The role of toxicity testing in identifying toxic substances: A framework for identification of suspected toxic compounds in water

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List of abbreviations

ADME	Absorption, Distribution, Metabolism and Excretion
ATSDR	Agency for Toxic Substances and Disease Registry
DTA	Direct Toxicity Assessment
ECVAM	European Centre for the Validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic Acid
GC	Gas Chromatrography
HPLC	High Pressure Liquid Chromatography
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ITS	Intelligent Testing Strategy
MS	Mass Spectrometry
OECD	Organisation for Economic Co-operation and Development
PBPK	Physiologically-Based Pharmacokinetic
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SAR	Structure-Activity Relationship
SPE	Solid Phase Extraction
TEF	Toxic Equivalency Factor
TEQ	Toxic EQuivalent concentration
TIE	Toxicity Identification Evaluation
TRE	Toxicity Reduction Evaluation
TTC	Threshold of Toxicological Concern
USEPA	United Stated Environmental Protection Agency

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Executive summary

The Australian Drinking Water Guidelines provide guidance on acceptable drinking water concentrations of recognised toxicants. While the guidelines are extensive, not all possible natural and anthropogenic toxicants are included. Chemical monitoring alone may therefore be insufficient to identify all potential hazards, and additional methods may be required. The need to embark upon extensive toxicity identification should be carefully assessed at the outset, and could be driven by factors such as the presence of a contaminant of uncertain toxicity (prospective approach) or well-defined human health impacts or a demonstration of ecological toxicity such as deaths of fish or other aquatic fauna for which all other possible causes have been considered and eliminated (retrospective approach).

Toxicity testing (Chapter 2) can identify toxicants by their biological activity and/or their effect on biological systems, and offer an additional tool for water quality monitoring and risk assessment. Toxicity can be tested at the cellular levels with *in vitro* bioassays and in whole organisms with *in vivo* bioassays. These methods of course also have their limitations, and it is important to understand that toxicity in a cell or a non-human species does not necessarily indicate a risk to humans or other organisms. However, a combination of toxicity testing and chemical analysis can provide a powerful tool for investigative water quality monitoring (Chapter 3). If toxicity is discovered in source waters, it is necessary to determine the identity of the toxicant to determine what (if any) risks are posed to human health.

Toxicity identification evaluation procedures (Chapter 4) are based on a combination of chemical fractionation and toxicity testing, and can often identify the chemical class or even the identity of the toxicant. They are conducted in three phases: toxicity reduction evaluation (phase I) attempts to identify the class of the toxicant (*e.g.* metal, volatile compound, organic), toxicity identification (phase II) involves extensive chemical characterisation of the toxic fraction to identify the toxicant, and toxicity confirmation (phase III) verifies that the toxicant has indeed been properly identified.

Once a chemical is identified, a drinking water guideline can be obtained from the published Australian guidelines. If no guideline exists, an interim guideline can be derived from the available toxicological information and compared with the likely exposure from drinking water to determine if the toxicant is likely to pose a risk to human health (Chapter 5). It is important to understand the limitations of all available evidence to produce a meaningful risk assessment.

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1 Introduction

This document outlines an approach towards identifying the presence (or lack) of a health hazard in a drinking water supply based on the occurrence of a suspected toxicant. It is based on an assumption that toxicity has been identified as a concern but the nature and identity of a toxic substance has not been identified by conventional water sampling and chemical analysis and/or the chemicals that have been found are in compliance with the health-based guidelines for drinking water supplies in Australia (NHMRC/NRMMC, 2004).

The most commonly-used method to screen water quality at present is through targeted chemical analysis and comparison with the relevant guidelines. Most of the chemicals likely to be of concern are included in the guidelines, but with more than 100,000 chemicals in commercial use and many more natural compounds, chemical testing of regulated chemicals alone may be insufficient to identify all potential hazards and additional methods may be required. It is proposed that biological testing is also conducted as part of an integrated approach to assessment of water quality based on a weight-of-evidence approach.

The need to embark upon an extensive process of toxicity identification should be carefully assessed at the outset, and could be driven by factors such as the presence of an identified substance or contaminant of uncertain toxicity, or evidence of ecological impacts ("prospective approach") or well-defined human health impacts for which all other possible causes have been considered and eliminated ("retrospective approach"). The retrospective approach depends on clearly identified health outcomes based on epidemiological data, while the prospective process tries to identify potential issues early to prevent the possibility of undesirable health outcomes.

The first step in conducting any risk assessment is to identify an issue in order to establish a context by identifying what is the concern, how the concern was raised and whether the issue is amenable to risk assessment (enHealth, 2004). Issues identified may have dimensions relating to perceptions, science, economics and social factors. For example it may be reported that a toxicant could be present in a water supply. This could be intermittent or be a regular occurrence. In some cases the cause for concern may be known and can therefore be managed. If the contaminant of concern cannot be identified by chemical analysis then a program of investigation is required using investigative monitoring methods. In order to investigate the issue further, biological testing (bioassays) that are targeted towards identifying hazards to human and/or ecological health can be used as part of an integrated process of investigation. The approach discussed here is to screen the water using alternate methods (*in vitro* and/or *in vivo* biological testing methods) to determine if exposure of humans to a potential hazard is likely and to direct further investigation. It is important to realise that the perception of harm does not always translate into fact, and the basis for the health concern needs to be carefully and objectively examined at the outset, and may in some instances negate the need for further investigation (as was the case with the George River Water Quality Investigation; George River Water Quality Panel, 2010).

The intent of this document is to outline the steps that can be taken to identify an unknown toxicant in a drinking water source. The document briefly describes toxicity testing (Chapter 2) and its

application to water quality monitoring (Chapter 3), outlines the standard protocol for toxicity identification evaluation (Chapter 4) and discusses how to bring the newly-generated information together to produce a more comprehensive risk assessment (Chapter 5).

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2 Toxicity testing

The purpose of toxicity testing is to determine whether a compound or water sample has the potential to be toxic to biological organisms, and if so to what extent. Toxicity can be evaluated in whole organisms (*in vivo*) or using molecules or cells (*in vitro*). The main advantage of toxicity testing is that it detects toxic compounds based on their biological activity, and as such does not require *a priori* knowledge of the toxicant to identify its presence (unlike chemical analysis). The same characteristic is also of course a disadvantage, because while toxicity testing can determine if toxic compounds are present it does not identify them. Identification of the toxic component is then required, as outlined in Chapter 4.

Once a suspected toxicant is identified, modelling approaches (*in silico*) can sometimes be used to predict its toxicity based on the physico-chemical properties of the compound and its likely fate and transport in the environment.

2.1 Direct toxicity assessment

2.1.1 *In vivo* bioassays

Conventional toxicity testing relies on direct toxicity assessment in whole organisms (algae, shrimps, sea urchin, fish, rats etc) (Blaise and Férard, 2005). The organisms are exposed to the chemical(s) or mixture(s) of interest and monitored for any sign of adverse health effect. This can be either a gross morphological effect (such as weight loss, visible lesions, death) or more subtle biochemical markers, whether biomarkers of exposure (an indicator of the internal dose, such as a metabolite in urine) or biomarkers of effect (an indicator of a health effect, such as enzyme activity). The duration of the exposure depends on the type of toxicity monitored, from short-term acute effects (96h or less), sub-acute (a couple of days), sub-chronic (a couple of weeks) to chronic effects (a significant portion of the organism's life expectancy).

Depending on the species used, *in vivo* toxicity testing is generally seen as the most relevant predictor of human health effects. This is because *in vivo* tests include a measure of absorption, distribution, metabolism and excretion, all of which could modulate the toxicity of the sample. There are a few disadvantages to *in vivo* testing however:

- **Interspecies extrapolation.** *In vivo* toxicity tests are done in whole organisms from species other than humans, and the results are extrapolated to human health predictions. The greater the difference of the species to humans, to more tenuous this extrapolation becomes. For example, a herbicide targeting photosynthesis would be particularly toxic to algae but much less so to non-photosynthetic organisms such as humans. Likewise it would be difficult to extrapolate from an effect in shrimp to humans, due to the very significant differences in toxicokinetics (*i.e.* absorption, distribution, metabolism and excretion) between the two organisms. Even species widely used for assessment of human health risks such as rats, dogs or monkeys exhibit significant differences in metabolising enzymes compared to humans (Martignoni et al., 2006), which could result in significant differences

in toxicity between different species. It is therefore important to understand the mechanism of toxicity to meaningfully extrapolate *in vivo* toxicity to potential human health effects.

