School of Environmental Biology

Toxicological Impact of Agricultural Surfactants on Australian Frogs

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of the

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Declaration

I declare that all work presented in this thesis is that of myself alone unless otherwise acknowledged. The content of this thesis has not been submitted previously, in whole or in part, in respect of any other academic award.

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Abstract

Surfactants are one of the more ubiquitous contaminants in aquatic systems. Their importance as toxic components of pesticide formulations has, however, been largely overlooked. Amphibians particularly, as inhabitants of shallow, temporary and often lentic aquatic environments may be at risk from exposure to these chemicals when they enter aquatic systems. This thesis presents data on the toxicity of surfactants to amphibians. Several experimental exposures were conducted with embryo-larval, tadpole and adult developmental stages of the Australian species- *Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis* and *Litoria moorei* and the exotic species- *Bufo marinus* and *Xenopus laevis.* Animals were variously exposed to glyphosate formulations that contain a high proportion of nonionic surfactants, or commercial pesticide wetting agents (alcohol alkoxylate and nonylphenol ethoxylate (NPE) surfactants).

Feeding stage tadpoles of *C. insignifera*, *H. eyrei*, *L. dorsalis* and *L. moorei* were exposed to three commercial glyphosate formulations, glyphosate isopropylamine and glyphosate acid in static-renewal acute toxicity tests. The 48-h LC50 values for Roundup® Herbicide (MON 2139) tested against tadpoles of *C. insignifera*, *H. eyrei*, *L. dorsalis* and *L. moorei* ranged between 8.1 and 32.2 mg/L (2.9 and 11.6 mg/L glyphosate acid equivalent (ae)), while the 48-h LC50 values for Roundup® Herbicide tested against adult and newly metamorphosed *C. insignifera* ranged from 137-144 mg/L (49.4-51.8 mg/L ae). Touchdown® Herbicide (4 LC-E) tested against tadpoles of *C. insignifera*, *H. eyrei*, *L. dorsalis* and *L. moorei* was slightly less toxic than Roundup® with 48-h LC50 values ranging between 27.3 and 48.7 mg/L (9.0 and 16.1 mg/L ae). Roundup® Biactive (MON 77920) was practically non-toxic to tadpoles of the same four species producing 48-h LC50 values of 911 mg/L (328 mg/L ae) for *L. moorei* and >1000 mg/L (>360 mg/L ae) for *C. insignifera*, *H. eyrei* and *L. dorsalis.* Glyphosate isopropylamine was practically non-toxic producing no mortality amongst tadpoles of any of the four species over 48 h, at concentrations between 503 and

684 mg/L (343 and 466 mg/L ae). The toxicity of technical grade glyphosate acid (48-h LC50, 81.2-121 mg/L) is likely to be due to acid intolerance.

Feeding stage tadpoles of *B. marinus*, *X. laevis*, *C. insignifera*, *H. eyrei*, *L. dorsalis* and *L. moorei* were exposed to NPE and alcohol alkoxylate in static renewal acute toxicity tests. All species exhibited non-specific narcosis following exposure to both these surfactants. The 48-h EC50 values for NPE ranged between 1.1 mg/L (mild narcosis) and 12.1 mg/L (full narcosis). The 48-h EC50 values for alcohol alkoxylate ranged between 5.3 mg/L (mild narcosis) and 25.4 mg/L (full narcosis). *Xenopus laevis* was the most sensitive species tested. The sensitivity of the other five species was size dependent with larger species displaying greater tolerance. Replicate acute toxicity tests with *B. marinus* exposed to NPE at 30°C over 96 hours indicated that the narcotic effects were not particularly time dependant. The mean 24, 48, 72 and 96-h EC50 (mild narcosis) were 3.6, 3.7, 3.5 and 3.5 mg/L respectively. The mean 24, 48, 72, and 96-h EC50 (full narcosis) values were 4.0, 4.1, 4.2 and 4.0 respectively. Acute toxicity tests with *B. marinus* exposed to NPE at 30°C under conditions of low dissolved oxygen (0.8-2.3 mg/L) produced a two to threefold increase in toxicity. The 12-h EC50 values ranged from 1.4 to 2.2 mg/L.

