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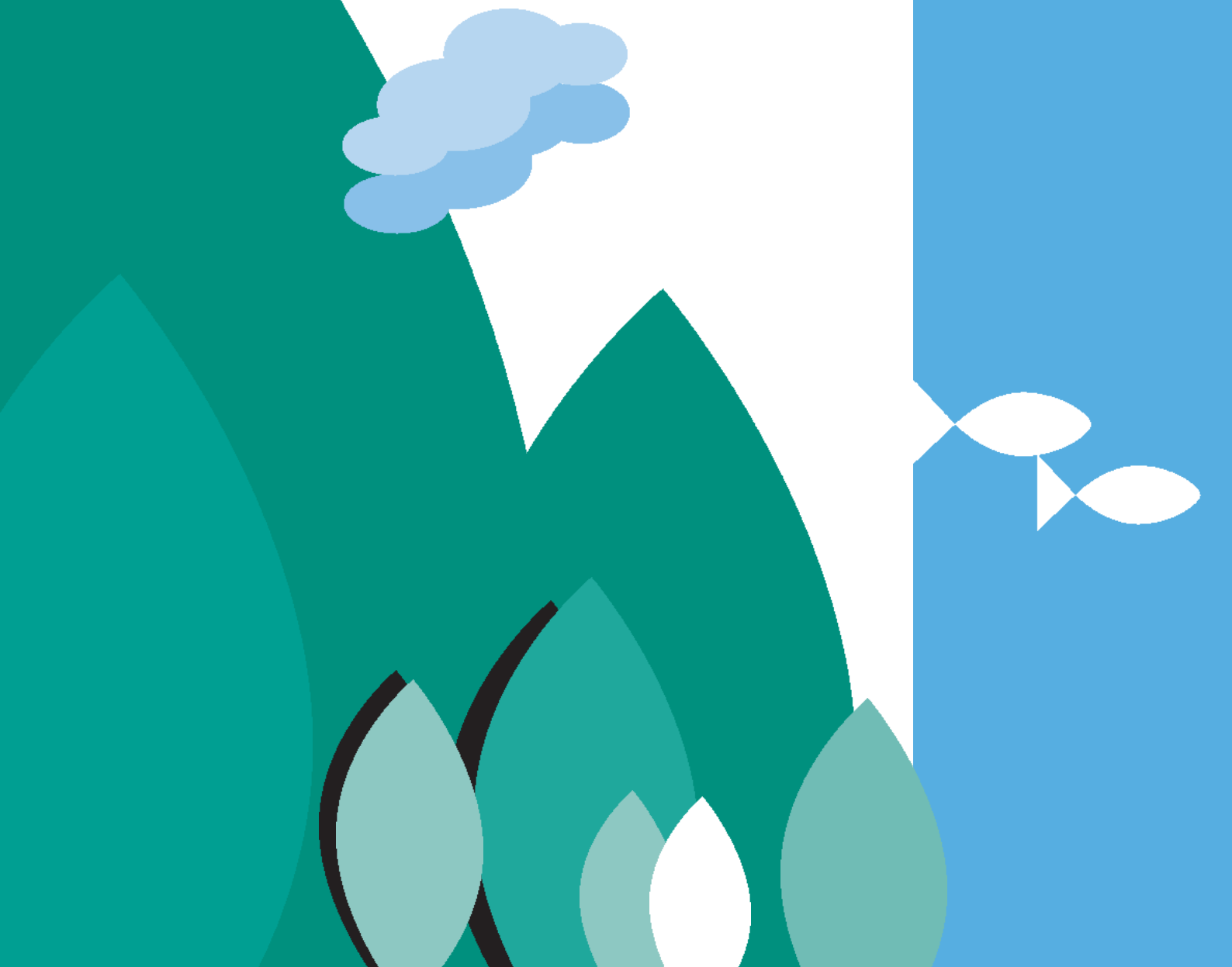

AUSTRALASIAN JOURNAL OF ECOTOXICOLOGY
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AIMS AND SCOPE

The Australasian Journal of Ecotoxicology is an international journal published three times a year by the Australasian Society for Ecotoxicology. It is dedicated to publishing scientifically sound research articles dealing with all aspects of ecotoxicology. This includes acute and chronic toxicity studies measuring lethal and sublethal responses, ecological impact studies pertaining to ecotoxicology, studies relating to the mechanisms of chemical toxicity, discussion of ecotoxicological theory and use of ecotoxicology information to develop and validate environmental criteria. Studies at all levels of biological organisation will be considered: from (sub-) cellular and individual organisms to population and community studies. All data must be generated by statistically and analytically sound research, and all papers will be peer reviewed by at least two reviewers prior to their being considered for publication. The Journal will give priority to the publication of original research that is undertaken on the systems and organisms of the Australasian and Asia-Pacific region, but papers will be accepted from anywhere in the world. As well as scientific papers, the Journal will contain short communications, to allow the publication of original data generated in small-scale projects, and letters to the Editor are most welcome. The Editor will commission and publish reviews from time to time. Authors interested in publishing review articles are invited to contact the Editors. Titles of completed PhD and MSc theses will also be published.

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AUSTRALASIAN JOURNAL OF ECOTOXICOLOGY

VOLUME 16, NUMBER 3, SEPTEMBER 2019

PAPERS

ECOTOXICOLOGY IN AUSTRALASIA AND THE AUSTRALASIAN JOURNAL OF ECOTOXICOLOGY: AN HISTORICAL PERSPECTIVE

John C Chapman
Formerly, Office of Environment and Heritage. PO Box 29, Lidcombe NSW 1825

ABSTRACT

As the *Australasian Journal of Ecotoxicology* (AJE) finishes its 16 volumes of publication with this issue, it is interesting to reflect back on its achievements since its inception in 1995 and how well it has addressed issues of importance to ecotoxicology in the Australasian region. Several editorials and reviews in the first few volumes addressed the need to fill knowledge gaps in such areas as method development, sensitivity of native species and developing regional test species, addressing environmental parameters that affect toxicity, developing environmental guidelines for water, sediment and soil, developing robust biomonitors, bioaccumulation and new and emerging chemicals. Progress has been made in many of these areas since 1995 and some of this has been reflected in AJE publications. Some reflections on the future areas for ecotoxicology are also provided.

Key words: Ecotoxicology; Australasian publications; historical development.

INTRODUCTION AND EARLY HISTORY OF ECOTOXICOLOGY IN THE REGION

In Australia, there was increasing activity in ecotoxicology through the 1970s and 1980s. The Commonwealth's Office of the Supervising Scientist facilities (now ERISS) at Jabiru in Kakadu National Park was established in 1976 for the purpose of monitoring the Ranger Uranium Mine, of which ecotoxicity tests formed a significant component. A range of tropical species were being developed for laboratory ecotoxicity tests and creekside monitoring of Magela Creek and other waterways. Some of the early work in Victoria was on the marine amphipod *Allorchestes compressa* (eg. Ahsanullah 1976) and copepods (Brand et al. 1986), and on a range of freshwater species at the Arthur Rylah Institute (Bacher and O'Brien 1990), as well as in Barry Hart's group at Chisholm Institute of Technology (now Monash University) (eg. Daly et al. 1990). There was also ecotoxicology undertaken at Griffith University, Queensland, on pesticides, metals and organochlorine compounds (eg. Mortimer and Connell 1994) and on pesticides in Tasmania (Davies et al. 1994). In NSW, ecotoxicology had been undertaken in at least two CSIRO groups on metals and pesticides (eg. Mann and Florence 1987; Korth et al. 1995) and at the NSW Institute of Technology (now University of Technology Sydney UTS) (Skidmore and Firth 1983; Mitchell et al. 1988). In fact, there had been extensive soil and plant toxicology studies throughout Australia for many years (Markich et al. 2002) and some aquatic ecotoxicology studies were being carried out in the 1960s (eg. Wisely and Blick 1967).

This concurrent work of ecotoxicology facilities scattered throughout Australia was recognised at the time and a workshop was held at the Chisholm Institute of Technology (later Monash University) in November 1984 (Hart 1986) to exchange knowledge, develop networks in the area of ecotoxicology and to advance the science. Dr Vince Brown, an 'elder statesman' of ecotoxicology from the UK, guided the proceedings with input from various ecotoxicology groups including those mentioned above, and the workshop proceedings (Hart 1986) outlined the activities in ecotoxicology in Australia and recommended progress forward, including establishment of an ecotoxicology database, which was published in AJE.

In 1984, Professor Margaret Burchett of UTS and Dr David Leece of SPCC were instrumental in establishing the Centre for Ecotoxicology (CET; then called Centre for Environmental Toxicology) as a joint venture between the UTS (then NSW Institute of Technology) and the, then, State Pollution Control Commission (SPCC; later NSW EPA, having gone through a number of name changes, and now two separate institutions: the NSW Office of Environment and Heritage and the NSW EPA). The Centre, initially directed by Dr Geoff Thompson, was originally intended to help develop ecotoxicity data for Commonwealth legislation on industrial and agricultural chemicals, and for NSW legislation on hazardous chemicals, although it was considered adequate to source much of the data from overseas (Johnston et al. 1990). The facilities, located at the UTS building at Gore Hill from 1984 until 2003, developed a range of ecotoxicity tests (eg. Julli et al. 1990; Mitchell et al. 1988), including acute and reproductive impairment tests with *Ceriodaphnia* cf *dubia*, a species originally sourced from Lake Parramatta (Julli et al. 1990) and sent to many laboratories around Australia. The annual seminars at CET, which drew in a variety of researchers, eventually morphed into the ASE conferences.

Around this time, ecotoxicology was gaining impetus in New Zealand (Hickey 1995a) with the influence of the New Zealand Resource Management Act (1991) (Hickey 1995b), which requires that discharges to receiving waters do not cause "adverse effects" after allowing for "reasonable mixing". Tests were developed with locally relevant freshwater and marine species (Hickey 1995a) and the major issues outlined were sensitivity of native species, detection of effects at low levels of contamination and addressing interpretation of the RMA Act. Research and consulting studies have encompassed both freshwater and marine environments with field monitoring around discharges, experimental manipulation

using chemically-dosed sites and laboratory-based toxicity bioassays with effluents and sediments. The small scale of sources αvδ ρelatively recent industrial development has meant that the extent of environmental contamination in NZ is limited, largely covering agricultural sources, mining, forestry, urban stormwater and geothermal issues. Terrestrial research has been undertaken at Landcare Research, Manaaki Whenua, Lincoln; there had been considerable research, including ecotoxicological studies, into effects of sodium monofluoroacetate (1080) on target and non-target species (Eason 1997) and on studies with marsupials, soils and other aspects of terrestrial biodiversity.

THE LEGISLATIVE ENVIRONMENT

As mentioned above, the work of the Office of the Supervising Scientist (now ERISS) was legislated in 1976 to monitor the Ranger Uranium Mine at Jabiru, within Kakadu National Park, although in recent years this work has expanded throughout the Northern Territory. Elsewhere in Australia, there is little direct legislative requirement for ecotoxicity testing, unlike in the USA (Bradbury 1995), although it has been incorporated in some specific licence conditions or pollution reduction programs (Chapman 1995a, b). The water quality guidelines (ANZECC and ARMCANZ 2000) have focussed work on the final tier of risk assessment for toxicants, where direct toxicity assessment is recommended to determine if a chemical causes toxicity at a specific site (van Dam and Chapman 2001).

In New Zealand the New Zealand Resource Management Act (1991) (Hickey 1995b) has stimulated research since 1986 into establishing techniques for assessing and mitigating adverse effects on the environment. This Act requires that discharges to receiving waters do not cause "adverse effects" after allowing for "reasonable mixing". This legislative requirement has further stimulated research to assess considers ecological effects of contaminants, whether they be toxic or natural indicators.

ASE AND AJE

The Australasian Society for Ecotoxicology (ASE) was established at its first conference at UTS in July 1994 to bring together the disparate groups in ecotoxicology in Australia and New Zealand and to facilitate interaction between scientific groups, government and industry. This was due in large measure to Dr David Leece of (then) EPA and Professors Margaret Burchett (UTS) and Des Connell (Griffith University) and Drs Graeme Batley of CSIRO and Chris Hickey of NIWA, NZ, as well as a number of others. Volume 1 of the *Australasian Journal of Ecotoxicology* was published in July 1995, featuring some of the papers presented at the Conference. ASE brought together ecotoxicologists and environmental chemists in the Australasian and Asia-Pacific region to a community that, in 2011, became the Australasian chapter of the Society of Environmental Toxicology and Chemistry (SETAC-AU), as part of SETAC-Asia-Pacific (SETAC-AP).

During the lifetime of AJE a number of editorials, opinion pieces and reviews were published that summarised the state of ecotoxicology in the region and speculated on the future of the science. I will reflect on many of these below and how these predictions and evaluations played out, with particular emphasis on papers published in AJE.

It must be recognised that much of the work of ecotoxicologists within ASE would have been published within high impact journals such as *Environmental Toxicology and Chemistry, Aquatic Toxicology* and others, but a review of the 208 papers published in AJE up to the second issue of Volume 16 demonstrates that AJE was certainly not "left with the dregs" and published a wide range of quality papers covering a representation of many significant and ground-breaking studies. A review of those papers over the 16 years, from around 600 authors (over 400 different authors), revealed that they were from some 150 institutions, with 97 institutions from the strict geographical Australasian region (Australia, New Zealand and Papua-New Guinea). At the outset, ASE sought to be interactive with neighbouring regions (and "Australasia" was defined rather loosely). Hence there were also papers representing 60 institutions and researchers from SE Asia and Japan (19), North America (15), Europe (19) and the Indian sub-continent (including Sri Lanka) (7). The papers are a reflection of the widespread collaborations that institutions undertook within the geographical region, and continue to undertake through SETAC-AU and SETAC-AP.

I found it difficult to clearly categorise each paper into clearly defined 'boxes' but I have attempted to do so (others may come up with different categories and allocations), noting that there is no intention to imply any ranking of importance, quality or value in these categories. The categories I devised were: Reviews (51 papers, 25% of total papers; some of these also presented original data); method development papers (33; 16%); field or mesocosm studies (15; 7%); papers on biomarkers (24; 12%); endocrine disrupting chemicals (EDCs as additional category; 15; 7%); biological monitoring (13; 6%); terrestrial ecotoxicology (31; 15%); complex effluent assessments (24; 12%); sediment testing (9; 4%); risk (12; 6%); environmental quality guidelines as a separate category (23, including 15 in Volume 7; 11%); how physiological parameters affect toxicity (13; 6%); residues in the environment (20; 10%); bioaccumulation as a separate category(17; 8%) papers dealing with policy issues (11; 5%). As many of these topics overlapped, the total is more than 100%, and I recognise that others may come up with different categories.

AJE has published over 50 review papers, some on broad topics covering major aspects of ecotoxicology (Bradbury 1995; Calow 1995; Manning 2005) or on focussed topics, such as particular chemicals (eg. endosulfan – Hose et al. 2003; copper – Kamunde and Wood 2004; the rodenticide 1080 – Eason 1997), types of tests (phytotoxicity – Burchett and Pulkownik 1995; mesocosms – Ward and Jacoby 1995) or endpoints (eg. fish immune responses – O'Halloran et al. 1998) or regional overviews (eg. Widianarko and van Straalen 1997). Many of these helped filled major knowledge gaps, including detoxification in marsupials (Bolton and Ahokas 1995; El-Merhibi et al. 2007), phytoremediation (Chaudhry et al. 1998) and endocrine disrupting chemicals (Manning 2005).

COMMENTARIES FROM AJE ON ECOTOXICOLOGY IN THE REGION

In my opening editorial (Chapman 1995a) I reflected on some aspects that were, at that time, not well addressed in ecotoxicology. These included the need to determine what ecological properties to protect and what test endpoints would reflect those properties. In addition there was the need to develop environmental quality criteria for water, sediment and soil, developing discharge limits, applying ecological risk as a framework for assessing impacts of new and existing pesticides and industrial chemicals, applying test data over different climatic regimes. At that stage the extent of endocrine disrupting chemicals in the environment was largely unknown. Hickey (1995a, b) came up with a similar view, noting also the need to understand the sensitivity of native species and developing practical biological monitoring techniques that complemented existing chemical analyses and better informed the environmental managers. Issues in New Zealand at that time included urban runoff into estuarine environments, sediment contamination (and the need to develop sensitive, sublethal sediment toxicity tests) and better relating laboratory and field effects from agricultural runoff.

Wu (1996), from a south-east Asian perspective, also noted the paucity of ecotoxicological data relevant to Australasia and the Asia-Pacific regions, particularly in the light of the fast economic growth, which frequently overrides environmental concerns. He raised the need for common key species and techniques to help arrive at comparable results within the region and how physiological, hydrographic and climatic scenarios affect test results. Widianarko and van Straalen (1997) also viewed ecotoxicology from a SE Asian perspective and observed the, at that time, very low participation rate in scientific publications by SE Asian scientists (<1% in six journals evaluated). The authors clearly spelt out the burgeoning environmental issues of the region, due to industrial growth and waste disposal, pressures on agricultural production and increased pesticide use, land clearing, increasing population coupled with greater affluence, increasing urbanisation. They noted the species richness of the region as being a potential scientific asset and the need for ecotoxicology in SE Asia to take into account the environmental complexity of the region to help determine the effects of chemicals on these systems. Over the 16 volumes, 19 papers could be attributed to SE Asian scientists (including Japan), which at 9% still leaves room for improvement and suggests that some of the barriers to publication that Widianarko and van Straalen (1997) identified are still there. I did not repeat the survey that they did for the six journals (it would be best carried out by the same authors using the same methodology) but a scan of just one of these, the first three issues of each year from 2008 to 2010 of the SETAC journal *Environmental Toxicology and Chemistry* (covering around the same number of articles as those in AJE) showed around 4% of papers from SE Asia (including Japan and Korea but excluding China, which was well represented there but not in AJE).

Peter Chapman (1998) canvassed a number of issues impacting on ecotoxicology, including hormesis, nutrient enrichment, the need to develop relationships between chemical toxicity and body burden, sediment toxicity. He concluded that there is a need to address multiple working hypotheses (ie. there are often "more than one potential explanatory/causative agent") and not to dismiss those results that don't fit our pre-conceived hypothesis.

Holdway (2006) lamented how we recycle old issues with new and emerging chemicals. These included bioaccumulation and biomagnification from brominated flame retardants and perfluorinated compounds, and groundwater contamination with arsenic.

HOW HAVE SOME OF THESE ISSUES WORKED OUT OVER THE ENSUING 15-20 YEARS?

I will be focussing mainly on publications in AJE, while recognising that many researchers in this region will have published in higher impact international journals; where appropriate for illustration, I will refer to some of these.

Developing regional methods and understanding the sensitivity of native species

One of the important functions of AJE was to report on the development of ecotoxicity test methods that may contribute to understanding sensitivity of native species (Hickey 1995b), matching test endpoints to ecological properties (Chapman 1995a) and contributing to regional test species (Wu 1996).

AJE papers with ecotoxicity methods have reflected just some of the developments in tests with species native to the region, sometimes adapting overseas tests to local species and/or conditions or pioneering new tests (eg. Kefford et al. 2007). By 2000 there were at least 18 freshwater species and 24 marine species available for general use within the region (van Dam and Chapman 2001), with more species developed for research purposes, and many more have been added to the list over that past 15 years. The number of species reported in AJE as having been tested in both laboratory and field studies (excluding those field studies that focus on diversity and abundance and excluding reviewed data) is over 120, comprising 50 freshwater, 43 marine species and 31 terrestrial species. The methods reported in AJE over the past 16 volumes covered a wide range of aquatic and terrestrial plants, invertebrates and fish, as well as a few mammals, birds, reptiles and amphibians (Table 1).

Most of the 33 papers that I classified as 'method development' papers examined the species' responses to a range of chemicals, often on complex effluents or field waters or as part of multispecies evaluations (eg. Binet et al. 2003; Ross and Bidwell 2006). Frequently, methods reported were used multiple times in AJE papers and elsewhere (eg. the marine diatom test with *Nitzschia closterium* reported by Stauber (1995) was picked up in at least four papers (eg. Ross and Bidwell 1999; Binet et al. 2003; van Dam et al. 2008). While no one species has been classified as a common key regional test species (Wu 1996), Nitzschia closterium has been most commonly cited in AJE for marine testing, while the most common freshwater test species reported was *Ceriodaphnia dubia* (Julli et al. 1990), in 6 papers, reflecting their common usage throughout the region.

Table 1. Numbers of species reported in AJE from Volume 1 to 16#2.

Organisms	Fresh/Mar/Terrest	No of species	Number native to region*	No of papers
Mammals	Terrestrial	4	2	7
Birds	Terrestrial	1	1	1
Reptiles	Fresh	1	1	1
Amphibians	Fresh	2	1	1
Fish	Fresh	12	5	14
Fish	Marine	9	7	13
Crustaceans	Fresh	11	11	17
Crustaceans	Marine	9	7+	9
Insects	Fresh	19	19	4
Molluscs	Fresh	4	3	3
Molluscs	Marine	13	9	15
Other aquatic invertebrates	Fresh	1	1	1
Other aquatic invertebrates	Marine	5	5	5
Terrestrial invertebrates	Terrestrial	9	4	10
Algae	Fresh	3	3	4
Algae/macro-algae	Marine	7	7	6
Macrophytes	Fresh	3	2	3
Terrestrial plants	Terrestrial	27	13	8
Bacteria	Marine	1	?	4
In vitro	N.A	3	0	5

* 'Native' does not imply 'endemic' (although some were endemic). + Excluding 1 Antarctic species; N.A = not applicable

Although aquatic ecotoxicology dominated the publications in AJE, there were a number of developments in other areas of ecotoxicology reported in AJE, starting with the review of plant ecotoxicology in Australia by Burchett and Pulkownik (1995). In general, terrestrial methods have been under-reported, and despite terrestrial species comprising around a quarter of all those reported in AJE, these were in just 26 papers, while only one of these I classified as a method development paper (Burchett and Pulkownik 1995), which summarised the progress in terrestrial plant tests, building on earlier work by Mitchell et al. (1988) and others. There were, however, a few papers that provided data on soil invertebrates that may help to contribute to soil quality guidelines (eg. Nursita et al. 2005; Iwai and Noller 2008). The review by Sheppard et al. (2005) was the only AJE paper that specifically touched on soil quality guidelines; such papers were published over this period in in grey literature and other journals (eg. Heemsbergen et al. 2009).

As mentioned earlier, around 24 papers used ecotoxicology to determine the response of species to effluents and complex mixtures, whether using one or two species (eg. Korth et al. 1995; Twining and Nowak 1996) or a full suite of multi-species and/or multiple-endpoint tests (eg. Binet et al. 2003; Ross and Bidwell 1999, 2006). It is in this area that ecotoxicology can greatly contribute in determining overall toxicity and helping to define discharge limits. One of the challenges of this approach is being able to obtain representative samples when the mixtures are non-homogeneous or vary with time.

