a science communication workshop as an educational tool. Urban Water Security Alliance - Technical Report # 72

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Chapter 2. Review of the application of bioanalytical tools in validation and verification monitoring of recycled water schemes

by Frederic Leusch

2.1 Introduction: Why do we need bioanalytical tools?

Conventional drinking water often contains a variety of organic and inorganic chemical contaminants commonly found in surface waters such as agricultural pesticides as well as chemicals formed during water treatment such as disinfection by-products. These chemicals have different human health risk profiles: some can be acutely toxic and result in immediate adverse health effects, others pose chronic health risks and only produce adverse effects after prolonged continuous exposure, and yet others may not be toxic to human health even after a lifetime of exposure. Drinking water standards are set by health authorities for specific chemicals that are likely to be found in water sourced from conventional sources such as surface water or groundwater. These standards are usually extrapolated (with highly conservative safety factors applied from toxicity experiments with animals combined with specific quantitative exposure assessment, and) provide "safe" concentrations of specific chemicals in drinking water (NHMRC/NRMMC 2011, WHO 2011). This approach is generally considered effective for drinking water sourced from pristine water sources or water sources that have been used for a long time without evidence of harm. However, those same drinking water standards are not appropriate for less traditional water sources such as water reclaimed from wastewater, which may contain very different sets of chemicals that introduce new, unaccounted risks.

Municipal wastewater often contains a wide range of natural and synthetic chemicals, including personal care products, household chemicals, industrial compounds, chemicals excreted by people such as natural and synthetic hormones and pharmaceuticals, and chemicals formed during wastewater treatment. Health authorities have therefore produced new guidelines documents specifically for water sourced from wastewater, which consider the much larger universe of chemical contaminants potentially present in source waters and derive more comprehensive lists of compounds (NWQMS 2008). Some water recycling schemes have conducted extensive chemical monitoring data on reclaimed water (e.g., Department of Health 2009, WaterSecure 2010), and these rich datasets can be used in combination with chemical exposure assessment techniques (NWC 2011) to determine the likelihood and significance of exceedance of chemical guidelines (Leusch et al. 2012, Rodriguez et al. 2009). However, even this extensive chemical monitoring of 300-400 different chemicals is only a subset of the vast number of chemicals that are likely present. For example, between 30,000 to 70,000 compounds are in daily use (Schwarzenbach et al. 2006), and there are more than 4,000 pharmaceutical compounds alone (Boxall et al. 2012), each likely to produce several different environmental transformation products. The actual number of chemicals potentially present in water is thus likely to be in the hundreds of thousands. Not only would it be impossible to write a drinking water guideline document that would consider all of those compounds, it is also not feasible to detect them by conventional chemical analysis.

Numerous decades of water quality monitoring have provided a reasonable (though always improving) understanding of which chemicals are likely to be present in drinking waters of traditional sources at significant enough concentrations to present an elevated level of risk (Leusch et al. 2007). However, we have much less experience in dealing with the diversity of chemicals that may be present in municipal effluents. Even if the operators of a water recycling scheme could identify all of the organic chemical components in a specific municipal effluent, there would be scant toxicological data available for most of them and thus little basis for assigning risks. Another important limitation of conventional chemical analysis is that chemicals present at concentrations below the analytical limit of detection may still contribute to the overall additive toxicity of a large number of chemicals present in a complex mixture, and so a long list of "nondetects" can still produce an adverse biological effect. This is particularly relevant for highly treated waters, where many chemicals fall below detection limit after reverse osmosis and advanced oxidation but biological activity is not necessarily reduced below detection limit (Escher et al. 2011). Because of these limitations, and because many compounds in

wastewaters are simply unidentifiable, many scientists have suggested that toxicity testing of recycled water may be the only way to ensure the water's chemical safety (NRC 1998).
Toxicity testing involves collecting whole water samples and subjecting these to tests for a range of toxicological endpoints, either using whole animals (in the case of direct toxicity assessment, DTA) or bioanalytical tools (also known as *in vitro* bioassays). Toxicity testing may include testing for mutagenic activity, carcinogenic activity, hormonal activity such as estrogenicity, or various forms of acute and chronic toxicity. Whole animal toxicity testing has been the cornerstone of toxicology for a long time, but ethical and financial drivers to reduce, refine and replace whole animal tests (Zurlo et al. 1996) combined with recent advances in molecular toxicology (Boelsterli 2009, Hartung and McBride 2011, Seidle and Stephens 2009, Shukla et al. 2010) have lead to an intense interest in alternative techniques, such as *in vitro* bioassays.
In vitro toxicity tests are tests performed at the molecular or cellular level in the laboratory, usually on concentrated water extracts. Examples of molecular endpoints include binding to specific enzymes or receptors, while cellular events could include cell death, maturation or growth. An advantage of *in vitro* assays is that they can be based on human cells, thereby eliminating the inter-species predicament of whole animal testing. *In vitro* tests detect the triggering molecular or cellular toxic event that occurs at much lower, environmentally relevant concentrations, often below detection limits of chemical analysis and *in vivo* testing (Asano and Cotruvo 2004). The main limitation of *in vitro* assays is that they lack some metabolism and transport mechanisms that may modulate toxicity in whole organisms, and that many cell-based assays are based on cancer cell lines. Also, *in vitro* bioassays were developed for screening purposes and there is still much debate about their ability to predict whole-organism effects (NRC 1998). But the gap in our understanding of the link between an *in vitro* response and an adverse outcome in whole organisms is getting narrower: the concept of Adverse Outcome Pathway (Ankley et al. 2010) provides a solid framework to link a molecular/cellular event (as measured using *in vitro* bioassays) to a whole organism effect (Shukla et al. 2010). *In vitro* bioassays are well-suited to monitoring of water quality, as they are significantly faster and cheaper than *in vivo* toxicity testing, are amenable to high-throughput screening, and allow the generation of relatively rapid toxicology data without the need for ethically and financially expensive whole-animal experimentation (Zurlo et al. 1996). In recent years, there has been a move towards standardising the various *in vitro* techniques available, with the creation of the European Centre for the Validation of Alternative Methods (ECVAM) in 1991 and the US National Toxicology Program Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) in 1998. These two programs, and similar efforts by Organisation for Economic Cooperation and Development (OECD), have published an ever-growing catalogue of defined operating protocols for the testing of chemicals.
In vitro bioassays are increasingly applied to water quality assessment (reviewed in Escher et al. 2012, Poulsen et al. 2011) and, considering the particular predicament of conventional chemical risk assessment with complex water sources such as treated sewage (too many chemicals, too many unknowns, no integration of mixture toxicity with the current testing regimes), it is only logical to apply bioanalytical techniques in the context of recycled water quality assessment. A few *in vitro* bioassays have in fact been applied since the 1960s to assessment of recycled water quality, but recent developments have greatly expanded the number and scope of bioanalytical tools available for recycled water quality testing. The rest of this document provides an overview of the application of *in vitro* bioassays for recycled water quality assessment from its early days in the 1960s to today.

2.2 Types of bioanalytical tools

There are five categories of bioanalytical methods based on the biological response that is monitored: three are measures of toxicity (non-specific, specific and reactive toxicity) and the other two (adaptive stress responses and induction of xenobiotic metabolism pathways) are measures of the response of a cell when exposed to foreign or toxic chemicals (Escher et al. 2012).
With heavy use of acronyms and technical language, understanding the purpose and endpoint of *in vitro* bioassays can be difficult to the neophyte. This section very briefly describes the different assays mentioned in this review, and may be useful as a quick reference.
Note that many more assays than those listed below may be suitable to water quality testing (Poulsen et al. 2011, WateReuse Research Foundation 2014).In fact, many (but not all) of the bioassays developed for drug discovery applications can be used with environmental samples, although modifications of the assay protocols are often necessary to make the assays compatible with water extracts. In a recent study, Escher et al. (2014) tested 103 different bioassays in a variety of water samples, including reclaimed water, and identified a few endpoints that have so far received little attention but appear to be highly relevant for water quality assessment.

2.2.1 Non-specific toxicity

Non-specific toxicity assays measure toxicity to all types of cells due to interference with basic cellular physiology or chemistry, such as damage to cell membranes or interference with intracellular homeostasis. Toxicity measured by non-specific assays is often termed "baseline toxicity". Many non-specific assays use bioluminescent marine bacteria, which are easy to quantify, while others use mammalian or human cells. The bacteria assays appear more sensitive (Escher et al. 2014), but of course it is more difficult to relate the results to health risks. On the other hand, some of the human cell based assays have been well correlated with conventional acute rodent toxicity tests (Konsoula and Barile 2005).

Bacterial growth inhibition: provides a measure of **bacterial cytotoxicity**. In its most simple form, bacteria are grown in liquid nutritious media with the test sample and growth is monitored by measuring the spectrophotometric absorbance at 600nm and compared to control bacteria cultures.

Caco2-NRU: provides a measure of **cytotoxicity to human gastrointestinal cells**. In this assay, human intestinal (Caco2) cells are incubated with the test sample for 24h, after which they are exposed to neutral red, a red-coloured dye. Live cells will pump neutral red inside the cell, while dead cells will not. After a short incubation, the amount of neutral red inside cells, which is directly proportional to the number of live cells, is measured by spectrophometric absorbance at 540nm.

HepaTOX: provides a measure of **cytotoxicity to human liver cells**. Human liver cells (C3A human hepatocellular carcinoma cells) are exposed to the test sample for 24h, after which they are exposed to the weakly fluorescent dye resazurin. Resazurin is reduced to the highly fluorescent resorufin in mitochondria of live cells, which can then be measured with a fluorometer set to 530nm excitation and 580nm emission. The amount of fluorescence is directly proportional to the number of live cells.

LDH leakage assay: provides a measure of **cytotoxicity**. Lactate dehydrogenase (LDH) is an enzyme present in the cytosol of many different cells. When the cell membrane is compromised, LDH leaks out of cells into the culture medium. The concentration of LDH in the culture medium, which is directly proportional to the number of damaged cells, can be measured by addition of a compound that reacts with LDH to produce a red product, which can be simply measure by spectrophotometric absorbance at 490nm.

Microtox: provides a measure of **bacterial cytotoxicity**. The assay relies on marine bacteria that are naturally bioluminescent. Bacteria are exposed to the test sample and luminescence is measured after a short period of incubation. If the sample is toxic to bacteria, the luminescence will decrease as the cells die. The extinction of bioluminescence is directly proportional to the toxicity of the sample to these marine bacteria. There is an ISO method for carrying out the Microtox assay with water samples (ISO 11348:2007).

ToxScreen3: provides a measure of **bacterial cytotoxicity**. Like its more famous cousin the Microtox, this assay relies on marine bacteria that are naturally bioluminescent. Bacteria are exposed to the test sample and luminescence is measured after a short period of incubation. If the sample is toxic to bacteria, the luminescence will decrease as the cells die. The extinction of bioluminescence is directly proportional to the toxicity of the sample to these marine bacteria.

WIL2NS TOX: provides a measure of **cytotoxicity to human white blood cells**. White blood cells (WIL2NS lymphoblast cells) are exposed to the test sample for 24h, after which they are exposed to the weakly fluorescent dye resazurin. Resazurin is reduced to the highly fluorescent resorufin in mitochondria of live cells, which can then be measured with a fluorometer set to 530nm excitation and 580nm emission. The amount of fluorescence is directly proportional to the number of live cells.

2.2.2 Specific toxicity

Specific toxicity assays measure interference with specific biological functions, such as photosynthesis, enzyme or receptor function, endocrine signalling, etc. An example of a well-studied specific toxicity endpoint is estrogenic endocrine disruption, which can lead to feminisation of male fish.

AChE (acetylcholinesterase) inhibition: provides a measure of **neurotoxicity**. Acetylcholinesterase (AChE) is an enzyme found mainly at neuromuscular junctions that is responsible for hydrolysis of the neurotransmitter acetylcholine. Toxic compounds such as organophosphate and carbamate pesticides can inhibit the action of AChE and cause paralysis and death by asphyxiation. In this assay, the test sample is incubated with a

preparation of isolated enzyme AChE. A compound is added that is converted to a yellow products by AChE. The amount of the coloured product, which is inversely proportional to the amount of AChE inhibition, can be measured by spectrophotometric absorbance at 405nm. Note that this is a naked enzyme assay system without a cell membrane, which provide a less meaningful toxicity assessment than cell-based assays (which provide some integration of toxicokinetics).

AR-CALUX: provides a measure of **androgenic endocrine activity**, part of the CALUX battery of assays. See "CALUX" for a description of this particular type of assay.

A-SCREEN: provides a measure of **androgenic endocrine activity**. In this assay, breast cancer cells that have been genetically modified to become sensitive to androgens (MCF7-AR1 cells) are exposed to the test sample and an estrogen and then counted after a period of incubation. If androgenic compounds are present in the sample, the modified breast cancer cells decrease their proliferation rate. Therefore the number of cells at the end of the incubation is inversely proportional to the androgenic activity of the sample.

CALUX (Chemical Activated Luciferase gene eXpression): a reporter gene assay battery developed by BioDetection Systems (Amsterdam, The Netherlands) to measure nuclear-receptor mediated responses, including **endocrine activity**. The cells used in this assay have been genetically modified to express the enzyme luciferase upon activation of a specific nuclear receptor (for example, the ER-CALUX detects compounds that bind to and activate the estrogen receptor ER). Cells are exposed to the test sample, and the amount of luciferase produced after a period of incubation is measured after addition of luciferin by measuring light intensity. The light intensity is directly proportional to amount of activation of the nuclear receptor.

ER-CALUX: provides a measure of **estrogenic endocrine activity**, part of the CALUX battery of assays. See "CALUX" for a description of this particular type of assay.

E-SCREEN: provides a measure of **estrogenic endocrine activity**. The assay relies on the principle that breast cancer cells require estrogens to proliferate. Breast cancer cells (MCF7-BOS cells) are exposed to the test sample and counted after a period of incubation. If the sample is estrogenic, the cancer cells will have proliferated, and therefore there will be more cells. The proliferation is directly proportional to the estrogenic activity of the sample.

GeneBLAzer: a reporter gene assay battery developed by Life Technologies (Carslbad, CA, USA) to measure nuclear-receptor mediated responses, including **endocrine activity**. The cells used in this assay have been genetically modified to express the enzyme β-lactamase upon activation of a specific nuclear receptor (for example, the ER-GeneBLAzer detects compounds that bind to and activate the estrogen receptor ER). Cells are exposed to the test sample, and the amount of β-lactamase produced after a period of incubation is measured after addition of a fluorescent substrate with a fluorometer set to 409nm excitation and 447nm emission. The amount of fluorescence is directly proportional to the activation of the nuclear receptor.