The embryotoxicity of NPE was determined in *X. laevis*, *L. adelaidensis* and *C. insignifera* using a Frog Embryo Teratogenesis assay-*Xenopus* (FETAX). The 96-h LC50, EC50 and MCIG (LOEC) values for *X. laevis* were 3.9 to 5.4 mg/L, 2.8 to 4.6 mg/L and 1.0 to 3.0 mg/L respectively. The 140-h LC50, EC50 and MCIG values for *L. adelaidensis* were 9.2 mg/L, 8.8 mg/L and 5.1 to 6.0 mg/L respectively. The 134-h LC50, EC50 and MCIG values for *C. insignifera* were 6.4 mg/L, 4.5 mg/L and 4.0 mg/L respectively. Teratogenicity indices for the three species ranged between 1.0 and 1.6 indicating either no or low teratogenicity. *Xenopus laevis* was the more sensitive of the three species and the only species that displayed indisputable terata.

The acute toxicity data indicated that the amphibian species tested were of similar sensitivity to fish and some invertebrates.

Developmental retardation and oestrogenic effects following exposure to nonylphenol ethoxylate were indicated by sublethal toxicity tests. *Crinia insignifera* embryos were exposed during early embryogenesis to sublethal concentrations of NPE. Exposure to NPE did not affect either weight nor size (snout-vent length) at metamorphosis. Exposure to 5.0 mg/L NPE resulted in a significant delay in the time required to reach metamorphosis. Also, exposure to 3.0 mg/L NPE for the first 6 days of embryonic development or exposure to 5.0 mg/L NPE from day 2 to day 6 resulted in a statistically significant predominance in the female phenotype amongst metamorphosing froglets. Exposure for the first five days to 1.5 mg/L or 3.0 mg/L NPE had no effect on sex ratio. The results indicated that exposure to NPEs has endocrine disruptive effects in this species and that a narrow window of susceptibility exists for the induction of predominantly female phenotype.

This study has also followed the degradation of a mixture of NPE oligomers and the concomitant formation of individual oligomers in static die-away tests with and without illumination in freshwater. Over 33 days in darkness there was a progressive and complete loss of long chain oligomers ($NPEO_{8-17}$), transient increases and subsequent loss of short to medium chain oligomers ($NPEO_{4-7}$), and large persistent increases (~1000%) in short chain oligomers ($NPEO_{1-3}$). In the presence of illumination, biodegradation was retarded and heterotrophic bacterial proliferation was inhibited. After 33 days there was complete loss of long chain oligomers ($NPEO_{9-17}$), incomplete loss of medium chain oligomers ($NPEO_{6-8}$) and increases in short chain oligomers ($NPEO_{1-5}$).

This thesis discusses the importance of persistent metabolites of NPE degradation as it pertains to the habitat, developmental time frame and ecology of amphibians. Degradation of NPE is likely to occur over a time frame that is longer than that required for complete embryogenesis and metamorphosis of many species of amphibians, and may easily encompass those critical stages of development during which oestrogenic metabolites can affect development.

Thesis Organisation

The thesis is divided into seven chapters and two appendices. In the General Introduction (Chapter 1) the paucity of information on the toxicity of chemicals to amphibians is highlighted. The greater part of the introduction, however, consists of a review of the literature dedicated to surfactant toxicity in aquatic fauna with references to its relevance to the research aspects of the thesis. Chapters 2 to 6 are research chapters. With the exception of Chapter 5, all these chapters are typed duplications of published articles (Chapters 2, 3, 4 & 6). Chapter 5 has not been published and is not currently submitted for publication. However, to maintain consistency of format, Chapter 5 has been written as though it were prepared for journal submission. Consequently, an unavoidable degree of replication exists within this thesis. Figure and table numbering, reference formats and some terminology has been altered in all Chapters as a concession to consistency of thesis format and continuity. Chapter 7 is a General Discussion that provides an overview of the research findings. A published review of the amphibian toxicology literature was written during the course of the research for this thesis and is included as an appendix. A tabulated database of published numerical toxicity data is also attached as an appendix.