AJE hosted a number of database papers that collated the Australasian ecotoxicity data on species relevant to the region for metals (Markich et al. 2002; Langdon et al. 2009; 343 species for fresh, marine, sediments and soil), pesticides (Warne et al. 1998; 65 species) and organic chemicals (Warne and Westbury 1999; 30 species). As well as underlining the extent of ecotoxicology work undertaken in the region, these database papers have provided a basis for deriving water quality guidelines (discussed below) using Australasian data and identifying significant data gaps, provide better understanding of the sensitivity of native species (Hickey 1995a, b) and helped to address the paucity of ecotoxicological data relevant to Australasia and the Asia-Pacific regions identified by Wu (1996). Identifying the relative sensitivity of native species has been addressed by papers published elsewhere (Johnston et al. 1990; Kwok et al. 2007); there is as yet still not a clear consensus on whether native species are inherently more sensitive or not but it may be that the literature so far on this topic has established that such a focus is no longer needed but that use of native species, especially for direct toxicity assessment, is preferable wherever possible. The database papers have identified regional tests on 41 pesticides, 119 industrial organic chemicals and 22 metals and metalloids.

There are times when those outside the discipline of ecotoxicology have expressed the thought that ecotoxicology is unrelated to real-world conditions, as it is confined to laboratory tests under controlled conditions, which do have

their value of course. Note that decisions will be made on chemicals and 'safe' levels whether data are available or not and laboratory studies provide a controlled estimate of likely cause-effect links that cannot be so easily determined in field studies. Direct toxicity assessment of effluents outlined above go some way to establishing actual effects in the field and they form an important step in the risk approach of the Australian and New Zealand water quality guidelines for toxicants (ANZECC and ARMCANZ 2000; van Dam and Chapman 2001). SETAC-AU and the AJE have always maintained a focus on the use of ecotoxicology in unravelling chemical effects in ecosystems (Chapman 1995b); at least 15 papers have had a direct focus on field ecosystem effects (eg. Connolly and Jones 1996; Roach 1997), while others have addressed this issue from the perspective of mesocosms or field microcosms (eg. Ward and Jacoby 1995; Cox et al. 2007), assessing the impact of natural indicators and physiological effects on toxicity (Thomas et al. 2010) or TIE testing (Golding et al. 2006). There is no one tool that can determine the effect of chemicals on the environment and ecotoxicity testing helps to supply the cause-effect link in determining chemical effects (Chapman 1995b).

Applying ecological risk assessment

The opening ASE conference in 1994 had contributions on ecological risk assessment from USEPA and European perspectives, which translated into review papers in the first AJE issue (Bradbury 1995; Calow 1995). Bradbury (1995) stressed the need for a predictive capability in ecotoxicology, with strategic laboratory and field studies in the context of a modelling strategy that includes ecotoxicological modelling, such as QSARs. The latter point was taken up by Rose et al. (1997) for non-polar narcotic chemicals, by Westbury et al. (2004) for substituted phenols, and by Sharifi and Connell (2003) on dietary accumulation of DDT and chlorobenzenes. The first two listed used laboratory tests with the Australian *Ceriodaphnia* cf *dubia* to validate the models. Calow (1995) outlined the European scene at the time, for which the practical outworking of the quotient method, with its arbitrary safety factors, did not match the formal probability statements in the text of legislation. There was considerable scope for ecotoxicology in making the practice closer to the principles, such as in better and more ecologically relevant tests, terrestrial tests to inform risk assessment and feedback from monitoring programs. I have expanded on these aspects elsewhere.

The authors of the dozen AJE papers on risk assessment used various approaches for assessing risk. Probabilistic Bayesian approaches were advocated by Pollino and Hart (2005) and Pollino et al. (2008). Although the mathematics can seem daunting, Bayesian approaches are valuable in that they allow incorporation of a variety of data including qualitative information on such poorly understood attributes as pollution pathways and mechanisms and interactions between physical, chemical and biological factors in complex ecosystems. The programs currently available are reasonably user-friendly. As the authors state, understanding of Bayesian Networks provides *"useful tools in risk assessments for quantifying ecological effects and evaluating the effectiveness of management scenarios in situations where the cause-effect relationships are complex and poorly known."*

Probabilistic approaches are favoured for the development and application of environmental quality criteria for water, sediment and soil; these could be considered as a subset of risk and a total of 23 papers covered that area. The ANZECC and ARMCANZ (2000) approach to water quality guidelines enshrines a risk assessment approach, both in their derivation and application (Chapman 2001). The 15 papers in the special issue of AJE (Volume 7) on these guidelines covered the full range of the Guidelines, not only for toxicants but also for sediments (with more recent developments by Batley and Simpson 2008), natural indicators, biological monitoring and speciation (Markich et al. 2001). The special issue also drew in viewpoints from The Netherlands (Crommentuijn et al. 2001) and Canada (Caux and Kent 2001). Bioaccumulation has not been well incorporated into the toxicant guidelines (Connell 2001) while some of the statistical improvements with the current revision have been flagged (eg. Fox 2008). The application of the guidelines has been evident in some more recent papers (eg. Hunt et al. 2007; Dunlop et al. 2008).

How physiological and climatic parameters affect toxicity

Chapman (1995a) and Wu (1996) touched on the issue of the influence of climatic factors. Climate change and its potential effect on the fate and effects of chemicals has not featured as strongly in AJE as elsewhere. Jeffree (2009) provided a review on this issue, posing the challenge that *"If ecotoxicologists want to remain relevant to the most important environmental issues and challenges then they need to find ways to scientifically face the 'elephant in the living room' viz. climate change and related effects of CO_2"*. For marine ecotoxicology, incorporation of increasing ocean acidity in ecotoxicological studies is one area of potential investigation (Jeffree 2009). Thermal tolerance in freshwaters and the effect of toxicants may be another (Patra et al. 2007) although the issue of adaptation over time is not one that is easily incorporated into ecotoxicology. There will be continuing and increasing need to incorporate climate change effects into ecotoxicological studies.

However, other papers have touched on related effects such as seasonal and geographic variability of the response of *Mytilus edulis* to chemicals (Williams and Hall 1999) or salinity in warm climates (Dunlop et al. 2008). Other studies have examined how parameters such as salinity and suspended matter in natural waters can affect chemical toxicity (eg. Thomas et al. 2010). The impact of natural indicators such as turbidity and salinity on chemical toxicity still remains a useful area of study.

Biological monitoring techniques

Hickey (1995a) noted the need to develop practical biological monitoring techniques that complemented existing chemical analyses. Biomonitoring was the topic of 13 papers in AJE. The review by Rainbow (2006) outlined the requirements of a good field biomonitor for metals: strong net accumulators of the metal concerned; sedentary; abundant; easily identified and sampled; large enough for analysis; a long-lived species; hardy and tolerant of raised metal bioavailabilities and of physicochemical variation in the habitat; and tolerant of handling in the laboratory and in the field. Not all of these are characteristics suitable for ecotoxicological testing organisms.

At least 10 later papers have applied these requirements to a number of species and situations, as well as introducing novel biomonitoring techniques such as fish ventilatory response (Nielsen and King 1995). Byrne and Vesk (1996) examined the value of analysis of granules freshwater mussel *Hyridella depressa* as a biomonitor of metals. Physiological changes (imposex) in marine molluscs have been used as a biomonitor of the antifouling agent tributyltin (Reitsema et al. 2003). Ross and Bidwell (2006) used biomonitors for lead in a marine environment near a smelter as one of a number of ecotoxicological evaluation tools. Biomonitoring tools have been valuable for the field approach to endocrine disrupting chemicals (Adams and Tremblay 2003; Miranda et al. 2010). Biomarkers, as a separate subset of biomonitors (Tremblay et al. 2000; 24 papers in AJE) and which include field assessments of endocrine disruption (eg. Jones et al. 2000; Ataria et al. 2004), provide a valuable tool for exposure to stressors such as endocrine disrupting chemicals (EDCs; including TBT), oils (Gagnon and Holdway 1998), pesticides (Boonthai et al. 2000; Hodge et al. 2000; Tremblay 2004), and non-specific contaminants in reef fish (Klumpp et al. 2007). The continuing challenge is to determine how changes in biomarkers relate to effects on individual species in the field or in whole communities or ecosystems.

EDCs have been well represented in scientific literature over the past 20 years and AJE has published 15 papers since 2000, including the important wide-ranging review by Manning (2005), which identified major knowledge gaps, including EDC effects on marsupials and monotremes. The EDC papers have covered method development (eg. Jones et al. 2000; Rawson et al. 2010), effects in complex effluents (Kashiwada et al. 2005; Gadd et al. 2005), biomonitoring and field assessments (Codi King and Hassell 2008; Miranda et al. 2010), and specific chemicals (eg. pesticides – Tremblay 2004; nonylphenol – Kim et al. 2005). The research activity in the wider EDC literature has remained strong and this issue should remain of interest to this region.

Bioaccumulation

The chemical measurement and monitoring of contaminants in organisms relies on the fact that certain organisms (or organs within organisms) bioaccumulate specific chemicals or groups of chemicals above the levels found in their environment, whether that be aquatic or terrestrial. AJE reported at least 17 papers dealing with aspect of bioaccumulation, plus three on passive samplers (eg. Muschal 1999; Rose and Kibria 2007). Jeffree (2006) reviewed metals and radionuclides bioaccumulation in marine and freshwater biota to address two questions: "i) *Are there general underlying chemical principles that can explain and predict their patterns of bioaccumulation?* [which has been largely answered]*; and ii) Are there modes of bioaccumulation that are characteristic of particular phylogenetic groupings of organisms?*", the latter a work in progress. For metals, the bioaccumulation studies covered bivalves (Jeffree et al. 1995), fish (Alquezar and Markich 2006), and land plants (eg. Sridokchan et al. 2005). Bolton and Ahokas (1997) reviewed the accumulation and detoxification of chemicals in marsupials. The actual issue, raised by Peter Chapman (1998), on the need to develop relationships between chemical toxicity and body burden, has only partially been addressed by studies in this region or those published in AJE, and there is still some progress to be made in this area.

Sediment toxicity

Hickey (1995b) expressed the need to develop sensitive, sublethal sediment toxicity tests, also repeated by Peter Chapman (1998). There has been considerable activity in the region in sediment ecotoxicology and developing sediment tests appropriate to the region (Simpson et al. 2005). AJE has published nine papers reporting activity in this area, including a review of marine whole sediment tests for temperate and tropical environments (Adams and Stauber 2008), and recent advances in sediment quality guidelines by Batley and Simpson (2008). Chironomids were proposed for freshwater sediment testing by Smith et al. (1999) and amphipods were a common saltwater test species, with the need recognised to develop rapid and sublethal sediment tests to replace the rather unwieldy 28-d acute test (Surtikanti and Hyne 2000). Amphipods were recognised as a useful Antarctic sediment test species (Lane and Riddle 2004), the low temperature extending the acute test period.

New and emerging chemicals

Holdway (2006) noted with dismay the tendency to recycle old issues with new and emerging chemicals. There was only one paper published in AJE that touched on new and emerging chemicals (Manning et al. 2008), such as brominated flame retardants and perfluorinated compounds. I suspect that the cost of analyses has skewed the publication of regional studies into specialist and higher impact journals, even though a number of researchers in the region have been working in these areas.

Environmental chemistry

While ASE did not specifically have an environmental chemistry focus, since corrected by the formation of SETAC-AU in 2011, chemistry has been implicit in almost all the papers published in AJE and the ecotoxicology community has recognised the importance of environmental chemistry in elucidating effects of chemicals on the environment. Understanding metal bioavailability (Kamunde and Wood 2004) with stepwise refinement of chemical tests has been a key feature of the risk approach for the Australian and

New Zealand water quality guidelines (Markich et al. 2001) and measurement of chemical concentrations in ecotoxicity testing is acknowledged as important in ensuring quality of test results (Warne et al. 1998).

Nevertheless, up to 21 papers in AJE have had their primary focus on measuring chemical residues in the environment, apart from those with a focus on bioaccumulation. Chemicals studied have included (but not limited to) hydrocarbons in Kakadu National Park (van Dam et al. 1998); at least five papers on pesticides in irrigation water (eg. Church et al. 2004; Wightwick and Allinson 2007; Davis et al. 2008), metals in pine forests (Denholm 2000) and mining (Baru and Buckney 2003; King et al. 2003), and dioxin in birds (Manning et al. 2008).

FUTURE OF ECOTOXICOLOGY IN THE REGION

As Ross Jeffree (2009) emphasised, climate change remains an issue that can either drive ecotoxicology research or else overwhelm the science (and funding) as concerns are diverted to this huge issue. However, there are opportunities for ecotoxicology here, and if concurrent issues increase, such as pesticide usage, there will be continuing need for ecotoxicology in that area alone.

Tropical ecotoxicology was addressed in the special AJE issue of Volume 14, 2008, as well as in a number of other papers throughout AJE. However, van Dam et al. (2008) noted the pressure on development in the Australian tropics and the "*paucity of fully-developed, regionally-relevant marine toxicity testing methods for Australian tropical marine species*" as well as the need to develop additional sub-lethal/chronic tropical toxicity tests to address site-specific risk assessments. Tropical ecotoxicology is an area of great interest to SE Asia and collaboration with ecotoxicologists from that region will be of mutual benefit. I also expect that there will be increased publications of Antarctic ecotoxicology in coming years.

Chemical issues often gain prominence when there are major spills or incidents but at other times they can remain hidden, which can inhibit chemical issues gaining traction with policy makers. Ecotoxicology is useful in cases of chemical incidents and in setting discharge limits for licences (obtaining representative samples when the mixtures are non-homogeneous or vary with time remains a challenge). However, it is in establishing cause-effect links where ecotoxicology can contribute significantly, particularly when combined with field studies. It is a continuing challenge to determine effects at low concentrations of chemicals, such as around guideline levels and a combination of techniques, including use of biomarkers and sensitive sub-lethal tests will remain useful. There is no one tool that can determine the effect of chemicals on the environment. The continuing challenge for biomarkers is to determine how changes in biomarkers relate to effects on individual species in the field or in whole communities or ecosystems. There has been significant effort in the Northern Hemisphere to establish this relationship for endocrine disrupting chemicals and, with different ecosystems and hydrology in our region, there is need for continued research on EDCs. Manning (2005) highlighted major knowledge gaps in this area, including EDC effects on marsupials and monotremes.

Bayesian approaches can be valuable in risk assessment and in ecotoxicology generally, and it would be beneficial to see a wider adoption of such approaches in this field of study. The current revision of the ANZECC and ARMCANZ (2000) water quality guidelines is expected to result in a system that is more easily updated as new science contributes to this area and new chemicals or new data on older chemicals can be incorporated to develop more robust guideline trigger values. This can be seen in the foreshadowed improvements in the sediment quality guidelines (Batley and Simpson 2008), who I'm sure would agree that more work needs to be done on sediment ecotoxicology and guidelines development. The area of soil quality guidelines and soil ecotoxicology is an area where there are currently many gaps. One additional useful contribution to water quality guideline (WQG) development is the impact of natural indicators such as turbidity and salinity on chemical toxicity, as well as how to incorporate bioaccumulation into WQGs and groundwater guidelines, which have not been discussed here.

The actual issue, raised by Peter Chapman (1998), on the need to develop relationships between chemical toxicity and body burden, has only partially been addressed by studies in this region or those published in AJE, and there is still some progress to be made in this area. In addition, Jeffree (2006) noted the need to understand "*modes of bioaccumulation that are characteristic of particular phylogenetic groupings of organisms*". Biological monitoring tools, including, but not limited to, bioaccumulators is another continuing area of contribution by the science of ecotoxicology.

The issue of new and emerging chemicals, by their very nature, require ongoing ecotoxicological assessment, even if only to avoid repeating past mistakes, as highlighted by Holdway (2006).

With the formation of SETAC-AU in 2011, the area of environmental chemistry is expected to increase in recognition by ecotoxicologists in the region.

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VICTORIAN REPORT ON ENDOCRINE DISRUPTION RESEARCH — MAY 2018

Kathryn Hassell[1]*, Mayumi Allinson[1], Jackie Myers[1], Kallie Townsend[1], Kavitha Chinathamby[1,2], Ana Miranda[1,2], Shanthanagouda Admane[2], Dayanthi Nugegoda[1,2], Graeme Allinson[1,3], and Vincent Pettigrove[1]

[1]Centre for Aquatic Pollution Identification and Management (CAPIM), The University of Melbourne, Parkville, Victoria 3052, Australia.

[2]School of Science, RMIT University, PO Box 71, Bundoora, Victoria 3083, Australia.

[3]School of Science, RMIT University, PO Box 2476, Melbourne, Victoria 3001, Australia.

ABSTRACT

The occurrence of endocrine disrupting chemicals (EDCs) in waterways and their impacts on aquatic wildlife have generated a significant amount of scientific and public interest internationally, but there is little published information on their occurrence and/or impact in Victorian freshwater or estuarine environments. Hormonal activity in media and biota is generally measured as 17β-estradiol equivalent concentration (EEQ). The first measurements of hormonal activity in Victorian wastewater discharges were reported in 2005 and 2007, and generally, estrogenic and androgenic activity and the levels of measured steroidal estrogens and androgens in treated effluents were low (typically <10 ng/L EEQ). The first reports of hormonal activity in freshwaters appeared in 2009, and were also typically low (<10 ng/L EEQ). In addition to steroidal estrogens and androgens, there are likely to be other sources of hormonally active substances in Victorian aquatic environments as well, such as herbicides, some veterinary medicines and other pesticides, and any number of industrial chemicals. To date there have been only few published studies on the field use of validated biological indicators of endocrine disruption in Victoria, and work is ongoing to find suitable biomonitoring species for endocrine disruption research in both freshwater and estuarine environments. The native flathead gudgeon (*Philypnodon grandiceps*) has been identified as a potential candidate fish species for freshwaters and the native blue-spot goby (*Pseudogobius* sp.) has been identified as a potential candidate fish species for estuaries. Some non-native fish species from both freshwater and estuarine environments, including cyprinids and others, are also being investigated, and research is also underway to establish suitable invertebrate bioindicators for both fresh and estuarine waters (e.g. *Potamopyrgus* and some amphipod species). Given the paucity of data, it is clear that considerable research is needed to identify which chemicals pose the greatest potential risk to aquatic organisms in terms of endocrine disrupting activity, and to establish the real-world concentrations of these chemicals in Victorian waters.

Key words: EDCs; Victorian biota;

INTRODUCTION

The state of Victoria, in southern Australia, covers an area of 227 600 km^2 that contains a vast network of inland lakes, rivers and streams that cover a distance of more than 85 000 km (VRHS 2002), and has a 2000 km coastline containing more than 120 bays, inlets and estuaries (Barton et al. 2008). With a population of almost 6.4 million people (Australian Bureau of Statistics 2017), Victoria is the second most populous state in Australia. Victoria is also the second most densely populated of Australia's states and territories. The vast majority of people live in Melbourne, the largest city in the state, but there is also Greater Geelong, Greater Bendigo, Ballarat and other urbanised, regional centres that are rapidly growing in population size. Melbourne itself is a sprawling conurbation that today has a population of approximately 4.8 million people. Although it has some high-density living areas, Melbourne is essentially a suburban city, and one in which the vast majority of households are connected to a reticulated sewerage system, with stormwater directed into a separate system that directs the water into local streams and rivers. As such, Melbourne for the most part avoids an issue that is common to many cities; which is that of heavy rainfall overloading combined sewerage systems that in turn leads to the discharge of raw sewage into watercourses as authorities seek to protect valuable wastewater treatment plant (WWTP) assets.

Population growth and urbanisation modify natural landscapes and can lead to increased amounts of pollutants entering waterways. One such group that have been associated with population centres are endocrine disrupting chemicals (EDCs), and specifically natural and synthetic estrogens and pharmaceuticals from WWTP discharges (Matthiessen et al. 2006). EDCs can interfere with the normal functioning of the endocrine system through binding or blocking hormone receptors, or modulating ligand-receptor interactions leading to upregulation or downregulation of hormone production and cascading effects within specific endocrine pathways (Colborn et al. 1993).

Eukaryotic organisms have a nuclear receptor super-family with many different receptors, each member of which can mediate endocrine disruptive effects. Janošek et al. (2006) suggested there are almost 50 nuclear receptors divided into three subclasses: type I receptors, or those that interact with steroid hormones such as the estrogen and androgen

*Author for correspondence, email: khassell@unimelb.edu.au

receptors (ER, AR); type II receptors that interact with retinoic acid (RAR) and retinoids in general (RXR), and those that interact with planar aromatic chemicals (the aryl hydrocarbon receptor, AhR) albeit the AhR is more accurately described as being a member of the extra-nuclear bHLH-PAS (Basic-Helix-Loop-Helix Period Aryl hydrocarbon nuclear translocator Single minded) family of receptors (Kojima et al. 2010); and type III receptors, these being 'orphan' receptors, or those still awaiting recognition of specific ligands. Xenobiotic compounds interact with these receptors in many different ways, although it is apparent that such receptors are physiologically activated by low molecular weight environmental contaminants that display structural similarities to the many endogenous compounds that normally stimulate the receptors (e.g. the steroid hormones, vitamin A derivatives, planar aromatic chemicals). Much of the focus of investigations into the occurrence of EDCs has been on chemicals that interfere with the ER and AR receptors, e.g. natural estrogens such as 17β-estradiol (E2) and synthetic mimics such as ethinylestradiol (EE2), and their effects on the growth and development of organisms both *in utero* and *in ovo*. However, there are other developmental pathways that might also be affected by some of the myriad natural and synthetic chemicals observed in aquatic ecosystems.

For instance, vitamin A (retinol) and its biologically active metabolites (collectively known as retinoids) are necessary for visual development, control of growth, and differentiation of embryonic cells (Inoue et al. 2010; Janośek et al. 2006, 2008). Although the precise mechanisms by which retinoid signalling pathways are disrupted by xenobiotic chemicals is not fully understood, it is well known that both a deficiency and a surplus of endogenous retinoids, most notably retinoic acids (RAs), can lead to abnormal development of various organs such as the eye, brain, and heart of offspring. Similarly, the majority of known AhR ligands are coplanar aromatic compounds. The current understanding of the AhR signal transduction pathway is that a dioxin-like compound first binds to the extra-nuclear AhR, after which the complex is translocated to the nucleus of the cell, where it induces the transcription of a number of genes that influence basic cellular processes (such as growth, differentiation, and programmed cell death), and the production of proteins (including the mixed function oxidase, cytochrome P-450 (CYP 1A1; Behnisch et al. 2001). This AhR mechanism of action is believed to play a pivotal role in the onset of many aspects of dioxin-like toxicity. For instance, in laboratory studies, exposure of vertebrates to dioxin-like chemicals (Janośek et al. 2006) results in a range of conditions, including reproductive and developmental disorders (e.g. reduced numbers of offspring, malformations) and wasting syndrome (the progressive weight loss until death).