GR-CALUX: provides a measure of **glucocorticoid endocrine activity**, part of the CALUX battery of assays. See "CALUX" for a description of this particular type of assay.

I-PAM (Imaging Pulse-Amplitude Modulated fluorometry): provides a measure of **phytotoxicity** (toxicity to plants). Healthy photosynthetic organisms convert most of light energy into photosynthesis, however certain toxic chemicals such as herbicides can block photosynthesis, forcing the organisms to release the energy as fluorescence instead. This assay measure fluorescence emitted by photosynthesis organisms such as microalgae after a brief pulse of light. The amount of fluorescence measured is inversely proportional to photosynthesis: a sample with high fluorescence indicates that the test sample is blocking photosynthesis, which eventually leads to death for organisms that depend on it for their energy (such as photosynthetic plants and bacteria). The **combined algae test** combined photosynthesis inhibition (as measured in a PAM) with growth after 24h.

PR-CALUX: provides a measure of **progestagenic endocrine activity**, part of the CALUX battery of assays. See "CALUX" for a description of this particular type of assay.

TR-CALUX: provides a measure of **thyroid endocrine activity**, part of the CALUX battery of assays. See "CALUX" for a description of this particular type of assay.

YAS (Yeast Androgen Screen): provides a measure of **androgenic endocrine activity**. In this assay, a gene that expresses the enzyme β-galactosidase in the presence of androgenic compounds has been inserted into yeast cells. The genetically modified yeast cells are exposed to the test sample and the amount of β-galactosidase produced is measured by addition of a substrate that produces a coloured product in the presence of β-galactosidase.

The amount of the coloured product, which is directly proportional to the androgenic activity in the sample, can be measured by spectrophotometric absorbance at 570nm.

YES (Yeast Estrogen Screen): provides a measure of **estrogenic endocrine activity**. In this assay, a gene that expresses the enzyme β-galactosidase in the presence of estrogenic compounds has been inserted into yeast cells. The genetically modified yeast cells are exposed to the test sample and the amount of β-galactosidase produced is measured by addition of a substrate that produces a coloured product in the presence of β-galactosidase. The amount of the coloured product, which is directly proportional to the estrogenic activity in the sample, can be measured by spectrophotometric absorbance at 570nm.

2.2.3 Reactive toxicity

Reactive toxicity is caused by the reaction of the chemical with endogenous molecules, such as protein or DNA. Several reactive toxicity pathways lead to cancer, including mutagenicity (change in DNA code) and genotoxicity (physical damage to DNA).

6-Thioguanine resistance assay: provides a measure of **mutagenicity in mammalian cells**. In this assay, Chinese hamster cells (V79 cell line) that have only one functional copy of the gene that encode for the HGPRT enzyme (hypoxanthine-guanine phosphoribosyltransferase; an enzyme responsible for producing DNA nucleotides from hypoxanthine or guanine) are exposed to the test sample in a growth medium that contains 6-thioguanine, a toxic form of guanine that poisons cells with a functional HGPRT enzyme. This means that any cells that grow must be mutants that have lost the ability to produce the HGPRT enzyme. Thus, counting the number of cells colonies present at the end of the exposure is a measure of the mutagenic activity in the test sample.

Ames test: provides a measure of **mutagenicity in bacteria**. The strain of bacteria used in the Ames test carry mutations in the gene involved in histidine synthesis. This means that these bacteria cannot produce histidine themselves and therefore require histidine-supplemented media to grow. However a lucky mutation in the gene can restore their ability to synthesize histidine, and those mutants (called "revertants") can grow on histidine-free media. The principle of the assay is very simple: histidine-deficient bacteria are plated on histidine-free media and incubated with the test sample. Only "revertants" will be able to survive on the histidine-free media, and the number of bacterial colony growing after the incubation period is a measure of the mutagenic potential of the sample. Because metabolism can create reactive compounds, the assay can be run with or without metabolic activation, described as "+S9" and "-S9", respectively. There are generally two strains used, named TA98 and TA100. The TA98 strain detects frameshift mutation, while the TA100 detects base-pair substitution.

Cytokine production assay (CPA): provides a measure of **immunotoxicity**. Cytokines are small proteins released by a broad range of cells to communicate with other cells. Immune cells in particular release cytokines to coordinate the immune response. In CPA, the amount of cytokines released by white blood cells (lymphocytes or macrophages) after exposure to the test sample is quantified by enzyme-linked immunosorbent assay (ELISA) methods. The tests can either measure induction of cytokine production (*i.e.*, induction of an immune response) or inhibition of cytokine production (*i.e.*, interference with normal immune signalling). These assays can be conducted with immortal cell lines (such as the THP1 human monocyte cells) or with primary white blood cells derived from whole blood.

Mammalian cell transformation assay: provides a measure of **carcinogenicity in mammalian cells**, capable of detecting both genotoxic and non-genotoxic carcinogens. This assay can be run with many different cell types, but all references to this assay in this review refer specifically to the assay performed with the mouse cell line C3H/10T1/2. The assay measures the change in phenotype due to conversion of cells from normal to malignant (*i.e.*, cancerous). For example, malignant cells grow uncontrollably, while normal cells exhibit close contact growth inhibition.

Sister chromatid exchange assay: provides a measure of **genotoxicity in mammalian cells**. The assays detects double-stranded DNA breaks by quantifying the amount of DNA exchange between pairs of DNA strand (chromatids) on chromosomes.

umuC (Umu Chromotest): provides a measure of **genotoxicity in bacteria**. Bacteria have a DNA repair mechanism called the "SOS response" that is activated when the bacteria detect DNA damage. Part of this SOS response includes activation of the *umuC* gene. This assay uses *Salmonella typhimurium* bacteria that have been genetically modified to produce the enzyme β-galactosidase when the *umuC* gene is activated. The genetically modified

bacteria are exposed to the test sample and the amount of β-galactosidase produced can be measured by addition of a subtrate that produces a yellow colored product in the presence of β-galactosidase. The amount of the coloured product, which is proportional to the amount of DNA damage, can be measured by spectrophotometric absorbance at 570nm. Because metabolism can create reactive compounds, the assay can be run with or without metabolic activation, described as "+S9" and "-S9", respectively. There is an ISO method for carrying out the umuC assay with water samples (ISO 13829:2000).

WIL2NS FCMN (Flow Cytometry MicroNucleus): provides a measure of **genotoxicity to human cells**. The micronucleus is a small nucleus that forms when a chromosome fragment is not integrated into the nucleus of daughter cells during cell division, and is an indication of DNA damage (genotoxicity) *in vitro*. The micronucleus formation assay has been used as a reliable indicator of genotoxicity, and this version of the assay used flow cytometry to separate cells with or without micronuclei. In this assay, human lymphocytes (WIL2NS cell line) are exposed to the test sample and the proportion of cells with micronuclei is quantified by flow cytometry

2.2.4 Adaptive stress response

Adaptive stress response assays measure the defence mechanisms that cells can initiate to protect against chemically induced damage. These include production of proteins and enzymes to repair DNA damage, isolate reactive oxygen species, etc. Assays for adaptive stress response do not measure toxicity *per se*, but rather the cell's early response to toxic injury.

Assays for adaptive stress response have only recently been tested for water quality assessment, and none of the studies reviewed in this document have used it for recycled water assessment. Adaptive stress response assays appear sensitive and relevant to water samples, particularly oxidative stress and inflammation (Escher et al. 2014), and future work will likely explore these endpoints in the context of recycled water.

2.2.5 Xenobiotic metabolic pathway

Finally xenobiotic metabolism pathway assays measure the induction of biological pathways involved in metabolising xenobiotics, such as the aryl hydrocarbon receptor (AhR) or the peroxisome proliferator-activated receptor (PPAR) responses. This type of assay also does not measure toxicity *per se*, but the cell's attempt to detoxify foreign compounds.

AhR-CAFLUX: provides a measure of **AhR induction**. The Aryl hydrocarbon Receptor (AhR) is a protein that is involved in detoxification of foreign compounds, particularly dioxins and aromatic hydrocarbons (PAHs). In this assay, a rat liver cell (H4IIE cell line) has been genetically modified to express a green fluorescent protein (EGFP) upon stimulation of the AhR gene. The cells are exposed to the test sample, and fluorescence is measured in a fluorometer set to set to 490nm excitation and 535nm emission. The amount of fluorescence is directly proportional to the induction of the AhR gene.

YDS (Yeast Dioxin Screen): provides a measure of **AhR induction**. The Aryl hydrocarbon Receptor (AhR) is a protein that is involved in the biological response to dioxins and aromatic hydrocarbons (PAHs). In this assay, a gene that expresses the enzyme β-galactosidase in the presence of dioxin-like compounds has been inserted into yeast cells. The genetically modified yeast cells are exposed to the test sample and the amount of β-galactosidase produced is measured by addition of a substrate that produces a coloured product in the presence of β-galactosidase. The amount of the coloured product, which is directly proportional to the dioxin-like activity in the sample, can be measured by spectrophotometric absorbance at 570nm.

HepCYP1A2: provides a measure of **cytochrome P450 1A2 (CYP1A2) induction**. Cytochrome P450s are a group of multi-function oxidase enzymes that are involved in a variety of biological functions, including detoxification of foreign compounds in the body. Cytochrome P450 1A2 (CYP1A2) in particular is involved with metabolism of foreign compounds, and its expression is induced in liver cells exposed to toxic compounds. In this assay, liver cells (C3A cell line) are exposed to the test sample and the amount of CYP1A2 present in the cells after a period of exposure is quantified by addition of a precursor, which CYP1A2 converts into luciferin. The amount of luciferin, which is directly proportional to the amount of CYP1A2 activity, can be measured in a luminometer after addition of luciferase.

2.3 Application of bioanalytical tools to validation and/or verification monitoring of water reclamation projects

Since the 1980s, bioanalytical tools have been incorporated in validation and/or verification monitoring of water recycling schemes (NRC 1998). Prior to 2000, most applications of bioanalytical tools to recycled water were to detect reactive toxicity, specifically mutagenicity and genotoxicity. In particular, the Ames test for mutagenicity (Ames et al. 1973) was widely applied. The Ames test, also known as the *Salmonella* mutagenicity test or the Bacterial Reverse Mutation Assay, measures the mutagenicity of a test sample by its ability to induce mutations in specific *Salmonella* bacteria strains. Unfortunately, bacterial cells have an inherently high degree of gene mutation, and the Ames test has a relatively high rate of both false positive and false negatives, which has challenged the value and significance of Ames test results (NRC 1998). After much initial enthusiasm in the promise of *in vitro* methods, the limitations of the Ames test had a negative impact on the perceived value of *in vitro* bioassays as a whole in the 1990s (NRC 1998).

The development and application of new bioassays since then have led to renewed recognition of the value of bioanalytical tools for water quality monitoring, and bioassay batteries used for testing of water quality have since the mid-2000s expanded in both application and complexity (Escher et al. 2012, Poulsen et al. 2011).

Table 2-1 presents an overview of the *in vitro* bioassays applied during validation and/or verification monitoring at a variety of water reclamation schemes in various countries, reviewed more thoroughly in the remainder of this document.

Table 2-1. Bioanalytical tools applied to recycled water assessment.

Scheme name	Country	Endpoints	Assays	See section	Reference
Montebello Forebay Groundwater Recharge Project (1962-present)	USA (CA)	Mutagenicity	• Ames test • Mammalian cell transformation assay	2.3.1.1	(NRC 1998)
Orange County Water Factory 21 (1975-2004) and Groundwater Replenishment System (2004-present)	USA (CA)	Mutagenicity	• Ames test	2.3.1.2	(McCarty et al. 1982, NRC 1998)
Potomac Estuary Experimental Water Treatment Plant (1980-1982)	USA (VA)	Mutagenicity	• Ames test • Mammalian cell transformation assay	2.3.1.3	(NRC 1984, 1998)
San Diego Total Resources Recovery Project (1981-1999)	USA (CA)	Mutagenicity Genotoxicity	• Ames test • Micronucleus test • 6-Thioguanine resistance assay • Mammalian cell transformation assay	2.3.1.4	(NRC 1998, Olivieri et al. 1996)
Tampa Water Resource Recovery Project (1987-1989)	USA (FL)	Mutagenicity Genotoxicity	• Ames test • Sister chromatid exchange test	2.3.1.5	(NRC 1998)
Tucson Reclaimed Water System (1989-present)	USA (AZ)	Mutagenicity	• Ames test	2.3.1.6	(Quanrud et al. 2003)
Windhoek Direct Potable Reuse Scheme (1968-present)	Namibia	Cytotoxicity (bacteria) Cytotoxicity (human cells) Mutagenicity Neurotoxicity Immunotoxicity	• Bacterial growth test • LDH leakage assay with whole blood cells • Ames test • AChE inhibition • CPA in whole blood cells	2.3.2	(Faul et al. 2013, Iiputa et al. 2008, Menge and Slabbert 1999)
Dan Region Sewage Reclamation Project (1960-present)	Israel	Mutagenicity	• Ames	2.3.3	(Gruener 1978)

Scheme name	Country	Endpoints	Assays	See section	Reference
Perth Groundwater Replenishment Scheme (2009-present)	Australia (WA)	Cytotoxicity (bacteria) Genotoxicity Estrogenicity Androgenicity Phytotoxicity	• Microtox • umuC • E-SCREEN • AR-CALUX • I-PAM	2.3.4.1	(Leusch et al. 2014a, Reitsema et al. 2010)
Qld Western Corridor Recycled Water Scheme (2009-present)	Australia (Qld)	Cytotoxicity (bacteria) Genotoxicity AhR induction Estrogenicity Phytotoxicity Neurotoxicity	• Microtox • umuC • AhR-CAFLUX • E-SCREEN • I-PAM • AChE inhibition	2.3.4.2	(Macova et al. 2010a, Macova et al. 2011)
Nine unidentified water reclamation plants in Australia	Australia	Cytotoxicity (human cells) Mutagenicity Genotoxicity Endocrine activity Neurotoxicity Immunotoxicity MFO induction	• Caco2-NRU • WIL2NS TOX • HepaTOX • Ames test • WIL2NS FCMN • ERα-CALUX • AR-CALUX • GR-CALUX • PR-CALUX • TRβ-CALUX • AChE inhibition • CPA with THP1 cells • HepCYP1A2	2.4.3	(Leusch et al. 2014b, NWC 2011)
Landsborough Water Reclamation Plant	Australia (Qld)	Cytotoxicity (bacteria) Estrogenicity	• Microtox • E-SCREEN • ERBA	2.4.3.2	(Leusch et al. 2005, Reungoat et al. 2012)
South Caboolture Water Reclamation Plant	Australia (Qld)	Cytotoxicity (bacteria) Estrogenicity AhR induction Neurotoxicity Phytotoxicity Genotoxicity	• Microtox • E-SCREEN • AhR-CAFLUX • AChE inhibition • I-PAM • umuC	2.4.3.2	(Macova et al. 2010b, Reungoat et al. 2012, Reungoat et al. 2011, Reungoat et al. 2010)