Abbreviations

ABS	Alkylbenzene sulphate, Dodecylbenzene sulphonate
AE	Alcohol ethoxylate
AEO_n	Alcohol ethoxylate with n ethylene oxide units
AES	Alkyl ethoxysulphate, Alkyl ether sulphate
AEO_nS	Alkyl ethoxysulphate with n ethylene oxide units
AOS	Alpha olefine sulphonate, -olefine sulphonate
APE	Alkylphenol ethoxylate
$APEO_n$	Alkylphenol ethoxylate with n ethylene oxide units
AS	Alkyl (alcohol) sulphate
BO	Butylene oxide
C_n	Alkyl group with n carbons
DO	Dissolved oxygen
EC50	Concentration effective to 50% of test organisms
EO	Ethylene oxide
HPLC	High performance liquid chromatography
LAS	Linear alkylbenzene sulphonate
LC50	Concentration lethal to 50% of test organisms
LOEC	Lowest observable effect concentration
NOEC	No observable effect concentration
NP	Nonylphenol
NPE	Nonylphenol ethoxylate
NPE_nC	Nonylphenoxy carboxylate with n ethylene oxide units
$NPEO_n$	Nonylphenol ethoxylate with n ethylene oxide units
OP	Octylphenol
OPE	Octylphenol ethoxylate
OPE_nC	Octylphenoxy carboxylate with n ethylene oxide units
$OPEO_n$	Octylphenol ethoxylate with n ethylene oxide units
PO	Propylene oxide
SAS	Secondary alkane sulphonate, Sodium alkanemonosulphonate

Chapter 1

General Introduction

"Amphibians were here when the dinosaurs were here, and they survived the age of mammals. They are tough survivors. If they're checking out now, I think it is significant."

-David Wake

The Puzzle of Declining Amphibian Populations

Amphibian populations are undergoing a global decline in numbers. The phenomenon of amphibian decline came to light in 1989, at the First World Congress of Herpetology held in Canterbury, England. A number of delegates presented largely anecdotal accounts of missing species and declines in frog numbers at sites where frogs had previously been numerous (Barinaga, 1990). The evidence for declines in the USA, Central and South America, Europe and Australia was further addressed at a National Research Council (USA) sponsored workshop held at Irvine, California (Blaustein and Wake, 1990). The absence of long-term census data for many of the species and populations, prompted some researchers to advocate natural population fluctuations as a default hypothesis (Pechmann and Wilbur, 1994). The apparent severity and widespread nature of many of the declines, however, subsequently pointed to a phenomenon of unprecedented magnitude (Richards *et al.*, 1993; Fischer and Shaffer, 1996; Laurance, 1996; Pounds *et al.*, 1997; Lips, 1999). The individual cases of population declines pointed to numerous potential causes, including exotic predators (Hecnar and McLoskey, 1997; Bradford *et al.*, 1998; Gillespie and Hero, 1999), increased UVB radiation as a consequence of ozone depletion (Blaustein *et al.*, 1997; 1998; Broomhall *et al.*, 2000), disease (Bradford, 1991; Crawshaw, 1997; Berger *et al.*, 1998; 1999), loss of habitat (Johnson, 1992; Dupuis, 1997; Thumm and Mahony, 1999), and water contamination (Beebee *et al.*, 1990; Bellemakers and van Dam, 1992; Beasley *et al.*, 1995; McAlpine *et al.*, 1998; Bishop *et al.*, 1999; Rouse *et al.*, 1999).

Environmental Contamination and Amphibians

Are amphibians particularly sensitive to environmental pollutants?