Much of the early work on EDCs focussed on discharges from WWTPs since these are the most significant source of much of the steroidal input into many aquatic environments (Matthiessen et al. 2006). In that context, in early 2006, there were approximately 185 WWTPs in Victoria (not including plants under construction, in process of being decommissioned, and those private plants for which no information was available) (Allinson et al. 2010). Of these WWTPs, 56% were in the northern part of the state (defined as being north of the Great Dividing Range), and 44% in the southern part; the majority (67%) were lagoon-based plants, that used maturation lagoons, facultative lagoons, and oxidation ponds for sewage treatment. The remaining WWTPs (33%) were mechanical treatment plants, including those using various forms of activated sludge processes, extended aeration/flocculation technology, and trickle filters (Allinson et al. 2010). In 2006, there was a five order of magnitude difference in reported annual outflow between the smallest and largest WWTPs Victoria (<1 – 150 000 ML). At that time, 59% of the WWTPs recycled their effluent onto land, 29% discharged some portion of their effluent to freshwaters, and 11% discharged directly to the ocean. Unsurprisingly the largest WWTPs in terms of annual discharge serve the larger urban areas, with most of Melbourne's sewage treated by its Western and Eastern Treatment Plants (~180 and 125 GL/annum; discharge to Port Philip Bay and the Southern Ocean, respectively). The next largest WWTPs are Geelong's Black Rock (20 GL/annum; discharge to Southern Ocean), Bendigo (7 GL/annum; discharge to land and Bendigo Creek), and Ballarat South (7 GL/annum; discharge to Yarrowee River).

Victoria has a diverse range of agricultural landscapes, including irrigated horticulture, irrigated pastures, dryland cropping, and animal production. These landscapes cover ~61% of Victoria, accounting for approximately 13.9 million ha of land, with over 95% of agricultural land devoted to dryland agriculture. While representing a relatively small area, irrigated agriculture is the most intensive form of agriculture in Victoria, accounting for over half of Victoria's water consumption, and producing over 30% of the total value of agricultural commodity production (DPI 2005). Unlike dryland agricultural areas, surface and/or sub-surface drainage systems are generally present within irrigated areas to control waterlogging and salinity. Many of these drains discharge into rivers and water bodies, potentially accelerating the transportation of chemicals to these aquatic environments. Agricultural sources of hormonally active contaminants also include run off from confined animal feeding operations (CFOs) and extensive dairy and beef agriculture (sources of naturally occurring hormones such as estradiol and testosterone, as well as hormonally active veterinary medicines (VetMeds)) (Hanselman, et al. 2003; Johnson et al. 2006; Kahn et al. 2007; Allinson 2008). Almost two thirds of Australia's dairy farms are located in Victoria, with most of the farms located in the higher rainfall areas of southern Victoria and the irrigation regions of north-eastern Victoria (Dairy Australia 2017). Dairy manure and dairy farm effluents contain high levels of estradiol and its breakdown product, estrone (combined load up to 4000 ng/L in effluents) (Sarmah et al. 2006). Recycling of these resources onto land creates the potential for contamination of surface waters by hormones through run-off, and although steroid hormone transport from Victorian agricultural systems is quite likely (Allinson 2008), albeit no major studies of endogenous and/or synthetic hormonal inputs into creeks and rivers from Victorian agricultural systems have been conducted to date.

AIMS

Here we provide a review of the existing information available on measured concentrations of known endocrine disrupting chemicals, specifically steroidal estrogens and androgens, as well as the overall level of 'hormonal activity' as measured using recombinant receptor-reporter gene and enzyme-linked immunosorbent assays (ELISA) in Victorian aquatic environments, as well as providing a summary of the limited data currently available on biological effects in aquatic organisms. This information is summarised in order to highlight existing knowledge gaps and to prioritise future research needs.

RESULTS

Measured concentrations of endocrine disrupting chemicals in Victorian aquatic environments

The effluent from municipal wastewater treatment plants (WWTPs) is often considered to be the source of much of the EDC input into aquatic environments. Mispagel et al. (2005) published the first measurements of estrogenic activity in Victorian WWTP discharges, reporting that estrogenic activity was detected in most samples tested [6/7 samples: <LOD - 55 ng/L 17β-estradiol equivalents (EEQ)]. In a follow up survey, Mispagel et al. (2009) noted that most of the twelve WWTP effluents investigated showed estrogenic activity in the range 1 - 10 ng/L EEQ, with 17β-estradiol (E2) concentrations for the most part in the range 2 - 5 ng/L.

In 2006-2007, two detailed surveys of the treated effluent from 45 WWTPs located across Victoria sorted by treatment type (lagoon-based plants and those with activated sludge-based processes) was undertaken (Allinson et al. 2010). Grab water samples were obtained in winter 2006 and summer 2007, and sample extracts were used to challenge a yeast-based (*Saccharomyces cerevisiae* Y190) recombinant receptor-reporter gene bioassay into which the human estrogen receptor α (hERα) or the medaka (*Oryzias latipes*) estrogen receptor α (medERα) had been inserted (Shiraishi et al. 2000). Measurement of specific hormone concentrations (E2, estrone (E1), and 17α-ethinylestradiol (EE2)) was also undertaken using commercial ELISA kits. The authors noted that almost all of the effluents examined in that study showed estrogenic activity in both winter 2006 (hERα: <0.1 - 7.9 ng/L EEQ; medERα: <0.5 - 11 ng/L EEQ, with one sample 73 ng/L EEQ) and summer 2007 (hERα: <0.1 - 16 ng/L EEQ; medERα: <0.5 - 18 ng/L EEQ) surveys. Across all sites and treatments, steroid concentrations were: winter 2006, E2 0.1 – 12.4 ng/L, E1 0.1 – 32.0 ng/L; summer 2007, E2 <0.05 – 18.5 ng/L, E1, 0.2 – 18.4 ng/L. EE2 was observed sporadically, at concentrations below 0.5 ng/L (Allinson et al. 2010).

A range of other receptor activities and concentrations of specific chemicals were also measured in the samples collected in 2006 and 2007. For instance, Allinson et al. (2008) noted that when using yeast cells into which the human androgen receptor (AR) had been stably integrated, none of the WWTP effluents produced a response. The lack of response in the androgen assay may reflect lack of test sensitivity, rather than lack of androgenic activity *per se*, since the authors also noted that concurrent ELISA testing suggested that there were low levels of testosterone and androstenedione in the WWTP discharges (testosterone: winter 2006, <LOD – 2.85 ng/L; summer 2007, <LOD – 2.90 ng/L; androstenedione: winter 2006, <LOD – 9.30 ng/L; summer 2007, <LOD – 14.65 ng/L). Allinson et al. (2011a) also reported the RAR and AhR activity of the same water samples. Using yeast cells into which the human RARγ receptor had been integrated (Kamata et al. 2008), or YCM3 yeast cells carrying the response element for the AhR complex, XRE5 (Miller 1999), Allinson et al. (2011a) reported that more than 90% of the effluents examined elicited RAR activity (<0.5 – 198 ng/L all-trans-retinoic acid equivalents, ATRA-EQ), and that all of the effluents had AhR activity (16-279 ng/L β-naphthoflavone equivalents, βNF EQ).

A follow up study in 2008 found low levels of estrogenic activity (< 2 ng/L EEQ) in the discharge from Melbourne's Eastern and Western WWTPs (ETP and WTP) but none in the WTP's class-A recycled water delivered to farmers in the Werribee Irrigation District (Allinson et al. 2008). The ETP and WTP effluent had the same level of RAR activity (1.3 ng/L ATRA-EQ), but there was no RAR activity in the recycled water samples. AhR activities were slightly higher in WTP discharge (68 ng/L βNF EQ) than ETP (63 ng/L βNF EQ), although the highest AhR activity was observed in the WTP recycled water (129 ng/L βNF EQ). The WTP recycled water sample did not induce thyroid receptor (TR) activity, but the ETP and WTP effluents did (16 and 18 ng/L triiodothyronine equivalents, T3EQ, respectively).

The first information on the above-mentioned recombinant receptor-reporter gene bioassay (hormonal) activity of freshwaters in Victoria emerged in 2009 (Allinson et al. 2009a). The project involved the collection of water samples from six stations on the main stem of the Yarra River in the city of Melbourne, Australia in April 2008 and April 2009. No estrogenic or thyroid, and <1 ng/L retinoic acid receptor activity was observed in the Yarra River. However, AhR activity increased with progression downstream. AhR activity was higher in April 2009 (mean 23.2 ng/L βNF EQ) than at the same time in 2008 (mean 16.5 ng/L βNF EQ), perhaps as a result of extensive bushfires in the catchment in the months immediately prior to sampling. About 24% of the total AhR activity observed was associated with suspended solids (Allinson et al. 2011a).

In 2010 water samples were collected by CAPIM field staff from 24 wetland sites in and around the city of Melbourne in April, and sample extracts were prepared for measurement of hERα and medERα activity (Allinson et al. 2015). No estrogenic activity was observed in 23 of the samples, and <1 ng/L EEQ (medERα) observed in the remaining sample. The results of the positive controls run at the same time as the samples suggest the bioassay was performing to expectation, and capable of measuring low levels of hormonal activity (≤1 ng/L EEQ). Consequently, the lack of response in the bioassay results obtained from the wetlands sites must be due to a lack of estrogenic compounds.

Water samples were collected from four creeks in and around Melbourne in April 2008 (Allinson et al. 2009b) and from a further 16 creeks and rivers in a follow up study between February 9 and April 1, 2009 (Chinathamby et al. 2013). No estrogenic activity was observed in most of the urban, peri-urban and rural water samples using the hERα and medERα bioassays, and where estrogenic activity was observed, the level was low (<2 ng/L EEQ), with the exception of a single WWTP-impacted site (12.0 ng/L EEQ) and an urban creek (39 ng/L EEQ). A total estrogen (ES: ∑E1, E2, E3) response was observed in most of the 16 samples measured using ELISA, but where observed, the ES concentrations were also low (<2 ng/L EEQ), with the exception of the WWTP-impacted site (ES, 11.0 ng/L EEQ). ES concentrations at rural sites were not noticeably different from those observed at the urban sites or the majority of the WWTP sites. RAR activity was in the range <0.4 – 8.1 ng/L ATRA-EQ, with the highest level observed in the samples from the WWTP impacted site; AhR activity was in the range 7- 180 ng/L βNF EQ, although in this case the highest levels were generally observed in the rural and clean site samples. The reason for this latter is not yet clear, but as Allinson et al. (2011b) noted, testing was only on a limited number of samples on a limited number of occasions, and there is still a need for further extensive on-ground research, particularly in the tributaries of the Yarra River, to provide data for higher-level risk assessment by water suppliers and government agencies.

In as yet unpublished work, CAPIM staff collected water samples from a catchment in south west Victoria identified by EPA Victoria as being under threat from dairy effluent pollution in November and December 2013 (Allinson 2014). Chemical concentrations were measured using ELISA and both E2 and E1 were observed in every sample. Steroid concentrations were: E2, 0.4 – 2.1 ng/L; and E1, 2.5 – 16.0 ng/L. Overall, the concentrations of both E2 and E1 were generally slightly higher in spot water samples in November 2013 than in December 2013 (E2, November, 0.6 – 1.8 ng/L; December, 0.4 – 2.1 ng/L; E1, November, 3.1 – 16.0 ng/L; December, 2.5 – 15.9 ng/L).

Estuaries are often the final repositories for aquatic pollutants, so Ferguson et al. (2013) investigated how estuarine hydrology influenced natural and synthetic estrogen concentrations within the Little River, a tidal estuary in close vicinity to the major discharge point of Melbourne's Western Treatment Plant. Quantitative ELISA tests were used to determine concentrations of total natural estrogens (ES: ∑E1, E2, E3) and the synthetic estrogen, 17α-ethinylestradiol (EE2). The highest concentrations were measured in samples taken from the WWTP effluent discharge channel (29.0 ng/L and 0.35 ng/L, respectively). The concentrations of ES within the estuary were in the range 2.25-23.16 ng/L. In the upstream freshwaters, ES concentrations (2.95-7.26 ng/L) were lower than in the estuary, although their presence suggests an additional source of ES to the environment, most likely of agricultural origin. The EE2 concentrations measured in both the estuarine and freshwater areas were below 0.20 ng/L (Ferguson et al. 2013).

Biological effects of endocrine disrupting chemicals in Victorian biota

Useful biomonitoring programs should incorporate organisms from low to complex levels of organisation for biologically relevant assessments on receiving environments (Batzias and Siontorou 2006). In practice, selecting a suitable species that is available in the receiving environments can be challenging, let alone establishing relevant endpoints that would be practical for use on the selected species. Here, we describe some of the species that have been examined to date to assess the biological effects of EDCs in Victorian aquatic environments.

The effects of 17α-ethinylestradiol on Australian Chironomidae

Aquatic macroinvertebrates are functionally important in aquatic ecosystems and have many uses in ecotoxicology and biomonitoring. In Australia, the species-diverse family Chironomidae (chironomids or non-biting midges) are frequently used in biomonitoring to indicate aquatic ecosystem impairment. However, the effects of EDCs on Australian chironomids are unknown and existing studies in the literature come from single species laboratory studies with international *Chironomus* species. Two field-based microcosm experiments were done to understand the effects of a known potent EDC, 17α-ethinylestradiol (EE2) on multiple Australian chironomid species. The approach was based on that described by Pettigrove and Hoffmann (2005). The first experiment was run at a disused farm dam in Cottles Bridge, Victoria, with five concentrations of EE2 tested ranging from 1 ng/L to 10 µg/L. The second was conducted at Starvation Creek, Victoria, with only a single high concentration tested (10 µg/L).

Assemblages, populations, development rate and sex ratios of chironomids were unaffected by EE2 exposure. These results were consistent across both experiments and were similar to those seen in ecotoxicity assays using European and North American *Chironomus* species (Meregalli et al. 2001; Watts et al. 2001; Dussault et al. 2008). From both EE2 experiments, larvae from the genus *Procladius* exhibited a novel antennal deformity that has not been previously described in the literature. Deformed antennae appeared "buckled" or with small constrictions in the chitin, and were associated with the 10 µg/L EE2 treatment in both experiments (Figure 1). While the induction of deformities at high EE2 concentrations suggests chironomids underwent some physiological disturbance due to EE2, the results also indicate that environmentally relevant concentrations of EE2 would not directly affect Australian chironomids. As such, chironomids are not considered as good candidate organisms for indicating ecological impairment by this synthetic estrogen in biomonitoring programs.

Intersex gonads in wild caught black bream from Victorian estuaries

The black bream (*Acanthopagrus butcheri*) is a native Australian fish belonging to the Sparidae family, which is widely distributed throughout estuaries and sheltered coastal areas in southern Australia (Gomon et al. 1994). In studies

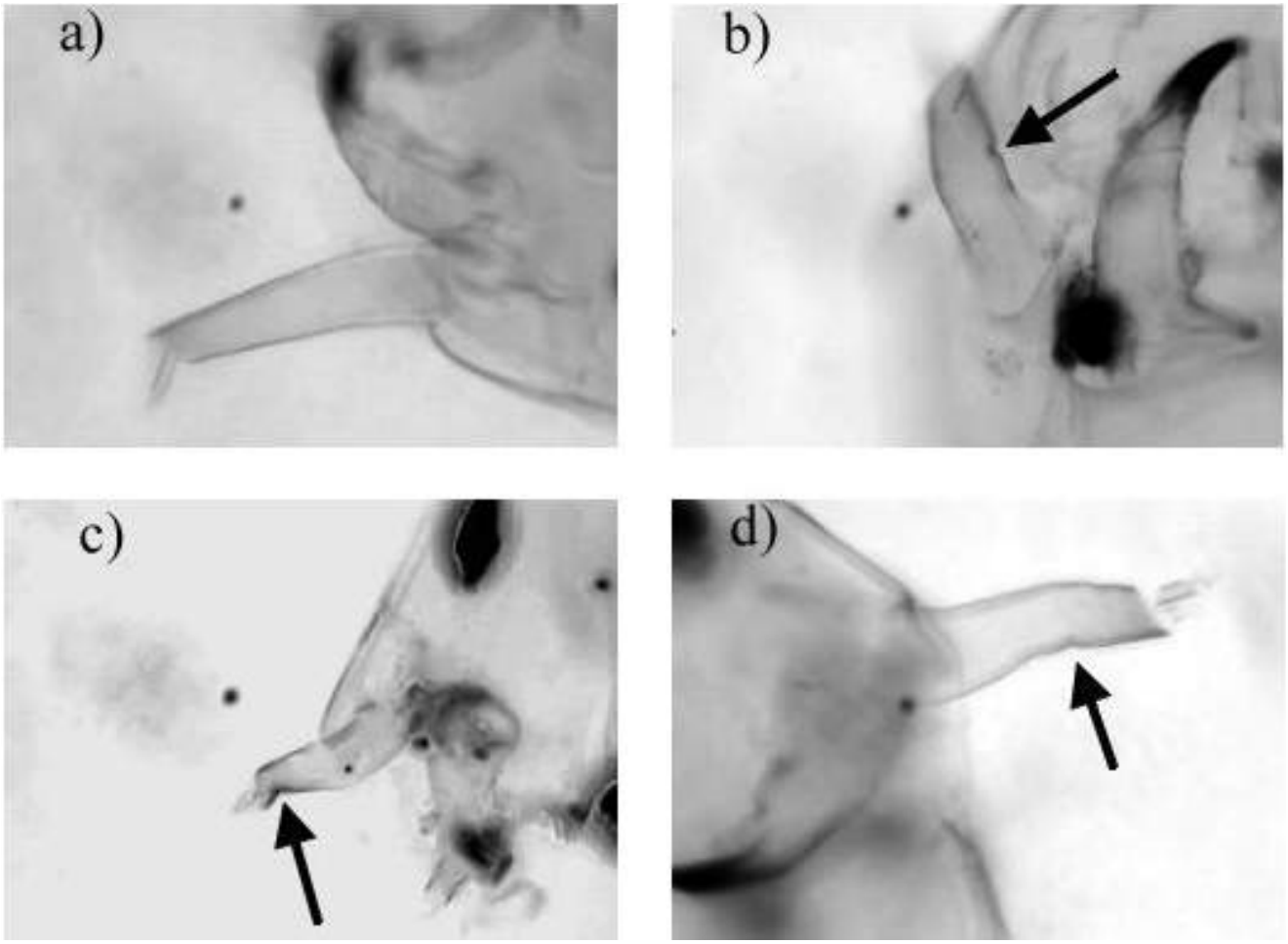

Figure 1. Deformities of the first antennal segment in *Procladius paludicola* larvae where (a) normal and (b) – (d) show "buckling". The arrow points to the deformity. This novel deformity was observed in organisms exposed to 10 ng/L 17α-ethinylestradiol (EE2).

of black bream to date, sexual development is not clearly defined. Some authors suggest they may be protogynous (Rowland and Snape 1994) and others suggest they may be rudimentary hermaphrodites, with residual ovarian tissue in a fully developed testis, or residual testis in a fully developed ovary (Haddy and Pankhurst 1998; Sarre and Potter 1999). To investigate if Victorian black bream exhibit any signs of exposure to estrogenic EDCs, and to establish if the black bream would be a suitable candidate species for biomonitoring EDCs in estuaries, samples were collected from various estuaries throughout Victoria between 2007-2009, with known differences in land use and likely contamination sources and types.

Intersex fish were observed at all field locations, however the percentage of males containing oocytes in otherwise normal testes was greater in fish collected from sites close to a major sewage treatment facility (Werribee River and Little River), and an estuary with a known history of industrial contamination (Yarra River) (Table 1) (Jobling et al. 2008). All intersex fish exhibited focal intersex, whereby oocyte strings were restricted to small, well defined and discrete areas of the testis (Figure 2). Intersex fish were observed from all age classes, and there did not appear to be any trend towards one sex or the other with age (no sex-skewing, as would be observed in protogynous or protandrous fish). Based on the data analysed so far, it is presumed that black bream might be rudimentary or seasonal hermaphrodites, and we are unable to conclude whether or not any of the sampled fish exhibit signs of exposure to estrogenic EDCs. Plasma vitellogenin (Vtg) levels were measured in all fish, using semi-quantitative ELISA and Western blot, as described in Codi King et al. (2008). Vitellogenin was not detected in any male black bream, however the protein was detected in females (Figure 3) (Hassell 2009). The Vtg concentrations in females varied, as was expected, given that fish of a range of sizes and stages of maturity were sampled. Further sampling has been conducted, and work continues to determine if there are seasonal differences in the rates of hermaphroditism amongst Victorian black bream populations.

Differences in gonopodium indices in Gambusia holbrooki *from Victorian freshwater streams*

The Eastern mosquitofish, *Gambusia holbrooki* is a sexually dimorphic, live-bearing poeciliid fish species that was introduced into Australia in the 1920s primarily for mosquito control. During sexual maturation, males develop an elongated anal fin, known as a gonopodium, which is used during copulation to transfer sperm packets, called spermatozeugmata to the female (Grier 1981; Doyle and Lim 2002). Development of the gonopodium occurs

Table 1. Incidence of intersex gonads in male black bream from five Victorian estuaries.

Site	No. of female fish	No. of 'male' fish	No. of normal males	No. of males with oocytes present (%)
Mallacoota Inlet	70	34	29	5 (14.7)
Yarra River	22	19	2	17 (89.5)
Maribyrnong River	15	9	7	2 (22.2)
Werribee River	17	25	5	20 (80.0)
Little River	22	19	4	15 (78.9)

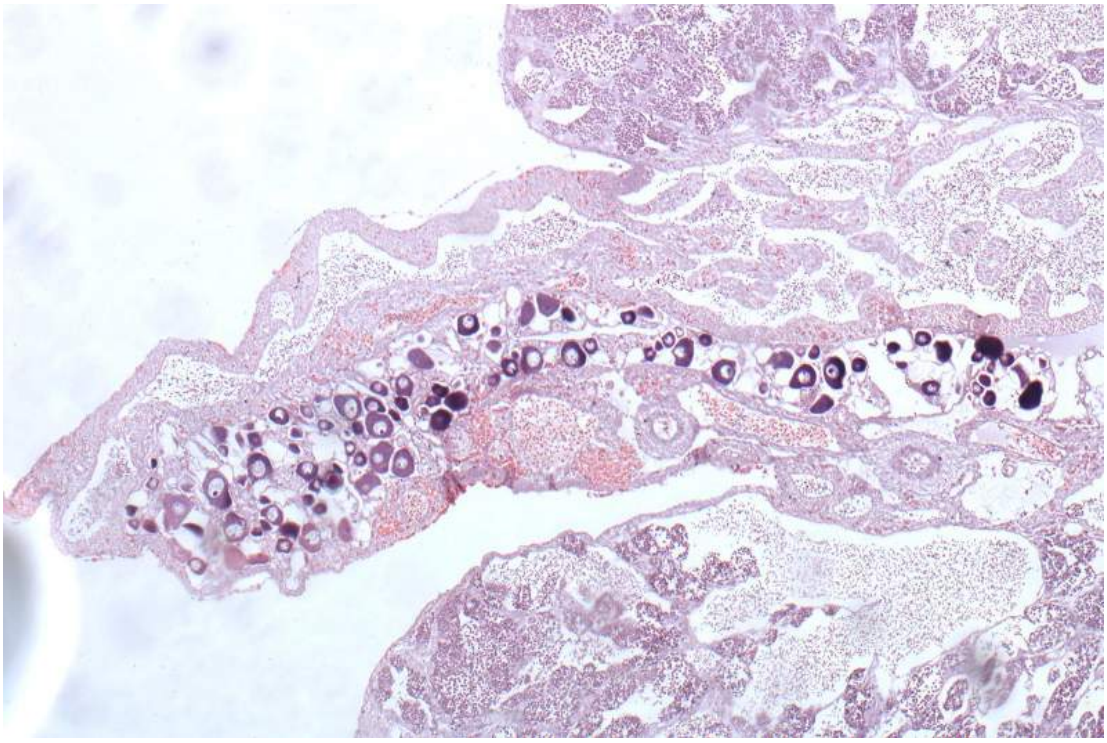

Figure 2. Example of intersex gonad in male black bream. The mature or maturing testes of some male fish had small, discrete areas containing primary oocytes. Magnification x100, stained with H&E.

in response to circulating androgens, and females can be masculinised (formation of a gonopodium) by exposure to exogenous androgens (Turner 1942; Angus et al. 2001), and likewise, males can be demasculinised (reduction in size and width, or inhibited maturation of the gonopodium) by exposure to exogenous estrogens (Batty and Lim 1999; Doyle and Lim 2002; Dreze et al. 2000; Game et al. 2006). Thus, gonopodial indices have been established as a useful measure of endocrine disruption in both male and female mosquitofish, and in Australia, field studies have been able to demonstrate endocrine disruption in this species near both sewage treatment facilities (Batty and Lim 1999; Leusch et al. 2006; Rawson et al. 2008) and urban wetlands (Game et al. 2006).