Scheme name	Country	Endpoints	Assays	See section	Reference
Gerringong Water Reclamation Plant	Australia (VIC)	Cytotoxicity (bacteria) Estrogenicity	• Microtox • E-SCREEN	2.4.3.2	(Reungoat et al. 2012)
One unidentified Qld water reclamation plant	Australia (Qld)	Cytotoxicity (bacteria) Androgenicity Estrogenicity Genotoxicity	• ToxScreen3 • AR-CALUX • ER-CALUX • umuC	2.4.3.3	(Watson et al. 2012)
Five unidentified US water reclamation plants	USA	Estrogenicity Androgenicity	• E-SCREEN • YES • A-SCREEN • YAS	2.4.2	(Drewes et al. 2006)
Two unidentified Australian water reclamation plants	Australia	Cytotoxicity Phytotoxicity Endocrine activity Neurotoxicity Immunotoxicity Mutagenicity Genotoxicity Adaptive stress response Xenobiotic metabolism	• 103 different bioassays	2.4.3	(Escher et al. 2014)

2.3.1 Water reuse projects in the United States

2.3.1.1 Montebello Forebay Groundwater Recharge Project (1962 - present)

The Montebello Forebay Groundwater Recharge Project is a managed aquifer recharge project in California in operation since 1962. A health effects study was conducted in 1984, which applied two *in vitro* tests to a variety of water samples, including stormwater (wet and dry weather), groundwater, imported water and reclaimed water: the Ames mutagenicity test and mammalian cell transformation assay (NRC 1998).

Low-level mutagenic activity was detected in most water samples (concentrated 10,000 - 20,000×), but interestingly the storm runoff water contained more mutagenic activity than the reclaimed water, which itself contained more activity than ground water or imported water. More than half of the mutagenic activity in the reclaimed water samples appeared to be due to the chlorination process (NRC 1998).

2.3.1.2 Orange County Water Factory 21 (1975 - 2004) and Groundwater Replenishment System (2004 - present)

This project is a managed aquifer recharge project in Orange County, CA. Operated as Water Factory 21 from 1976 to 2004, the facility was upgraded and the new water reclamation plant, operational since 2007, produces approximately 250 ML/d with an advanced treatment train based an MF/RO/UV system.

The Ames test was applied in the 1980s to water extracts collected from various treatment stages at Water Factory 21 (McCarty et al. 1982). The results show significant mutagenicity in the influent (*i.e.*, treated wastewater) but a significant decrease after GAC treatment, where no mutagenicity was detected. Mutagenicity was however again detected after chlorination. Fractionation experiments suggested that the mutagenic activity was associated mostly with hydrophobic organic compounds, but the exact compounds could not be identified.

2.3.1.3 Potomac Estuary Experimental Water Treatment Plant (1980 - 1982)

The Potomac Estuary Experimental Water Treatment Plant was a US Army Corps of Engineers pilot project to provide highly treated water by blending Potomac estuary water with secondary effluent from a municipal WWTP in Washington DC (NRC 1984). The treatment train combined filtration, carbon adsorption and disinfection (NRC 1998). Two *in vitro* tests were applied to both the reclaimed water and local drinking water: the Ames test for mutagenicity and mammalian cell transformation assay for carcinogenicity.

The results showed low level activity in Ames test, with the reclaimed water exhibiting less activity than local drinking water. The cell transformation assay also showed a small number of positive samples with both the reclaimed water and the local drinking water (NRC 1984). The study concluded that the reclaimed water did not indicate any increase in potential chronic health effects compared to local drinking water, although a subsequent review emphasized that while the toxicity testing showed that the water produced by the advanced water plant was of high quality, the limited number of toxicity tests were insufficient to clearly establish human health effects (NRC 1984).

2.3.1.4 San Diego Total Resources Recovery Project (1981 - 1999)

The City of San Diego Total Resources Recovery Project was a pilot plant to reclaim water for indirect potable reuse. The advanced water treatment plant train included UV, RO and GAC (NRC 1998).

Four *in vitro* bioassays were applied to reclaimed water and a reservoir acting as local drinking water source: the Ames test for mutagenicity, the micronucleus test for genotoxicity, the 6-thioguanine resistance assay for mutagenicity in mammalian cells and mammalian cell transformation assay for carcinogenicity (Olivieri et al. 1996).

The results show weak but statistically significant mutagenic activity in both reclaimed and drinking water source waters, with lower activity in reclaimed water compared to the conventional alternative. The results with the mammalian cell transformation assay were not repeatable and were thus rejected, and the remaining two assays did not show any mutagenic or genotoxic activity in either water samples (NRC 1998). Based on these *in vitro* results and additional chemical and microbiological tests, the study concluded that the health risks associated with the use of reclaimed water as a raw water supply were less or equal to the use of the (then) current raw water source (Olivieri et al. 1996).

2.3.1.5 Tampa Water Resource Recovery Project (1987-1989)

The advanced water treatment plant of the Water Resource Recovery Project in Tampa, FL, was a pilot plant to evaluate the acceptability of using reclaimed water to augment the city's water supply. The final treatment train included GAC and disinfection with ozone. Two *in vitro* bioassays were used to test the reclaimed water: the Ames test for mutagenicity and a sister chromatid exchange assay (NRC 1998). No mutagenic or genotoxic activity was observed in any of the samples.

This project provides an interesting insight into some of the power of quick and rapid *in vitro* bioassay use during the early design stage. Three different treatment trains were initially trialled (GAC, RO and UF), but the project proponents settled on GAC based on better results with the Ames test. Likewise, ozonation was selected as disinfection agent instead of chlorine because the latter produced mutagenic activity in the final water. Extensive toxicity testing during validation, including chronic toxicity tests in whole animals, confirmed that the selected treatment train had no adverse effect on any of the endpoints monitored (NRC 1998).

2.3.1.6 Tucson Reclaimed Water System (1989 - present)

The Reclaimed Water System in Tucson, AZ, is a soil aquifer transfer (SAT) scheme that infiltrates reclaimed water from secondary effluent into a managed aquifer for non-potable uses via numerous recharge basins. The Sweetwater Recharge Facilities in particular have been in operation since 1989. Quanrud et al. (2003) tested reclaimed water sample extracts using the Ames assay for mutagenicity. There was a small (but not statistically significant) increase in mutagenicity associated with the hydrophobic acid fraction from the recharge ponds (up to 2.25× increase in the number of revertants), and hydrophobic acid extracts from nearby monitoring wells did induce a significant increase in mutagenicity as measured in the Ames test (up to 8.27× increase in the number of revertants). The results suggest that mutagenic compounds were less biodegradable during SAT than other bulk organics, or that mutagenic by-products are created during SAT.

2.3.2 Windhoek Direct Potable Reuse Scheme (1968 - present)

The Goreangab Water Reclamation Plant has had several upgrades since the start of operations in 1968. The current advanced water treatment train, upgraded in 2002, produces 21 ML/d and consists of O_3/BAC+GAC/UF followed by chlorination.

A regular monitoring programme included testing water quality with *in vitro* assays such as the Ames test and a bacterial growth inhibition assay once a month (Iiputa et al. 2008). Mutagenicity in the source water (treated sewage) was on occasion mutagenic (up to 2.9× increase in number of revertants), however the reclaimed water never induced significant mutagenicity (all results <2× increase) (Menge and Slabbert 1999). Inhibition of bacterial growth was evident with both the source and product waters, with up to 34% inhibition of bacterial growth in reclaimed water. The authors attribute this inhibition to occasionally high iron, aluminium and manganese concentrations (Menge and Slabbert 1999).

More recently, Faul et al. (2013) collected five grab samples over a 1 year period from March 2010 - April 2011 and applied four *in vitro* assays to samples concentrated by solid phase extraction: an AChE inhibition assay (neurotoxicity), an LDH leakage assay with whole blood cells (cytotoxicity), and two cytokine production assays (CPA; for IL-6 and IL-10) in whole blood cultures (immunotoxicity). The results show a reduction of biological response in the final effluent compared with the secondary treated sewage influent, up to 6% activity in the AChE inhibition assay (72->95% decrease from secondary treated sewage), <1% cytotoxicity in the LDH leakage assay (>96% decrease), up to approximately 110 pg/mL IL6 in the first CPA (84->99% decrease), and <1pg/mL IL-10 in the second CPA (>99% decrease).

2.3.3 Dan Region Sewage Reclamation Project (1960s – present)

The Dan Region Sewage Reclamation Project is 100-150 ML/d groundwater recharge scheme established in the 1960s that receives treated wastewater from 8 WWTP in Tel Aviv, Israel. After a basic mechanical and biological treatment step, the water is injected into the local aquifer. Groundwater is then used mostly for agricultural use.

A 1978 study applied the Ames assay to test the effect of ozonation on the mutagenicity of Dan Region groundwater (Gruener 1978). There was no significant difference in mutagenicity as determined by the Ames test between groundwater (reclaimed from wastewater) and distilled water, but ozonation of groundwater led to a 3-6× increase in mutagenicity. The specific mutagens could not be identified

2.3.4 Water reuse projects in Australia

2.3.4.1 Perth Groundwater Replenishment Scheme (2009 - present)

The Perth Groundwater Replenishment Scheme at the Beenyup Advanced Water Recycling Plant in Western Australia is currently being expanded to a capacity of approximately 75 ML/d. The scheme treats secondary treated wastewater for managed aquifer recharge. The treatment train consists of microfiltration followed by reverse osmosis (MF/RO).

A three year trial of the system (Groundwater Replenishment Trial) was conducted from 2009 to 2012 after an extensive chemical monitoring campaign (Department of Health 2009). A National Water Commission study investigated source and reclaimed water quality testing with five *in vitro* bioassays (Leusch et al. 2014a, Reitsema et al. 2010). Grab samples were collected every 2-3 months from March 2008 to April 2009 and analysed with bioassays for non-specific toxicity (Microtox), reactive toxicity (umuC) and specific toxicity (I-PAM, E-SCREEN and AR-CALUX). The MF/RO treatment significantly reduced biological response in all assays, and only minimal basal toxicity was detected in the final effluent: up to 0.41 TU in the Microtox (56->82% decrease from secondary treated sewage), <0.04 GTU in the umuC+S9 (>55% decrease), <0.04 GTU in the umuC-S9 (>90% decrease), <0.03 µg/L DEQ in the I-PAM (>40% decrease), <1 ng/L EEQ in the E-SCREEN (>39% decrease) and <2.5 ng/L DHTEQ in the AR-CALUX (also undetectable in the secondary treated effluent).

Overall, the bioanalytical results show MF/RO treatment was very effective at removing biologically active chemicals, and the reclaimed water was comparable in quality to ultrapure laboratory grade water (Leusch et al. 2014a, Reitsema et al. 2010).

These findings were confirmed by a more recent study for the Australian Water Recycling Centre of Excellence, with MF/RO treatment leading to a reduction in the bioassay response of 92% in the Microtox assay, 89% in the AREc32 oxidative stress assay, and >90% (to below limit of detection) in both the I-PAM and umuC-S9 assays (Tang et al. submitted).

2.3.4.2 Qld Western Corridor Recycled Water Scheme (2009 - present)

The Queensland Western Corridor Recycled Water Scheme was constructed in the later part of the 2000s to provide drought relief for Southeast Queensland. The scheme includes three advanced water reclamation plants located at Bundamba, Luggage Point and Gibson Island, which draw water from existing wastewater treatment plants in the region. The scheme is designed to provide up to 250 ML/d for indirect potable reuse, although it is currently not in operation due to currently high levels of conventional water supply.

Alongside an extensive chemical monitoring campaign (WaterSecure 2010), a variety of *in vitro* bioassays have been applied to water produced from the Western Corridor Recycled Water Scheme, including Microtox, AChE inhibition, I-PAM, E-SCREEN, AhR-CAFLUX, and umuC bioassays (Macova et al. 2010a, Macova et al. 2011). A grab sample taken in October 2009 at the Bundamba Water Reclamation Plant showed very low activity with all bioassays for the final effluent (after advanced oxidation): 0.12 mg/L TEQ in the Microtox (87% decrease from secondary treated effluent), <0.06 µg/L PTEQ in the AChE inhibition assay (>96% decrease), 0.05 µg/L DEQ in the I-PAM (81% decrease), <0.01 ng/L EEQ in the E-SCREEN (>97% decrease), 0.08 ng/L TCDDEQ in the AhR-CAFLUX (93% decrease), <0.05 µg/L 4NQOEQ in the umuC -S9 (>79% decrease) and <0.8 µg/L BaPEQ in the umuC +S9 (>86% decrease).

Interestingly, the same study also applied the same assays to a variety of other water samples from the urban water cycled, including surface, wastewater, drinking water and ultrapure laboratory blanks. The water produced by the Bundamba Water Reclamation Plant was better than current drinking water in all bioassay results, and almost identical to the ultrapure laboratory blank (Macova et al. 2010a, Macova et al. 2011).

2.4 Additional studies using bioanalytical tools for recycled water quality assessment

In addition to these validation and/or verification monitoring applications, bioanalytical tools have also been applied to recycled water by a growing number of scientists. Often times this is done at particular plants to test a specific treatment train.

2.4.1 Pre-2000: focus on mutagenicity and genotoxicity

As previously stated, most bioanalytical testing prior to 2000 focused on reactive toxicity, and specifically mutagenicity and genotoxicity. Several studies have tried identifying mutagenic and genotoxic compounds in water (mostly drinking water, reviewed in Loper 1980, Meier 1988, Stahl Jr 1991). Those studies confirmed that chlorination by-products were likely the cause of the reactive toxicity in water. Several highly mutagenic compounds were identified, such as MX (NRC 1998), but even those compounds could not account for the total reactive toxicity in water samples, and the identity of the causative compound(s) is still unclear to this day. The results however clearly emphasized that exposure to chlorination disinfection by-products in water should be minimized, although not at the cost of adequate disinfection and removal of pathogens.