- **Sensitivity.** In general, *in vivo* effects are detectable at µg/L concentrations (Asano and Cotruvo, 2004). When the purpose of toxicity testing is purely to detect toxicants, other more sensitive methods such as *in vitro* testing may be necessary.
- **Artefacts and confounding factors.** When testing whole water samples, physico-chemical parameters such as temperature, pH, turbidity, colour and dissolved organics and inorganics can cause artificial toxicity in the test organisms, which would not otherwise occur in the environment (*i.e.* false positive) (Postma et al., 2002).
- **Ethical cost.** There is an ethical need to reduce, refine and replace *in vivo* methods with alternatives, such as *in vitro* and *in silico* methods wherever possible (Balls et al., 1995).
- **Financial cost.** *In vivo* experimentation can be costly in financial terms as well, and high-throughput low-cost alternatives are sometimes necessary on those grounds alone.

Despite these limitations, *in vivo* assays are commonly used in assessing risks to human health as they can provide a reliable indicator of potential toxic injury to the population, in particular when the toxicity is novel.

2.1.2 *In vitro* bioassays (bioanalytical methods)

In vitro bioassays have been in use for drug discovery by the pharmaceutical industry for decades. In *in vitro* bioassays, molecules (*e.g.* enzymes) or whole cells are exposed to the chemical(s) or mixture(s) of interest and monitored for specific responses. There is of course more to health than cellular health and human beings are more than simply billions of independent cells. However, for chemically-induced toxicity the initial interaction of the chemical at the molecular or cellular level is a necessary (but not sufficient) prerequisite for toxicity (Escher and Hermens, 2002). This is because toxicity occurs at the site of interaction of the toxicant (which can be either the parent compound or a metabolite) with the target biomolecule ("primary effect"). Organisms however have defence and detoxification mechanisms to cope with a certain degree of primary toxicity, and it is only when those defence mechanisms are overcome that observable toxicity occurs ("secondary effect"). This means that *in vitro* toxicity is likely to occur at significantly lower doses than *in vivo* effects (Figure 1), but also means that a substance can be toxic *in vitro* but not *in vivo*.

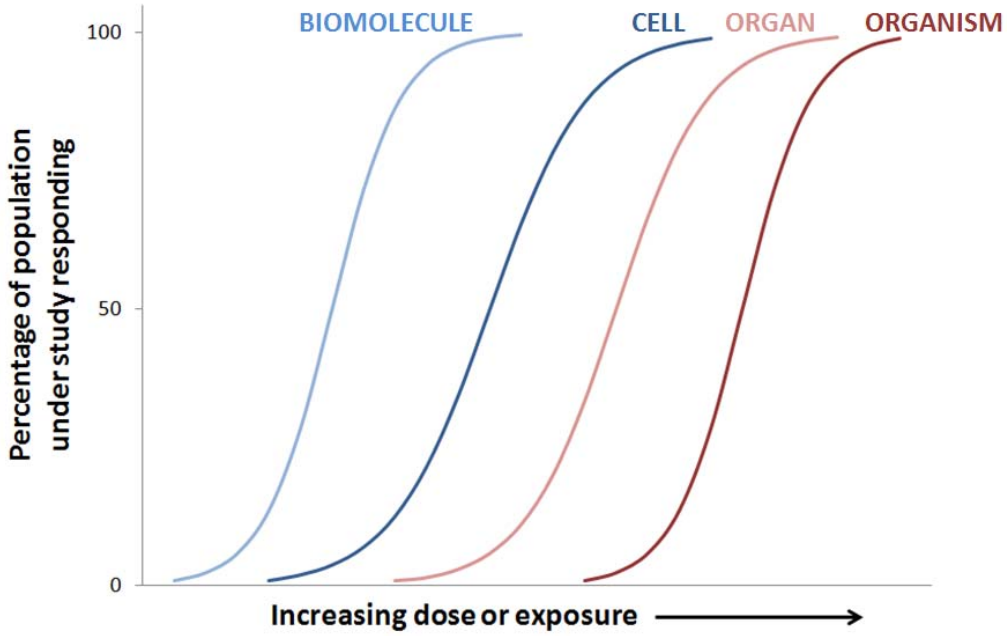

Figure 1. A continuum of toxicity.

A variety of toxic effects can be monitored *in vitro*, from basal toxicity (cytotoxicity) and reactive toxicity (interaction with protein or DNA, which can then lead to carcinogenicity) that can potentially affect all cells, to specific toxicity that may only affect certain cells or organs (*e.g.* endocrine effects, neurotoxicity, immunotoxicity, liver toxicity etc). Typically, *in vitro* tests are carried out with specific cell types depending on the endpoint of interest. Some assays can be more variable than others, and thorough quality assurance / quality control procedures such as consistent use of positive and negative controls, monitoring of assay performance with control charts, quantification of detection limits, determination of reproducibility and robustness, use of inter-assay samples, intra- and inter-assay duplication and adoption of Good Laboratory Practices (OECD, 1998; OECD, 2004) can ensure the production of reliable high-quality data. Each type of bioassay has its advantages and limitations, and no single assay can provide a complete assessment of the biological activity of a sample. Therefore a battery of bioassays is required to rigorously assess the potential of a sample to cause biological effects in exposed organisms.

In vitro assays are generally high-throughput short-term (<1 week) assays that provide a quick measurement of potential toxicity in the sample. These methods are presently at different stages of development and not all are presently suitable for inclusion in a monitoring program.

There are a few important limitations to *in vitro* assays that need to be very clear:

- **No incorporation of toxicokinetics.** Toxicokinetics include absorption, distribution, metabolism and excretion (ADME), all of which can significantly affect the toxicity of a substance. For example, if a substance is not absorbed by the lining of the gastro-intestinal tract it will be excreted without interacting with cells within the body and thus will not be harmful to whole organisms, even if it is toxic to individual cells. Or if the compound is

quickly metabolised to a less toxic form by liver enzymes, again the substance would be significantly less toxic *in vivo* than might be suggested by *in vitro* tests. Conversely, some compounds can be bioactivated by metabolism, and they may be more toxic *in vivo* than *in vitro*. The presence of barriers to distribution within the human body (*e.g.* the blood-brain barrier, the blood-testis barrier, the placenta etc) can also restrict the ability of the absorbed compound to affect specific organs. And finally the compound may be excreted rapidly by human kidneys, resulting in far shorter exposure than would occur *in vitro*.

- **Higher sensitivity but lower relevance.** As discussed above, *in vitro* assays measure the primary effect, which is the initial interaction between a chemical and a biomolecule. In whole organisms, defence and detoxification mechanisms can overcome a certain amount of this primary effect with no significant health consequence. It is only when those defence mechanisms are overcome that toxicity occurs *in vivo*. This means that *in vitro* bioassays can detect toxicants at lower doses than *in vivo* bioassays do, but also that this *in vitro* toxicity does not necessarily mean any adverse effect will occur *in vivo*, and thus overestimate the actual toxicity of the substance. *In vitro* assays were developed for screening purposes and there is still much debate about their ability to predict whole organism effects (NRC, 1998) and regulatory agencies have generally been wary of using *in vitro* bioassay data to predict human health effects (Nielsen et al., 2008).

Because of these limitations, *in vitro* bioassays should not be used as a measure of effect. However, they are well suited to monitoring water quality (exposure assessment), as they are significantly faster and cheaper than *in vivo* exposures and are amenable to high throughput screening. They also allow the generation of relatively rapid toxicology data without the need for ethically and financially expensive whole-animal experimentation (Balls et al., 1995). A concerted international effort by the US (National Toxicology Program - Interagency Coordinating Committee on the Validation of Alternative Methods ICCVAM) and European Union governments (European Centre for the Validation of Alternative Methods ECVAM) is underway to progress the development of these alternative methods and address their shortcomings, and a few have become accepted OECD testing methods (OECD, 2009). These methods are highly specific in application and guarantee standardized outcomes. A the time of writing, 9 *in vitro* methods were approved by the OECD for skin absorption and corrosion, phototoxicity and genotoxicity (methods 428, 430, 431, 432, 435, 473, 476, 479 and 482) (OECD, 2010). ICCVAM and ECVAM are currently validating *in vitro* tests methods for acute oral toxicity, genetic toxicity, biologics, immunotoxicity, dermal corrosion and irritation, ocular toxicity, developmental toxicity, pyrogenicity and endocrine disruptor effects (Nielsen et al., 2008).