The first assessment of endocrine disruption in Victorian populations of mosquitofish, through the analyses of biomarkers such as gonopodial indices, as well as measurement of ethoxyresorufin-*O*-deethylase (EROD) activity in livers, and induction of vitellogenin (Vtg) protein was conducted in 2007 by researchers at RMIT University. They conducted field surveys in 2007, 2008 and 2009, in which male mosquitofish were sampled from several different locations with different land uses. The land use areas were urban and industrial, rural, sewage treatment plant discharge, and reference sites. Estrogenic activity of surface waters was found at all sites in this study, including reference sites (Chinathamby et al. 2013). This ranged from 0.1-1.7 ng/L EEQ, except for one site (WWTP) at 12 ng/L EEQ. EROD activity was measured in pooled samples of five individual liver homogenates, with a total of three sets per field site, adapted from Holdway et al. (1993). Vtg protein was measured semi-quantitatively in whole body homogenates using a dot blot (immunobinding) assay that was trialled with several different commercially available anti-Vtg antibodies. No strong associations were found among the biomarkers selected for this study. EROD activity in fish ranged from 0.42 pmol/min/mg protein to 10.85 pmol/min/mg protein. Fish from industrial/urban

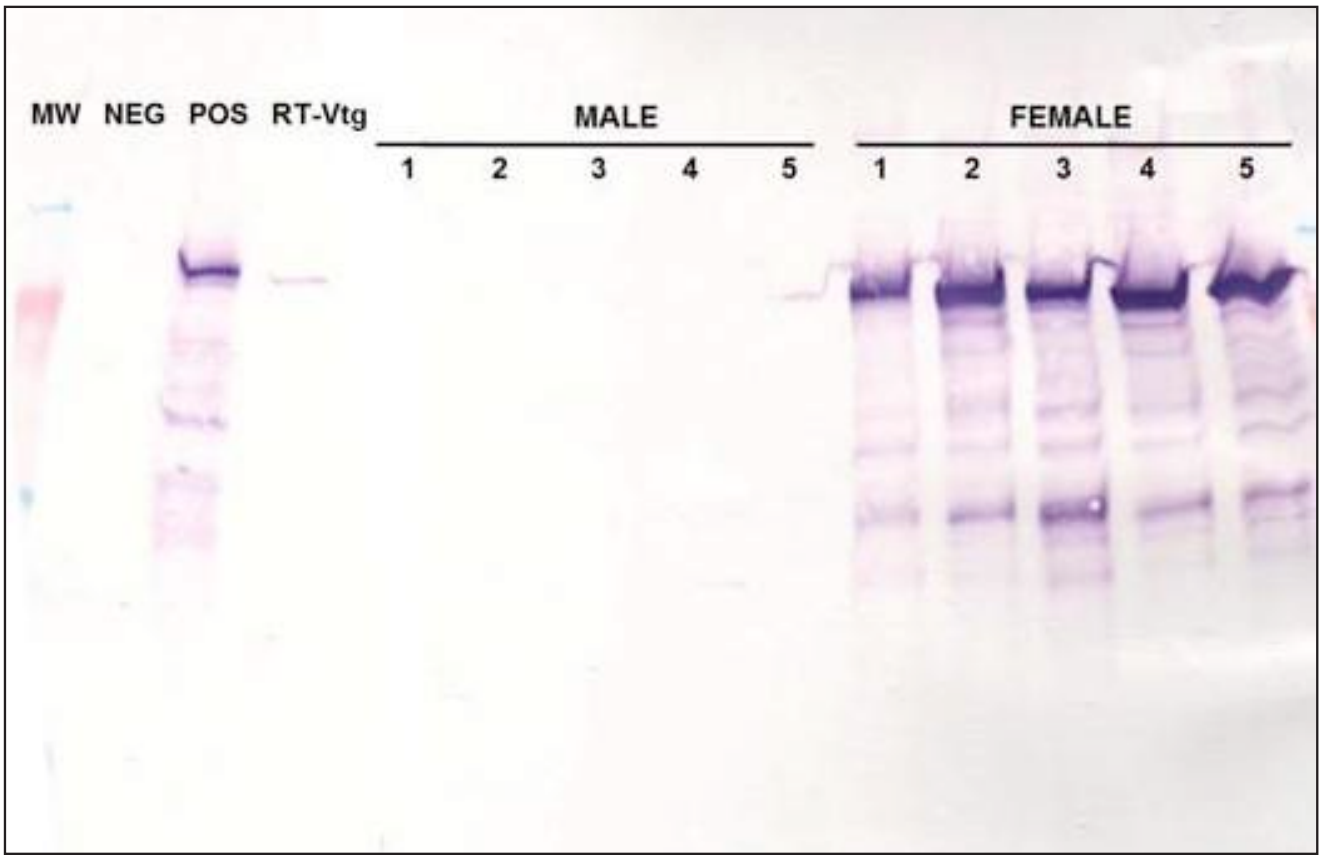

Figure 3. Vitellogenin detection in male and female black bream captured from Little River during December 2007. Positive (POS) and negative (NEG) black bream and rainbow trout Vtg (RT-Vtg) controls are also shown.

sites or sites receiving urban stormwater showed generally higher EROD activity (Chinathamby, unpubl). Vtg protein levels in fish ranged from 4.70 ng/µL S-VtgEq to 22.83 ng/µL S-VtgEq (Salmon vitellogenin equivalents). Fish from three out of four WWTP sites exhibited the highest Vtg levels suggesting that fish from those sites could be exposed to estrogenic environmental contaminants. In particular, Vtg protein levels were high in fish from the WWTP site with a high water concentration of 12 ng/L EEQ.

In 2010 a larger scale investigation of reproductive morphology in male mosquitofish was conducted in conjunction with extensive surveys of pesticides and hormonal activity from sites across Victoria (Myers, unpubl). Mature mosquitofish were collected from a total of 23 sites, and water samples were also collected at each site for pesticide and hormonal analyses. A range of morphometric measurements were made on each fish and used to calculate gonopodial indices (gonopodium length/standard body length, the index of elongation (length ray 4:6 ratio) and the percentage of males with hooks and serrae) to assess for signs of EDC exposure. The study showed significant differences in the morphological indices between populations of mosquitofish inhabiting the selected sampling sites suggesting that endocrine disruption may be occurring in some Victorian freshwater systems (Myers, unpubl). The highest levels of change appeared to be occurring at sites impacted by two different land use activities, namely STP and dairy. Increased gonopodium lengths, relative to males collected from reference sites and a lack of hooks and serrae on the distal region of the gonopodium suggested the presence of hormonally active substances with estrogenic, androgenic or anti-androgenic effects at those land use sites. To better understand what was causing the observed effects, a more comprehensive survey, including chemical screening, was started in early 2011 throughout Victoria to determine whether other regions with the same land uses would produce similar effects on gonopodial indices in mosquitofish.

Effects of EDCs on Murray rainbowfish in laboratory studies

Several EDCs have been evaluated by RMIT researchers to determine possible effects of these compounds on the reproductive health of the native Australian Murray River rainbowfish (*Melanotaenia fluviatilis*). Among those EDCs were 17α-trenbolone, atrazine, pyrimethanil, estrone, 17β-estradiol and perfluorooctanoic acid (PFOA) as well as mixtures of EDCs (Miranda 2018).

In fish exposed to the feedlot contaminant 17α-trenbolone, several effects were observed however there was a lack of dose response. Among the effects, the exposures led to an overall decrease of vitellogenin in females, an increased number of atretic follicles and, in breeding groups of fish, it caused a reduced total cumulative number of eggs and led to several eye and spinal deformations in about 20% of the hatched larvae.

Further to this, the exposure of rainbowfish eggs to 17α-trenbolone (until hatching) resulted in abnormalities in eggs and hatched larvae similar to those observed in offspring of exposed adults, yet there was no dose-response in the percentage of abnormalities observed. This indicates that 17α-trenbolone may have effects beyond its androgenic action, and may also act as a genotoxic compound at concentrations commonly found in effluents from feedlots. The effects of 17α-trenbolone on both egg and larvae can additionally impair population fitness at relatively low concentrations.

When 17α-trenbolone was mixed with an estrogen, 17β-estradiol, it was demonstrated that 17α-trenbolone was partially able to counteract the effects of the estrogen 17β-estradiol, although no counteraction was observed at the molecular level.

Exposure to atrazine caused disruption of normal gonadal development and maturation of rainbowfish. Since these effects are also under hormonal control it is proven that pathways other than the estrogen receptor binding are also susceptible to disruption. This was clear at low atrazine concentrations with atrazine causing effects on gonadal maturation in females, and a significant increase of spermatogonia in the male gonads whereas there were no effects on vitellogenin levels.

Similarly, when fish were exposed to mixtures of chemicals, in long-term exposures, alterations in gonads and liver were observed while vitellogenin levels remained unaltered. While only estrone and 17α-ethinylestradiol induced vitellogenin production in male rainbowfish, deleterious effects on the gonads were evident from tissue histopathology in exposures to pyrimethanil and atrazine. This is indicates that these chemicals have the ability to disrupt multiple pathways.

Further evidence of this was also demonstrated after exposure of rainbowfish to perfluorooctanoic acid (PFOA). PFOA was found to be slightly estrogenic, leading to the induction of vitellogenin in male fish, as well as inducing changes in antioxidant processes that may be linked to the thyroid pathway. From all of this research with rainbowfish it can be concluded that the effects of EDCs are broader than expected and that investigations into possible effects on other physiological targets are required too.

Recently, a review was published on the effects of environmental stressors on the fish thyroid cascade. This review highlights the effects of several environmental toxicants on fish thyroid function, including PBDEs, PCBs, PCDDs and PCDFs, PAH/oils, phthalates, metals, pesticides, mixed pollutants/chemicals, cyanide and other stressors including acid (low pH) and ammonia (Nugegoda and Kibria 2017).

Another research project at RMIT University evaluated the effects of selected EDCs on aromatase and Vtg gene expression in the Australian native Murray River rainbowfish, *Melanotaenia fluviatilis*. The ovarian (*cyp19a1a*) and brain (*cyp19a1*b) aromatase gene isoforms were isolated and characterised from *M. fluviatilis* (Shanthanagouda et al. 2011 a, b). The sequence information enabled the construction of a phylogenetic tree and it was observed that the aromatase gene isoforms of the Murray River rainbowfish showed a high identity with pejerrey, a closely related atherinid species. The effects of selected EDCs (E2, nonylphenol (NP), bisphenol A (BPA) and the aromatase inhibitor fadrozole) on aromatase and Vtg gene expression, as well as Vtg protein expression were investigated in laboratory experiments. The detection of the Vtg protein in plasma following exposure to EDCs was not consistent, however, the expression of the *vtg* gene in the liver and testes of males exposed to EDCs was clearly evident as analysed by qPCR. The results of this study suggested that exposures to the tested EDCs have a disruptive effect on the steroidogenic pathways of *M. fluviatilis* and therefore are likely to affect sex differentiation, sexual behaviour and reproductive cycles in this species. It was concluded that *M. fluviatilis* is sensitive to EDCs, and both aromatase isoforms and *vtg* gene expression have potential as biomarkers of exposure to such chemicals in this species.

Alternative candidate species

Work is ongoing to find valid biomonitoring species for EDCs in both freshwater and estuarine environments in Victoria. A review of potential candidate fish species for freshwater biomonitoring identified Australian smelt (*Retropinna semoni*), flathead gudgeon (*Philypnodon grandiceps*) and the Southern pygmy perch (*Nannoperca australis*), since all are common and widespread species, they are small and sexually dimorphic and they have relatively rapid generation times (Miranda et al. 2010). Flathead gudgeons have been used in both laboratory exposure studies (McDonald 2017) as well as field studies to assess pollution impacts (Kellar et al. 2013) at the University of Melbourne, and work is ongoing to establish breeding populations of this species in the laboratory environment for further EDC-based research. The native blue-spot goby (*Pseudogobius* sp.) has been identified as a potential candidate fish species for estuaries, and laboratory exposures of embryos to 17β-estradiol showed alterations in gonad development (Hallein 2012), whilst wild caught adult blue spot gobies from an agricultural site in south-eastern Victoria were the first to be observed with testicular oocytes (testis-ova), albeit the incidence levels were low (13%) (Sharley et al. 2013). In field studies when native fish are not present in great enough abundance for testing, non-native fishes such as carp, roach and goldfish have been used instead (Hassell et al. 2016; Kellar et al. 2014). Carp (*Cyprinus carpio*) and roach (*Rutilis rutilis*) that were sampled from the Yarra River showed no elevation of plasma vitellogenin levels in males and no incidence of intersex gonads; it was thus concluded that there was no evidence of exposure to environmental estrogens in these fish (Hassell et al. 2016). On the other hand, in goldfish (*Carassius auratus*) sampled from Dandenong Creek in eastern Victoria, low incidences of testis-ova in males and oocyte atresia in females were observed, yet the values were similar between the impact and reference sites, and therefore it was concluded that EDCs were not the cause of biological impairment observed in fish and other aquatic biota in that catchment (Kellar et al. 2014). Clearly there remains a need to further characterise native fish

responses to endocrine disruption in laboratory settings, as well as continuing monitoring of wild caught fishes (native and non-native) from different freshwater, estuarine and marine waterways in Victoria.

Additionally, there is a need to develop alternatives to fish as indicators both for ethical reasons, and because many Australian native fishes are either slow growing and/or long lived, and are thus not appropriate for use in bioassays that rely on reproductive endpoints. Research is currently occurring at the University of Melbourne to establish suitable invertebrate bioindicators for both fresh and estuarine waters. More rapid responses may be achieved using invertebrates, such as gastropod molluscs or amphipods, which are easier to culture and occur in greater numbers. Furthermore, unlike most fishes, invertebrate species can be easily transplanted to different locations in field-based studies to gather robust, environmentally-relevant experimental data.

SUMMARY

There are a large number of known and potential hormonally active compounds in Victorian waterways. Estrogenic steroid hormones in freshwater samples can be reliably and definitively quantified using instrumental methods based on gas or liquid chromatography – mass spectrometry (GC- or LC-MS). However, because of the resource implications of using such technology (including the need for expensive instrumentation and high level technical expertise in its operation), preliminary screening of samples using rapid assessment tools, such as in vitro assays, is an attractive prospect for waterways managers. Recombinant receptor-reporter gene bioassays, such as the yeast two-hybrid assay described above, measure the activation of a receptor, and so they can provide a broad measure of the hormonal potency of samples. In addition, ELISAs have potential utility as a method for the rapid mapping of contaminant levels across large areas, and to optimise the design of monitoring networks (Morozova et al. 2005). One of the major advantages of using in vitro assays is that the total receptor activity observed when the assays are challenged by sample extracts can be expressed relative to a standard compound, e.g. for estrogenic activity as 17β-estradiol equivalents (EEQ), which allows for quantification of receptor activity without having to know the precise chemical composition of the sample. In a recent study, Allinson et al. (2011b) compared the performance as screening tools of two yeast-based recombinant receptor-reporter gene bioassays with a commercial ELISA kit for measurement of total estrogens. For WWTP effluents, there was a very good correlation between the measured total estrogen concentrations (ELISA) and estrogenic activity by the hERα bioassay ($r^2 = 0.93$), but not for the medERα bioassay ($r^2 = 0.50$). For freshwater samples, the correlations between bioassay response and ELISA ES measurements were also very good ($r^2 > 0.95$). However, there was no correlation between bioassay response and ELISA ES measurements for estuarine samples. While the reasons for the lack of correlation of the recombinant receptor-reporter gene assay activity and that of the ELISA for estuary-derived water samples is not yet understood, their study has shown that where there is a high probability that estrogenic activity is the result of steroid hormones, e.g. domestic effluent from WWTPs or animal feedlot operations, or where information on only the levels of steroidal estrogens is required, the use of commercial ELISA kits in conjunction with extensive sample preparation methods may be a cost-effective way to rapidly screen samples prior to more definitive (and more expensive) testing. Where there is no such *a priori* understanding of the source of potential estrogenic activity, or where information on the prevalence of anti-estrogenic materials in samples is required, the use of other in vitro assays is recommended as a screening technique.

It also needs to be acknowledged that steroidal estrogens and androgens are unlikely to be the only sources of hormonally active substances in Victorian aquatic environments. Other sources of potential EDCs in Victoria include herbicides, some veterinary medicines and other pesticides, and any number of industrial chemicals. These chemicals may be in current use, or be legacy issues from the past. Agriculture relies heavily on pesticides to produce high yields of crops of consistent quality, and to minimise the impacts of unwanted pests e.g. locusts. Many pesticides are known or suspected EDCs (McKinlay et al. 2008), and a significant number of these chemicals are in widespread and high-volume use throughout Victoria. Overall, although the occurrence of EDCs and their impacts on aquatic wildlife have generated a significant amount of scientific and public interest, there are still only a small number of studies that have assessed endocrine disruption in wild caught fish and invertebrates in Victorian waterways, and only a limited group of test species in laboratory studies. Clearly much more work needs to be conducted to both identify which chemicals pose the greatest potential risk to aquatic organisms in terms of endocrine disrupting activity, and to establish what the real-world concentrations of these chemicals are in Victorian waters.

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FATE AND ANALYSIS OF ENDOCRINE DISRUPTING CHEMICALS, PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN A MEMBRANE BIOREACTOR

Heather Coleman[1*], Martin Troester[2], Stuart Khan[2], James McDonald[2], Graeme Watkins[3] and Richard Stuetz[2]

[1]School of Pharmacy and Pharmaceutical Sciences, Ulster University, Coleraine, BT52 1SA, N. Ireland, UK.
[2]UNSW Water Research Centre, University of New South Wales, Sydney, NSW 2052, Australia.
[3]MidCoast Water, PO Box 671, Taree, NSW 2430, Australia.

ABSTRACT

The removal efficiency of endocrine disrupting chemicals (EDCs), pharmaceuticals and personal care products (PPCPs) of a package plant membrane bioreactor (MBR) treating raw municipal sewage was assessed using biological and chemical analysis. Recombinant yeast oestrogen and androgen bioassays were employed to determine oestrogenic and androgenic activities, and Gas Chromatography–Mass Spectrometry was used to determine the concentrations of ibuprofen, salicylic acid, triclosan and 4-tert-octylphenol in samples before and after MBR treatment. The MBR was shown to have removed over 78% of the oestrogenic activity and over 98% of androgenic activity from raw sewage samples. Removal rates for ibuprofen, salicylic acid, triclosan and 4-tert-octylphenol were 99.9%, 99.8%, 93.1% and 98.1%, respectively. All removal rates were comparable or better than those previously reported from conventional activated sludge processes and support the application of this treatment for decentralised treatment of domestic wastewater from single households or clusters of homes and the use of the treatment effluent for alternative water management practices such as water reuse and recycling.

Key words: sewage treatment; yeast screen bioassay; GC-MS, oestrogens; androgens membrane bioreactors.

INTRODUCTION

The term "endocrine disrupting chemicals" (EDCs) includes all substances that may affect the endocrine system (the communication system of glands, hormones and cellular receptors that control the body's internal functions). This includes natural oestrogens, synthetic steroidal oestrogens, oestrogen mimics and phytoestrogens (plant oestrogens) (Manning 2005). Synthetic steroidal oestrogens are mass produced as pharmaceutical agents. Biologically significant levels of EDCs have been found in water bodies, and have been linked to adverse effects on wildlife (Tyler et al. 1998). The long-term effects of human exposure to most of these chemicals are unknown, but currently hotly debated.

Another large class of chemicals that has received increasing attention during the last decade comprises pharmaceuticals and personal care products (PPCPs) (Ternes and Joss 2006). These substances are used in large amounts throughout the world, often on a par with agrochemicals, and have been linked to ecological impacts at trace concentrations. The majority of PPCPs are more polar than conventional pollutants and have acidic or basic functional groups. These properties, coupled with their occurrence at trace concentrations, create unique challenges for both removal processes and analytical detection. To date PPCPs are not required to undergo the same level of testing for possible environmental effects as priority pollutants and as a result their effect on the environment is largely unknown. A wide range of PPCPs and oestrogenic and androgenic hormones are identified in the Australian National Guidelines for Water Recycling, 2006, as substances 'potentially found in water, which could pose a risk to the environment'.

The aim of this work was to assess the suitability and efficiency of submerged membrane bioreactors (MBRs) for the removal of these key contaminants of concern for future decentralised water management practices. MBRs have attracted a significant amount of interest for package plant applications due to their ability to produce high effluent quality in terms of domestic sewage treatment over conventional activated sludge systems. MBRs comprise a combination of a conventional activated sludge process and microfiltration/ultrafiltration membrane separation, which has the advantages of a small footprint and reduced sludge production (Qin et al. 2006). The application of package plant systems as either single or cluster formations has been shown to produce treated effluent of sufficient quality (in terms of BOD and SS) to meet environmental regulations for direct discharge into watercourses (Daude and Stephenson 2003). However, like centralised wastewater treatment systems, there remains concern as to the fate and removal of trace contaminants by package plant treatment processes. This is particularly important in regions with no suitable receiving waters or where the treated wastewater is for reuse.

Samples were collected from the raw sewage influent, mixed liquor from the MBR, and the effluent from an operational package MBR plant located at a regional sewage treatment plant (STP) in NSW, and subjected to both biological and chemical analysis. Biological analysis involved the use of yeast screen bioassays (Routledge and Sumpter 1996), which test for both oestrogenic and androgenic activities to give an indication of endocrine disrupting potential at each stage of the treatment process. Chemical analysis by Gas Chromatography-Mass Spectrometry (GC-MS) was used to determine the concentrations of some pharmaceuticals and personal care products.