2.4.2 Post 2000 non-Australian studies at full scale plants

A study funded by the Water Environment Research Foundation applied four bioassays for estrogenic and androgenic endocrine activity (E-SCREEN, A-SCREEN, YES and YAS) to test water from five unspecified water reclamation facilities in several US states (Drewes et al. 2006). The results show that estrogenic (0.2 - 7.9 ng/L EEQ in the E-SCREEN) and androgenic activity (1.6 - 9.1 ng/L TEQ in the A-SCREEN) was detected in treated sewage, but that soil aquifer treatment (SAT) and high pressure membranes (such as reverse osmosis) were very effective at reducing the residual endocrine activity to below detection limits (<0.04 ng/L EEQ and <1 ng/L TEQ in the E-SCREEN and A-SCREEN, respectively). The results of the estrogenic bioassays were well correlated with chemical analysis of estrogen hormones, but androgenic activity was higher than predicted, indicating the likely presence of unknown androgenic compounds.

Escher et al. (2009) applied a battery of *in vitro* bioassays to test the efficacy of ozonation to remove the residual toxicity of treated wastewater at a full-scale Swiss WWTP. Six bioassays were used: the Microtox and a green algae growth inhibition assay for non-specific toxicity, the YES, I-PAM and AChE inhibition assyas for specific toxicity, and the umuC for genotoxicity. Ozonation removed 65-76% activity in the non-specific endpoints, 86-95% specific activity, and completely removed genotoxicity in the final effluent.

Stalter et al. (2011) investigated the effect of ozonation or PAC trains at WWTPs in Switzerland and Germany using four *in vitro* bioassays: cytotoxicity to rat pituitary cells (GH3 cell line), estrogenic and androgenic endocrine activity (YES and YAS, respectively), and AhR induction (YDS). The bioanalytical results show that both methods effectively removed estrogenicity and AhR activity (63-99%), but achieved only minimal removal of androgenic and anti-estrogenic activity. Cytotoxicity was better removed by PAC (61%) than ozonation (32%). The results also showed that a dose of 0.7 g O_3/g dissolved organic carbon (DOC) was optimal to achieve most of the removal.

2.4.3 Australian studies at full scale plants

2.4.3.1 Reverse osmosis systems

A study funded by the National Water Commission (Leusch et al. 2014b, NWC 2011) investigated five **unidentified reverse osmosis (RO)-based water reclamation plants in several Australian states**. Grab samples were collected every second day for a week in April/May and July 2010, concentrated 1,000× by SPE and analysed in a battery of 13 *in vitro* bioassays: three assays for human cell cytotoxicity (Caco2-NRU, WIL2NS TOX and HepaTOX), two reactive toxicity assays (Ames and WIL2NS FCMN), six assays for specific toxicity (ERα-CALUX, AR-CALUX, GR-CALUX, PR-CALUX, TRβ-CALUX and acetylcholinesterase inhibition assay), one adaptive stress response (CPA in THP1 human monocyte cells) and one xenobiotic metabolism assay (HepCYP1A2). Biological activity was detectable in 8 out of 13 assays in the secondary treated effluent, but only in 3 bioassays in the final RO effluent: up to 0.87 ng/L EEQ and 4.4 µg/L TMXEQ in the ER-CALUX assay (66->99% decrease from secondary treated sewage), up to 0.61 µg/L DEXAEQ in the THP1-CPA (15->98% decrease), and up to 0.09 TU in the WIL2NS TOX assay (less than 2× above the limit of detection of the assay at

0.05 TU). The remaining biological response in the final RO effluent was tentatively attributed to plasticizers from the RO membranes and disinfection by-products (Leusch et al. 2014b, NWC 2011).
A large inter-laboratory study (Escher et al. 2014) screened a water sample from **an unidentified Australian RO-based water reclamation plant** with a battery 103 different *in vitro* bioassays: 10 assays for cytoxicity (including Microtox and Caco2-NRU), 46 for specific toxicity (including the I-PAM and various CALUX and GeneBLAzer assays for endocrine activity), 12 for reactive toxicity (including Ames and umuC tests), 16 for adaptive stress response and 19 for xenobiotic metabolism (including the AhR-CAFLUX). The objective of the study was to evaluate cell-based bioassays for their suitability to benchmark water quality and to assess efficacy of water treatment processes. The study found that source water (treated sewage) produced a biological response in 53 out of 103 bioassays, but that MF/RO treatment reduced the biological response in all bioassays (although it was still above detection limit in 13 assays) and that the subsequent advanced oxidation (AO) step removed it in all but five bioassays: the Microtox and another bacteria cytotoxicity assay, the Ames test, and two assays that detect induction of xenobiotic metabolism (specifically the AhR and CAR pathways).
In addition, the studies on large-scale systems described above for the **Perth Groundwater Replenishment Trial** (section 2.3.4.1) and the **Qld Western Corridor Recycled Water Scheme** (section 2.3.4.2) both relate to RO-based systems.
Conclusions on RO systems from Australian studies: The above studies clearly showed that reverse osmosis, which is an effective technique to remove organic contaminants, is likewise highly efficient at removing the biological response in *in vitro* assays. Some low residual activity is sometimes detected in membrane-based systems (Escher et al. 2011, Leusch et al. 2014b) indicating that RO is an effective but not absolute barrier to biologically active compounds, as had been previously demonstrated for individual chemicals (Snyder et al. 2007). Reverse osmosis should be used in combination with source control and complementary treatment options (such as AO).

2.4.3.2 Ozonation systems

Leusch et al. (2005) measured estrogenic activity at the **Landsborough Water Reclamation Plant** (O_3/BAC/UV). A grab sample was collected in August 2000 and two bioassays were used: an estrogen receptor binding assay (ERBA) and the E-SCREEN. Both assays clearly detected estrogenic activity in the influent, but the treatment train was very effective and no activity was detected in the final effluent: <0.75 ng/L in the ERBA (>98% decrease from raw sewage influent) and <0.03 ng/L in the E-SCREEN (>99% decrease). That same plant was investigated again in September 2010 using the Microtox and the E-SCREEN assays (Reungoat et al. 2012). Three grab samples were taken, and activity was detected in both bioassays: up to 0.94 mg/L TEQ in the Microtox (51-60% decrease from secondary treated effluent) and up to 0.07 ng/L EEQ in the E-SCREEN (94-96% decrease).
In a series of studies, Reungoat and co-workers (Macova et al. 2010b, Reungoat et al. 2011, Reungoat et al. 2010) investigated the **South Caboolture Water Reclamation Plant** (O_3/BAC/O_3) using a combination of chemical and *in vitro* bioassay analysis. Grab samples were collected over a four week period in July and August 2008, and the following bioassays were used: Microtox, E-SCREEN, AhR-CAFLUX, AChE inhibition, I-PAM and umuC. Treatment was very effective at removing the biological response, but the final effluent of the advanced water treatment plant had detectable activity in many of the assays: up to 0.72 mg/L TEQ in the Microtox (67-84% decrease from secondary treated effluent), < 0.06 ng/L EEQ in the E-SCREEN (>99% decrease), up to 0.36 ng/L TCDDEQ in the AhR-CAFLUX (46-69% decrease), up to 0.04 $GTU_{ECIR1.5}$ in the umuC -S9 (83->92% decrease), up to 1.2 µg/L PTEQ in the acetycholinesterase inhibition (57->90% decrease), and 0.05 µg/L DEQ in the I-PAM assay (50->91% decrease). The plant was sampled again in September 2010, and two bioassays were used to determine water quality: Microtox and the E-SCREEN (Reungoat et al. 2012).
Reungoat et al. (2012) tested grab samples collected over three days in September 2010 from **Gerringong Water Reclamation Plant** (O_3/BAC/MF/UV) using the Microtox and the E-SCREEN bioassays. Non specific toxicity was detected in the recycled water with up to 0.57 mg/L TEQ in the Microtox (69-75% decrease from secondary treated effluent) but there was no estrogenic activity in the recycled water, with all results <0.03 ng/L EEQ in the E-SCREEN (>98% decrease from the secondary treated effluent).
A large inter-laboratory study (Escher et al. 2014) screened a water sample from **an unidentified Australian ozone/BAC water reclamation plant** with a battery 103 different *in vitro* bioassays: 10 assays for cytoxicity (including Microtox and Caco2-NRU), 46 for specific toxicity (including the I-PAM and various CALUX and GeneBLAzer assays for endocrine activity), 12 for reactive toxicity (including Ames and umuC tests), 16 for adaptive stress response and 19 for xenobiotic metabolism (including

the AhR-CAFLUX). As previously stated, the objective of the study was to evaluate cell-based bioassays for their suitability to benchmark water quality and to assess efficacy of water treatment processes. The study found that the source water (treated sewage) produced a biological response in 60 out of 103 bioassays, but that O_3/BAC treatment reduced the biological response in all bioassays, although the product water still produced a small biological response in 13 bioassays: the Microtox and another bacteria cytotoxicity assay, two Ames tests, the ER-CALUX assay, two assays for oxidative stress (a type of adaptive stress response) and six assays that detect induction of xenobiotic metabolism (specifically the AhR, CAR and PXR pathways).

Conclusion on ozonation/BAC systems from Australian studies: Where ozonation and BAC were used, all of the tested final effluents produced only minimal biological response, if any, in the deployed *in vitro* bioassays. When biological activity was detected, it was always less than 10× above the assay quantification limit or activity in the ultrapure laboratory blank. This suggests that even in those cases where biological activity was detected in the final effluent, that activity is unlikely to be of significant health concern. Bioanalytical tools thus provide additional evidence that ozonation and BAC are effective technologies to produce high quality purified recyled water.

2.4.3.3 Ultrafiltration systems

When pathogen removal is the primary aim of treatment as for example in water reclamation for irrigation, less extensive treatment is sometimes used, such as ultrafiltration (UF) and UV.

A National Water Commission study (Leusch et al. 2014b, NWC 2011) investigated two **unidentified UF-based Water Reclamation Plants in different Australian states**. Grab samples were collected every second day for a week in April/May and July 2010, concentrated 1,000× by SPE and analysed in a battery of 13 *in vitro* bioassays: three assays for human cell cytotoxicity (neutral red uptake with Caco2 cells, WIL2NS TOX and HepaTOX), two reactive toxicity assays (Ames and WIL2NS FCMN), six assays for specific toxicity (ERα-CALUX, AR-CALUX, GR-CALUX, PR-CALUX, TRβ-CALUX and acethylcholinesterase inhibition assay), one adaptive stress response (CPA in THP1 human monocyte cells) and one xenobiotic metabolism assay (HepCYP1A2). Biological activity was detectable in 10 out of 13 assays in the secondary treated effluent, and UF/UV treatment had only minimal (if any) effect on the measured activity. The trends were comparable with results from chemical analysis, suggesting that ultrafiltration did not remove trace organic contaminants or their associated biological response (Leusch et al. 2014b, NWC 2011).

Watson et al. (2012) applied four *in vitro* bioassays to composite samples collected in 2011 at an **unidentified Qld Water Reclamation Plant** (MF/UF/UV) for non-potable reuse, specifically to investigate the effect of chlorination on effluent toxicity. The bioassays used were the ToxScreen3, AR-CALUX, ER-CALUX and umuC bioassays. The study showed that chlorination increased non-specific toxicity of the samples slightly, possibly due to the formation of disinfection by-products. The effluent of the water reclamation plant prior to chlorination had minimal biological activity, with 1.3 $TU_{REFIC50}$ in the ToxScreen3 assay, <3.5 ng/L DHTEQ in the AR-CALUX, <0.13 ng/L EEQ in the ER-CALUX, <0.01 $GTU_{REFIR1.5}$ in the umuC -S9 assay and 0.03 $GTU_{REFIR1.5}$ in the umuC +S9 assay.

Conclusion on ultrafiltration systems from Australian studies: Ultrafiltration is an effective technique to remove pathogens but is not effective at removing trace organic contaminants (Snyder et al. 2007) or their associated biological response. This was clearly shown in the NWC study (Leusch et al. 2014b, NWC 2011), with UF/UV treatment having negligible effect on biological response associated with trace organic contaminants.

2.4.4 Small (laboratory scale) experiments

Bioanalytical tools can be very useful in small-scale experiments to determine treatment efficacy, in particular because these bioassay tools provide a measure of the total biological response. This can provide a considerable improvement over the commonly accepted method of conducting these tests, which only include chemical analysis of a select number of compounds. This standard type of analysis can show the removal of a specific chemical structure, but does not indicate at all whether any (potentially more toxic) transformation products have been formed during treatment. Applying standard chemical analysis for targeted compounds in combination with *in vitro* bioassays can overcome this limitation and provide a more comprehensive assessment of treatment efficacy. This can provide a useful and comparatively cost-effective method to compare different treatment configuration, as was done with the Tampa Water Resource Recovery Project (see 2.3.1.5). A recent review of advanced oxidation processes in water and wastewater treatment strongly emphasized the need to combined chemical analysis with bioassay testing to detect toxic by-product formation from advanced oxidation processes (Rizzo 2011).

There is a large and growing body of scientific studies that have applied bioassays to small-scale experimental water reclamation treatment, and the following are provided as an illustration.

Petala et al. (2006a) conducted batch experiments using water from a WWTP in Thessaloniki (Greece), comparing the effect of iron and aluminium coagulation using the Microtox and Ames assays. The WWTP effluent was toxic to the Microtox bacteria and produced significant mutagenicity in the Ames test. Addition of ferric chloride and a flocculant resulted in a decrease in toxicity to bacteria but at the price of a significant increase in the mutagenicity of the effluent. Addition of aluminium sulfate and a flocculant also resulted in a slight decrease in toxicity to bacteria, but also decreased mutagenicity of the effluent. Finally, addition poly-aluminium chloride (PAC-18) and a flocculant greatly reduced the toxicity to bacteria but did not reduce (or increase) mutagenicity of the effluent. The same research group also tested the effect of ozonation on the same secondary effluents in the Microtox and a range of *in vivo* assays (Petala et al. 2006b). Ozonation was found to be effective to remove toxicity *in vivo*, but increased toxicity in the Microtox assay. This toxicity was likely due to the highly reactive environment after ozonation, and the toxicity completely disappeared after 48h of storage.