2.1.3 Epidemiology

If toxicity testing reports measurable toxicity in a drinking water source, epidemiological study of the exposed population may be warranted to determine if potential exposure to the contaminant has resulted in human health effects. Although epidemiology is the most relevant measure of human health (compared to *in vivo* or *in vitro* toxicity testing), designing and conducting these types of studies to detect the impact of drinking water on human health has proved challenging (NRC, 1998). This is because a large population study group is required to accurately quantify whether a true difference exists between exposed and unexposed subjects, and many other socioeconomic and health risk factors as well as environmental factors may contribute to differences between these two cohorts (such as exposure to environmental contaminants from other sources, differences in health

surveillance between different populations etc) (NEPC, 2008; enHealth, 2004). There can also be significant time delays between study initiation and a final result – particularly if a longitudinal cohort study is required over many years to demonstrate health outcomes with a latency period. Epidemiological studies are not always feasible or practical, and if they are to be undertaken it is essential to carefully design the study from the onset and rely on clear health outcome measures that are plausibly related to exposure to the toxicant (which will depend on its mechanism of action, if known, and evidence from experimental animals) (enHealth, 2004).

2.2 *In silico* approaches

Some of the shortcomings of *in vitro* bioassays, particularly the lack of integration of toxicokinetics, can be partly overcome by combining them with computer (*in silico*) modelling using structure-activity relationships (SAR). In SAR, the chemical structure and other physico-chemical properties of the substance (once it is known) can be used to predict its toxicokinetics. Based on available toxicity databases, a predicted threshold of toxicological concern (TTC) can be assigned to the chemical (Kroes et al., 2004), which can then be used to derive a provisional drinking water guideline (NRMMC/EPHC/NHMRC, 2008).

In silico methods are very useful in the absence of other toxicological data, but are based on data from other chemicals and as such should be viewed with appropriate caution.

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3 Screening for toxic compounds in water

3.1 Conventional analysis of regulated chemicals

The standard approach to water quality assessment is outlined in the relevant guideline documents that make up the National Water Quality Management Strategy. For drinking water this is the Australian Drinking Water Guidelines (NHMRC/NRMMC, 2004), while for most other types of water use and ecosystem impacts it is the Australian Guidelines for Water Quality Monitoring and Reporting (ANZECC/ARMCANZ, 2000). The first step in screening water for toxic compounds is to measure the concentration of all likely chemicals with a specified guideline in the appropriate guideline document. If none of the regulated chemicals are found above guideline values, this provides a degree of confidence in the safety of the water. However it does not rule out the possibility that an unmeasured (and possibly unregulated) toxicant may be present. Toxicity testing however can fill that gap by following a tiered approach described as “intelligent testing strategy”.

3.2 Intelligent testing strategy: dealing with mixtures and unknowns

As previously stated, toxicity testing measures total biological activity in a given water sample, but does not provide identification of the causative chemicals. Chemical analysis on the other hand only allows measurement of selected chemicals, and biologically active compounds may be missed because they were not originally targeted. But combining the two techniques provides significantly more analytical power than each individual method alone.

In an intelligent testing strategy, a tiered approach is used to screen for toxicity, starting with physico-chemical characterisation of the water in tier 1 (including parameters such as pH, conductivity, turbidity, hardness as well as analysis of regulated chemicals) to *in vitro* toxicity testing and finally *in vivo* toxicity testing if required.

In this proposed approach, water samples are first tested using conventional chemical analysis targeting individual chemicals with a guideline (NHMRC/NRMMC, 2004) (Step 1, Figure 2).

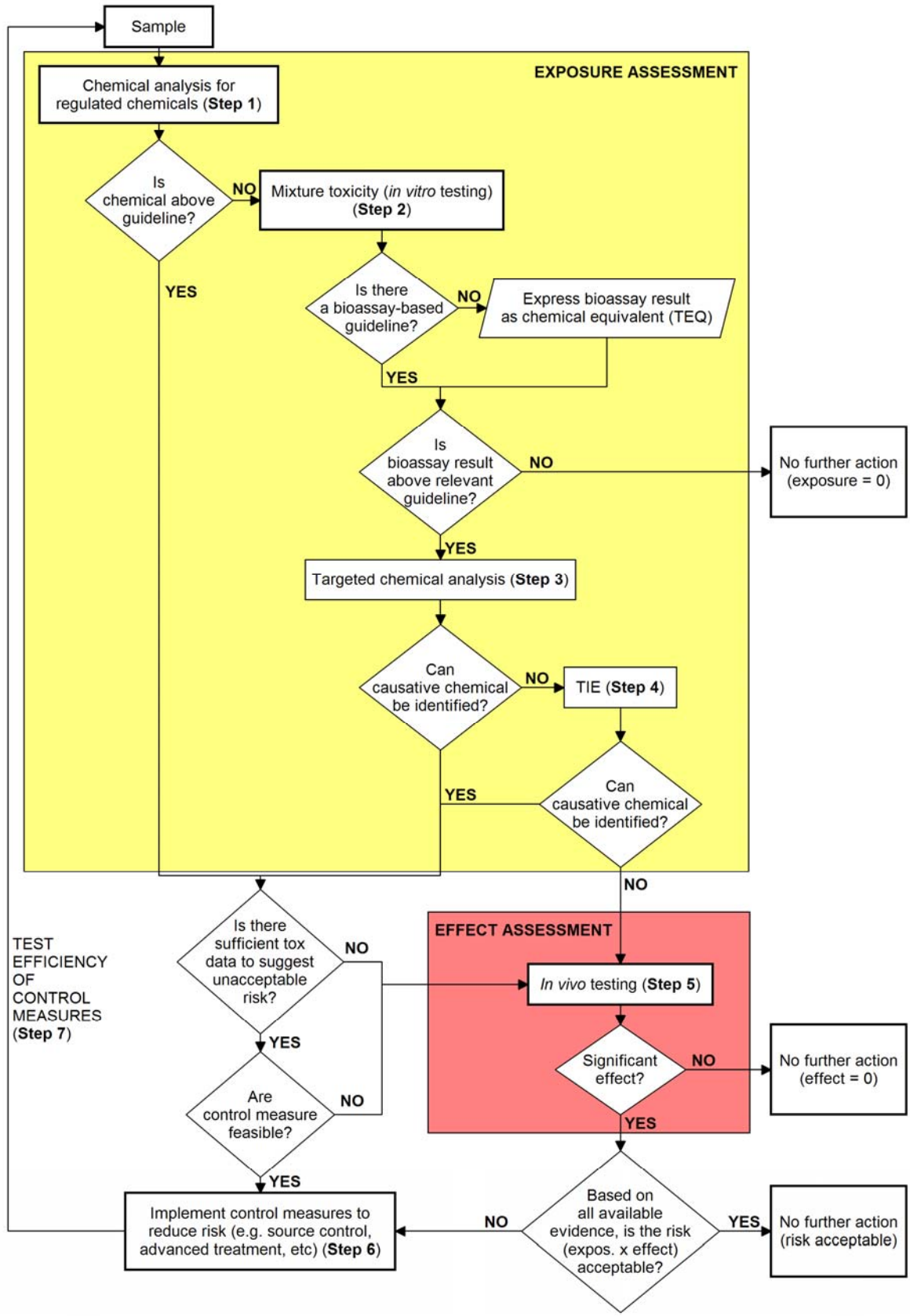

Figure 2. Proposed toxicity testing framework (modified from NEPC 2008)

If none of the measured chemicals are above their respective guideline values, then *in vitro* bioassays are used to screen the samples for additional unexpected biologically-active compounds as well as provide a limited measure of mixture toxicity (Step 2, Figure 2). Relevant *in vitro* bioassay selection is critical at this stage, and should cover a wide range of modes of action and potential health effects (Escher and Hermens, 2002). The bioassay battery should at least cover some measures of:

- **Non-specific toxicity.** Basal cytotoxicity caused by non-specific effects (*e.g.* membrane damage, generation or reactive oxygen species, etc).
- **Reactive toxicity.** Toxicity caused by DNA or protein damage (*e.g.* genotoxicity, carcinogenicity).
- **Specific toxicity.** Toxicity caused by specific interaction or interference with an enzyme or a receptor site (*e.g.* endocrine effects, enzyme function, etc).