*Author for correspondence, email: h.coleman@ulster.ac.uk

MATERIALS AND METHODS

Samples (7.5 litres) of raw sewage, mixed liquor from the MBR and MBR effluent from a sewage treatment plant in NSW, Australia were collected in 2.5-L glass bottles. Details of the sewage treatment plant and MBR are given in Table 1 below.

Triplicate samples (1 litre) of the influent raw sewage, MBR supernatant and MBR effluent were subjected to biological and chemical analyses. Deuterated surrogate standards (300 ng) were added to each sample used for chemical analysis. Because these surrogate standards (which included known endocrine disrupting chemicals) would interfere in the biological analyses, different samples had to be used for the biological and chemical analysis. For quality control (chemical analysis, biological analysis) and recovery calculation (biological analysis), triplicates of spiked MilliQ water were extracted parallel to the treatment of the real samples and analysed with the respective method. Unspiked MilliQ water was used as a blank. Fenoprop (m = 4.3 μg) was added to each sample, blank and calibration standard as an internal standard prior to derivatization and chemical analysis.

Sample preparation

For filtration of the samples, Whatman No.1 filter paper was used followed by Whatman GF/A glass fibre filter paper (pore size 1.6 μm) and finally Whatman GF/F filter paper (pore size 0.7 μm). The GF/F filtrates were then acidified with sulfuric acid to pH 2-3. Solid phase extraction was used for extracting and concentrating the samples. Waters Oasis HLB 6cc cartridges were conditioned with 5 mL of acetone, 10 mL of methanol and then with 10 mL of MilliQ water adjusted to pH 2-3 prior to extraction. One litre of the acidified GF/F filtrate from the mixed liquor of the lab scale MBR was spiked with the respective standard solutions of the analytes. All filtered samples were then passed through the cartridges at a flow rate of 5 mL/min. The cartridges were dried with a gentle flow of nitrogen and then eluted with 4 mL of acetone/methanol mixture 1/1 v/v and the extracts were dried to complete dryness under a gentle stream of nitrogen. The sample extracts were re-dissolved in 1 mL of acetonitrile prior to analysis as outlined below.

Biological Analysis - Analysis of oestrogenic and androgenic activities

A yeast screen bioassay was used to test for oestrogenic and androgenic activity in samples. Details of the yeast screen procedure can be found in Routledge and Sumpter (1996). The yeast assays were carried out in a type II laminar air flow cabinet to minimise aerosol formation. Aliquots (four replicates, 10 μL each) of each sample were transferred to a 96-well optically flat bottom plate (NUNC). Each plate used for analysis contained 4 x 10 μL sample, at least one row of blanks (200μL assay medium only) and one row of ethanol (10 μL ethanol water and 200 μL assay medium), as well as two rows of standard curve for 17β-oestradiol (concentration range 10 μg/L to 9.53 ng/L) or testosterone (50 μg/L to 195.3 μg/L) for the oestrogenic and androgenic assay respectively. 200 μL of the seeded assay medium (medium containing recombinant yeast and the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG)) were then dispensed to each sample well using a multichannel pipettor. The plates were sealed with autoclave tape and shaken vigorously for 2 minutes on a titer plate shaker and read at 570 nm (for colour) and 620 nm (for turbidity) prior to incubation at 32°C in a naturally ventilated heating cabinet. For the next two days, the plates were shaken vigorously on the plate shaker for 2 minutes, to mix and disperse the growing cells, read at 570 nm and 620 nm and returned to the 32°C incubator. On the fourth day, after incubating for 3 days, the plates were shaken for 2 minutes and left for approximately 1 hour to allow the yeast to settle. The plates were then read at an absorbance of 570 nm and 620 nm using the plate reader. The plates were left at room temperature and read again later to verify the results. Photographs were taken of each plate with a Nikon D50 SLR camera every day after each plate reading. For the androgenic assay, the assay plates were incubated at 32°C for 48 hours and then incubated at room temperature and read when the data with the best contrast was obtained. The actual oestrogenic and androgenic activities were then calculated based on the dilution factor. Blanks were run on each plate for all samples in each assay and were always less than the limit of detection, i.e. < 0.02 ng/L in the oestrogenic assay and < 0.1 ng/L in the androgenic assay.

Chemical analysis - Analysis of PPCPs

Chemical analysis was carried out using GC-MS using a Hewlett-Packard HP5973 mass spectrometer with negative electron impact ionization combined with a Hewlett-Packard HP 6890 gas chromatograph. A HP-5MS capillary column was used (5% Phenyl Methyl Siloxane; nominal length 30 m, nominal internal diameter 250 μm, nominal film thickness 0.25 μm) with helium as the carrier gas at a constant flow rate of 1.2 mL/min and pressure of 12.43 psi. The temperature program was 100°C, held for 3 minutes, ramped to 150°C at 30°C/min, held for 1 minute, ramped to 205°C at 3°C/min, ramped to 260°C at 10°C/min and finally held for 23 minutes. An injection volume of 1 μL was used in splitless mode with an inlet temperature of 210°C, using an Agilent

Table 1. Details of the sewage treatment plant, NSW, Australia.

STP	Treatment Processes	Flow (ML/d)	Details
Old Bar	Activated sludge, continuous alum dosing for phosphorus removal & UV disinfection	0.8	Load about 3500EP (capacity 6000EP); average age of population =35
	Membrane Bioreactor (MBR) with electrochlorination	0.004	Load about 25EP

Footnote: EP = Equivalent population

7683B autosampler. All quantitative results were calculated by integration of the peak areas obtained by monitoring the respective ion fragments (m/z) using selective ion monitoring mode (SIM). Peak identifications and calculations of the peak areas were carried out using HP MS ChemStation, Data analysis application G1701DA and the embedded NIST library. Due to the high polarity and the low volatility, the analytes needed to be derivatised prior to analysis by GC-MS. Bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane (BSTFA-TMCS) was used as the derivatising agent to form the trimethylsilyl (TMS) derivatives by replacing the hydrogen atom of the respective functional group (i.e. carboxylic and hydroxyl) with TMS groups to decrease polarity and increase volatility of the compounds. Aliquots of analyte (i.e. 450 μL of standard solutions, or redissolved dried solid phase extraction extracts in 450 μL acetonitrile) and 80 μL of BSTFA-TMCS (99:1) were heated at 70°C for 100 min prior to analysis with GC/MS.

RESULTS AND DISCUSSION

Biological analysis – Analysis of oestrogenic and androgenic activities

After incubation, the control wells appear light orange in colour, due to background expression of β-galactosidase (β-gal), and turbid, due to growth of the yeast. Positive wells are indicated by a deep red colour accompanied by turbid yeast growth. Clear cells (containing no growth) indicate lysis of the cells, and the colour may vary. To correct for turbidity the following equation was applied to the data obtained for each well:

Corrected value = Chemical Absorbance at 570 nm – [Chemical Absorbance at 620 nm – Blank absorbance (i.e. acetonitrile) at 620 nm]

An average value was then taken for the four results obtained for each sample and for the two results obtained for each calibration curve. Calibration curves developed for 17β-oestradiol and testosterone for each plate were plotted giving concentration versus absorbance at 570 nm. The concentration of oestrogenic or androgenic activity in each well was then calculated as 17β-oestradiol equivalents (EEq) or testosterone equivalents (TEq) from the standard curves in μg/L. These values were then divided by 1000 (since the samples were pre-concentrated 1000 times), giving final concentrations in ng/L. A mean value was calculated for each sample in four wells. The mean and standard error were then calculated for each set of triplicate samples. Fate and levels of oestrogenic and androgenic activities in influent, MBR and effluent samples are shown in Table 2.

The raw wastewater showed an oestrogenic activity of 4.82 ± 0.38 ng/L EEQ (Table 2). Coleman et al. (2007) investigated the same MBR previously and detected slightly lower influent concentrations of 2.41 ng/L. Results in a similar range were reported in Singapore with raw wastewater activity of 2.5-7.1 ng/L (Hu et al. 2007). Studies in England by Kirk et al. (2002) reported activities in mainly domestic wastewater that were generally one order of magnitude higher, ranging from 15 ng/L EEQ up to 80 ng/L EEQ for different sewage treatment plant influents. This study also revealed differences in oestrogenic activities for samples taken at different times, ranging from 15 ng/L up to 40 ng/L for one particular plant. Leusch et al. (2006) conducted a study of STPs in Queensland, Australia and found levels of <4-185 ng/L oestradiol equivalents in raw sewage samples. Factors such as differences in population densities, treatment technologies, socioeconomic factors and climatic differences should be considered when comparing different studies. Temperature can significantly affect the rate of degradation of hormones during activated sludge treatment (Layton et al. 2000).

Androgenic activity levels in the raw influent were found to be 1177 ng/L (Table 2). Coleman et al. (2007) investigated the same MBR in 2006 and reported a value one third of this (478 ng/L). The recent increase in androgenic activity may be due to an increase in population in this area over time as well as seasonal variations. Kirk et al (2002) reported lower levels of oestrogenic and androgenic activity in influent and effluent after rainfall events. The androgenic values for the raw samples are significantly higher than those of the oestrogenic component, being approximately 240 times higher than oestrogenic activity. Coleman et al. (2007) reported values of 74-240 times higher for androgenic activity compared to oestrogenic activity in raw samples from four sewage treatment plants operated by MidCoast Water. Leusch et al. (2006) also reported that androgenic activity in raw and treated sewage in Queensland was much higher than oestrogenic activity being on average 50-100-fold higher. Kirk et al. (2002) have suggested that most of the androgenic activity in municipal sewage with a predominantly domestic input is most likely caused by androgens excreted by humans. Androgen levels in humans are generally higher than oestrogen levels (Leusch et al. 2006). Concentrations of androgens in sewage would therefore be expected to be much higher than those of oestrogens.

Wastewater treatment with MBR technology lead to a significant decrease in oestrogenic activity (78.4%) and in androgenic activity (98.2%) (Table 2). These results are comparable to a previous study by Coleman et al. (2007) on the same MBR where removal rates of 88% for oestrogenic activity and 98% for androgenic activity were observed. The measured removal of 78% is also comparable to those MBR removal efficiencies measured in similar studies reporting removal rates of 68% (Hu et al. 2007).

Coleman et al. (2007) also compared removal rates with secondary treatment (activated sludge). The results showed that the conventional activated sludge (CAS) process and the MBR process are comparable for removal of oestrogenic and androgenic activities from raw sewage. General literature comparison and studies investigating both MBR and CAS treatment technology (Holbrook et al. 2002; Drewes et al. 2005) do not reveal significant differences between MBR and CAS for the capability of removing oestrogenic activity. Drewes et al. (2005) showed that the total oestrogenic activity was removed by an average of 96% during secondary treatment and that two pilot-scale MBRs achieved the lowest concentrations of individual EDCs in secondary treated effluents, showing a better performance than the conventional

full-scale facility operated in the same service area.

Values of 21 ng/L TEq and 1.04 ng/L EEq were observed in the final effluent, which may be biologically significant since some studies have shown reproductive abnormalities in fish at ng/L levels (0.1 ng/L oestrogenic activity and >1 ng/L androgenic activity) (Jenkins et al. 2001; Ellis et al. 2003). It may therefore be prudent to identify the specific chemicals responsible for the observed biological activity, which may provide more information on toxicity and the source of the pollution. This will be the subject of a future publication.

Chemical analysis - Analysis of PPCPs

Table 3 shows the levels of PPCPs detected in influent, MBR and effluent samples. The high concentrations detected in raw wastewater for the analgesic ibuprofen (6474 ± 862 ng/L or 6.47 ± 0.86 μg/L), and for salicylic acid (32 152 ± 400 ng/L or 32.15 ± 0.40 μg/L) (Table 3) as an active metabolite of the analgesic aspirin, are on the upper end but still in the same range as reported in other studies investigating raw wastewater where concentrations ranging from 1.2 μg/L (Clara et al. 2005) to 6.77 μg/L (Radjenovic et al. 2007) for ibuprofen and from 2.72 μg/L (Lee et al. 2005) up to 38.5 μg/L (Al-Rifai et al. 2007) for salicylic acid were reported. The high values are not surprising, as ibuprofen and aspirin are highly consumed pharmaceuticals in Australia with annual dispensed masses of 20 389 kg and 14 196 kg (data from 2004) respectively (Khan and Ongerth 2004). The antibacterial agent triclosan which is widely applied in soaps and detergents was found at a concentration of 1288 ± 160 ng/L or 1.29 ± 0.16 μg/L in the raw wastewater (Table 3). This concentration is of the same magnitude as those reported in Canada (Lee et al. 2005) and in the USA (Snyder et al. 2007). Gatidou et al. (2007) detected 9 μg/L in hospital effluent in Greece. Studies from Japan report concentrations lower than those detected in most of the western countries; this has been attributed to the lower usage of triclosan in Japan (Nakada et al. 2006).

Octylphenol is a degradation product of octylphenol ethoxylate which is a non-ionic surfactant widely used in both domestic and industrial applications, e.g. the manufacture of plastics and elastomers (Gadzala-Kopciuch et al. 2008). The concentration of 4-tert-octylphenol of the investigated MBR influent was 839 ± 41 ng/L (Table 3) which was slightly lower than reported in most literature (Lee et al. 2005; Quintana et al. 2007; Céspedes et al. 2008). The levels of 4-tert-octylphenol reported in wastewater range from several hundred nanograms per litre up to several micrograms per litre depending on the wastewater source. Reports indicate that the higher the industrial fraction of the sewage, the higher the concentration of 4-tert-octylphenol. The fact that the source of the wastewater in this study is domestic would explain the results. Clara et al. (2005) measured concentrations of 118–680 ng/L of 4-tert-octylphenol in raw sewage of a rural area whereas Lee et al. (2005) reported concentrations of up to 3.08 μg/L for wastewater with a high industrial fraction.

All the detected contaminants were removed efficiently from the wastewater during treatment using a MBR with removal efficiencies ranging from 93% (triclosan) up to 99% (salicylic acid, ibuprofen) (Table 3).

Ibuprofen was removed almost completely from the wastewater during treatment, with a removal efficiency of 99%. Other studies reported similar findings during MBR treatment whereas removal with CAS treatment was reported to be in a lower range of 60 – 95% (Kim et al. 2007; Radjenovic et al. 2007; Reif et al. 2008). The excellent removal efficiency of ibuprofen for MBR treatment and the higher values compared to CAS treatment can be explained by the longer sludge retention time (SRT) that can be achieved when using a MBR compared to CAS. Clara et al. (2005) observed a strong influence of the sludge retention time (SRT) for the biodegradation of ibuprofen and determined a critical SRT of 5 days for significant biodegradation of ibuprofen (at 10°C). The studied MBR has a SRT of 30 days which would explain the high removal rates. An almost complete removal of 99% was achieved for the salicylic acid. Similarly, high removal efficiencies were reported for CAS treatment (Lee et al. 2005; Al-Rifai et al. 2007; Quintana et al. 2007) indicating that salicylic acid is easily biodegraded. The investigated MBR showed a triclosan removal rate of 93%. A study investigating the fate of triclosan during MBR treatment carried out by Snyder et al. (2007) reported efficient and nearly complete removals of > 99%. This is higher than removal rates reported during CAS treatment ranging from 58% (Bendz et al. 2005) up to 90% (Lee et al. 2005) with most reported removals being between 80% and 90%. Interestingly, in this study, twice as much triclosan was detected for the final effluent compared to the MBR (Table 3). This could be due to the fact that grab sampling was undertaken, meaning that influent, MBR and effluent samples may not necessarily be representative of each other. It could also be due to matrix effects during GC/MS analysis. The decrease from the influent concentration of 839 ± 41 ng/L down to the effluent concentration of 16 ± 4 ng/L for 4-tert-octylphenol during MBR treatment gave a removal rate of 98%. Clara et al. (2005) investigated the removal of 4-tert-octylphenol by MBR treatment reporting 45%-98% removal. A broad range of removal efficiencies have been reported in different studies for CAS treatment, from 30% (Nakada et al. 2006) up to almost complete removal (Céspedes et al. 2008). The MBR removal efficiencies reported in the literature (Clara et al. 2005; Kim et al. 2007; Radjenovic et al. 2007; Snyder et al. 2007; Reif et al. 2008) are similarly high to those in this study and in general slightly higher than those measured for CAS treatment, indicating that MBR treatment is a promising technology for the removal of the investigated trace organics.

Despite the high removal rates for the investigated PPCPs, environmentally-relevant concentrations for ibuprofen, salicylic acid, triclosan and 4-tert-octylphenol of 11 ± 2 ng/L 15 ± 5 ng/L, 89 ± 11 ng/L and 16 ± 4 ng/L, respectively, were found in the effluent samples. Several studies reported the occurrence of these compounds in surface water with major sources being sewage treatment plant effluents (Bolz et al. 2001; Céspedes et al. 2008). In general, PPCP concentrations measured in surface waters are well below concentrations that are known to cause acute toxicity to aquatic organisms. However, chronic exposure to pharmaceutically active substances and/or endocrine disrupting chemicals such as octylphenols has the potential for more subtle effects, such as

Table 2. Fate, levels and % removal of oestrogenic and androgenic activities in a membrane bioreactor (MBR).

	Influent (ng/L)	MBR (ng/L)	Effluent (ng/L)	% removal
Oestrogenic Activity	4.82 ± 0.38	1.24 ± 0.20	1.04 ± 0.21	78.4
Androgenic Activity	1177 ± 180.7	289 ± 4.52	21.1 ± 4.46	98.2

Table 3. Fate, levels and % removal of pharmaceuticals and personal care products in a membrane bioreactor (MBR).

	Influent (ng/L)	MBR (ng/L)	Effluent (ng/L)	% removal
Ibuprofen	6474 ± 862	12 ± 1	11 ± 2	99.8
Salicylic acid	32 152 ± 400	46 ± 5	15 ± 5	99.9
Triclosan	1288 ± 160	43 ± 2	85 ± 10	93.1
4-tert-octylphenol	839 ± 41	74 ± 4	16 ± 4	98.1

metabolic or reproductive changes on non-target organisms (Daughton and Ternes 1999). Besides the aqueous effluents, another major environmental source of the trace organics could be the use of the digested sludge in agriculture, especially for chemicals with high tendency to adsorb to biosolids and a high stability against biodegradation such as alkylphenols (Céspedes et al. 2008). Studies by Lapen et al. (2008) detected several PPCPs after land application of municipal biosolids in tile drainage, including triclosan and ibuprofen.

CONCLUSION

The efficiency of a MBR for the removal of EDCs and PPCPs was assessed using biological (YES and YAS screens) and GC-MS analysis. Raw sewage samples, MBR supernatant and MBR effluent were analysed for oestrogenic and androgenic activities, ibuprofen, salicylic acid, triclosan and 4-tert-octylphenol. Results showed that the MBR removed over 78% of oestrogenic activity and over 98% of androgenic activity from raw sewage samples. Removal rates for PPCPs ibuprofen, salicylic acid, triclosan and 4-tert-octylphenol were 99.9%, 99.8%, 93.1% and 98.1%, respectively. The high removal rates are comparable with conventional full-scale activated sludge processes and are attributed to the high SRT of the MBR of 30 days. These observations are highly encouraging from the point of view of implementing these systems as 'package plant' units for the decentralised treatment of effluent from single households or clusters of homes and the potential application of the treatment effluent for alternative water management practices such as water reuse and recycling.

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DEVELOPMENT AND APPLICATION OF TOXICITY TESTS FOR THE EFFECTS OF AN INSECT GROWTH REGULATOR, TEBUFENOZIDE, ON A MAYFLY NYMPH, *ATALOPHLEBIA* SP.

Anne Colville[1*], Ross V. Hyne[2], Richard Lim[1] and Peter Petocz[3]

[1]School of Life Sciences, University of Technology Sydney, PO Box 123, Broadway, NSW 2007, Australia.

[2]Ecotoxicology and Environmental Contaminants Section, Department of Environment and Conservation, New South Wales, c/- Centre for Ecotoxicology, PO Box 29, Lidcombe, New South Wales 1825, Australia.

[3]Department of Statistics, Macquarie University, North Ryde, NSW 2109, Australia. ppetocz@efs.mq.edu.au

ABSTRACT

To determine suitable test conditions for laboratory experiments, studies were carried out to develop culturing and toxicity testing methods for the nymphs of the mayfly *Atalophlebia* sp. AV13. A diet of very soft, conditioned leaves produced the highest moulting and growth rates. Growth occurred between moults, but increased on the day following a moult. Moulting frequency decreased with increasing nymph size. Models fitted to growth data suggested that mayfly nymphs approximately 2 mm long could be used for growth tests in the laboratory for up to 40 days before growth rates slow and the adult mayflies begin to emerge. Experiments on growth rates should use small mayfly nymphs and the test period should extend over at least seven days, to reduce the influence of growth peaks during moulting. Nymphs longer than 9 mm are unsuitable for growth tests, because of large moult/intermoult variations, and because growth almost ceases in final instar nymphs.

A 14-day test examined the effects of the Insect Growth Regulator tebufenozide on survival, growth and moulting rates of 2-3-mm-long mayfly nymphs. At concentrations from 0.1 to 1 mg/L, tebufenozide caused incomplete and abnormal moulting leading to death. At 2 mg/L, tebufenozide caused mortalities but by a mechanism that apparently did not involve abnormal moulting. The LC50, LOEC and NOEC values obtained were: 96-h: 0.63 mg/L, 0.60 mg/L, 0.32 mg/L; 7-day: 0.39 mg/L, 0.60 mg/L, 0.32 mg/L; and 14-day: 0.28 mg/L, 0.32 mg/L, 0.11 mg/L, respectively. There was no effect on growth rates at tebufenozide concentrations below the LC50, but there was a significant increase in moulting rates at 7 days in 0.05 mg/L.

A test of 0.05-0.3 mg/L tebufenozide on large nymphs found no adverse effects on emergence success. Female fecundity was reduced in 0.3 mg/L tebufenozide, but the number of nymphs was too small to be statistically significant.

The results suggest that mayfly nymphs are among the most sensitive of aquatic invertebrates to the effects of tebufenozide.

Key words: Ephemeroptera; growth; moult; diet; tebufenozide, IGR

INTRODUCTION

This study was carried out to examine the effects of the pesticide, tebufenozide, on an Australian mayfly nymph in freshwater streams. Tebufenozide, a bis-acylhydrazine, is one of a group of pesticides known as Insect Growth Regulators (IGRs), which act by mimicking arthropod hormones. They target hormonal functions specific to arthropods, and therefore have low toxicity to vertebrates (Dhadialla et al. 1998; Smagghe et al. 2012). Tebufenozide mimics the ecdysteroid hormones such as 20-hydroxyecdysone, which occur in all arthropods, and which have been shown to have hormonal regulatory functions in arthropods and possibly other invertebrate phyla (Lafont and Koolman 2009). The best-known effects of ecdysteroids are on moulting, growth and gametogenesis. However, ecdysteroids have also been found to affect sleep patterns in *Drosophila* (Ishimoto and Kitamoto 2010); memory creation, storage and retrieval in *Drosophila* (Ishimoto et al. 2009); stress resistance and lifespan in *Drosophila* (Simon et al. 2003); and neuronal activity in crayfish (Haskell and Moorhouse 1963; Tomaschko 1999; Bacqué-Cazenave et al. 2013).