Kontana et al. (2008) tested a combination of various treatment options (chlorination, coagulation and GAC) to reclaim water from wastewater using several whole animal and *in vitro* bioassays: the Microtox, a cytokine production assay (CPA; for IL-1, IL-2, IL-10, IFNγ and TNFα) in mouse spleen cells and a thymidine uptake assay in mouse spleen cells. The results show that chlorination and coagulation have little effect on effluent toxicity, but that the inclusion of GAC greatly reduced effluent toxicity in all bioassays (although the effluent still caused statistically significant induction of some cytokines, in particular TNFα). A follow-up study evaluated the effect of ozonation (only or in combination), using the same location and bioassays (Kontana et al. 2009). The results show that ozonation alone or in combination with GAC greatly reduced the effluent toxicity in all bioassays, including the cytokine production assays.

Cao et al. (2009a) used *in vitro* bioassays to study the effect of chlorination and ozonation on genotoxicity of WWTP effluents in batch experiments using highly concentrated samples (10,000× by SPE). Using the umuC bioassay, the study showed that (not surprisingly) chlorination of WWTP effluents produced a variety of disinfection by-products and a concomitant increase in genotoxicity, up to 5-7 µg/L 4NQOEQ. Ozonation, however, reduced the genotoxicity of the chlorinated effluent, down to 4µg/L 4NQOEQ at a dose of 1 mg/L O_3 and as low as 1 µg/L 4NQOEQ at a dose of 10 mg/L O_3. This study illustrates the usefulness of *in vitro* assays in fine-tuning treatment variables.

In another study, Cao et al. (2009b) also conducted batch experiments with highly concentrated (10,000× by SPE) effluent from a WWTP in Beijing (China) to evaluate various water reclamation technologies (such as O_3, UF, RO, UV, chlorination and PAC/sand filtration) using two *in vitro* bioassays: the umuC for genotoxicity and a yeast reporter gene assay for retinoic acid receptor (RAR) activity. Ozonation (8.5 mg/L O_3) and RO were the most effective methods to reduce genotoxicity in the effluent, almost completely removing the activity. Chlorination caused an increase in genotoxicity, and the other treatments had no significant effect on this endpoint. Ozonation was also very effective at removing the weak RAR activity in secondary effluent. Chlorination was somewhat effective, but did not remove all activity, while the other treatments (including RO) had no significant effect on this endpoint.

Lundstrom et al. (2010) conducted batch experiments with different train configuration at a pilot plant next to WWTP in Stockholm (Sweden) using *in vitro* tests (Microtox, algal growth inhibition) in combination with short term *in vivo* tests and chemical analysis. Chemical analysis alone indicated that sand filtration was an effective method to polish the wastewater, but the toxicity tests showed that toxic compounds were present in sand filter effluent (even if the monitored compouds were not). The study highlights how conclusions from chemical analysis alone could incorrectly suggest inadequate treatment options.

Looking at the impact of soil-aquifer transfer (SAT), Zhang et al. (2011) conducted lab-scale experiments passing ozonated treated sewage through soil columns and using *in vitro* tests (umuC for genotoxicity and the YES assay for estrogenicity) as well as short term *in vivo* tests. Ozonation removed 56% of estrogenic activity, 70% anti-estrogenic activity and 99.8% genotoxicity, while 28d residence in the soil columns removed an additional 22% of estrogenic activity and 15% of anti-estrogenic activity.

2.5 Current limitations

There are of course limitations to bioanalytical tools. The limitations do not mean that bioanalytical methods cannot be used for human health risk assessment, but that care must be taken when relying on *in vitro* data for *in vivo* extrapolation.

2.5.1 Bioanalytical tools in risk assessment: measure of exposure *vs.* effect?

While future progress in this field may one day allow their use as "measures of effect", it is important to recognize that bioanalytical tools cannot at this moment accurately predict whole organism effects. One exception is the good correlation between cytotoxicity in the Caco2-NRU assay and rat *in vivo* acute toxicity (Konsoula and Barile 2005). So while *in vitro* assays can be used to a certain extent to predict the likelihood of whole organism effect, the correlation is generally poor. However, there is no doubt that they can already be used as "measures of exposure", *i.e.*, as an additional surrogate measure of chemical water quality that overcomes the limitations of chemical analysis, especially issues of non target chemicals and mixture toxicity.

2.5.2 Practical limitations

Limitations of sample preparation: *In vitro* bioassays are commonly conducted with concentrated water samples, which have been extracted either by liquid liquid extraction (LLE) or solid phase extraction (SPE). Extraction is carried out for two main reasons: 1) to concentrate the sample and thus more easily detect potential contaminants, and 2) to focus bioassay responses on the world of organic chemicals and not inorganic substances (which can be comprehensively analysed by chemical methods). It is important to ensure that a suitable extraction technique is used that retains as wide a spectrum of chemical compounds as possible (WateReuse Research Foundation 2014).
Limitation of using cancer cell lines: Note that most cell lines are cancerous cell line, which (as opposed to primary cells) easily proliferate under laboratory conditions. Cancer cells can however exhibit morphological and genetic changes compared to normal cells, and these need to be taken into account when analysing bioassay results.

2.5.3 Bioanalytical tools and regulations

There are currently no bioanalytical guidelines in drinking or recycled water regulation (although it should be noted that some dioxin guidelines are based on bioanalytical toxic equivalency, and that bioassays for dioxin-like activity, such as the DR-CALUX, have been used to provide a sum-measurement of all dioxin-like compounds in water). In the section on recycled water monitoring, the Australian Guidelines for Water Recycling: Phase 2 Augmentation of Drinking Water Supplies (NWQMS 2008) state that "biological tests can be used as a screening and prioritisation tool for subsequent chemical analysis", and that "due to ethical considerations and speed of completion, *in vitro* tests should take priority" [over *in vivo* toxicity testing methods].
There is currently significant scientific effort to develop bioassay-based "guidelines", termed "effects-based trigger values (EBT)" to clearly indicate that these are not meant to be enforceable standards but rather screening tools that would trigger further conventional chemical analysis to identify causative chemicals and, if deemed necessary, effective treatment options. Brand et al. (2013) proposed several EBT for endocrine activity, as measured by several CALUX assays. Tang et al. (2013) and Escher et al. (2013) proposed an approach to derive EBT for non-specific assays, such as the Microtox assay and the oxidative stress response. Other projects are currently underway, such as the DEMEAU project funded by the European Commission, that aim to provide guidance on EBT. All of these proposals are still very novel, and require some time to be fully evaluated and tested by regulators before they can be more widely used.

2.6 Conclusions and future directions

The studies presented in this review clearly show that bioanalytical tools have a valuable place in risk assessment of reclaimed water. This development is a consequence of the realisation that we cannot monitor every potential constituent in reclaimed water, and that a rational approach that takes into account the inherent limitations of different monitoring strategies is needed (Asano and Cotruvo 2004). A recent review by the National Research Council remarks that while *in vitro* bioassays should not be used in isolation for the determination of human health risks, a battery of *in vitro* bioassays can provide a powerful approach to screening water samples (NRC 2012), an suggestion echoed in the Australian Guidelines for Water Recycling (NWQMS 2008).
One issue that is limiting greater uptake of bioanalytical methods is the lack of bioassay-based guidelines to compare bioanalytical results to. While there have been several proposals in this area,

these still need to be evaluated by health regulators. However, it has long been recognized that at the very least bioanalytical tools can be used to compare alternate water supplies such as reclaimed water with current conventional drinking waters to give information on the relative toxicities of the two water supplies (NRC 1998).

It is important to keep in mind that adoption of bioanalytical tools for recycled water monitoring will most likely not lead to lower monitoring costs. The cost of testing samples in a thorough *in vitro* bioassay battery is equivalent to current chemical analysis costs. Bioanalytical tools do not replace chemical testing, but rather they present an important addition to our current monitoring strategies by providing a means to detect non-target chemicals and unexpected transformation products, and provide a sum measure of toxic chemicals acting via the same mode of action. However, recent developments in high-throughput testing are likely to lead to a reduction in the per sample cost of *in vitro* testing, and application of intelligent testing strategies combining tier 1 screening with bioanalytical tools and suitable surrogate and indicator chemicals would most likely to lead to a reduction of total analytical costs.

Based on the information currently available, the following endpoints appear particularly well-suited for recycled water quality assessment (Escher et al. 2014):

- From a health endpoint, assays for endocrine activity, in particular **estrogenic** and **glucocorticoid activity**. Reporter gene assays such as the CALUX and GeneBLAzer batteries are exquisitely sensitive to hormonally active compounds, and provide a sensitive measure of potential endocrine disruption, which is of high public concern.
- While obviously not an issue specific to reclaimed water, it is important to continue to monitor disinfected water with assays for reactive toxicity such as **mutagenicity** and **genotoxicity**. Although the results from these assays have been and will continue to be difficult to fully comprehend without clearly identified causative chemicals, comparison with other water sources and drinking water provide an important context for the activity in reclaimed water. It is also important to understand the limitation of the current (mostly bacteria-based) assays for reactive toxicity in a human health perspective, and development of novel assays better able to detect human carcinogens should be encouraged.
- More difficult to connect to a health outcome at the moment (although future developments in molecular toxicology may fill in the gaps), **adaptive response** assays (particularly oxidate stress) and **xenobiotic metabolism** assays (particularly AhR and PXR pathways) appear highly sensitive to compound in both source and reclaimed waters. It is particularly important with these assays to compare the results with currently accepted water sources, as even highly treated water is likely to produce a biological response in those assays, which can respond to compounds that may not be toxic to whole organisms due to downstream defence and repair mechanisms.
- Finally, **bacterial toxicity** assays are more sensitive than cytotoxicity assays with human cells, although of course less relevant to human health assessment. Nevertheless, their sensitivity to a wide range of compounds (Tang et al. 2013) may make them ideally suited as performance indicator bioassays, especially when applied online.

It should be noted that this list should not be seen as a comprehensive and final list, and future research may well identify other mode of toxic action that are relevant to drinking water.

The next stage of evolution of *in vitro* bioassays will be application to online monitoring. Some bacterial and algal assays have already been adapted to online format (reviewed in Storey et al. 2011), although issues of sensitivity in particular remain to be overcome (Woutersen et al. 2011). Once validated, these techniques would provide a real-time and sensitive tool to perform screening-level toxicity testing for routine monitoring.

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Chapter 3. Translating and communicating the Science

by Heather Chapman

3.1 Communication relating to the use of bioanalytical tools in water quality assessment

3.1.1 Introduction

From 2008 the research focus discussed in Chapter 1 of this report began to include broader considerations than the technical issues associated with the measurement of individual chemicals in recycled water. Questions were being raised about what was being done about chemicals that no one thought to look for (and therefore would not find), what happened in a mixture of chemicals and what was in wastewater from hospitals for example. These were questions that few organisations (industry and government) were equipped to respond to, and that was a significant driver in the development of the bioanalytical methods through research projects in an effort to address or explain these concerns. As discussed previously, bioanalytical methods address the very issues of mixture toxicity and the so called 'unknown unknowns', by assessing biological activity of a water sample integrating all of the chemicals present in addition to chemical analyses itself, because by chemical analysis you only find what you look for. This, along with the acknowledgement that the process of risk assessment, risk perception and risk communication was as important, if not more important, than management of the technology and regulation of some (but not all) individual chemicals. It was also apparent that investment in understanding the way we communicate risk was also required. How recycled water for drinking is viewed across Australia depends on several factors including the management of health risks, cost effectiveness and public perceptions and that of these factors will be unique to particular regions and communities.

3.1.2 Science in policy making and regulation

A number of studies involving the use of science in policy making have identified some key elements that need to be considered when developing effective communication. In a report from Green et al (2010) the authors discuss key elements that need to be considered when developing effective communication. These include those relating to communication, audience, framing, messages, messengers, channels and effect. While it is not intended to go into the communication theory here there are some important considerations. While many professionals acknowledge that communication of science is important there is also a perception that scientists while they may be skilled in science are not skilled in communication. And there is also a perception that communicators, such as the media are not skilled at science. Holmes and Clark (2008) discuss the role of interpreters. The need to enhance the quality of interaction between researchers and policy makers was a point they identified. Differences between policy makers and researcher's culture, time frames, reward structures and motivations were identified as obstacles to good communication. Similar obstacles have also been identified by research in Australia as described in the case studies below. For a more comprehensive discussion on risk communication from science to policy and regulation refer to Ross et al (2013). For a review of the literature on communicating scientific and technical information about alternative water supply options refer to Green et al (2010). Terminology and language is a theme that has been identified in several fora (e.g. see Simpson and Stratton 2011). Participants in a study by Black and Veatch (2010) concluded that in order to gain the trust and respect of the public, the politicians, the regulators and members of the water industry need to work together to build a bank of credible, robust data that they can use to demonstrate the recycled water is safe and acceptable for public use. It is believed that clear and consistent message to the public is important in generating trust in the water provider that is underpinned by good policy and good science.

It has been recognised that in the uptake and use of new knowledge is an active process and an essential outcome of water quality research. The target audience in each of these case studies was

the educated professional (project participants and other invited professionals). There was no attempt in these projects to address communication to the public as it was not within the scope of this work, however this could be used if the message and messengers were appropriate to various audiences. Three case studies are presented below each of which aimed to understand and improve communication aspects of the use of bioanalytical tools in water quality assessment. These projects are similar in that they were all conducted along-side the technical development of bioanalytical tools with the additional aim of informing, educating and facilitating the uptake and dissemination of new knowledge and technologies for water quality monitoring. The goal of each of the projects developed over time, all based on prior learnings as follows.

Case study 1 Risk communication from science to policy and regulation and implementation of recycled water in Australia" Ross et al (2011)

- This component of the parent project was based on technology transfer and uptake being a desired outcome of applied research. It was also recognised that some of science was seen as poorly understood and that for water reform (as identified by the NWC) new methods of assuring water safety was required. In order to progress this, it is important for proper communication channels to exist between researchers, policy and regulation staff and water industry itself.
- The primary aim of this project was therefore to understand barriers to communication between scientists, policy makers and regulator and managers of recycled water.