If there is a bioassay guideline available, the bioassay response is compared directly to that guideline. Otherwise the bioassay results have to be expressed in terms of the equivalent concentration of a reference chemical that would induce a similar biological response. This is the concept of toxic equivalent concentrations (TEQs), which was used initially for dioxin-like activity. For example, a response in an assay to measure aryl hydrocarbon receptor (AhR) activity could be expressed as TCDD-equivalents, while a response in a bioassay to measure estrogenic endocrine disruption could be expressed as 17β-estradiol or nonylphenol equivalents. This allows a translation of the bioassay response to an equivalent chemical concentration, which can then be compared to the relevant chemical guideline (see section 5.1.1). The reference chemical(s) must be chosen carefully based on a thorough understanding of the bioassay system as well as the potency and relevance of the chemical to the measured biological endpoint. If the response in the bioassay exceeds the available guideline value, then the sample is forwarded for targeted chemical analysis based on the type of toxicity measured and the most likely candidate chemicals (Step 3, Figure 2).

If the causative chemicals cannot be identified through a targeted chemical analysis, then a full toxicity identification evaluation (TIE) may be necessary (Step 4, Figure 2) (see Chapter 4). Once identified, a confirmation step is usually performed to ensure that the causative pollutant has been correctly identified by testing the activity of the chemical compound in the bioassay. If after a TIE the causative chemical can still not be identified, then a full effects assessment may be required (Step 5, Figure 2).

Once the chemical has been identified (at Steps 1, 3 or 4; Figure 2) or the effects assessment has been conducted (Step 5, Figure 2), then an informed decision can be made on the need for further risk mitigation and the implementation of control measures (Step 6, Figure 2). Of course, the efficiency of those control measures then needs to be tested using the full framework (Step 7, Figure 2).

3.3 Sampling considerations

Sampling is an important and often underestimated component of the overall process, and the final analysis is only as good as the sampling. An inadequate sampling schedule or method could provide samples that are not representative of the system. It is therefore crucial to understand the system to be sampled before sampling starts to determine the appropriate sampling locations, frequency and

type. After collection, the samples must be preserved in such a way that prevents further degradation of its chemical contents but also does not interfere with the testing methodologies. Chapters 9 and 10 of the Australian Drinking Water Guidelines (NHMRC/NRMMC, 2004) provide thorough guidance on these critical questions.

The sampling frequency should seek to capture toxicants that are only intermittently present, whether because of anthropogenic activities (*e.g.* release of an industrial compound influenced by process cycle) or natural events (*e.g.* run-off from rain events remobilising chemicals from the soil to receiving waterways). In some instances the toxicants are only released in short pulses, which could even be missed by frequent sampling. Composite or proportional sampling, where a small water sample is taken at regular intervals by an automated sampling device, can help in these instances. While this technique allows some integration for the variation in chemical contaminant concentrations over time, its most significant limitation is the fact that biodegradation can occur over the sampling time taken to achieve a composite sample. Therefore chemical contaminant concentrations may be underestimated. Passive accumulation devices (also called passive samplers) can be submerged in the monitored water and accumulate chemical contaminants by absorption or adsorption in a trap, usually a membrane, which provides some protection against biodegradation. The sampling devices can be submerged in the water for several days/weeks and the concentration of chemical contaminants in the trap is integrated over the whole exposure time. There are still issues of accurate quantification to be resolved, but passive samplers have been used successfully, including in Queensland to identify short pulses of pesticide discharges in surface waters (Stephens et al., 2009).

Toxicants can be either dissolved in the water phase or bound to particulate matter suspended in the water. Particulate matter has the capacity to concentrate materials because of it ability to bind a number of compounds, including metals and organic chemicals. Foams are specific examples of particulates, largely comprising surfactants, which generate non-water soluble complexes to which materials can bind. Particle-bound toxicants generally have lower bioavailability and are usually removed by filtration, sedimentation and or coagulation processes during drinking water treatment. They can, however, pose a greater risk to ecosystem health. Which component of the water to sample is determined by the focus of the risk assessment.

The type of sampling is dependent on both the target analyte and the matrix in which it is contained. Information sheet 2.1 in the Australian Drinking Water Guidelines (NHMRC/NRMMC, 2004) provides advice on appropriate sampling handling and preservation methods. Samples are preserved to ensure they maintain their chemical composition for as long as possible. A combination of filtration, acidification and addition of a preservative (such as methanol) are common, depending on the analyte, and how long a sample can be stored until analysis very much depends on the analyte. When screening for an unknown toxicant (see Chapter 4 below), filtration to remove microorganisms is common and the samples are usually analysed within 48h of sampling (or as soon as is practical).

4 Toxicity identification evaluation

Toxicity identification evaluation is a technique used to identify the source of toxicity in a complex environmental sample or mixture. It relies on sequential iteration of physico-chemical fractionation combined with toxicity testing to separate and identify the biologically-active compound(s).

TIE is conducted in three phases: toxicity characterisation (Phase I) (USEPA, 1992), identification (Phase II) (USEPA, 1993a) and confirmation (Phase III) (USEPA, 1993b).

4.1 Sample preservation

The first stage of the process prior to carrying out TIE is to remove bacteria by filtration or addition of bacteriocidal compounds like formaldehyde or methanol. The preferred technique is by filtration, as this does not introduce further chemicals into the water sample. This is to ensure no further biological degradation of the chemicals in the sample. Once microorganisms are removed, the TIE process can proceed. It is crucial to conduct toxicity testing in TIE as soon as practical, and usually within 48h of sampling.

4.2 Phase I – Toxicity characterisation

During phase I, the sample is physically and/or chemically-altered by a variety of methods in an attempt to remove different classes of toxicants. The toxicity of the resulting sample is compared with that of the original sample to determine if treatment has had any effect on toxicity. This approach is also sometimes referred to as Toxicity Reduction Evaluation (TRE).

The following methods are often used to manipulate the sample and remove specific groups of toxicants:

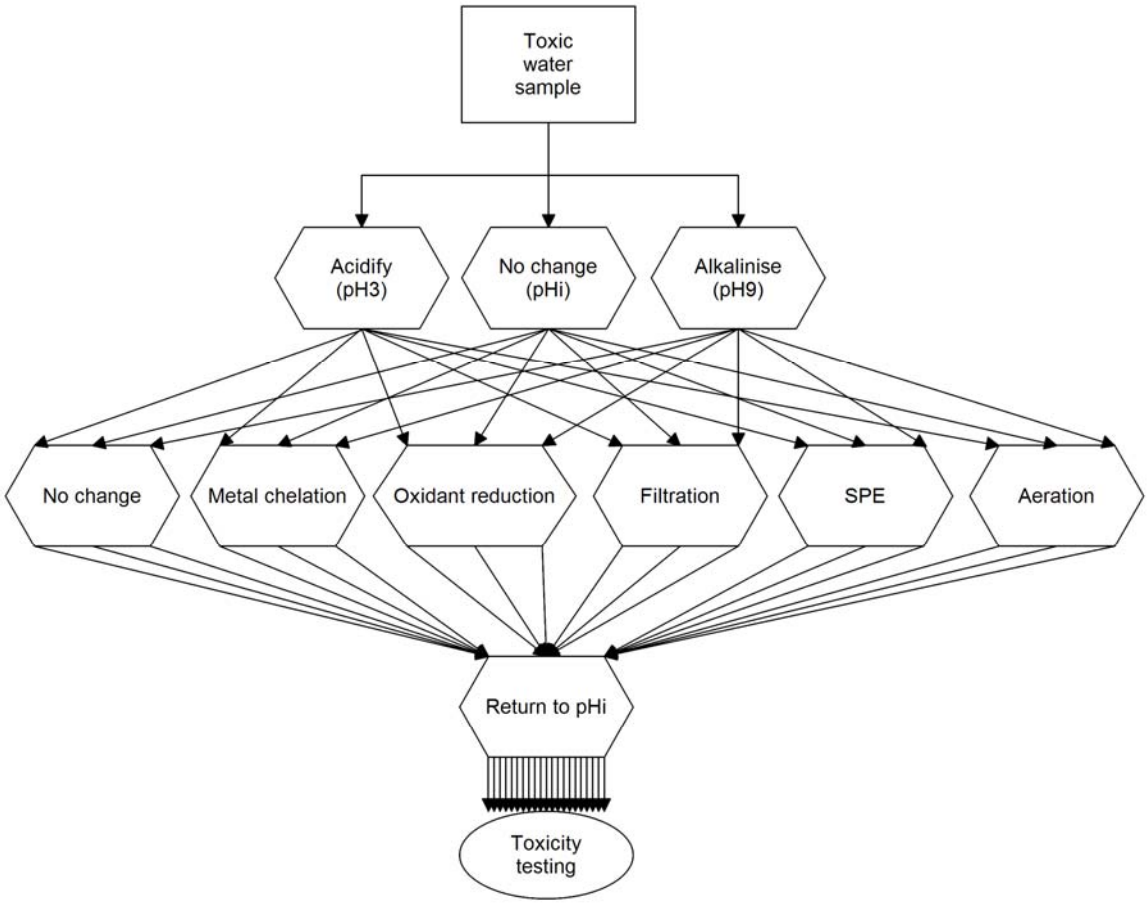

Figure 3. Example of sample manipulation in toxicity reduction evaluation.

It is important to conduct the TRE as soon as possible, and sample manipulations are generally performed within 2 days of sampling to reduce the impact of degradation. The TRE procedure can potentially produce dozens of sub-samples from one original water sample, and short-term high-throughput toxicity tests (such as carefully-selected *in vitro* bioassays) are usually preferred to keep the required time and cost at a reasonable level.

4.2.1 pH adjustment

The pH can have a significant effect on the chemistry and toxicity of a water sample, and pH adjustment is often carried out in phase I. Three pH values are usually tested: acidic (pH 3), basic (pH 9) and the natural pH of the water sample. This extreme change in pH can result in significant degradation of hydrolytically unstable compounds. The pH of the water sample is brought back to the initial (natural) pH of the sample prior to toxicity testing.

The pH is also adjusted prior to other treatments, as it can influence their effectiveness. Again, three pH values are generally used for this: pH 3, pH 9, and the natural pH of the water sample. The pH is returned to the initial pH of the water sample after the treatment and immediately prior to toxicity testing.

4.2.2 Removal of metals

Natural waters will frequently contain dissolved metals contingent on their geological origin and contact with materials in the catchment generated by human activity, all of which can be influenced by the pH of the water. Cationic metals (such as aluminium, cadmium, copper, iron, lead, manganese, nickel and zinc) can be removed by addition of ethylenediaminetetraacetic acid (EDTA) or by passing the sample through a cation exchange column. The latter would be preferable, as cation exchange removes the metal from the water while the EDTA-metal chelate remains in the water sample. EDTA chelation is nevertheless most commonly used, and can be strongly affected by the pH value.

Excess EDTA can lead to false positive due to its toxicity, which depends on the species and cell line used - for example exposure to 7-8 mg/L for 7 days caused 50% toxicity in *Ceriodaphnia dubia* (USEPA, 1992), while *in vitro* exposure to 700 mg/L for 30 minutes caused 50% toxicity in V79 (Chinese hamster lung fibroblast) cells (Ballal et al., 2009). The proper sample process controls (such as blank sample with the same EDTA concentration) need to be tested in parallel to confirm the change in toxicity is not due to the intrinsic toxicity of the chelating agent.

4.2.3 Sodium thiosulfate reduction

Addition of sodium thiosulfate can reduce the toxicity of oxidative compounds (such as chlorine, bromine, ozone) and also certain cationic metals (such as cadmium, copper, silver, mercury). Excess sodium thiosulfate can however be toxic, and the proper sample process controls need to be tested in parallel to confirm that the change in toxicity is not due to sample manipulation.

4.2.4 Removal of volatile and sublatable compounds by aeration

Aeration of the sample can remove substances that are oxidisable or volatile, and concentrate substances that are sublatable in surface foam (a sublatable compound is one that is adsorbed on the surface of gas bubbles in a liquid). The pH of the sample can affect the rate of oxidation or volatilisation. If this treatment reduces toxicity, bubbling with nitrogen gas can determine whether oxidation or volatilisation/sublation was the effective process.

4.2.5 Removal of particulate matter by filtration or centrifugation

If toxicity is reduced by filtration or centrifugation, this indicates that the toxicant is associated with suspended solids or removable particles. This however provides little specific information on the nature of the toxicant, and further testing (such as accelerated solvent extraction) will need to be carried out on the filtrate or pellet to determine its specific nature. It may however provide valuable information on treatment plant processes likely to remove the toxicant (see section 4.7).

4.2.6 Removal of mid-polar to non-polar organics by solid phase extraction

Mid- to non-polar organic compounds (such as some pharmaceuticals, hormones, industrial compounds, natural toxins etc) can be removed from the water phase by solid phase extraction (SPE). The chemicals retained can also be separated into different fractions by gradient elution of the SPE cartridge, a simple form of liquid chromatography separation.

4.2.7 Procedural blanks – an important consideration

It is important to run a procedural blank for each treatment used, to ensure that any changes in toxicity are due to an effect of the treatment on the chemical composition of the sample and not

simply due to the treatment itself. For example, excess EDTA or sodium thiosulfate can cause toxicity in both *in vitro* and *in vivo* bioassays. It is recommended to use a physical alternative (*e.g.* cation exchange *vs.* EDTA) when available.

4.2.8 Artefacts and confounding factors

As previously discussed (section 2.1.1), *in vivo* methods can be sensitive to confounding factors such as temperature, pH, turbidity, dissolved oxygen, colour, and innocuous dissolved organic and inorganic compounds, and it is important to ensure that the observed toxicity is not an artefact of these characteristics (*i.e.* a false positive) (Postma et al., 2002). Standardised methods should have clear criteria for general water quality parameters (which may need to be adjusted prior to testing) to ensure the validity of the toxicity test. The role of potential confounding factors can be investigated by the inclusion of appropriate control samples and correlation analysis between the putative confounding factor and the toxicity result, and may require further testing (Postma et al., 2002).

4.3 Phase II – Toxicity identification

By the end of phase I, a rough chemical class can be assigned to the toxicant based on which treatment resulted in a reduction of toxicity. This information can then direct intensive chemical screening in phase II using methods suited to the putative chemical class of the toxicant. For example, if EDTA chelation resulted in a reduction of toxicity, metal analysis using ICP-MS or ICP-AES would be warranted. If SPE resulted in toxicity reduction, organics analysis using HPLC-MS (/MS) or GC-MS (/MS) would be appropriate. If volatilisation results in reduced toxicity, then headspace GC-MS analysis would be used, etc.

With chemically-complex samples, further fractionation and toxicity testing may be necessary. For example, different organic compounds can be separated by liquid chromatography, and testing the toxicity of the different fractions can help narrow the number of candidate toxicants.

4.4 Phase III – Toxicity confirmation

Phase III is an oft-overlooked but critical part of TIE. This last phase confirms whether the toxicant identified in phase II is indeed responsible for the observed toxicity in the environmental sample. There are four methods for toxicity confirmation: correlation, symptom, species sensitivity and spiking. Obviously the application of more than one method results in greater confidence in the toxicity confirmation.

4.4.1 Correlation analysis

If several environmental samples with different toxicities are available (*e.g.* samples of the same water body but on different days), then it becomes possible to compare the measured concentration of the suspected toxicant in each sample with the toxicity measurement. If the suspected toxicant is indeed responsible for the toxicity, then this comparison will show a significant correlation. Correlation analysis should always be confirmed with at least one other method below, as it may be the result of co-occurring pollutants or events.

4.4.2 Symptom analysis

This method involves testing the toxicity of the suspected toxicant either *in vitro* or *in vivo* to confirm that this exposure results in similar symptoms as exposure to the environmental sample. If the suspected toxicant is indeed the source of toxicity, then the symptoms should be similar.

4.4.3 Species sensitivity analysis

This method relies on the fact that different species and different assays show different sensitivities to the same toxicant. For example, the microalgae *Monoraphidium arcuatum* is significantly more sensitive to arsenic V than *Chlorella* sp (Levy et al., 2005). If arsenic V was the suspected toxicant, one would predict that the water sample would also be significantly more toxic to *M. arcuatum* than to *Chlorella* sp.