Very early in the dialogue on declining amphibian populations, it was suggested by numerous researchers that these animals were sensitive indicators of environmental contamination by virtue of their unique physiology and ecology (Wake and Morowitz, 1990; Dunson *et al.*, 1992; Blaustein and Wake, 1995). Amphibians are the highest vertebrate group to retain an essentially naked egg, and the only vertebrate group that has an aquatic larval stage and a terrestrial adult phase (Duellman and Trueb, 1986). Furthermore, the skin of an adult amphibian is a permeable organ used for respiration and water-balance (Duellman and Trueb, 1986). Their dual life cycle and unique physiology, implies that amphibians have more opportunities for exposure and more modes of exposure to environmental contaminants than other vertebrates.

The notion that amphibians exhibit greater sensitivity to environmental contaminants than other phylogenetic groups has yet to be verified. Several studies have attempted to evaluate the relative sensitivity of amphibian larvae to various chemicals when compared to other taxa (Jolly *et al.*, 1978; Birge *et al.*, 1979; Slooff and Baerselman, 1980; Thurston *et al.*, 1985; Holcombe *et al.*, 1987; Thybaud, 1990; Herkovits *et al.*, 1995; Deyoung *et al.*, 1996; McCrary and Heagler, 1997), but the issue remains contentious since the results of such studies are often dependent upon the toxin to which test species are exposed (Birge *et al.*, 1979; Slooff and Baerselman, 1983; Thurston *et al.*, 1985; Holcombe *et al.*, 1987). It is noteworthy, however, that some aqua-culture industries rely on the apparent sensitivity of amphibian larvae to specific chemicals, to eliminate them from fish-culture ponds (Kane *et al.*, 1985; Kane and Johnson, 1989).

From the perspective of potential exposure to xenobiotics, a more important aspect of amphibian biology, is the reliance of many species on shallow, ephemeral or lentic ponds for reproduction (Wassersug, 1974; Tyler, 1994). Such environments do not afford appreciable dilution or rapid egress for pollutants, and

it is in these environments that contaminants such as pesticides may rise to toxic levels.

Herbicides in particular, have increased in use in Australia over the last two decades (Barratt, 1998), and while the active ingredients of herbicides may be of low toxicity, even at relatively high concentrations, formulation additives such as surfactants may present a toxic hazard to aquatic fauna in shallow aquatic environments (Watkins *et al.*, 1985). In Australia, numerous anecdotal reports have suggested that frog populations are at risk from the toxic effects of herbicides or the surfactant additives in herbicide formulation (NRA, 1996).

Are Australian amphibians more sensitive to environmental pollutants than other amphibians?

Australian native amphibians are phylogenetically distinct from those species most frequently used for toxicity studies (Tyler, 1994). More than 80% of the amphibian toxicological literature describes studies using representatives of only three genera- *Rana, Bufo* or *Xenopus* (Power *et al.*, 1989; Mann and Bidwell, 1999a). In Australia, only one species belongs to the genus *Rana* (a relatively recent colonist from New Guinea, restricted to Cape York Peninsula) and the only *Bufo* species is represented by the introduced pest species *Bufo marinus* (cane toad).

Consequently, The Action Plan for Australian Frogs, recommended further research into the toxicity of pollutants to Australian frogs (Tyler, 1997). While the amount of research dedicated to toxicity testing in amphibians as a group, has been considered inadequate (Hall and Henry, 1992; Boyer and Grue, 1995), there are virtually no studies that have focused on the toxicity of environmental pollutants in Australian frogs (Mann and Bidwell, 1999a; Appendix 2).

The view that Australian amphibians may display differences in sensitivity to environmental contaminants when compared to species from the Northern Hemisphere (i.e. *Bufo* spp., *Rana* spp.) or Africa (i.e. *Xenopus laevis*) may be valid, since species variation has been demonstrated even amongst a