Case study 2 "Evaluating a science communication workshop as an educational tool" (Ross and Chapman, 2012)

- Communication is characterised by the message, the messenger, framing, the audience, channels and effect (Holmes and Clark, 2008). In this research the goal was to conduct a workshop to inform and educate policy makers and regulator and managers of recycled water about bioanalytical tools. The messengers were researchers on the project and audience was the regulators, policy makers and the water managers.
- The primary aim of this project was to evaluate the transfer of knowledge on the application and interpretation of bioanalytical tools for industry, policy and regulation in recycled water quality monitoring programs through a workshop using semi-structured interviews.

Case study 3 "Evaluation of an online survey to assess the effectiveness of technology transfer workshops on acceptance of bioanalytical methods" (Chapman et al, 2012)

- A depth of information is obtained using direct survey methods as reported in Case study 2. Another method of measuring the effect of technology workshop messages is through the use of online survey techniques. This is a quicker and less expensive method but is considered to provide more superficial information. There was an opportunity during the delivery of another project on bioanalytical methods development using workshops to compare the two approaches.
- The primary aim of this project was to use an online survey to assess the effectiveness of technology transfer workshops on acceptance of bioanalytical methods.

3.2 Case study 1 "Enhancing risk communication from science to policy and regulation and implementation of recycled water in Australia" (Ross et al, 2011)

3.2.1 Background

In 2008 the National Water Commission (NWC) under the Raising National Standards (RNS) program co-funded a research project with Water Quality Research Australia to include risk assessment and risk communication in addition to the technical development of bioanalytical (Chapman et al, 2011). There was acknowledgement by the NWC that research on new and emerging methods of including *in vitro* bioassays into water quality assessment was critically important, but so was the use of the new knowledge in risk assessment, policy development and management of water. This project was undertaken subsequent to those conducted previously under Water Quality Research Australia's

(WQRA) predecessor the Cooperative Research Centre for Water Quality and Treatment (CRCWQT), Global Water Research Coalition (GWRC 2008) and Uniquest (EPHC/NRW/NWC 2008) and in parallel with a partner project conducted in Western Australia (Reitsema et al. 2010). This project extends the previous work by including additional endpoints, primarily of human health relevance.
Chapter 1 of this report introduces the challenge (and proposes some possible solutions) to the presence of biologically active chemicals in recycled water and risk perception. Even in the absence of clear evidence of human health impacts, concerns remain and it became necessary to have tools to have risk assessment and risk management tools to manage the manage perception as well as science.
Chapter 2 reviewed the risk assessment and regulation of chemicals in Australia to provide us with a starting point for consideration of how they may be regulated in the future. At the time of the review (2010) federal government regulatory agencies were responsible for the risk assessment of chemicals introduced into Australia, but only for some agricultural chemicals are exposure guidelines set (i.e. acceptable daily intakes (ADI)). There was no environmental assessment of pharmaceuticals or good additives and no assumption of water reuse in the environmental assessment of industrial chemicals. The move towards water reuse therefore poses some unique challenges in the risk assessment of new and existing chemicals in Australia, which continue to be regulated on a one by one basis.
Chapters 3-5 provided an overview of health outcomes that need to be considered, validation of extraction and chemical analysis and the bioanalytical methods for the selected chemicals and for samples from a range of treatment technologies and locations around Australia.
Chapter 6 Enhancing risk communication from science to policy and regulation and implementation of recycled water in Australia (Ross et al, 2011)
This work examined issues regarding communication between research scientists, policy officers in government, regulators and the water industry. As public perceptions of risk have been shown to be influenced by perceptions of the credibility of the responsible agency (policy maker/regulator or water manager) it is vital that decisions made on water supply are based on the best-available science and communicated effectively. The research comprised semi-structured interviews with stakeholders and the data was analysed using qualitative methods. A summary of the research follows.

3.2.2 Study aims and methods

This study aimed to understand barriers to communication between scientists, policy makers and regulator and managers of recycled water. Based on a literature review and the identified need to establish stronger links and better communication between scientists, policy makers and managers of recycled water in Australia, the following research questions were formulated:

- What are the perceived obstacles to improving links between science, policy and regulation, and practice with regard to water recycling in Australia?
- What are the best sources of technical information to guide decision making about recycled water and how are these accessed?
- Are difficulties experienced in accessing and interpreting scientific information to inform policy, regulation and practice?
- Are the science, policy and implementation processes consistent across Australian states and territories?

Semi-structured, individual, face-to-face interviews were conducted with water industry representatives from Queensland, New South Wales, Australian Capital Territory, Victoria, South Australia and Western Australia. Potential interviewees were recommended initially by senior water industry representatives as having significant knowledge and experience in the industry. Interviewees were purposefully selected from this group by the researchers to provide a broad range of perspectives on science, policy and practice in water in Australia. The semi-structured questionnaire was designed to gain perspectives on the way water science is transferred through to policy and regulation, and then to the implementation of the policy in Australia. It also aimed to identify any key issues and investigate whether issues experienced overseas (e.g. difficulties accessing and keeping up with scientific information, communication issues) were also a concern in Australia. The semi-structured format was chosen because it provided enough consistency across interviews for points of comparison, while still being open ended to elicit in-depth responses and flexible enough to be tailored to different perspectives of the participants.

3.2.3 Summary of results

A range of issues were identified by the research. Although these issues are broad in nature and relate to a range of considerations it was apparent that in order to gain public acceptance of water supply solutions, there needs to be clear regulatory guidance and a clear process for communication to the public. Policy and regulatory need to be (and be seen to be) based on the best available science. While Chapter 6 summarised a number of issues regarding communication between and within different stakeholder groups, it did not propose solutions. In order for better communication and acceptance of the science and the issues by the public it is considered that the water industry itself needs access to reliable science and information in which to base decision making and to be seen as a credible source of information.

Among interviewees in this study it was generally thought that confusing and inconsistent use of language was a barrier to communicating about recycled water; both within the industry and when communicating with the public. A number of interviewees mentioned that there was confusion about terminology within the water industry and that there was a need for a consistent and common language. This was felt to be particularly important when industry professionals are working together to discuss policy and regulation. As one participant said "*I think this is a real problem because people use the same terms and mean different things. It can be quite difficult, I think, to get conformity across the industry. So you think you're talking about the same thing, when in actual fact you're not. So I think there's a lot of difficulty in getting a coherent policy platform out there*".

The WateReuse Research Foundation undertook research in 2009 into how water terminology affects the community's acceptance of reclaimed water especially for drinking (WRF 07–03) and explored the relationship between the community's interest in and knowledge of what we put into water and how we take it out again, and their attitude to potable recycling. The research showed that knowledge of water science in the community is not robust and that the provision of information improved the acceptance of water recycling.

The National Water Commission provided funding for similar research to be conducted in south-east Queensland. The research investigates how much knowledge the community has of water science and the impact that words, images and concepts have on their attitude to alternative water management proposals. It also includes an appraisal of the effect that knowledge and understanding of water and wastewater quality have on the acceptance of reclaimed water (Simpson and Stratton, 2011).

3.3 Case study 2 "Evaluating a science communication workshop as an educational tool" (Ross and Chapman 2012)

3.3.1 Background

This social science project was a component of the UWSRA project 'Bioassays and risk communication' and aimed to evaluate the transfer of knowledge on the application and interpretation of bioanalytical tools for industry, policy and regulation in recycled water quality monitoring programs. Part 1 of the project focused on further technical development of bioanalytical tools. The research conducted was based around a science communication workshop presented as an educational tool to invitees from government, academia and industry. Industry professionals representing science, policy, regulation and industry. Persons were interviewed before and after the workshop, and 12 month later to test their understanding of the science before and after the workshop and retention of that knowledge over a 12 month period.

3.3.2 Study Aims and Methods

Invitations to attend the bioassays workshop were emailed to relevant State and National representatives from water research, policy and regulation, and industry. The goal of the workshop was to facilitate communication about the application and interpretation of bioassays in water quality assessment, and was designed to provide practical information for water providers, regulators and researchers. Topics covered over the two hour presentation were: the risk assessment framework and water recycling guidelines; chemical analysis and direct toxicity testing; dose response and TEQ concept; mixtures, practical application, including both the endpoint and mode of action approach; strengths and weaknesses; and putting knowledge into practice.

Of the workshop invitees, 23 local representatives from research, policy/regulation, and industry were also invited to participate in the workshop evaluation research. Information regarding the purpose and

scope of the research, including assurance of confidentiality and anonymity, were provided to participants in advance. A total of 11 respondents were both willing and available to participate in all three of the interviews comprising the research, a 48 per cent response rate. The sample consisted of three females and eight males with experience in the water industry ranging from two and a half years to 19 years. The average water industry experience across the sample was just under ten years. Four participants worked in policy roles, five in regulation, and one each from research and industry respectively. The research participants were interviewed during the two months before the workshop and then again during two months following the workshop. A third set of interviews was conducted with the same 11 participants approximately 12 months after the workshop. All interviews were semi-structured, individual, and face-to-face.

The interviews conducted prior to the bioassays workshop aimed to gain an understanding of participants' current knowledge of and views about the applicability and limitations of bioassays. An additional aim of the pre-workshop interviews was to also identify communication barriers between scientists, regulators and policy makers, and industry representatives.

The second set of interviews was conducted with the same participants after the bioassays workshop to provide qualitative feedback and thus assess its effectiveness. Using evaluation criteria, the workshop was assessed based on participants' responses across a number of key points, which included:

- communication of key messages;
- if and how the workshop had improved knowledge levels about the use of bioassays;
- whether participants felt better equipped to locate scientific information and bioassay experts; and
- whether their views had altered regarding the applicability and limitations of bioassays.

3.3.3 Summary of results

This section is a summary of the main findings from the interviews. Further details and the full report is available online (http://www.urbanwateralliance.org.au/publications/UWSRA-tr72.pdf).

3.3.3.1 Pre-workshop interviews

The following is a brief summary of the topics raised by the interviewer (numbered headings) and the main points (dot points) from the respondents who were interviewed prior to attending the workshop.

1) Current level of knowledge of bioassays
   - Self-reported knowledge among the group was evenly spread with 4/11 reporting a good knowledge, 5/11 moderate levels and 2/11 no knowledge.
2) Workshop expectations
   - Participants generally expressed a desire to know more about bioassays and how they could be applied. Questions were raised regarding whether the use of bioassays could be used to provide an extra level of assurance to the public and therefore aid in acceptance of recycling schemes.
3) Perceived communication barriers
   - Terminology was the most frequently cited as a barrier to effective communication. Respondents reported that terminology differed between guidelines, legislation and community education programs leading to mixed messages that cause confusion and nervousness. A trend towards 'content free management' was identified as a barrier between regulators and policy writers. The issue of commercially confidential data was also cited as a barrier to communication on this topic.
4) Views on bioassays prior to the workshop
   - Several participants felt that the link between bioassays and human health was tenuous, particularly from cell based assays. The view was also that the tools were seen to be most useful in screening programs and not as a replacement to traditional chemical by chemical approach to management of water quality.
5) Other issues

- Concerns were raised regarding decision making not always being based on the best available science. It was generally considered that effective risk communication about water treatment and safety was a vital element in securing public support.

3.3.3.2 Post workshop interviews

Subsequent to the workshop, the same participants were again interviewed. A brief summary of the main topics and the responses include

1) General comments
   - All participants reported that attending the workshop was beneficial to them in terms of improving their knowledge of bioanalytical tools. The workshop presenters were described as knowledgeable and that they built a good rapport and interaction with the audience. They reported that was useful to gain a broader knowledge of bioanalytical tools and their application. Several respondents felt that bioanalytical tools could be useful in promoting public confidence in water recycling and that cost saving could be made by using them for broad screening of water quality.
2) Did views on bioassays change after the workshop?
   - Five of the eleven participants reported that since attending the workshop their views had altered more favourably towards the bioanalytical tools. Others reported already being supportive prior to the workshop but at least one felt there was still uncertainty in their application.
3) Risk perceptions
   - When asked if their views about the health risks associated with recycled water had altered since attending the workshop, almost all participants stated that their views had not changed because they were already confident with the treatment processes and safety of recycled water. However, one respondent said he found some of the information confusing and that he now perceived the health risks to be higher than he had thought prior to the workshop. Others felt that the workshop had showed that bioassays could be used to effectively demonstrate and communicate that the health risks in relation to recycled water.
4) Constructive comments
   - The most significant feedback from workshop participants (on the workshop itself) was that the component showing comparisons of different water treatment technologies and water quality needed to be explained in context (regarding the different water sources). Several people described the workshop handouts as a useful tool to take away, particularly in terms of being able to show the information to colleagues. However, some were disappointed that some of the detailed information from the power point presentation had been left out and would like to see this included. Others were keen to know more about how these results could be effectively communicated to the public to provide a more objective picture on the risks. It was noted that more work needs to be done in terms of providing advice for people in the profession on how to communicate risk to the general public. Most of the participants felt that it would be useful to further develop the workshop for specific audiences to make the information more accessible to a wider variety of people. Several interviewees mentioned that it would have been beneficial to have had more industry representatives in the audience.
5) Key messages from the workshop

During the post workshop interviews, participants were asked if they could describe what they perceived as the key messages that they took home from the workshop. These are summarised below.

- *Bioassays are an important tool to expand our understanding of water quality risks. They can be used to do things that traditional analyses have problems doing, for example, the cocktail effect, unknown unknowns and the mode of toxicity.*
- *Bioassays complement the other methodologies and can help to reinforce the results that are obtained through chemical analysis and biological analysis etc.*
- *Applying bioassays will give us a more comprehensive way of targeting our sampling and a more comprehensive view of what might be there rather than just looking for a single compound.*
- *Bioassays have other applications than just recycled water. They have strengths and weaknesses against traditional techniques.*
- *Bioassays are an under-utilised and unexplored resource for determining risk, toxicology or the effects and harm of hazards in the environment.*
- *There are a wide variety of bioanalytical tests available and they cover a range of parameters that can be estimated at different levels. The results are repeatable and reliable.*
- *Bioassays may have a substantial contribution to make, there is a lot of work already happening in the area and still a lot more work to be done.*
- *Bioassays are not the silver bullet that will guarantee public acceptance, but they are powerful tools that could be developed to enhance public confidence.*

3.3.3.3 Long term interviews

One year after the workshop was held, study participants were again interviewed to understand how information was retained over time as reported below.