4.4.4 Spiking

In this method, the suspected toxicant is added to the toxic water sample in increasing amounts and toxicity is determined to see if it increases proportionately to the amount of toxicant added.

4.5 Simplified TIE approach based on existing data

In some instances, prior chemical analysis may suggest a suspected toxicant. In that case, a full TIE may not be necessary and a simplified TIE may be appropriate. In this simplified approach, the water sample would be treated to remove the chemical class of the suspected toxicant (*e.g.* chelation if the suspected toxicant is a metal, SPE if it is an organic compound etc) and the toxicity of the resulting sample tested to confirm reduction of the toxicity. The identity of the suspected toxicant would then be confirmed by phase III principles, as described above.

Some researchers are also avoiding complicated toxicity reduction evaluation procedures (phase I of the TIE process) and directly analysing metals and organic compounds (by ICP-MS, HPLC-MS and/or GC-MS) (Yang et al., 1999; Hogenboom et al., 2009). Particularly when dealing with relatively clean water matrices (such as drinking and reclaimed water), this approach may allow for a quick identification of a few suspected toxicants. It is crucial to conduct a thorough phase III confirmation analysis to ensure that the suspected toxicants are indeed responsible for the detected toxicity.

4.6 Recent studies

There is a large number of scientific studies that have relied on TIE procedures to attempt to identify a variety of toxic compounds (reviewed in Hewitt and Marvin, 2005), including pesticides (Amato et al., 1992; Bailey et al., 2005; Bailey et al., 2000), metals (Burgess et al., 1995), estrogenic endocrine disrupting compounds (Hewitt et al., 1998; Quinn et al., 2004; Thomas et al., 2004a; Thomas et al., 2004b; Desbrow et al., 1998), in a variety of water matrices including pulp mill effluents (Dubé and MacLatchy, 2001) and industrial effluents (Yang et al., 1999; Yu et al., 2004) as well as sediments (Houtman et al., 2004).

Identification of chemical classes associated with the measured biological endpoint is frequently achievable, but confirmation of individual compounds has been more difficult (Hewitt and Marvin, 2005). The latter is, however, not always necessary, and chemical class identification alone can often provide sufficient information to determine appropriate treatment or source control options. In Australia for example, TIE procedures were successfully used in identifying the pesticide chlorfenvinphos as the cause of acute toxicity in treated wastewater from municipal sewage

treatment plants (Bailey et al., 2005). Source-control measures were then successfully implemented to eliminate chlorfenvinphos (and associated toxicity) from the discharge.

4.7 TIE findings to predict efficacy of water treatment technologies

In some instances the TIE process can provide insights into the expected efficacy of water treatment technologies, and this information should be used to prioritise risk management. This is because many of the treatment options used at a small scale in the TIE process are often used in full scale at water treatment plants. For example, if toxicity was reduced by solid-phase extraction, one would expect carbon filtration to be effective at removing the toxicant; if aeration was the effective treatment, dissolved air floatation would most likely remove the toxicant; if filtration was effective at removing the toxicant in the lab, then it is likely to do so in a full-scale water treatment plant as well.

These assumptions can easily be confirmed by testing a water sample pre- and post-treatment in the drinking water treatment plant, as done in treatment validation studies. Disappearance of toxicity in the post-treatment samples can clearly demonstrate the effectiveness of the treatment step itself. Almost all reticulated Australian drinking water is disinfected and will contain chlorine residual. Chlorine can contribute to increased toxicity and therefore must be removed prior to any bioassay. Chlorination by-products are generally stable and may be generated throughout the distribution supply depending on the organic content of the source water. It is therefore important to carefully select sampling points in the drinking water treatment train that are relevant to the specific research question.

If the toxicant cannot be removed by an available treatment process, then depending on the risk either an alternative treatment type may be required, or source controls need to be implemented if feasible.

5 How toxic is it to humans?

Once a toxicant has been identified, whether through an extensive or simplified TIE process, it is important to determine the risk in drinking water.

It is important to keep in mind that toxicity in a cell system or in other organisms does not necessarily equate to toxicity in humans (as discussed in chapter 2). Toxicity data in humans is, however, generally not available, and all other sources of information must be prioritised based on the relevance of their outcomes to human health outcomes.

The Australian strategy for assessing human health risks from environmental hazards is set out the enHealth guidelines (enHealth, 2004), summarised in Figure 4 below:

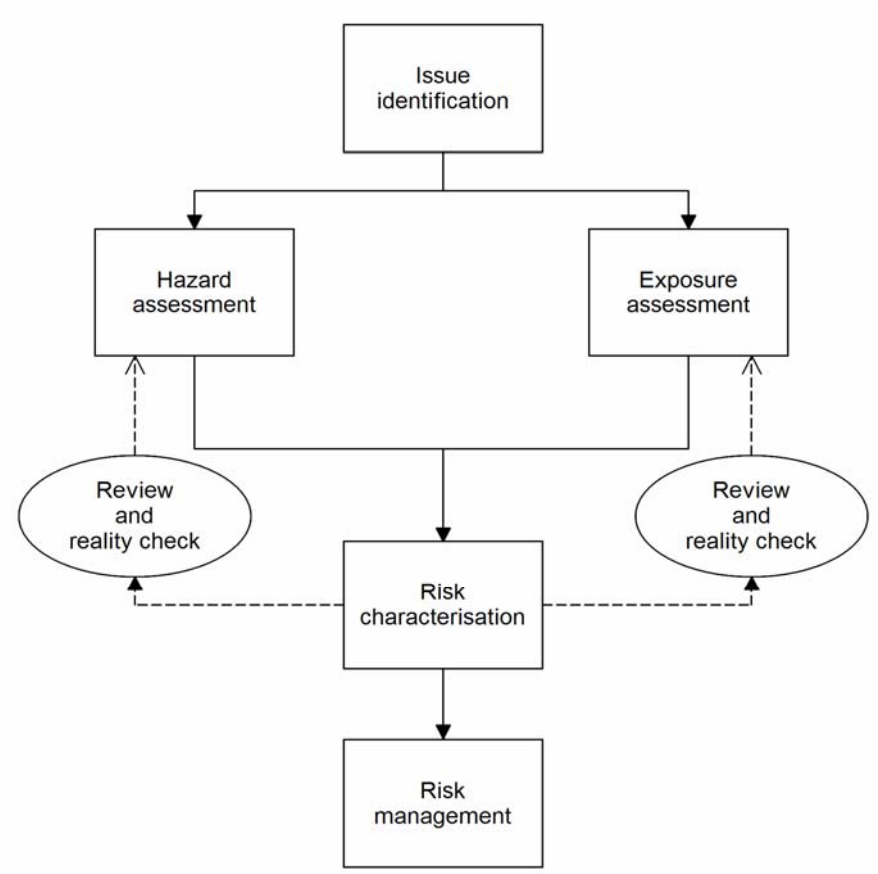

Figure 4. Risk assessment model proposed in enHealth (2004).

5.1 Hazard assessment – deriving a guideline

In the context of drinking water, the purpose of hazard assessment is to define an acceptable drinking water concentration based on the toxicity of the identified toxicant. The Australian

Guidelines for Water Recycling (Phase 2) (NRMMC/EPHC/NHMRC, 2008) propose a thorough framework for determining a drinking water guideline value for chemicals.