Tebufenozide, and more recently the related analogue methoxyfenozide, are particularly selective towards lepidopteran species (Smagghe and Degheele 1994a; Carlson 2000; Carlson et al. 2001), and are registered by the Australian Pesticides and Veterinary Medicine Authority in Australia for control of a range of lepidopteran pests (https://portal.apvma.gov.au/permits). Several studies on the effects of tebufenozide on aquatic species have been carried out in Canada on plankton (Kreutzweiser and Thomas 1995; Kreutzweiser et al. 1998; Gómez de Barreda Ferraz et al. 2004) and macroinvertebrates (Kreutzweiser et al. 1994), and a study in Germany on chironomids (Hahn et al. 2001). There have been some published studies in Australia on the effects of tebufenozide on terrestrial insects (Valentine et al. 1996; Gurr et al. 1999; Nicholas and Thwaite 2003; Lewis and Gorton 2007), but there is only one known Australian study on non-target aquatic invertebrates (Townsend 2013). The recently-revised Australian Water Quality Guidelines emphasise the need for data from a large number of species and taxa to derive national guideline values, rather than prioritising local species (Warne et al. 2014). However, they

*Author for correspondence, email: Anne.Colville@uts.edu.au

encourage the use of local, site-specific investigations when guideline values are exceeded, so there is a need for testing methods and data for Australian species.

This study examined the effects of tebufenozide on mayflies, because of their ecological importance and sensitivity (Chessman 2003). The species chosen was *Atalophlebia* sp. AV 13 (Ephemeroptera: Leptophlebiidae), a morphospecies of medium sized mayflies (maximum length of about 12 mm) with a widespread distribution throughout eastern Australia (Dean 1999; Baker et al. 2004). These mayflies are predominantly found in slow-flowing streams and pools, on both stones and leaf litter substrates (Peters and Campbell 1991; Christidis 2003). Because the species is accustomed to low-flow conditions, it is a suitable test animal for static-renewal laboratory testing.

To give a comprehensive assessment of the potential toxicity of tebufenozide it was necessary to test for a range of sublethal effects in addition to acute toxicity. There was no information available on suitable culture methods for mayfly nymphs of *Atalophlebia* species, so some preliminary tests were carried out to find an appropriate diet to support long-term growth. Growth rates and moulting frequencies were also investigated, because changes in growth rates may be an indicator of sublethal toxic effects.

A potential difficulty in measuring growth in insect larvae is the effect of moulting on growth rates. The classical model of arthropod growth is that growth occurs predominantly in the period immediately following moulting while the new exoskeleton is soft; once the cuticle has hardened the size remains relatively fixed until the next moult (Dyar 1890; Imms 1961). This is generally true of heavily sclerotised structures such as the head capsule or carapace, which are usually used in determination of the number of instars following the recommendation of Dyar (Dyar 1890), e.g., (Kondratieff and Voshell 1981; Alencar et al. 2001; Kosnicki and Burian 2003; Hamid et al. 2016). However, soft-bodied larval insects can grow in length between moults because the epicuticle is deposited in folds that can unfold and expand (Nijhout et al. 2014). The effects of moulting on growth rates were also examined to determine if it is necessary to take account of moults in the measurement of growth rates. The growth curves of a range of nymphs of different sizes were also used to model the predicted lifespan of nymphs in the laboratory, to estimate the possible time frame for long-term experiments.

The mayfly nymphs were then used as a representative test species to examine the effects of tebufenozide on growth rates, moulting rates, female fecundity and emergence success in non-target aquatic invertebrates.

MATERIALS AND METHODS

Test animals

Mayfly nymphs were collected by kick sampling in Alderley Creek (-32.448, 151.9668) or the Karuah River (-32.4058, 151.9518) near the town of Stroud, New South Wales, Australia. The samples were transferred to an insulated container containing aerated creek water and nylon netting to provide a substrate for the nymphs to cling to, and transported to the laboratory at the University of Technology, Sydney (UTS) on the same day. The water temperatures ranged between 17-25°C and the conductivity was approximately 250 µS cm-1. The nymphs were held in a constant temperature room at 25°C to acclimate before use in experiments. Medium and large Atalophlebia nymphs in the collection were identified as *Atalophlebia* sp. AV13, using the key in Dean (Dean 1999). Smaller nymphs used in these experiments could not be identified to species with this key, but they were similar in appearance to the larger nymphs, so it was assumed that they were probably also *Atalophlebia* sp. AV13

Body length measurements

Length measurements were taken in triplicate, using a Flinders Imaging MD-20 Image Analysis System as above. To measure body lengths, nymphs were transferred to a Petri dish containing just enough water to cover the body, so they could not raise their tail filaments, and examined under minimum illumination that allowed an image to be captured. Body length was measured from the front of the labrum to the end of the abdomen, using images taken while the animal was stationary, and not appearing stressed. If nymphs were disturbed, they tended to draw their legs in under the body and shorten the abdomen; this behaviour was interpreted as a sign of stress. Length measurements were chosen, rather than head width as in some studies (Hanes and Ciborowski 1991; Corkum and Hanes 1992; Tada and Hatakeyama 2000), because live nymphs did not always hold their heads in a suitable orientation for measurement, so length measurements were more reliable.

COMPARISON OF DIETS

Small *Atalophlebia* nymphs (approximately 2-3 mm length excluding tail filaments) were selected and placed in individual 250-mL beakers containing 150 mL dechlorinated Sydney tap water adjusted to a conductivity of 250 µS cm^{-1} with seawater. Test beakers were covered with cling film to reduce evaporation, placed on trays, and aerated gently (approximately 60 bubbles per minute) through Pasteur pipettes inserted through the cling film. The trays were placed in environmental chambers set at 25°C, and kept in the dark except when they were removed for maintenance and measurements.

Six replicates of five different food treatments were tested in the first diet experiment. Treatments were: (1) very soft conditioned leaves (approximately 15 cm^2, which covered half the bottom of the beaker; (2) leaves which appeared well conditioned but were still firm and intact (approximately 15 cm^2); (3) five-cm length of the pond weed, *Myriophyllum*

sp.; (4) Corn Flakes (Sanitarium brand) (approximately 3 cm^2); and (5) Vita Brits (Sanitarium brand) (approximately 3 cm^2**).** Food was renewed as required when the water was changed. A second test compared microwaved lettuce with very soft conditioned leaves as a food. Corn flakes, VitaBrits and lettuce were tested as potential artificial diets that could be obtained more easily, and would provide greater consistency, than conditioned leaves.

The conditioned leaves were collected from some stream mesocosm troughs at the UTS Stroud field station. Since the leaves that were sufficiently conditioned were no longer recognisable, the plant species could not be identified. The *Myriophyllum* sp. was collected from Alderley Creek; the stems were covered with natural black biofilm. Cornflakes and Vita Brits were obtained commercially. Lettuce was prepared by packing outer green leaves of iceberg lettuce into a 1-L microwave-safe plastic container, covering with cling-film, and heating for 3 minutes on full power (600W) in a domestic microwave oven.

Beakers were allocated randomly to different food treatments, with three replicates of each treatment repeated on two trays. Water was changed twice weekly, except for the Corn Flakes and Vita Brits treatments. After two days it was found that these had to be changed daily or the water became cloudy. Physicochemical measurements (pH, dissolved oxygen (DO), conductivity and temperature) were taken in the beakers before renewals, and in the fresh culture water. Moults were recorded daily. Mayfly nymph body lengths were measured at days 0, 7 and 14 of the first experiment, and on days 0, 3 and 6 for the lettuce vs soft leaves comparison. Growth was calculated as the ratio of the length at time x to the initial length.

Mayfly nymph moulting and growth patterns

Culture and measurements

The moulting and daily growth patterns of 45 individual *Atalophlebia* sp. AV13 nymphs were recorded. Nymphs were placed in 250-mL beakers containing 170 mL dechlorinated Sydney tap water with an adjusted conductivity of 250 μS cm^{-1}, aerated, and covered in cling film to reduce evaporation. Nymphs were fed *ad lib* with conditioned leaves from the mesocosm system at Stroud. The beakers were placed in environmental cabinets at 25°C in the dark.

Because of limited space in the cabinet, most nymphs were housed in pairs of very different sizes, so there would be no confusion as to the identity of each animal. Observations indicated that the paired nymphs did not interfere with each other and there was enough space, food and shelter for both. The remaining nymphs, which were of similar sizes, were housed individually. Culture water was renewed three times weekly. When water was renewed, the pH, temperature, DO and conductivity were measured in four randomly-selected beakers on each shelf in the environmental cabinet before the water change, and in the renewal water.

For most nymphs, measurements were carried out over 12 days. On the thirteenth day there were electrical problems in the building, which was transferred to a limited emergency power supply, so part of the experiment had to be terminated. Some recently-moulted nymphs were maintained for a further 1 or 2 days to obtain data on post-moulting growth. For a selection of large nymphs, measurements were continued until they emerged to subimagos and imagos.

Effect of moulting on daily growth rates

From the growth curves of 46 individual nymphs over 12 or more days, we selected 45 sets of body length measurements which covered a period of three days before and three days after a moult (for small nymphs, this sometimes included another moult). The data sets were sorted into three groups according to the initial length of the nymph (small, 2.2 - 4.49 mm, n = 13; medium, 4.5 - 5.99 mm, n = 19; large, ≥6.00 mm, n = 13). For each group, mean body length, and the mean percentage daily increase in body length, were calculated for each day. Percentage increase in body length for a given day x was calculated as [(length on day x) – (length on day x-1)]*100/(length on day x-1).

Estimation of mean growth curves and life span

The individual growth curves obtained from the 46 *Atalophlebia* sp. AV13 nymphs were adjusted to the left or right along the time axis (by addition to or subtraction from the time axis values) until the maximum degree of overlap was obtained, to provide a "composite data set". Two alternative models were derived mathematically: first, straight lines were fitted to data from individual mayflies and the slope of each line was graphed against the mayfly length at the midpoint of the line. The resulting quadratic pattern suggested a logistic curve (with or without a non-zero lower asymptote), and the equations of these curves were derived from the fitted quadratics. The individual growth curves were then adjusted to follow these curves, forming two more composite data sets. Nonlinear regression methods were then used to fit the appropriate curve to the each data set (Ratkowsky 1990). The success of the estimation and fitting process was measured by the residual mean square from the regressions, and an equivalent process was carried out with a linear model, clearly inappropriate, but able to provide a useful comparison.

The size of newly-hatched *Atalophlebia* sp. AV13 nymphs was not known, but was estimated from measured egg size, mean ± standard error of mean = 0.163 ± 0.001 mm (n = 20). By analogy with *Atalophlebia* sp. AV6, where the new-hatched young are approximately twice the length of the egg (R. V. Hyne, pers. observation), the young of *Atalophlebia* sp. AV13 were assumed to be approximately 0.3 mm long. The three-parameter logistic model was adjusted so that it corresponded to an initial mayfly length of 0.3 mm, and the resulting model and data set were used to estimate the life span of nymphs in culture in the laboratory.

Acute and subacute toxicity of tebufenozide

Two tests of the toxicity of tebufenozide were carried out: a 14-day toxicity test with small mayflies, and a test with large mayflies examining the effects on emergence and female fecundity.

Test animals

Mayfly nymphs for the toxicity experiments were collected from the Karuah River near Stroud, NSW, and acclimated in a constant temperature room at 25°C before commencement of the test. For the acute test, small nymphs were selected (mean length of the nymphs, excluding caudal filaments, was 2.6 mm; range 1.7-3.6 mm). For the test on emergence and female fecundity, nymphs >8 mm long were selected.

Experimental conditions

Tests were carried out in 250-mL beakers under conditions as for the growth pattern experiment. Test solution volumes were 160 mL for the acute testing with small mayflies, and 200 mL for the tests with large mayflies. Solutions were renewed three times weekly, and food (very soft conditioned leaves) was renewed as required.

Test solutions

The test solutions were prepared by spiking stock solutions of technical grade tebufenozide at appropriate concentrations in acetone, into dechlorinated Sydney tap (final acetone concentration 50 µL/L). Dechlorinated Sydney tap water, and dechlorinated Sydney tap water containing 50 µL/L acetone, were used as water and solvent controls.

14-day test of tebufenozide on small mayfly nymphs

Tebufenozide test solutions were prepared at final nominal concentrations of 0.02, 0.05, 0.1, 0.3, 0.6, 1.0 and 2.0 mg/L. The experimental design was a randomised block, with 12 blocks each containing all nine treatments. The nymphs were allocated randomly to test beakers, using random numbers generated with the program Qbasic in the statistical application SYSTAT.

Observations

Mayfly nymphs were examined daily, and dead nymphs and exuvia were removed and recorded. Incomplete moults were not included in the counts. Mayfly lengths were measured on days 0, 7 and 14. Three times weekly, pH, temperature, dissolved oxygen and conductivity were determined in selected beakers before solutions were renewed, and in the fresh solutions. Samples for tebufenozide analysis were collected from the freshly-made culture solutions on three renewal days, and from culture beakers at the end of two of those renewal periods, to determine mean initial concentrations and change in concentration over the renewal period. The samples were centrifuged for 15 min at 2500 rpm to settle any debris, and analysed within 12 hours.

Effects of tebufenozide on emergence success and female fecundity

Test animals

Test animals were large (>8 mm long) *Atalophlebia* sp. AV13 nymphs selected from the same collection used for the 14-day test, acclimated in the laboratory for four weeks before commencement of the test.

Experimental conditions

Test solutions were water control, solvent control and nominal tebufenozide concentrations of 0.05, 0.1 and 0.3 mg/L. Fifteen 250-mL beakers each containing 200 mL culture solution and one large female *Atalophlebia* sp. AV13, and fifteen beakers each containing one large male and one large female were set up. Soft conditioned leaves were added *ad lib*. for food and shelter. A 7-10 cm length of eucalyptus twig was placed in each beaker to allow the mayfly nymphs to climb out of the water when emerging.

Observations

Mortalities and successful emergence were recorded. Emerged female mayflies were dissected, and the eggs were flushed from the abdomen with culture water, and counted using a Sedgwick-Rafter counting chamber.

Tebufenozide analyses

Tebufenozide analysis was carried out using a Waters HPLC, Alltech C18, 5 µm mesh column (Cat No 88054, dimensions 250 mm x 4.6 mm) and C-18 guard column, using an isocratic elution solvent (80% HPLC grade methanol, 20% polished RO water) at flow rate 1 mL/minute, at room temperature (approximately 22°C). Standards were prepared from analytical grade tebufenozide, dissolved in 10% methanol/90% polished RO water to ensure that all the tebufenozide remained in solution (Colville 2003). Aliquots of the aqueous samples were injected directly into the HPLC. The presence of methanol shifted the retention time of the standards slightly compared with the aqueous samples, but the peaks were still clearly identifiable.

Statistical analyses

Unless otherwise specified in the results, statistical tests and growth modelling analyses were carried out using the statistical package SPSS Version 12.0. The normality and homogeneity of variances of the data were tested using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance, to determine whether parametric or non-parametric statistics were appropriate. The tests used are noted in the results section. All statistical analyses were tested against a significance level of $\alpha = 0.05$, with Bonferroni adjustment for multiple comparisons (Moore and McCabe 1999).

LC50 values (lethal concentration required to kill 50% of the population) were calculated by the trimmed Spearman-Karber method (Hamilton et al. 1977, 1978). NOECs (No Observed Effect Concentrations) and LOECs (Lowest Observed Effect Concentrations) were determined by Fisher's Exact test, using the statistical package Toxstat 3.3 (Gulley et al. 1988), according to the instructions given in the US EPA guidance document EPA-821-R-02-012 (US EPA 2002).

RESULTS

Comparison of diets

Except for the dissolved oxygen (DO) in the Vita Brits treatments on Day 2, the physicochemical conditions in all treatments were within acceptable ranges for the mayflies (ranges for conductivity, 250-372 μS cm^{-1}; temperature, 23-26 °C; DO, 69-100% saturation and pH, 7.5-8.0, respectively. After the frequency of water changes was increased, the DO in the Vitabrits treatment was also acceptable.

In the first test of diets, after 14 days the order for growth rates (calculated as the ratio of body length at 14 days to initial body length) was (hard leaves, Corn Flakes, Vita Brits) < *Myriophyllum* < soft leaves (Figure 1a).

The bodies of the nymphs fed Corn Flakes were covered with an unidentified growth. Nymphs fed Vita Brits grew slowly in the first week, but in the second week, when the water conditions were managed better, their growth rates increased considerably. Mayfly nymphs fed on microwaved lettuce grew at only 66% of the rate of nymphs fed soft leaves (two-tail t-test, $p < 0.001$) (Figure 1b).

The type of diet had a significant effect ($p < 0.005$) on the moulting frequency (Figure 2). Because the variability in the number of moults was large, most pairwise differences were not significant ($p > 0.05$). However, the nymphs fed on soft leaves and *Myriophyllum* moulted more frequently (Tukey's HSD, $p < 0.05$) than those fed on hard leaves.

Mayfly nymph moulting and growth patterns

Physicochemical conditions in the test beakers were similar to the values reported for the food type experiment, except the mean water temperature was 23°C. Figure 3 shows the overall pattern of growth curves obtained from all the mayfly nymphs measured. Growth rates varied from around 4% per day for nymphs 2-6 mm long, to 2.2% for nymphs 6-8 mm long, and 1.3% for nymphs above 8 mm long.

A composite growth curve was derived from five individual growth curves arranged so the lengths and moults were aligned as closely as possible (Figure 4), providing a clearer display of the growth pattern. The slopes of the growth curves increased around the time when a moult exuvium (the shed exoskeleton) was recorded, especially for the larger nymphs, but growth also occurred between moults. Growth was almost linear from about 2.5 mm until the final instar, when the rate decreased significantly ($p < 0.001$). The mean ± standard error of mean of the growth rate at 23°C for mayflies in the second-last instar was 0.22 ± 0.04 mm/day; this decreased to 0.03 ± 0.03 mm/day for the final instar.

Comparison of the lengths of seven male and seven female mayfly nymphs on the final day before emergence found no significant difference in length between the sexes (mean lengths (mm): female 10.22, sd 0.728; male 10.47, sd 0.543; t-Test: Two-sample assuming unequal variances p = 0.490).

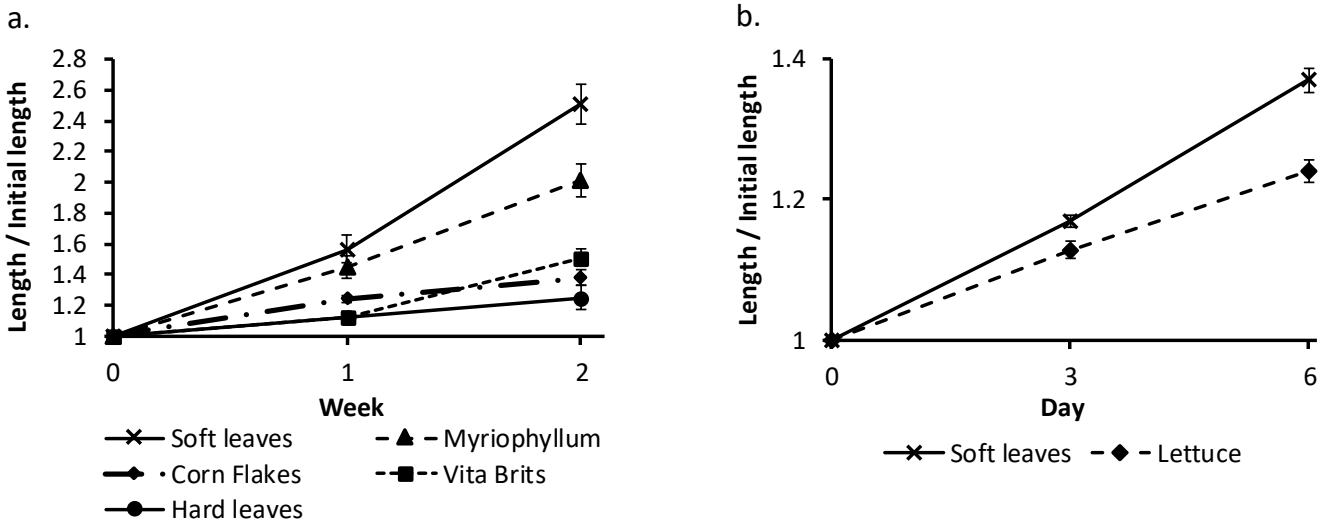

Figure 1. Effect of diet on growth of mayfly nymphs.
a. Mean growth on diets of soft leaves, *Myriophyllum* sp., Corn Flakes, Vita Brits and hard leaves over 2 weeks. n = 5 or 6 for each diet.
b. Mean growth on diets of soft leaves and microwaved lettuce over 6 days. n = 15 for soft leaves; 16 for lettuce.
Growth for each nymph was calculated as the proportion of the initial length. Error bars = standard error of the mean.