- Key information recalled
  - During the follow-up interviews almost one year after the bioassays workshop, all participants were able to recall specific aspects of the workshop that were particularly useful or of interest to them, and were able to identify at least some of what they felt were key messages. These comments clustered under themes that directly echoed responses during the first post-workshop interviews. Several participants recalled that the discussion on the benefits and weaknesses of the different assessment techniques was also very useful to their understanding of water quality assessment.
- Viability of bioanalytical tools
  - Possibly the most noteworthy feedback from this phase of the research was reports from several participants that, since the presentation of the workshop, some Queensland water regulators were currently investigating options for how they could apply bioanalytical tools in the regulatory area. It was suggested that, at this stage, bioassays would be likely to be used as an additional “screening tool” rather than as a regulatory tool. A number of respondents described bioassays as being valuable as a screening tool and in providing additional assurance of the safety of the water.
- Perceived barriers to uptake of tools
  - Despite the positive feedback on the applicability of bioassays, when prompted, participants also described some significant barriers to their adoption by regulators and industry. One health regulator cited the time restrains imposed on government as an issue for regulators adopting new techniques. It was acknowledged that the use of bioanalytical tools was a complex area and that it was important that results are not misinterpreted. It was also noted that some regulators may not have the expertise to correctly interpret the results and/or to share this information with non-technical policy professionals.
- Thoughts on science communication
  - As with all of the earlier interviews, participants felt strongly about the importance of science communication. It was reported that there were not enough opportunities for people to learn about current research, and to generally interact and network with other industry professionals. A number of respondents stressed that there is a great need for more water related science

communication workshops/forums. The importance of educating industry and regulators about the use of bioanalytical tools was noted by several respondents. As with the earlier interviews, all interviewees stated that they found attending the bioassays workshop to be beneficial, and all participants said they would be interested in attending more similar workshops. Also consistent with the earlier interviews, a number of participants felt that it would be useful to further develop the workshop for specific audiences to make the information more accessible to a wider variety of people.

3.4 Case study 3 "Evaluation of an online survey to assess the effectiveness of technology transfer workshops on acceptance of bioanalytical methods" Chapman et al (2012)

3.4.1 Background

In 2011 at the conclusion of the research project "*A National Approach to Risk Assessment, Risk communication and Management of Chemical Hazards from Recycled Water*"(Chapman et al, 2011) a series of was developed and presented by Water Quality Research Australia (WQRA) in partnership with National Water Commission (NWC) throughout Australia.
The purpose of the workshops, titled *"Health Risk Assessment of Recycled Water Using Bioanalytical Techniques – The Science and its Application"* was

1. To inform and educate members of the water industry and government on the science underpinning the use of the emerging method of biological monitoring that specifically targets bioactive organic chemicals.
2. To present the research outcomes of a recently completed project called *'A National Approach to Risk Assessment, Risk communication and Management of Chemical Hazards from Recycled Water'* jointly funded by the NWC Raising National Standards program, WQRA and industry partners.
3. To present the information within the context of regulatory risk assessment and monitoring as briefly discussed in the *Australian Water Recycling Guidelines, Phase 2 Augmentation of drinking water supplies (2008)* and to explore opportunity for incorporation of the techniques into monitoring programs.

Presenters at these workshops were lead investigators in the research that was presented. Chris Davis a then commissioner with the NWC also attended the workshops and provided a NWC perspective. Five workshops were conducted in Sydney, Melbourne, Adelaide, Perth and City of Gold Coast, Queensland. The format of the workshops was to present the technical aspects of the subject in a morning session and to present the research findings in the afternoon. In parallel a research project was conducted to evaluate the use of an online survey to assess the effectiveness of technical transfer workshops on acceptance of new analytical methods.
The workshops were designed to be generally educational about bioanalytical methods, but also presented the results of a recently completed project conducted by Water Quality Research Australia (WQRA) and the National Water Commission (NWC) (Chapman, 2011). The survey itself sought to canvass perspectives on the understanding and acceptance of bioanalytical methods for water quality and assessment and monitoring. It was also designed to provide valuable information back to WQRA on how to communicate research outcomes.
In addition to the workshops held in 5 states/territories, a hands-on demonstration of the bioanalytical laboratory techniques was held in Brisbane. In other cities a power-point presentation of the laboratory methods was given. In addition two videos were produced for those participants who could not attend the workshops and/or the laboratory demonstration.
A number of methods are available to obtain feedback including feed-back forms distributed on the day of the workshop, online surveys and direct interviews before and after the workshops. Feedback and online survey methods are more rapid than in-depth, one-on-one interview methods (refer to chapter 6 of Chapman et al. (2011) for more detail) Quite often, however, it is not practical to conduct the direct interviews, particularly with a representative number of participants. The dissemination and management of knowledge is widely acknowledged as a necessary component of research activity by

a number of agencies within Australia and overseas. The following have been identified by the Raising National Standard (RNS) program of the NWC as essential activities to encourage adoption of the knowledge and uptake of tools developed under the program.

- Industry education and training – towards best practice water management
- Interpretation of the bioanalytical data and its use in risk assessment
- Risk communication and consistency in the use of language
- Knowledge adoption and communities of practice
- Evidence based policy decision making and regulatory reform where required

3.4.2 Study aims and methods

The primary aim of this project was to examine the feedback that could be obtained via an online survey instrument about the value of technical transfer workshops. This was conducted to contribute to knowledge about how best to deliver research outcomes and to facilitate uptake. The survey was conducted with participants who attended workshops on the application of bio-analytical techniques to water quality monitoring that delivered the research outcomes of a WQRA/NWC project conducted from 2008-2010 (Chapman et al, 2011).

3.4.2.1 Survey background

The survey was conducted with participants of a series of workshops during October and November 2011. This was achieved by using web based (online) survey methods both prior to and after attendance at the workshops.

3.4.2.2 Survey design

The survey was designed based on the nature of information being sought. The evaluation criteria that were considered in designing the survey were:

- whether the workshop was tailored to the training needs of the participants
  - breadth of information presented
  - depth and degree of technical detail
  - relevance to day to day business of the participants;
- suitability of the training material for the target audience; and
- suitability of the training venues.

A series of questions asked individuals to rate their own level of knowledge about workshop topics as well as their perceptions of various issues, before and after the workshop. These were designed to assess whether there was a “shift” in these ratings as a result of being exposed to the training.

3.4.2.3 Survey administration

The on-line version of the survey was produced and administered by Clearwater Software Australia, a professional software development provider based in North Queensland. Clearwater set up the survey online and participants were invited to connect via a link in the email invitation. Clearwater also monitored the participation and completion rates of the survey and collated the information which was then returned to the research team. The responses were anonymous to the project team.

3.4.3 Summary of results

There were 29 respondents to the online survey, 38% females and 62% males. Most (79%) of respondents had a scientific background with tertiary qualifications. Job roles included managers/directors, consultants, researchers/scientists, water analysts and policy advisors. Shifts in perceptions were elucidated clearly through the use of Likert Scale responses and statistical analysis using SPSS (IBM, 2010). There was a statistically significant shift towards an increase in knowledge and perceived value of the methods after the workshop, compared to before.

The first set of questions related to knowledge and learning regarding water quality monitoring, *in vivo* toxicity testing, *in vitro* toxicity testing methods and the value of using *in vitro* methods for water quality monitoring and assessment. Looking at matched responses everyone felt that their level of knowledge about water quality monitoring, *in vivo* and *in vitro* toxicity testing increased, or remained the same as a result of attending the workshop. No-one felt they had a low level of knowledge compared to percentages of ~20% prior to attending. With regard the value of *in vitro* methods in predicting impacts to human health there was a significant shift in the median perceived value from medium to medium high and a decrease from 10% responding that their perception of the value of the

assays before the workshop to 3% after the workshop. Similarly for the perceived value of in vitro methods for water quality monitoring there was a significant shift in perception of value from medium and medium high, to medium high to high.
Prior to the workshop, views about the cost-effectiveness of using *in vitro* methods to identify hazardous chemicals in water were fairly evenly spread, with equal numbers of people rating that to be low, medium and unsure. Just over one-half considered that the cost-effectiveness would be low to medium. After participating in the workshop, a majority (58.6%) felt that their cost-effectiveness would be medium-high or high. Specifically, three participants lowering their rating; 11 increased it, while 15 did not change their initial perception.
Some specific comments included

- *"Difficult to quantify in an accurate way at this stage until commercially available" and*
- *"This is still in the earlier stages so a realistic "commercial" valuation is not yet available. This will come when there is greater clarity about the use of this technique, the applications (what is being assessed and why) and the mode of operation."*

A high proportion (>75%) of the respondents considered that the information presented at the workshops to be relevant or highly relevant to their current water related work. Additionally 48% felt that there was a likelihood of incorporating bioassays into their work in the future. Seventy nine percent either agreed or strongly agreed that they could see how *in vitro* methods could be incorporated into water quality assessment programs. The remainder were either unsure or disagreed. Nineteen of the 29 respondents expressed interest in attending a laboratory demonstration of bioanalytical methods.
Although there was a significant improvement in knowledge, perceived value and utility of *in vitro* methods being incorporated into water quality monitoring program, the participants reported perceived barriers to acceptance of the *in vitro* methods by regulators (83%) and industry (76%). There were a number of regulators present at the workshops, so this appears to be at odds to the opinion overall on the acceptance of the methods. However the numbers of each group were small so this may not be significant. It is unclear if the regulators themselves see barriers or there is just a perception of others that they would be. Numbers of water provider participants at the workshops was low so it is unclear if this is a reliable result for this group.

3.4.4 Key conclusions from this study

Key conclusions based on feedback from the workshop include

- The bioassay workshops appear to be an effective method of increasing knowledge and acceptance of new and emerging technologies for the audience surveyed
- The workshops served to increase the perceived value and acceptance of bioanalytical methods.
- There are unknowns regarding the cost effectiveness of incorporating the methods into routine water quality monitoring that need further investigation.
- There continues to be perceived (or actual) barriers to the uptake of the new methods by industry and regulators.
- A lack of laboratories with appropriate experience in procedures and uncertainty with data interpretation with respect to human health are seen as barriers to uptake.
- Practical demonstrations are seen as a useful activity
- Supporting material (copies and power points, fact sheets etc.) need to be clear and appropriate to the audience.

3.5 Summary

The studies described above have identified some of the key barriers to communication about water recycling (Case study 1) and more specifically barriers to uptake of bioanalytical tools in water quality assessment (Case studies 2&3). Ross et al (2013) examined some social science theories regarding communication (e.g. cultural cognitive theory of risk). This theory recognizes that there is an influence of social and cultural factors on risk perception and refers to the tendency of persons to form perceptions of risk and related facts that cohere with their self-defining values (Kahan et al, 2011). Cultural cognition theory proposes that psychological theory proposes that psychological mechanisms

predispose individuals to credit or dismiss evidence of risk in patterns that fit values they share with others (Kahan and Braman, 2006).

Some of the key issues identified in case study 1 are summarized in Table 3-1 and the key messages from the direct interview and online survey methods that relate to those issues are also shown in the table. Other issues that had previously been identified including political nervousness, language/terminology, consistency of guidelines and cost effectiveness were not included in the surveys but these are all important factors to be considered to enhance the use of science in environmental policy making and regulation.

In Case study 2 where there was direct interviews conducted there were several responses regarding communication to the public. Several respondents felt that bioanalytical tools could be useful in promoting public confidence in water recycling and that cost saving could be made by using them for broad screening of water quality. Others were keen to know more about how these results could be effectively communicated to the public to provide a more objective picture on the risks. It was noted that more work needs to be done in terms of providing advice for people in the profession on how to communicate risk to the general public. Other comments include "*Bioassays are not the silver bullet that will guarantee public acceptance, but they are powerful tools that could be developed to enhance public confidence."*

As reported by Ross (2011) one of the major challenges to the implementation of recycled water projects in Australia has been a lack of both public acceptance ad consistent polices and regulation for the management of the treated water. Perceptions of risk regarding recycled water is influenced by the credibility of the responsible authority (Baggett et al, 2008) and it is therefore important to have a good communication process so that policy decisions can be based on good science.

Table 3-1. Barriers to risk communication in water recycled (Column A) and key messages from survey projects (Columns 2 & 3)

A. Enhancing risk communication from science to policy and regulation and implementation of recycled water in Australia	B. Evaluating a science communication workshop as an educational tool	C. Evaluation of an online survey to assess the effectiveness of technology transfer workshops on acceptance of bioanalytical methods
Aim: To identify barriers to risk communication - water recycling	Aim: To identify changes in attitude regarding bioassays pre and post technology work-shops using face to face interviews	Aim: To identify changes in attitude regarding bioassays pre and post technology work-shops using an online survey
Recycled water research	Bioassays may have a substantial contribution to make, there is a lot of work already happening in the area and still a lot more work to be done.	A lack of laboratories with appropriate experience in procedures and uncertainty with data interpretation with respect to human health are seen as barriers to uptake.
Science to policy translation	Applying bioassays will give us a more comprehensive way of targeting our sampling and a more comprehensive view of what might be there rather than just looking for a single compound.	The bioassay workshops appear to be an effective method of increasing knowledge and acceptance of new and emerging technologies for the audience surveyed
Implementation	Bioassays complement the other methodologies and can help to reinforce the results that are obtained through chemical analysis and biological analysis etc.	Supporting material (copies and power points, fact sheets etc.) need to be clear and appropriate to the audience.
	Bioassays are an under-utilised and unexplored resource for determining risk, toxicology or the effects and harm of hazards in the environment.	Practical demonstrations are seen as a useful activity
	There are a wide variety of bioanalytical tests available and they cover a range of parameters that can be estimated at different levels. The results are repeatable and reliable.	
	Bioassays are an under-utilised and unexplored resource for determining risk, toxicology or the effects and harm of hazards in the environment.	
	Bioassays have other applications than just recycled water. They have strengths and weaknesses against traditional techniques.	
Risk perceptions	Bioassays are an important tool to expand our understanding of water quality risks. They can be used to do things that traditional analyses have problems doing, for example, the cocktail effect, unknown unknowns and the mode of toxicity.	The workshops served to increase the perceived value and acceptance of bioanalytical methods.
	Bioassays are not the silver bullet that will guarantee public acceptance, but they are powerful tools that could be developed to enhance public confidence.	There continues to be perceived (or actual) barriers to the uptake of the new methods by industry and regulators.
Cost effectiveness		There are unknowns regarding the cost effectiveness of incorporating the methods into routine water quality monitoring that need further investigation.