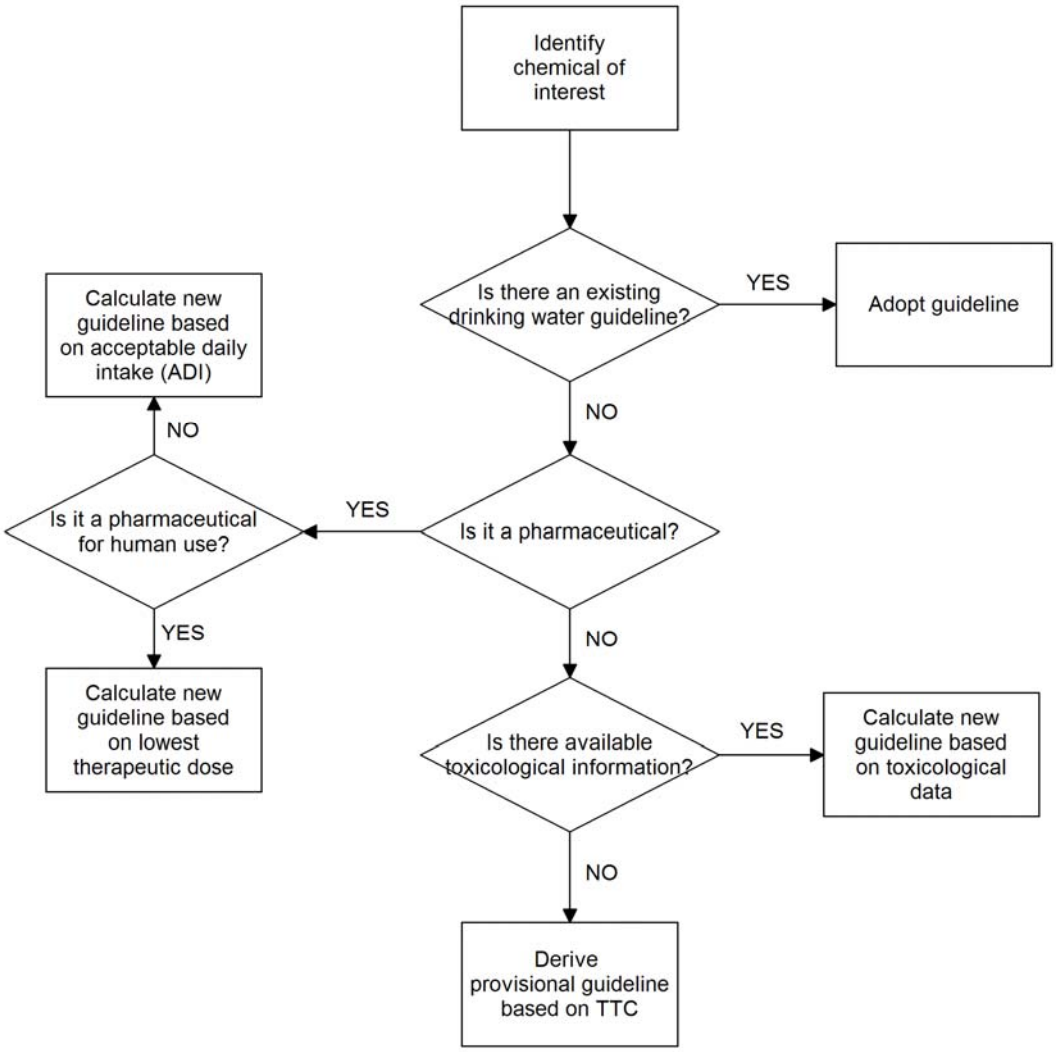

Figure 5. Step-wise decision tree to adopt a drinking water guideline for a new chemical.

5.1.1 Is there an available guideline?

The first step is to determine whether a guideline exists for the toxicant. There are several guidelines for chemical safety in drinking water, including, (in order of authority in an Australian context):

- Australian Drinking Water Guidelines (NHMRC/NRMMC, 2004)
- Australian Guidelines for Water Recycling (NRMMC/EPHC/NHMRC, 2008)
- World Health Organisation (WHO) Guidelines for Drinking Water Quality (WHO, 2004)
- European Union Drinking Water Directive 98/83/EC (EU, 1998)
- New Zealand Drinking Water Standards (Ministry of Health, 2005)
- Guidelines for Canadian Drinking Quality (Health Canada, 2008)

- USEPA Drinking Water Contaminants List (USEPA, 2009a)
- USEPA Drinking Water Health Advisories (USEPA, 2009b)

Australian guidelines should be used in the first instance. However, it is important to appreciate that all of the above guidelines are derived from the best available toxicological data (at the time) and are independently reviewed and updated periodically. As such, all of these authoritative guidelines have been set to a high standard and can be used with confidence in assessing toxicity.

If there is no available guideline value, one may need to be derived.

5.1.2 Deriving an interim guideline value for drinking water

Chemicals may be detected in the water supply for which no guideline value has been established. In such cases, interim guideline values can be set by toxicologists or other health professionals, for the protection of public health. The process involves consideration of information on exposure and dose-response relationships and is broadly depicted in Figure 5 and outlined in the World Health Organization Guidelines for drinking-water quality (WHO, 2004).

Expert judgement is required to derive a guideline value as it is necessary to select the most appropriate study from the available database. The two principal sources of toxicological information are studies on human populations and studies using laboratory animals – data from well conducted studies, where a clear dose-relationship has been demonstrated, are preferred.

Most toxicants are 'threshold chemicals' – i.e. there is a dose below which no adverse effects will occur. For such chemicals, a tolerable daily intake (TDI) should be derived using the most sensitive end-point in the most relevant study (preferably involving administration in drinking water), and the incorporation of uncertainty factors to allow for sources of uncertainty or database deficiencies. The guideline value is then derived from the TDI taking into account default assumptions such as the body weight of individuals, the proportion of total intake attributed to drinking water, and daily drinking water consumption volume. For 'threshold chemicals' (mostly genotoxic carcinogens), guideline values are derived using mathematical models that estimate risk at a particular level of exposure. In this case, guideline values are described as the concentration in drinking water associated with an estimated upper-bound excess lifetime cancer risk of 10^{-4}, 10^{-5}, or 10^{-6} (i.e. one additional cancer per 10,000/100,000/1,000,000 of the population ingesting drinking water containing the toxicant at the guideline value for 70 years) .

In some instances, guideline values can be set for toxicants for which there is uncertainty in the toxicological data. In setting interim guideline values, consideration needs to be given to other sources of the toxicant, such as food or air, as drinking water may only be a minor contributor to overall intake of the toxicant.

The overall process of deriving a guideline value for a given toxicant requires expert judgement and careful consideration of the available scientific evidence. International risk assessments need to be considered, along with the published, peer-reviewed scientific literature, and as such, the derivation of guideline values should not be attempted without the appropriate expertise.

5.1.3 Mixture toxicity

Toxicity testing can provide a measure of the combined effects of mixture of toxic compounds. *In vitro* bioassays generally do not integrate complex mixture interactions (*e.g.* where multiple cell types or organ systems are involved), and as such cannot provide a complete evaluation of mixture toxicity - they do, nevertheless, provide a measure of mixture toxicity for compounds with a similar mode of action.

Where mixtures are of similar compounds with the same mechanism of action such as with the dioxins, furans and co-planar PCBs (Polychlorinated Biphenyls), then the additive effect of the compounds can be assumed using the sum of the toxic equivalency factors (TEFs). Where the mechanistic process is different this is not possible. In general the likely toxicities are assumed conservatively to be additive despite the possibility that the interaction is antagonistic.

The need to evaluate interactions of the components within a mixture has been recognized by the US Agency for Toxic Substances and Disease Registry (ATSDR) in their development of interaction profiles. In one of those interaction profiles, they note: "*Weight-of-evidence analyses of available data on the joint toxic action of mixtures of these components indicate that scientific evidence for greater-than-additive or less-than-additive interactions among these components is limited and inadequate to characterize the possible modes of joint action on most of the pertinent toxicity targets. Therefore, it is recommended that additivity be assumed as a public health protective measure in exposure-based screening assessments for potential hazards to public health from exposure to mixtures of these components*" (ATSDR, 2004). Synergy is an unlikely event and in this context extremely rare (Borgert, 2004).

5.2 Exposure assessment – how much are humans exposed to?

The risk posed by a toxic compound is minimal if there is no exposure. For example, if a toxicant present in source water is effectively removed by water treatment processes, then there is no exposure to humans from drinking water and the risk to humans drinking the final treated water is minimal (largely residing in the risk of engineering breakdown of the water treatment process). Likewise, if a toxicant is degraded at low pH such as occurs in the stomach, the human exposure can be significantly decreased. Exposure assessment must relate to actual exposures, and not to the mere presence of materials in source waters.