narrow suite of North American species. For example, Berrill *et al.* (1993; 1994; 1995) and Berrill and Bertram (1997) reported differences in the sensitivity of toads (*Bufo* spp.), frogs (*Rana* spp.), and salamanders (*Ambystoma* spp.) to the organophosphate pesticide fenitrothion, the pyrethroid pesticide permethrin and various herbicides in acute tests. Hall and Swineford (1981) reported differences in sensitivity of up to one order of magnitude amongst *Bufo* spp., *Rana* spp., *Ambystoma* spp. and the frog *Acris crepitans* following exposure to the halogenated pesticides endrin and toxaphene in acute tests. Hoppe and Mottl (1997) noted species-specific differences in the occurrence, type and severity of malformations amongst field collected animals in Minnesota. Glooschenko *et al.* (1992) and Wyman (1988) correlated species distribution with various water/soil chemistry parameters, including soil pH, conductivity, alkalinity and the occurrence of metals. Also, a number of studies have reported variation in acid tolerance (for review see Pierce, 1985; Freda, 1986). Similar or greater variation in sensitivity to environmental pollution, may occur amongst Australian frog.

Toxicity of pesticide formulation additives

Pesticides are a major water contaminant; however, the contribution that pesticide formulation additives make to their overall toxicity has been largely overlooked. Few pesticides are sufficiently water miscible on their own and must be formulated with solvents and surfactants to produce fine suspensions and emulsions when added to water. Pesticide formulation ingredients are generally proprietary information. Typically, however, the emulsifying agents include a combination of anionic and nonionic surfactants (Friloux and Scott Tann, 1992). Even water-soluble pesticides frequently require the inclusion of nonionic surfactants to act as wetting agents and spreaders (Dodd *et al.*, 1993). Furthermore, extra nonionic surfactants are added to spray-tank mixtures as wetting agents and dispersants (Dodd *et al.*, 1993).

In many pesticide-surfactant mixtures (herbicide-surfactant mixtures in particular), the surfactant is frequently the more toxic component (Folmar *et al.*,

1979; Abdelghani *et al.*, 1997; Oakes and Pollak, 1999) and will dictate the toxicity of the mixture. Folmar *et al.* (1979) reported the glyphosate formulation, Roundup® Herbicide to be between 10 and 170 times more toxic than the active ingredient, glyphosate, and concluded that the formulation surfactant was primarily responsible for the formulation toxicity. In an examination of the toxicity of the aminocarb formulation, Matacil®, to juvenile Atlantic salmon (*Salmo salar*), McLeese *et al.* (1980) reported the formulation to be 2.5 times more toxic than the active ingredient, aminocarb. Moreover, nonylphenol (the adjuvant which constitutes 50% of the formulation) was 9.7 times more toxic than the active ingredient. The same authors, however, were unable to show a similar disparity in toxicity in marine shrimp (*Crangon septemspinosa*) (McLeese *et al.*, 1980). In a comprehensive comparison of technical active ingredients and formulation toxicity, Mayer and Ellersieck (1986) noted that formulation additives could increase toxicity by as much 2.5 orders of magnitude. The comparisons reported by Mayer and Ellersieck (1986) were criticised as being unreliable by Schmuck *et al.* (1994) because they were made between results from tests performed in separate laboratories under different test conditions. However, even in the comparisons reported by Schmuck *et al.* (1994), some pesticide formulations, particularly emulsifiable concentrates, displayed toxicities that were much higher than the active ingredients.

Few comparisons of formulated pesticides and active ingredients have been performed using amphibians as test species. Linder *et al.* (1990), noted a threefold increase in acute toxicity to leopard frogs (*Rana pipiens*) for a commercial formulation of the herbicide, paraquat, when compared to that of technical grade paraquat. The azinphosmethyl formulation, Guthion 2S was up to 21 times more toxic than technical grade Guthion to tadpoles of Pacific tree frogs (*Pseudacris regilla*) (Schuytema *et al.*, 1995; Nebeker *et al.*, 1998). Swann *et al.* (1996) found the emulsifying agents in the chlorpyrifos formulations, Dursban™ and Lorsban™ to be responsible for increased toxicity of the formulations in leopard frogs (*R. pipiens*), and at least partially responsible for mitachondrial swelling in the

ciliated palate of the same species. Oakes and Pollak (1999) similarly found that the surfactant incorporated into the herbicide formulation Tordon 75D® was solely responsible for reduced respiratory function in rat liver mitochondria exposed to the formulation.