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Chapter 4. Lessons learned and frequently asked questions (FAQ)

by Heather Chapman and Frederic Leusch

4.1 Lessons learned

When recycled water for indirect potable reuse was first proposed for the Sunshine Coast in Queensland in the 1990s there were concerns raised in the local community about the safety of the water, primary regarding endocrine disruptors. At that time evidence was emerging that some animal groups were being impacted by the presence of chemicals in water that had estrogenic properties. Despite the lack of an indication for impacts to humans from the concentrations found in water, there remained concerns that if it could happen to fish it could happen to humans. The worsening drought in Australia led to a number of policy changes and support from governments for the implementation of water recycling schemes for indirect potable reuse. Coincidental to this there was a surge in research activity globally on the topic of endocrine disruption and in Australia water recycling focussed on potential impacts on human health as a driver for research. The focus was initially on the technical development of bioanalytical tools but it soon became apparent that we were faced societal issues as well as technical. Of particular significance is the subject area involving risk perception and trust in the water provider.

The studies presented in Chapter 2 clearly show that bioanalytical tools have a valuable place in risk assessment of reclaimed water. A combination of chemical and bioanalytical methods, each with its inherent limitations but complementary to each other, provides a more rational approach than the conventional chemical-by-chemical analysis (Asano and Cotruvo 2004). A recent review by the National Research Council remarks that while *in vitro* bioassays should not be used in isolation for the determination of human health risks, a battery of *in vitro* bioassays can provide a powerful approach to screening water samples (NRC 2012), a suggestion echoed in the Australian Guidelines for Water Recycling (NWQMS 2008).

It is important to keep in mind that adoption of bioanalytical tools for recycled water monitoring will most likely not lead to lower monitoring costs, and that bioanalytical tools are not intended to replace chemical testing, but rather to offer an important addition to our current monitoring strategies by providing a means to detect non-target chemicals and unexpected transformation products, and provide a sum measure of toxic chemicals acting via the same mode of action.

While the exact role of bioassays in an operational and regulatory framework is still unclear at this stage, it has long been recognized that at the very least bioanalytical tools can be used to compare alternate water supplies such as reclaimed water with current conventional drinking waters to give information on the relative toxicities of the two water supplies (NRC 1998).

Risk communication has a significant role to play in the management of recycled water and in the communication of science to enable policy change, as described in Chapter 3. This is a particularly complex area and generally poorly understood by scientists. On the same hand communication persons are not necessarily trained in science and therefore the role of science translator was identified by Holmes and Clark (2008) as important. A step towards bridging the communication gaps has identified that technology transfer workshops as discussed in Chapter 3 of this report are important in bridging some of the barriers.

4.2 Frequently asked questions (FAQ)

The following is a list of questions that are frequently asked when the topic of bioanalytical tools in water quality assessment comes up.

4.2.1 What do bioassays tell us about water quality?

In vitro bioassays (sometimes called bioanalytical tools) can provide a measure of water quality, just as chemical analysis can be used to determine water quality. Bioassays can detect chemical contaminants by their biological effect in the assay rather than by their chemical structure (which is how pollutants are detected by conventional chemical methods). Bioanalytical tools thus detect a wide range of contaminants and provide risk-scaled sum measure of all bioactive compounds that act via

the mode of action that the assay detects. *In vitro* bioassays detect the initial interaction of the contaminant at the molecular or cellular level, and as such do not accurately predict toxicity in whole organisms (where defense and compensation mechanisms can eliminate the toxic effect). In other words, *in vitro* bioassays can be used as measures of exposure (*i.e.*, to quantify chemical pollutants in water samples) but not measures of effect (*i.e.*, to predict whole organism toxicity). *In vitro* bioassays testing does not replace conventional chemical analysis or whole animal toxicity testing, however it can fill some of the gaps left by our current chemical-by-chemical approach (specifically detect unknown compounds and transformation products and provide a measure of mixture interaction) and, when used in an Integrated Testing Strategy, lead to a more rational and cost-effective assessment of water quality.
Bioanalytical testing can also be used to benchmark water samples (*e.g.*, compare current drinking water sources with alternative water sources, or current drinking water with reclaimed water) and to determine the efficacy of different treatment technologies to remove bioactive compounds, including whether the process produced toxic transformation products.

4.2.2 What bioassays are available and suitable to recycled water?

In vitro bioassay methods can be classified into five classes based on their mode of action: non-specific, specific, and reactive toxicity, as well as adaptive stress response and xenobiotic metabolism. A good bioassay battery should always include at least one assay from each of the five classes. A large number of *in vitro* bioassay methods have been developed for screening purposes in drug development, and many (but not all) can be adapted to water quality testing. Prior to 2000, most studies applied only a few bioassays to test recycled water quality, usually only to test mutagenicity (Ames test). In the last decade many more endpoints have been included, such as endocrine activity, bacterial toxicity, photosynthesis inhibition, genotoxicity, immunotoxicity, etc. A recent study (Escher et al 2014) tested 103 different bioassays with various water samples (including wastewater, surface water, stormwater, reclaimed water and drinking water) and concluded that the most relevant endpoints at this stage (although this of course may evolve with future research) were bacterial toxicity, estrogenic and glucocorticoid endocrine activity, oxidative stress, xenobiotic metabolism (specifically arylhydrocarbon and pregnane X receptor-mediated), mutagenicity and genotoxicity.

4.2.3 How do bioassays fit into and complement the suite of monitoring tools that can be used for water recycling?

In vitro bioassays are one suite of tools available for recycled water assessment, alongside conventional chemical analysis methods and whole animal toxicity testing. Each has its set of advantages and limitations. *In vitro* bioassay methods can complement conventional chemical methods in water quality assessment because they can 1) detect non-target chemicals, such as unexpected compounds and transformation products, and 2) provide a risk-scaled total measure of bioactive chemicals in the sample by combining potency (*i.e.*, how toxic a chemical is) with concentration for each compound. One of the limitations of *in vitro* assays, however, is that they do not clearly identify the causative chemical(s), although some bioassays are particularly sensitive to certain classes of chemical compounds and can thus direct subsequent chemical analysis. Conventional chemical analysis thus complements bioassay methods because they can identify and quantify individual chemical compounds.

4.2.4 What information is generated by bioassay testing?

In vitro bioassays provide a sum measure of the bioactive compounds present in a water sample that act via a specific mode of action. For example, bioassays for estrogenic activity such as the ER-CALUX can detect any compound that can induce an estrogenic effect through an estrogen receptor genomic mediated effect, such as natural and synthetic hormones, bisphenol A and alkylphenols; bioassays for photosynthesis inhibition such as the I-PAM can detect any compound that can interfere with photosynthesis II in plants, such as herbicides. Depending on the assay, the total activity can be expressed as a bioanalytical equivalent (BEQ), such as estradiol equivalent (EEQ) or diuron equivalent (DEQ), or expressed in terms of how much the sample had to be concentrated (or diluted) to reach a pre-determined bioassay response (such as a toxic unit, or a relative enrichment factor). Bioanalytical tools thus provide a quantitative assessment of the concentration of bioactive compounds present in a water sample.

4.2.5 What are we trying to protect with bioassay testing?

In vitro bioassay methods are widely used during drug development by the pharmaceutical industry, and there is therefore a wide selection of bioassays available. The decision of which bioassay to use

for a particular project is generally either driven by chemical consideration (*e.g.*, for dioxin-like compounds, one might choose the AhR-CAFLUX; for herbicides, one might choose the I-PAM), but can also be protection-goal oriented (*i.e.*, to assess recycled water quality for irrigation, one might pick an assay for bacterial toxicity such as the Microtox and an assay representative of important and sensitive plant function such as photosynthesis in the I-PAM). A battery of carefully selected bioassays can detect bioactive chemicals by their mode of action, where the mode of action is related to a negative health outcome (*e.g.*, genotoxicity can lead to tumour formation, and assays for genotoxicity can provide a measure of the potential for carcinogenicity). It needs to be absolutely clear however that bioassay methods only detect the potential for harm, and do not correlate fully with whole organism effects. This is due to toxicokinetic and toxicodynamic modifiers of toxicity (such as absorption, distribution, metabolism and excretion), as well as to the simple fact that *in vitro* methods only detect the primary molecular or cellular response to chemical exposure. That cellular injury does not always lead to whole organism toxicity ("secondary response") thanks to defense and compensation mechanisms in whole animals that can either repair or compensate for the cellular injury.

Bioanalytical methods also provide a sum measure of the bioactive compounds present in a sample. As regulators are acutely aware of the growing (but still incomplete) list of compounds of interest in complex water matrices such as treated sewage, there is a clear need for methods that are able to detect not just those compounds that we know we need to look for (by chemical methods), but also those bioactive compounds that may be present without our knowledge. Bioanalytical tools can provide a simple method to address this to some extent. Certainly they are not perfect methods and will not detect all compounds in all situations, but the improved assessment is still better than not doing anything.

4.2.6 How do we communicate that the inclusion of bioassays is appropriate for water recycling on a cost/benefit basis?

Most people believe that bioanalytical tools will replace chemical methods. This is not correct. Bioanalytical tools provide a way to overcome some of the limitations of conventional chemical methods, but likewise conventional chemical methods overcome some of the limitations of bioanalytical tools. The great cost advantage of including bioassays in routine recycled water quality assessment, however, is that using a combination of carefully selected bioassays and chemical surrogates and indicators can allow the use of a much streamlined analysis, and avoid the need to monitor hundreds of chemical compounds. The application of an intelligent testing strategy that combines tier 1 screening with bioassays and surrogates/indicators, only followed by a more comprehensive tier 2 chemical analysis if those measures are above a pre-determined trigger level, provides a more rational and cost-effective approach to recycled water quality monitoring.

4.2.7 How is that information used? What actions are taken based on the data from these techniques? Who takes action?

There is currently no clear regulatory guidance on how to use the information from *in vitro* bioassays, and there is thus currently no regulatory implications for bioassay testing. Decades of experience however suggests that bioassays can be used to provide an improved monitoring programme with clear operational implications. An effective approach would be to apply the concept of Integrated Testing Strategy (ITS) used in chemical risk assessment. In ITS, a sample is first tested in a screening battery (Tier 1), and only those samples that exceed pre-determined trigger levels proceed to a more systematic chemical assessment (Tier 2). The results of tier 2 then determine whether further action is required, such as a more comprehensive toxicity assessment (Tier 3). Establishing relevant trigger levels is not a simple and easy task, but several proposals have recently been published that offer a structured approach to establish so-called Effects Based Trigger levels (EBT) in *in vitro* bioassays.

A simple, rational and cost-effective approach would be as follows:

1. On-going Tier 1 screening: regular water samples are collected and tested using a combination of carefully selected bioassays and chemical surrogates and indicators. The results are compared with EBT and surrogate/indicator trigger levels. All samples below trigger levels do not require further testing. Samples that exceed any of the trigger levels would be re-tested to confirm that the exceedance is consistent. If confirmed, this then triggers further (Tier 2) investigations. As an acknowledgment that those trigger levels are not hard standards, the level of exceedance should drive the extent to which further investigations are conducted. For example, 100-fold exceedance of a carefully derived trigger level would

warrant thorough investigations, while a 2-fold exceedance may be able to stop after tier 2 even if no causative compounds were identified. The point of all further investigations is to either identify the causative chemical (which can then be compared with guideline levels in the conventional risk assessment approach) or to identify a simple and effective treatment modification that can reduce the tier 1 response below trigger level.

2. Tier 2 chemical analysis: more thorough chemical analysis is conducted on any sample that exceeded the tier 1 trigger levels. This chemical analysis can be directed by the results of tier 1. For example, if the sample showed high estrogenic activity in an estrogenic assay, natural estrogens (17β-estradiol and estrone), synthetic estrogens (ethinylestradiol), bisphenol A and alkylphenol polyethoxylates (nonylphenol and octylphenol) would be targeted first. From this tier 2 chemical data, it is then possible to calculate a predicted bioanalytical equivalent and determine how much of the biological activity detected in tier 1 can be explained by the detected compounds. If most of the biological response is explained by the detected compounds (say >80%), then the conventional guideline approach is used by comparing the chemical concentrations (determined in Tier 2) with the relevant guidelines or standards to determine the need for further action. If however the tier 2 analysis does not identify the causative chemicals, the investigations would advance to tier 3.
3. Tier 3 advanced investigations: there are several methods to proceed, and the operators could choose to use all or select their preferred method:
    a. Tier 3(a): Conduct full chemical analysis for all relevant compounds in the pertinent guideline document and consult with regulator to determine if further action is necessary.
    b. Tier 3(b): Toxicity Identification and Evaluation (TIE): a conventional technique to identify toxic compounds in complex samples (TIE) can be used to chemically identify the most bioactive compounds. In TIE, the sample is treated using various methods that remove specific classes of compounds (*e.g.*, air purging to remove volatile compounds, chelation to remove metals) or fractionated (*e.g.*, using liquid chromatography to separate organic compounds by size or polarity) and re-tested in the bioassay to identify the class of compound responsible for the toxicity, which can then be further fractionated to identify the compound(s). If the causative compound is identified, its concentration would be related to a chemical guideline value and the need for further action determined using the conventional risk assessment method.
    c. Tier 3(c): Identify an effective removal method: using bench-scale testing, the operator could identify what (if any) treatment method (*e.g.*, activated carbon, UV, sand filtration) or simple modification of current treatment can reduce the tier 1 response to below trigger level. If the changes can be easily implemented into the full scale treatment plant at minimal cost, then no further action is required. In some instances the additional treatment may not be possible, but determining an effective removal method may help to identify the class of compound that is responsible for the tier 1 response.

4.2.8 How is the information generated by bioassays translated into language that can be used by regulators?

There are currently no bioassay-based guideline values, and bioassay testing therefore currently has no regulatory implications. It is important to realise that properly carried out bioassay analysis (*i.e.*, including proper quality assurance and quality control samples, reference compounds, replication and analysing serial dilution series of positive samples) provides repeatable and quantitative results. Bioanalytical results can be expressed as bioanalytical equivalent (BEQ), such as estradiol equivalent (EEQ) or diuron equivalent (DEQ), or expressed in terms of how much the sample had to be concentrated (or diluted) to reach a pre-determined bioassay response (such as a toxic unit, or a relative enrichment factor). Bioassay results can be compared to Effects Based Trigger levels (EBT) to determine the significance of the bioassay result, and determine if further chemical characterisation of the sample is required (see Section 4.2.7). Several approaches to derive EBT values have recently been proposed in the scientific literature. EBT values for assays expressed as BEQ can be relatively

simply based on currently available chemical guidelines, while assay-specific EBT values can be determined for all other assays.

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