Once a toxicant has been identified and suitable analytical methods are available, it is relatively straightforward to determine its concentration in drinking water. This should be determined at the tap, as chlorination and residence in the distribution system can affect the chemical composition of the water. Combined with an estimate of ingestion, which for drinking water in Australia is generally assumed to be 2L / person / day (enHealth, 2004; NHMRC/NRMMC, 2004), the external exposure dose can be calculated as concentration in the water × daily ingestion rate. From this external exposure dose it is then possible to estimate the internal dose using physiologically-based pharmacokinetic (PBPK) models (Simmons et al., 2005). PBPK models are generally specific for individual chemicals, and may not always be applicable. It is also possible to estimate the internal dose empirically by measuring the concentration of the chemical (or its metabolite) in biological tissues or fluids (*e.g.* blood, urine, hair, adipose tissue, bound to a target molecule etc) or by measuring biomarkers of exposure (*i.e.* a biological effect that occurs as a result of human exposure to the chemical, such as alkylated haemoglobin or changes in enzyme induction etc) (IPCS, 1999). In

the absence of data on pharmacokinetics, it is conservatively assumed that 100% of the chemical is absorbed (*i.e.* external dose = internal dose).

It is this internal dose that is relevant to human health, as this is the dose that organs will be exposed to. The concentration of a chemical inside humans can be significantly lower than that in the source water because of the barriers (drinking water treatment plant, distribution system, gastric pH, absorption from gastro-intestinal tract, metabolism in the liver etc) between the two (see Figure 6 for a hypothetical example).

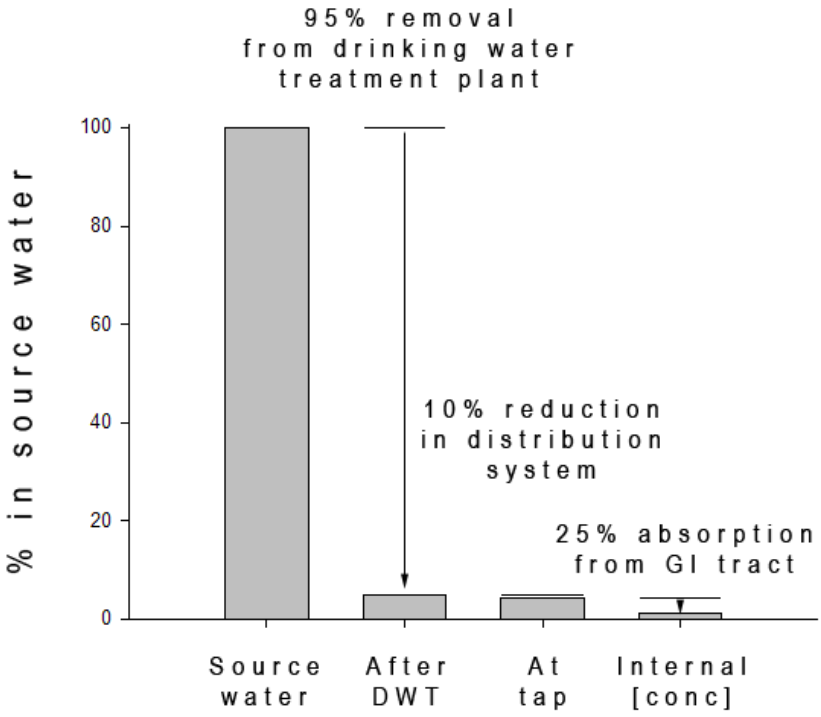

Figure 6. Concentration of a hypothetical chemical as a percent of the source water concentration.

As previously stated, this is an important limitation when extrapolating *in vitro* bioassays results to human health outcomes, because *in vitro* bioassays provide a measure of external exposure and do not take into account the possibly significant influence of toxicokinetics (absorption, distribution, metabolism and excretion) in the overall toxicity.

The final dosage is a result of both the concentration of the substance in water and the duration of exposure. The results of both *in vitro* and *in vivo* toxicity testing must therefore be extrapolated carefully, as there can be significant differences in exposure duration between a cell or an aquatic organism used in the test (exposed continuously, 24h a day) and an exposed human (who is only exposed while drinking, when the toxicant is present in the drinking water). Both *in vivo* and *in vitro* tests can thus overestimate toxicity. It is important to have an understanding of differences in

exposure duration between the test system and the human situation to critically evaluate direct toxicity tests.

For toxicants in water, the major route of human exposure is usually from drinking water. However, the human health risk from recreational use of the water should also be considered. The obligations in this respect are covered by the guidelines for recreational water quality (NHMRC, 2008), and a framework for risk assessment of recreational water is covered in the enHealth guidelines (enHealth, 2004).

5.3 Risk characterisation – what is the risk?

The final step of risk assessment is risk characterisation. The questions here are "What is the risk? To what? And from what?". The final evaluation combines information from all sources (*in silico*, *in vitro*, *in vivo*, epidemiology etc). All information is critically evaluated and weighed into a final measure of risk.

It is important to understand the limitations of the methods that were used and do a "reality check" to ensure a meaningful assessment of risk can be achieved with the available data:

- Was the toxicant conclusively identified? Were there any confounding factors?
- What is the nature of the toxicity data? Is it relevant to human health outcomes? Is the mechanism of toxicity understood? Is there enough information about the duration of exposure and the toxicokinetics of the toxicant(s) to meaningfully extrapolate bioassay data? Are PBPK models available, and are they valid for the toxicant? If there is epidemiological data, is it biased? How significant is the effect?
- How was the concentration of the toxicant in water determined? Was it measured in the relevant water matrix? Are environmental influences (*e.g.* partitioning, transformation) understood? Did it occur intermittently? If so, was it measured at the right time?

The final assessment is a weight-of-evidence assessment, based on a sound understanding of the data and its meaning.

While this document has focused on the identification of compounds that may pose a risk to human health from drinking water, it is important not to neglect other possible routes of exposure from water. The degree of risk is driven by the level of exposure, which generally means that the risks associated with gastrointestinal exposure to drinking water are higher than sanitary, pulmonary or recreational exposure. The outcomes of ingestion are also generally more profound than from dermal exposure. Nevertheless, risks from other routes of exposure should not be ignored.

Finally it must be highlighted that human health risk assessment is only a part of the full risk assessment. A toxicant in raw untreated water may also pose a risk to ecosystem health, and ecological risk assessment should be carried out within the framework of the guidelines for water quality and monitoring for freshwater and marine organisms (ANZECC/ARMCANZ, 2000). Is the ecosystem impacted? How does it compare with carefully-selected reference sites?

5.4 Risk management

Risk (the probability of harm) is the result of both hazard and exposure. Controlling exposure provides a means to mitigate risk. The risk can be managed by:

- Preventing the process producing the risk, in other words dealing with the problem at the source rather than attempting to remove it ("source control"). This is not always possible, particularly when dealing with a natural toxin, but clearly the method of choice when dealing with industrial contamination.

- Reducing or eliminating exposure. This is can be achieved by engineering / operational solutions (*e.g.* not drawing water during flood periods, moving offtake locations, additional treatment steps) and the implementation of critical control points.

Once instigated, it is important to monitor and evaluate the effectiveness of the actions taken, to ensure that the risk is indeed properly managed. Such actions are likely to require the cooperation of risk assessors, water authorities and regulators, and communication with consumers is an aspect that requires consideration throughout.

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6 Conclusions

- A tiered approach combining chemical analysis and toxicity testing can help screen a water source for regulated and unregulated toxicants
- If toxic effects are detected in wildlife, the identity of the toxicant needs to be determined because it could potentially have an effect in exposed human populations
- Toxicity identification evaluation (TIE) procedures can be used to attempt to identify the toxic substance
- Once identified, all sources of information (*in silico*, *in vitro* and *in vivo*) need to be considered to accurately evaluate the risk to human health, keeping in mind the limitations of each of these sources:
    - *In silico* methods are based on chemical structure and physico-chemical properties, and the information generated is highly dependent on the reliability of the model used
    - *In vitro* bioassays provide a measure of primary toxicity, without integration of toxicokinetics (absorption, distribution, metabolism and excretion) or detoxification mechanisms available *in vivo*. As such, they can overestimate toxicity
    - *In vivo* bioassays provide a more relevant measure of secondary toxicity, however possible differences between the test species and humans as well as exposure duration need to be considered
- It is important to understand the mechanism of toxicity to determine the relevance of each source of information
- Risk assessors will never have all the information they need, and real-life risk assessment must rely on a weight-of-the-evidence approach
- Risk assessors, water authorities and regulators will need to work together in response and risk management actions when required.

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