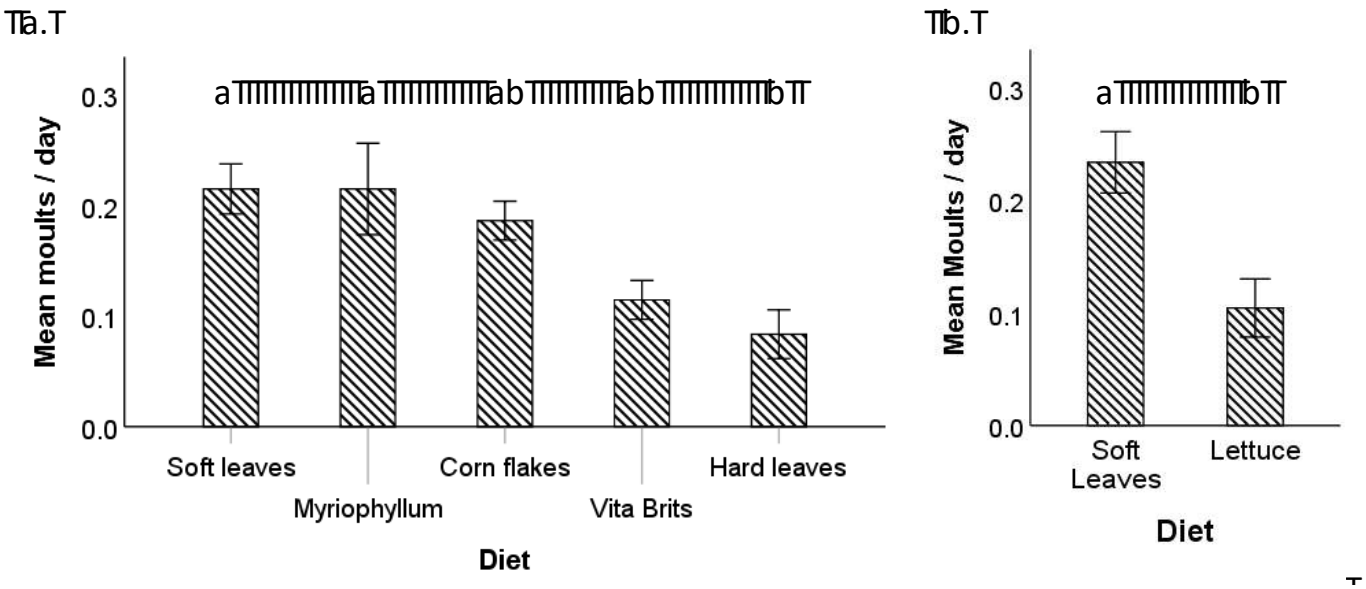

Figure 2. Effect of diet on the moulting rates of mayfly nymphs. Moulting rates were measured over 14 days for the first experiment, and over 6 days for the lettuce/soft leaves experiment. Bars with different superscript letters are significantly different.
a. Mean moulting rate on diets of soft leaves, *Myriophyllum* sp., Corn Flakes Vita Brits and hard leaves over 2 weeks. n = 5 or 6 for each diet. (Bonferroni multiple comparisons; Hard leaves were significantly different from soft leaves and *Myriophyllum*, $p < 0.05$.)
b. Mean moulting rate on diets of soft leaves and microwaved lettuce over 6 days. n = 15 for soft leaves; 16 for lettuce. (Mann Whitney test $p < 0.01$)
Error bars = +/1 1SE

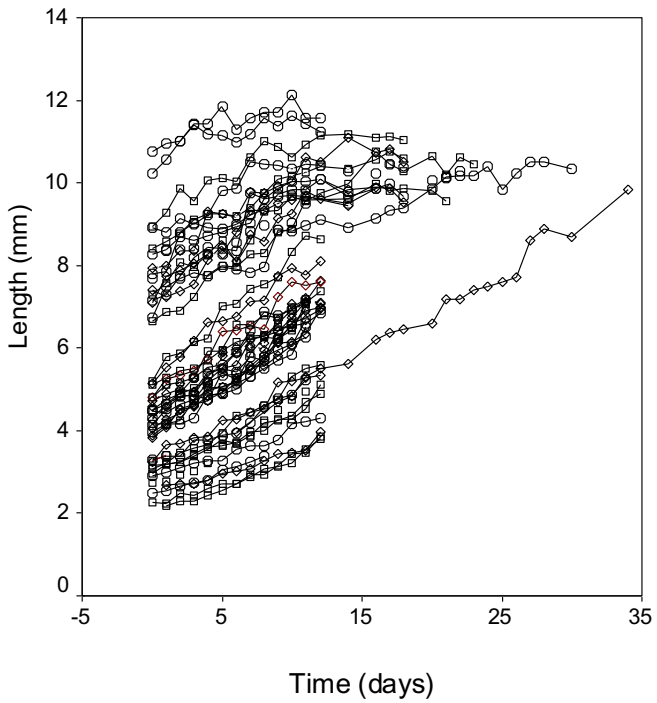

Figure 3. Growth curves for 46 mayfly nymphs in laboratory culture at 23°C. Each point represents the mean of three independent length measurements from three photographs for an individual nymph.

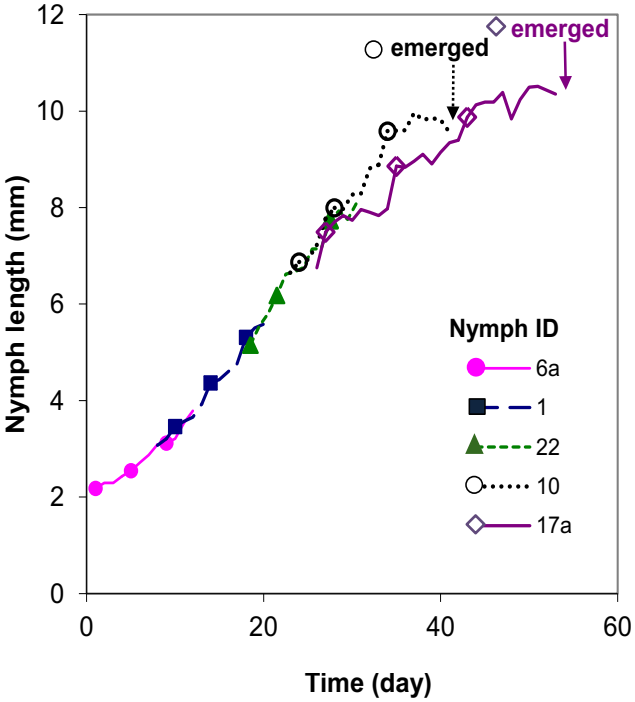

Figure 4. Composite growth curve derived from five mayfly nymphs, matched as closely as possible for body length and moults. Each point is the mean of three body length measurements taken from three photographs of a nymph. Markers indicate the days when moult exuvia were recorded for the respective nymph. Curves for two large nymphs (open markers) are shown in parallel; arrows labelled "emerged" mark the days of final moults and emergence to the sub-imago form.

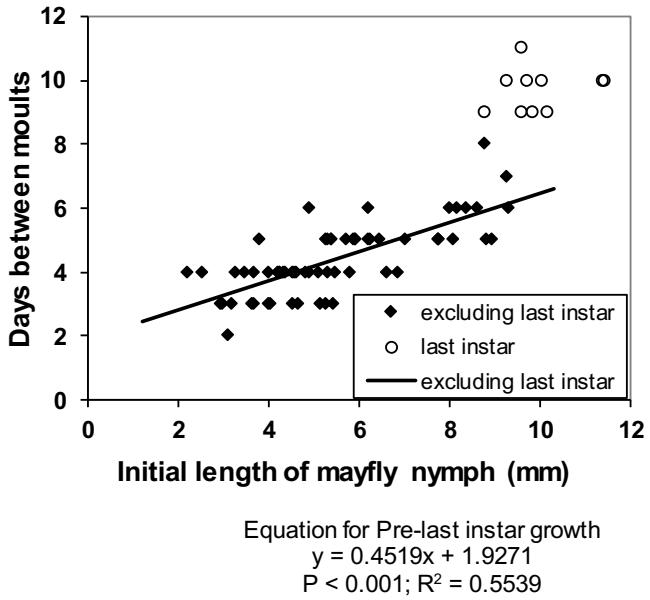

Figure 5. Moulting frequency for mayfly nymphs of different lengths. Values for nymphs in the final instar are plotted separately from earlier instars.

Moulting frequency

Moulting frequency decreased with increasing mayfly length in nymphs excluding to the final instar (Figure 5). The final nymphal instar lasted considerably longer than earlier instars. Small nymphs moulted more frequently than large individuals. The mean time between moults ranged from 3 days for 3-mm nymphs, to 6 days for 9-mm nymphs (Figure 5), while the final instar lasted an average of 10.1 days (s.e. 0.5, n = 16) before the nymphs moulted to emerge as winged subimagos.

Effect of moulting on mayfly nymph growth rates

The patterns of growth before, during and after moulting for pooled data for small, medium and large mayfly nymphs are shown in Figure 6. All mayflies showed a growth spurt with moulting (Figure 6a). In terms of absolute length increments, large and medium-sized mayfly nymphs grew more during moulting peaks than small mayfly nymphs did, but when comparison was made of growth as a percentage increase in length, the small and medium nymphs grew more than the large ones (Figure 6b). The difference between the intermoult growth rate and rate during the moulting peak was more marked for the large nymphs than for small ones. The growth peak at moulting for large nymphs (approximately 7% per day) was seven-fold higher than the intermoult growth rate (~1% per day). Small nymphs showed a peak growth of approximately 9% per day at moulting, but they grew faster between moults (approximately 3% per day), so the peak represented only a three-fold increase.

Estimation of nymph life span in laboratory culture

The results of the analyses of the mayfly growth curves are shown in Table 1. The best model was the mathematically derived three-parameter logistic curve (although the curve obtained by eye was close). The fitted 3-parameter logistic model (translated), (model 4 in Table 1) is plotted in Figure 7, over the points for the mayfly lengths. From this logistic model, it was estimated that the smallest nymphs used in this study (2.2 mm long) would have taken 30 days to grow from a hatch length of 0.3 mm to 2.2 mm under the culture conditions at a water temperature of 23°C.

The lengths of 14 mayfly nymphs undergoing the penultimate nymphal moult to the last instar ranged from 8.6-11.4 mm (mean 10.0 mm). These lengths correspond to modelled ages of 65 – 95 days (mean 75 days). The lengths of these nymphs before they underwent the final nymphal moult and emerged ranged from 9.5 – 11.6 mm (mean 10.3 mm).

Acute and subacute toxicity of tebufenozide – Lethality, moulting rates and growth

Experimental conditions

The mean initial length of the selected mayflies in this test was 2.61 mm, with standard deviation, 0.38 mm. There was no significant difference in initial length among the mayflies allocated to the different treatments (ANOVA, p =0.174). The experimental conditions (Table 2) remained within acceptable limits.

Table 1. Summaries of the models fitted.
The linear model was of the form: length = $A + Bt$, where A is the Y intercept and B is the slope.
The logistic curves were of the form: length = $D + \frac{A}{1 + e^{B-Ct}}$
where D = an optional parameter representing the minimum value, (D +) A = maximum value, B adjusts the value at t = 0, C = a parameter that adjusts the rate of increase, t = time (days).
RMS = Residual Mean Square.

		A	*B*	*C*	*D*	RMS
0.	Linear model for comparison	1.11	0.16			0.1417
1.	3-parameter logistic (by eye)	11.72	1.95	0.080		0.0858
2.	4-parameter logistic	10.08	2.98	0.107	1.51	0.1119
3.	3-parameter logistic	12.01	1.94	0.070		0.0848
4.	3-parameter logistic (translated)	12.01	3.62	0.070		0.0848

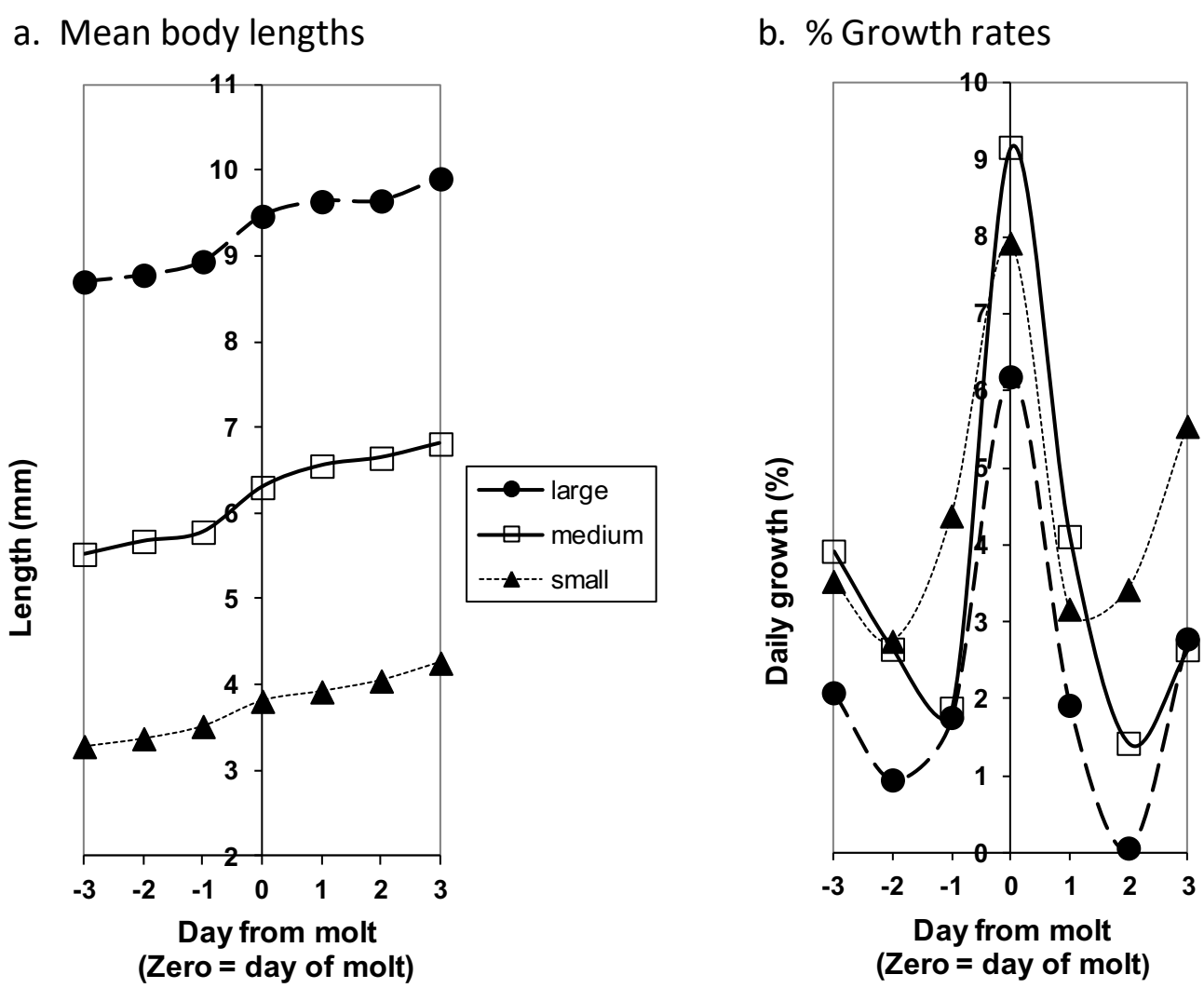

Figure 6. Effect of moulting on mayfly nymph growth rates.
Growth curves for small (2-4.49 mm) [▲] (n = 13), medium (4.5 – 6.99 mm) [□] (n = 19) and large (≥7 mm) nymphs [●] (n = 13), aligned to synchronise day of moulting as Day 0.
a. Mean body length of nymphs. b. Daily growth (percentage increase in body length in 24 hours).

All mayfly nymphs used in the acute/subacute test were *Atalophlebia* sp. and all nymphs large enough to be keyed out were identified as *Atalophlebia* sp. AV13 (Dean 1999).

The mean measured initial concentrations of tebufenozide in the test solutions were within ±10% of nominal values (Table 3). The mean change in concentration over the renewal periods was less than 10% for all concentrations except 2 mg/L, where the concentration decreased by 21%.

Acute toxicity

The time-response of the mayfly nymphs to tebufenozide is shown in Figure 8, and the calculated LC50 values, LOECs and NOECs are listed in Table 4. Death rates in 1 mg/L were higher than in 2 mg/L tebufenozide on days 1-4 (although the difference was not significant; Kaplan-Meier, Breslow (Generalised Wilcoxon test), p = 0.072), but from the fifth day the death rate in 2 mg/L caught up and passed that in the 1 mg/L tebufenozide solution. A number of the mortalities in the 0.3 and 0.6 mg/L tebufenozide treatments did not occur until the second week, so the toxicity was still increasing up to two weeks' exposure, indicating delayed toxicity.

The mode of action in 2 mg/L tebufenozide appeared to be different from that in the lower doses. At 1 mg/L or lower, animals appeared to behave normally when introduced into the test solution. Animals introduced into 2 mg/L tebufenozide solutions appeared shocked (curled up backwards and fell to the bottom motionless), although they recovered after some minutes and attached to the leaves in the beaker. However, they generally failed to feed.

Examination of dead nymphs also suggested a difference in cause of death. Of the 33 mayfly nymphs that died in concentrations of 1 mg/L or lower, 70% showed evidence of difficulties with moulting, such as short abnormal tail filaments, attached exuvia or missing limbs when limb tissues and new exoskeleton could not be withdrawn from the old exoskeleton. Of 11 dead nymphs in 2 mg/L tebufenozide, one was badly decomposed but might have shown abnormal moulting, and the remainder showed no evidence of moulting before death.

Table 2. Mean physicochemical measurements of test solutions in the acute/subacute test on mayfly nymphs.
Measurements were taken in beakers before solution renewals, and in the freshly-prepared replacement solutions. Replacement solutions showed little variation between concentrations on any given day, so measurements for all concentrations are averaged. The number of measurements varied with concentration because of mortalities in the higher concentrations. n =33 (controls, 0.02. 0.05, 0.1 mg/L), 28 (0.3 mg/L), 15 (0.6 mg/L), 9 (1 mg/L), 10 (2 mg/L).

Tebufenozide treatment	Conductivity µS/cm (s.e.)	Temperature °C (s.e.)	pH (s.e.)	DO (% saturation) (s.e.)
In beakers before renewals				
control	217 (1.8)	23.87 (0.11)	7.75 (0.03)	98.0 (0.2)
solvent control	221 (1.9)	23.87 (0.11)	7.74 (0.03)	97.5 (0.4)
0.02 mg/L	222 (2.0)	23.67 (0.09)	7.73 (0.03)	96.6 (0.6)
0.05 mg/L	223 (3.1)	23.71 (0.10)	7.73 (0.03)	97.7 (0.3)
0.1 mg/L	225 (2.2)	23.55 (0.08)	7.74 (0.03)	97.8 (0.2)
0.3 mg/L	226 (2.9)	23.62 (0.07)	7.75 (0.03)	97.8 (0.5)
0.6 mg/L	227 (6.1)	23.66 (0.08)	7.80 (0.04)	97.6 (0.9)
1.0 mg/L	228 (6.3)	23.59 (0.29)	7.83 (0.03)	98.8 (0.3)
2.0 mg/L	221 (2.9)	23.66 (0.19)	7.83 (0.02)	98.0 (0.6)
Replacement solutions (mean of all concentrations)	205.8 (0.2)	25.60 (0.18)	7.80 (0.02)	98.5 (0.1)

Table 3. Measured tebufenozide concentrations in test solutions in acute/subacute test on mayfly nymphs.

Nominal tebufenozide concentrations (mg/L)	Mean measured initial concentrations (s.e.) [a] (mg/L)	% decrease over renewal period [b]	Geometric mean concentration over renewal period [b] (mg/L)
0.02	0.022 (0.001) •••	9.5 ••	0.022 ••
0.05	0.055 (0.001) •••	3.3 ••	0.054 ••
0.10	0.108 (0.002) •••	-1.7 ••	0.111 ••
0.30	0.316 (0.003) •••	0.6 ••	0.318 ••
0.60	0.600 (0.007) •••	5.4 •	0.600 •
1.00	1.040 (0.007) ••	4.9 •	1.012 •
2.00	2.152 (0.054) ••	21.2 •	1.963 •

[a]Measured on 2 (••) or 3 (•••) renewal days (where treatments had surviving nymphs).
[b] Measured over 1 (•) or 2 (••) renewal periods (where treatments had surviving nymphs).

Because a different mode of action for 2 mg/L treatments seems likely, including the 2 mg/L data in the LC50 calculation probably overestimated the true value for lower concentrations, so the LC50 was recalculated with the 2 mg/L data excluded (Table 4).

Effect of tebufenozide on small mayfly moulting and growth rates

There were no significant differences in initial lengths of mayflies among the treatments (ANOVA, p = 0.252).

Tebufenozide treatment had no significant effect on growth at concentrations below those showing acute toxicity (Table 4). In week 1 there was no significant difference (ANOVA, p > 0.05) in growth rate between the solvent control and treatments up to 0.6 mg/L tebufenozide (nymphs in higher doses were dead at the time of measuring; one nymph in 1 mg/L was measured post-mortem) (Figure 9a). After two weeks, there was no significant difference (ANOVA, p > 0.05) between the controls and tebufenozide treatments up to 0.1 mg/L. The 0.3 mg/L tebufenozide treatment was significantly different from the control (Figure 9b).

There was no significant difference between the solvent controls and water controls after 7 and 14 days (Mann-Whitney U, Exact sig (asymp sig (2-tailed), p = >0.05), so these treatments were pooled to increase the sensitivity of the test. At low concentrations, tebufenozide cause a slight increase in moulting rates, which was significant at 0.05 mg/L in week 1 (Mann-Whitney test, p = 0.009). At higher concentrations in the first week, and at all concentrations over two weeks, tebufenozide did not affect moulting rates in surviving mayflies (Figure 10). Similar increases have been observed in concentrations up to 0.6 mg/L in pilot studies (unpubl. data).

Table 4. Lethality measures for mayfly nymphs exposed to tebufenozide. Concentration calculations were based on the geometric mean of measured concentrations in containers after and before water renewals.

Time	LC50 (95% CI) [a] mg/L	LOEC [b] mg/L	NOEC [b] mg/L
96-h (all treatments)	0.66 (0.23 – 1.93)	0.60	0.32
96-h (treatments 0 - 1.01 mg/L)	0.63 (0.42 – 0.93)		
7-day	0.40 (0.29-0.53)	0.60	0.32
14-day	0.28 (0.20-0.39)	0.32	0.11

[a] Trimmed Spearman Karber
[b] Fisher's exact test

Table 5. Successful emergence in final instar mayflies

Tebufenozide (mg/L)	No. of mayflies	Proportion emerging successfully
0	15	0.40
0.05	9	0.33
0.1	7	0.57
0.3	9	0.67

Table 6. Fecundity of female mayflies exposed to tebufenozide in the final instar before emergence.
No significant difference of tebufenozide treatments from pooled controls (ANOVA, p = 0.906, Duncan's 2-sided test, p > 0.896)

Tebufenozide (mg/L)	No. of surviving female mayflies	No. of eggs (s.e.)
0 (pooled controls)	8	1185 (317)
0.05	5	1174 (246)
0.1	6	1290 (372)
0.3	3	885 (224)

Effects on emergence success and female fecundity

The proportion of mayflies emerging successfully was low in all treatments (Table 5). Mayflies in the 0.3 mg/L tebufenozide treatment had slightly greater success in emerging than those in the controls, but the difference was not significant (Pearson χ^2, p = 0.449).

Because the number of mayflies that emerged successfully was low, the number of female imagoes available for egg counts was also low. The mean number of eggs per female in 0.3 mg/L tebufenozide was lower than in the other treatments (Table 6), but because variability was high within all treatments, there were no significant differences among treatments (ANOVA, p = 0.906).

DISCUSSION

Mayfly nymph diets

The results indicated that for *Atalophlebia* sp AV13, the best food was very soft conditioned leaves. If soft leaves are not available, *Myriophyllum* could provide a suitable diet if it has sufficient biofilm on the stems. The mayflies appeared to scrape the black biofilm off the surface of the stems. The artificial diets (microwaved lettuce, Corn Flakes and Vita Brits) could all support growth to some extent, but were less satisfactory than the soft conditioned leaves, and Corn Flakes caused an unidentified growth on the nymphs. Both cereals caused problems with clouding of the water and decreased dissolved oxygen. When this was overcome by more frequent water changes, the growth rate with Vita Brits in the second week increased nearly to equal the rate with *Myriophyllum*.

It was not possible to identify the species of the conditioned leaves used in this test, but unpublished observations suggested that the mayfly nymphs were not selective about species. If the leaves were soft enough, the nymphs fed upon the material between the veins, skeletonising the leaves. It seemed probable that at this stage of decomposition, much of the leaf matter was replaced by fungi and bacteria, and the original leaf species was not important as a food source.

Previous studies of mayfly nymph feeding by Chessman (1986) using unidentified species of *Atalophlebia* found that they vigorously processed eucalypt and willow leaves and wood in the laboratory. One species chewed from the edge of leaves and another skeletonised the leaves. Feeding methods, therefore, are quite varied, even within one genus, so feeding preferences should be confirmed for any test species used.

Growth patterns

The growth rates in this study (from 4% per day for smaller nymphs to 1.3% per day for nymphs above 8 mm long) are similar to rates found in other studies. Hatakeyama (1989) found growth rates of 20-30% increase in body length per week (2.9-4.2% per day) for nymphs of the mayfly *Epeorus latifolium* in model streams at 11.5°C. Wright and Mattice (1981) found growth rates in *Hexagenia bilineata* nymphs that varied from 0.5% to 2.2% per day at 22.5°C. Wright and Mattice (1981) and Rosillon (1988) also noted that little or no increase in body length occurred in mature nymphs, which agrees with the results in the present study.

Wright and Mattice (1981) found that growth in *H. bilineata* nymphs over a period of 49 days was reasonably described by linear functions over a range of temperatures and initial sizes, with R^2 (coefficient of determination) values from 0.81-0.90. A linear model in the present study also gave a high R^2 value (0.97), but the logistic model gave a slightly higher R^2 (0.98), and visual inspection suggested that the shape was more appropriate to the data.

Effect of moulting on growth

While growth rates did increase after moulting, growth also occurred between moults. If growth is measured over a short time span, such as 24 or 48 hours, it would be necessary to take into account moulting events when interpreting the results. However, if growth is measured over longer periods

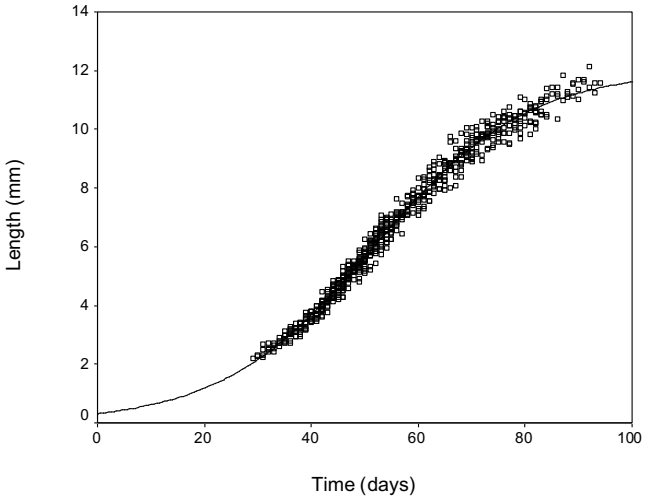

Figure 7. Mayfly lengths fitted to a logistic model to provide an overall growth curve.

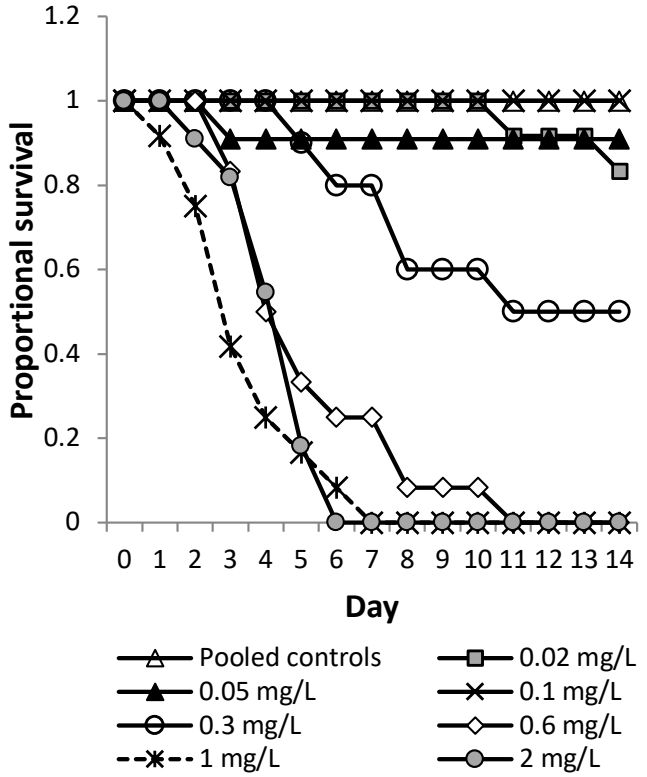

Figure 8. Survival curves of mayfly nymphs exposed to tebufenozide.

of a week or more, the variability introduced by differences of one or two moults in the period would be small compared with the overall growth, and could probably be ignored. This applies particularly to small mayflies, where moulting was more frequent and the differences between moulting and inter-moulting growth rates were less than in large mayflies.

Estimation of nymphal life span under laboratory conditions

These estimates of the life span of *Atalophlebia* sp. AV13 in the laboratory must be interpreted with caution, because i) the choice of overlap of the growth curves was subjective; ii) there were no data available for nymphs less than 2 mm long; and iii) the initial length at hatching is an estimate based on data from another species. In particular, the estimate of the time from hatch to 2.2 mm length is highly speculative, and more data on growth rates in this region are needed to refine the estimates. Definitive information on the shape of the curve could be obtained by hatching some eggs and growing the nymphs, but attempts to obtain eggs from field populations, and attempts to induce the adults to mate in the laboratory, were unsuccessful.

Examination of Figures 4 and 7 suggests that estimates of 35 – 40 days to grow from 2 mm to 9 mm are more realistic. Beyond 9 mm, there is an increasing probability that the mayflies will enter the last instar and the growth rate will decrease. Nymphs of this size would be unsuitable for tests involving measurement of growth rates. Growth measurements in the final instars were erratic and some nymphs decreased in length, presumably as body proportions changed to accommodate wing and gonad development. Beyond 10 mm, the modelled growth curve is reaching the asymptote and is not a reliable estimator of age. However, if it is assumed that the lengths at the penultimate nymphal moult range from 8.6-11.4 mm (mean 10.0 mm), corresponding to modelled ages of 65 – 95 days (mean 75 days), and the mean time spent in the final instar is 10 days, emergence probably occurs between 75-105 days (mean 85 days). An experiment starting with nymphs 2 mm long, and carried out under these experimental conditions, could continue for approximately 45 days before the adult mayflies began to emerge.

Published estimates of nymphal development times for mayfly species vary considerably. Many temperate species have univoltine life cycles (one generation per year), while some may have a slow-growing winter generation and one or more rapidly-growing summer generations (Brittain 1982). Campbell (1986) investigated the life histories of six mayflies from a temperate region of south-eastern Australia, and found a range of lifespans from about 6 months to three years, depending on species, and there was considerable variation within species. The large mayfly *Coloburiscoides giganteus* appeared to have a diapause period of 7-11 months over winter. (Marchant et al. 1984) followed the development of populations of mayflies in the La Trobe Valley in southeastern Victoria, and again found variable life spans. *Tasmanocoenis* sp. 2 was univoltine, taking a year to complete a generation, while *Tasmanocoenis tonnoiri* was trivoltine, with an overwintering cohort with a duration of about 8 months, and two summer cohorts each with a duration of about 2 months. Jackson and Sweeney (1995) found generation times ranging from 28 days to 159 days for five tropical mayfly species at 20°C, while Marchant (1982) estimated nymphal life spans of about a month at mean temperatures of about 30°C for two mayfly species in Magela Creek, Northern Territory. The estimated nymphal development time of 70-90 days at 23°C in the present study is similar to the times found for warmer-climate species, but more data are needed for the duration of early development stages.

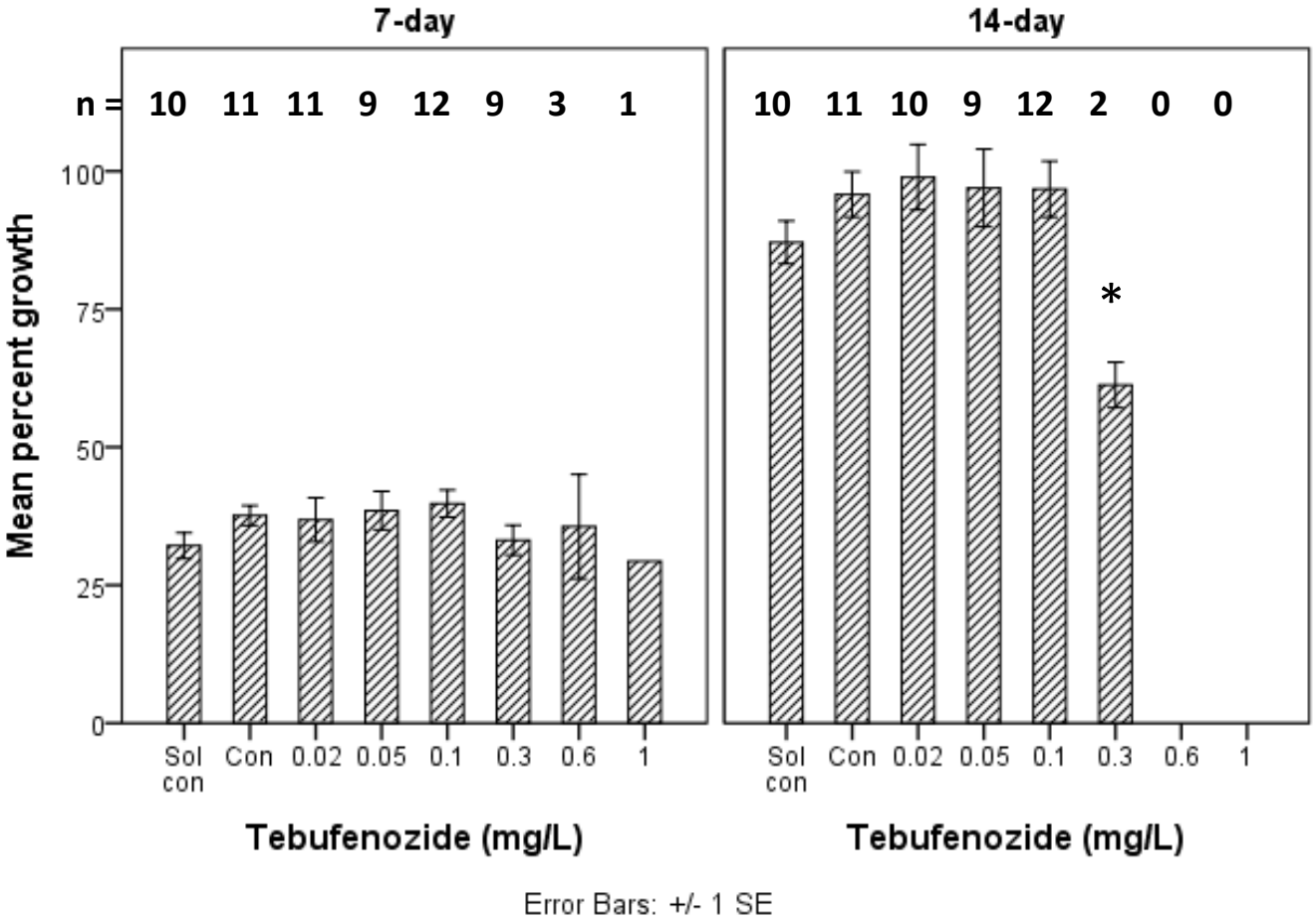

Figure 9. Effects of tebufenozide on percent growth in mayfly nymphs after 7 and 14 days.
n = no. of surviving mayflies
* = significantly different from control (Dunnett t (2-sided), p = 0.043).

Effects of tebufenozide

The major effect of tebufenozide seen in this study was death of nymphs, frequently associated with incomplete moulting in concentrations of 1 mg/L or lower, and in 2 mg/L, apparently by a mechanism not involving moulting.

Moulting rates were slightly increased at 0.05 mg/L, mainly because of increased moulting rates in the first four days of exposure. This mirrored similar peaks at low concentrations in preliminary trials. Stimulation of moulting is expected in this study, given the action of tebufenozide on moulting (Smagghe and Degheele 1995). However, stimulation of moult production has also been observed with other toxicants such as low pH (Rowe et al. 1989), and may be a more sensitive endpoint than measures of acute toxicity. Diamond et al. (1992) used moulting rates as index of subacute toxicity in the mayfly *Stenonema modestum*. They found that with a series of coal mine effluents, the stimulation of 7-day moult production often gave lower NOEC values than 14-day moult production or survival. The stimulation of moulting observed in *Atalophlebia* in this study occurred at concentrations below those causing acute toxicity. At higher concentrations the moulting rates fell because moults were initiated but not completed.

Rowe et al. (1989) noted that moulting may sometimes increase vulnerability to acid stress. Increased vulnerability could also be a factor in the effects of tebufenozide on mayflies, where a large proportion of mortalities was associated with difficulties in moulting. Tebufenozide has been shown to interfere with formation of the new cuticle by preventing deposition of the procuticle, so the new cuticle is structurally weak and may allow bleeding of haemolymph (Smagghe et al. 1996; Smagghe et al. 1999). In the mayfly nymphs, this may allow absorption of increased quantities of tebufenozide from the water. Structural weakness in the newly-formed cuticle would also explain why many limbs or tail filaments broke off during ecdysis. Death during moulting, therefore, could result from a number of causes, including exhaustion, inability to feed when exuvia remained attached to the mouthparts, haemolymph loss when limbs were lost or through the abnormal new cuticle, or from inability to walk or swim because of missing limbs.

The cause of mortality in 2 mg/L tebufenozide is not clear. These nymphs appeared visibly stressed when placed in the tebufenozide solution, failed to moult, and died with no apparent abnormalities. Given the rapid onset of the "shock" symptoms, a neurological mechanism would seem to be the most likely. Neurotoxicity could induce paralysis, and the analogue RH-5849 has been shown to block voltage-

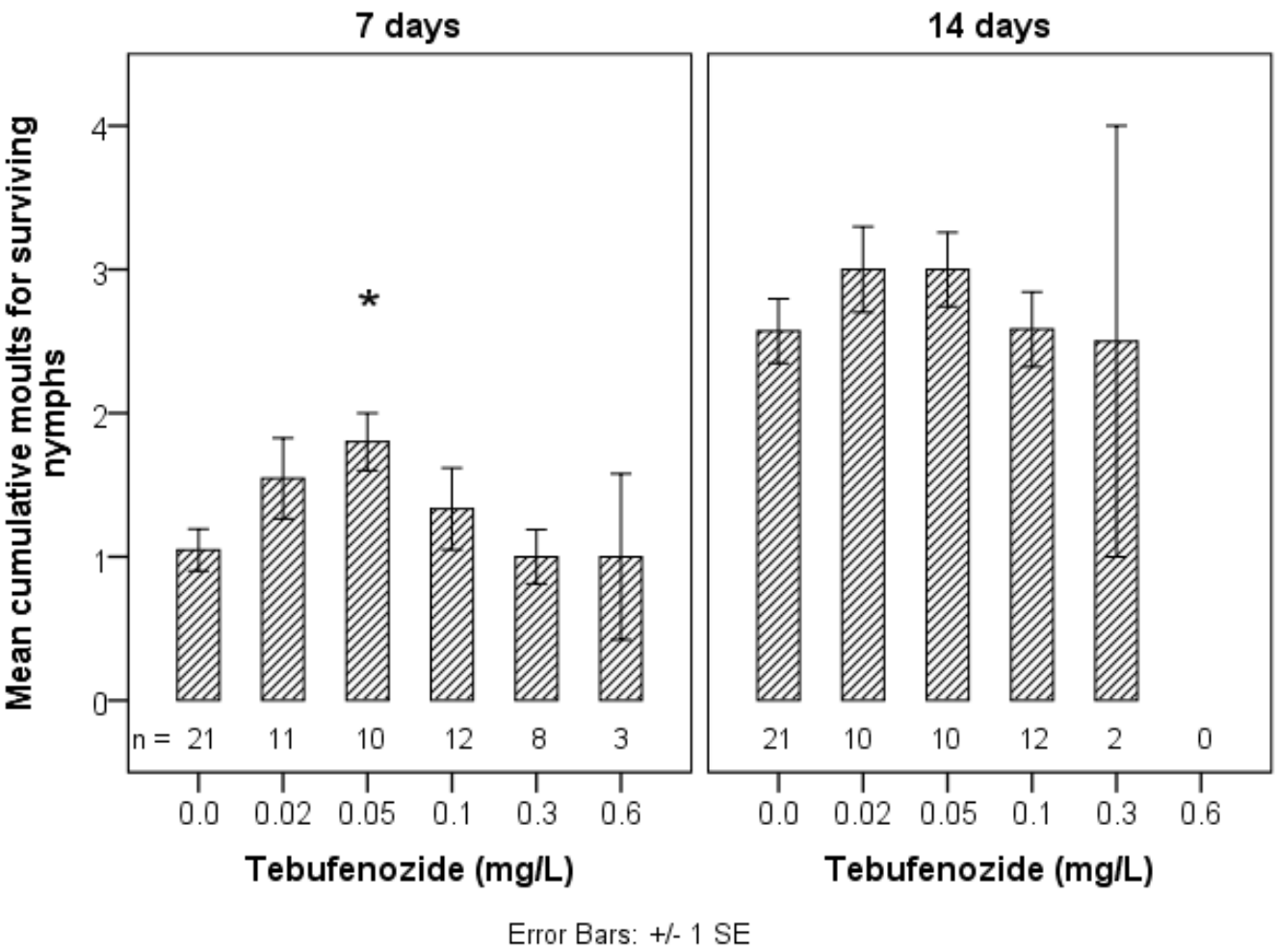

Figure 10. Mean moulting frequencies for surviving mayfly nymphs at 7 and 14 days.
n = number of surviving nymphs. * = significantly different from pooled controls (Mann-Whitney test, p = 0.009).

dependent K+ channels in nerve and muscle (Salgado 1992; 1998). Initial paralysis followed by recovery has been observed in tadpoles exposed to tebufenozide (Pauli et al. 1999), and tremors followed sometimes by recovery in the Colorado potato beetle *Leptinotarsa decemlineata* (Smagghe and Degheele 1994a,b). Neurotoxicity could also suppress feeding behaviour, but it is not clear why moulting was apparently suppressed (since ecdysteroids stimulate moulting directly) or what was the final cause of death.

Examination of the effects on emergence success unfortunately was limited by the high failure rate in all treatments including the controls. However, although the numbers were too low to detect significant differences between the treatments and the control, the success rate actually increased with tebufenozide concentration, so there was no indication that tebufenozide had adverse effects on emergence. Similarly, the measurements of female fecundity were limited by the low numbers emerging, and the fact that some mayflies did emerge successfully but shed their eggs into the water before they could be collected. The results suggested that fecundity might be affected in 0.3 mg/L, but a repeat test with larger numbers would be needed to confirm this.

The US EPA Ecotox database (US EPA 2017) has summarised toxicity data on the effects of tebufenozide. Most of the lethal concentrations reported for aquatic invertebrates are considerably higher than 0.83 mg/L (the reported solubility of tebufenozide (1996)). These concentrations can be achieved by solubilising the tebufenozide with an organic solvent such as acetone, but it is unlikely to be present at that concentration in the field. *Atalophlebia* in the present study, with a 14-day LC50 of 0.28 (95% C.I. (0.20-0.39) mg/L, is among the more sensitive organisms tested. The only invertebrates with similar sensitivities are the mosquitoes, such as *Aedes taeniorhynchus* and *Aedes aegypti* with 48 h LC50s of 0.15 (0.11 ~ 0.20) mg/L, and 0.92 (0.83~1.05) mg/L tebufenozide respectively (Song et al. 1997); and *Chironomus riparius*, in which chronic static exposure of 1st instar larvae gave an LC50 of 21.4 μg/L and semistatic exposure of 4th instar larvae an LC50 of 81.9 μg/L (Hahn et al. 2001).

Atalophlebia sp. AV13 and *C. riparius* appear to differ in the developmental stage or physiological function targeted by tebufenozide. In *C. riparius*, mortality in the larvae was low; most of the mortalities occurred in the process of pupation and emergence, and pupal mortality was higher for males (Hahn et al. 2001). In *Atalophlebia*, however, mortalities occurred in nymphs of all sizes, and although numbers were too low for determining statistical significance, there was no apparent sex bias or effect on emergence.

In these experiments, exposure would occur via both the oral and dermal routes, as tebufenozide would bind to the leaves provided for food. Since the most likely long-term exposure of mayfly nymphs in the field would be via ingestion of contaminated food and sediment, it would be useful to examine the effects of ingested tebufenozide on nymphs, and also to obtain field data on concentrations of tebufenozide in stream sediments and leaf debris after runoff from sprayed fields. Kreutzweiser et al. (1994) exposed specimens of shredding invertebrates, the stonefly *Pteronarcys* sp. and the caddisfly *Hydatophylax argus,* for 12 days to leaves sprayed with tebufenozide at a rate of 50 g/ha. The contaminated foliage did not cause mortality or affect final weights of the invertebrates, and feeding rates actually increased in treated units. These results suggest that there is probably little risk through this route of entry, but studies over longer periods and including reproductive endpoints would be desirable.

CONCLUSIONS

Moulting rates and growth rates could both be used as endpoints for sub-lethal toxicity testing with *Atalophlebia* sp. AV13. The highest growth rates were achieved when animals were fed on leaves that had been conditioned for long enough to be almost disintegrating. Moulting frequency was highest, and the relative sizes of the growth rate at moulting compared with growth rates between moults was least, in small nymphs. Tests examining moulting rates or growth rates should preferably use small mayflies if possible, and extend over more than a week. If growth rates are measured over shorter periods, it will be necessary to make allowance for the effects of moulting. Tests using nymphs of 2 mm length could be continued for about 35 - 40 days before the nymphs reached the final instars with corresponding decline in growth rates, and 40-45 days before emergence. Mayfly nymphs greater than approximately 9 mm in length are close to reaching the final instar and emerging. Nymphs of this size should only be used to examine effects on emergence, and in females, on fecundity by egg counts.

The results of the tebufenozide dosing provide useful toxicological data concerning sensitive endpoints for tebufenozide action in mayfly nymphs and the range of concentrations in which they might be observed. Continuous exposure to tebufenozide in the water (and probably bound to food) can cause slight increases in moulting rates at sublethal concentrations, and concentration-related increases in mortality. Effects on growth rates only occurred at concentrations causing acute toxicity. It is possible that concentrations of 0.3 mg/L cause reductions in fecundity in females, but repeat studies with much larger sample numbers, and improved conditions to increase the success of control emergences, are needed to confirm this.

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Dr Scott Markich
Editor-in-Chief
Australasian Journal of Ecotoxicology
Aquatic Solutions International
Level 1, 467 Miller Street
Cammeray NSW 2062, Australia
Facsimile: +61 2 9460 2711
Email: smarkich@optushome.com.au

Dr Alex Pulkownik
Associate Editor
Australasian Journal of Ecotoxicology
Department of Environmental Sciences
University of Technology, Sydney (UTS)
PO Box 123, Broadway NSW 2007, Australia
Facsimile: +61 2 9514 4079
Email: Alex.Pulkownik@uts.edu.au

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