An examination of surfactant toxicity in Australian frogs

This thesis has examined the toxicity of surfactants in Australian frogs, in response to the recommendation by Tyler (1997), and anecdotal reports of frog mortality or the cessation of frog chorus following the application of herbicides (NRA, 1996).

All experimental procedures that utilised live animals were performed with the approval of The Curtin University Animals Ethics Committee, The University of Adelaide Animal Ethics Committee, or The University of South Australia Animal Ethics Committee. In accordance with Animal Ethics requirements, all animals were euthenased at the termination of experiments by freezing, immersion in MS222 (Sigma®, USA) or halothane exposure (Fluothane , ICI Pharmaceuticals). All field-collected adult frogs and eggs were collected from the margins of two urban and one rural lake under permit from the Conservation and Land Management authority of Western Australia. Collection sites were characterised as being ephemeral lakes devoid of agricultural run-off with large healthy populations of frogs. Some urban storm-water contributed to the collection site, where *Crinia insignifera* and *Litoria Adelaidensis* were collected.

In Chapter 2 of this thesis, the issue of glyphosate formulation toxicity is re-visited with regard to Australian frogs. Once again, the results of that study pointed to the formulation surfactants as being wholly responsible for the herbicides toxicity.

Very little information is available on surfactant toxicity in amphibians, although there is an extensive literature for other aquatic organisms which has been comprehensively reviewed (Abel, 1974; A D Little Co., 1977; Sivak *et al.*, 1982; Lewis and Suprenant, 1983; Lewis and Wee, 1983; Lewis, 1991; 1992; Holt *et al.*,

1992; Painter, 1992; Talmage, 1994; Staples *et al.*, 1998). Chapters 3 to 5 have examined either the acute toxicity of two commonly used nonionic surfactant-additives, or the consequences of short term exposure for growth and development to metamorphosis to the adult stage. Chapter 6 examines the potential for environmental perisitence of one nonionic surfactant.

The remainder of this introductory chapter will discuss surfactant toxicity in aquatic fauna. Although the experimental aspects of this thesis deal almost exclusively with nonionic surfactants, the following review of the literature includes much information about anionic surfactants because it is both prerequisite and complimentary to an understanding of nonionic surfactant toxicity.

Surfactant Structure

Surfactants are used as emulsifiers, dispersants, spreaders and wetting agents with pesticides because of their ability to reduce the interfacial tension between aqueous and non-aqueous materials. The structure of surfactants is integral to this ability (Porter, 1994). Surfactants are usually described as possessing a hydrophobic "tail" and a hydrophilic "head" (Fig.1.1).

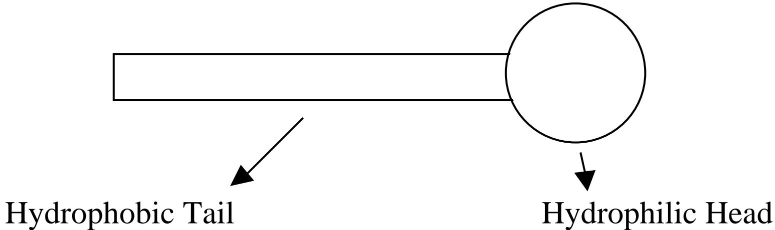

Fig. 1.1 Conceptualised structure of a surfactant molecule (Porter, 1994)

The hydrophobic tail groups of anionic and nonionic surfactants are either aliphatic hydrocarbon chains or a combination of aliphatic and aromatic hydrocarbons (Table 1.1). Anionic and nonionic surfactants differ in the kind of hydrophilic head-group they possess. For the purpose of this brief description, most anionic surfactants have either a sulphonate or sulphate head-group

(Table. 1.1), while nonionic surfactants have an polymerised alkoxylate (usually ethylene oxide) chain (Table 1.1).

Table 1.1 Surfactant structure for all surfactants described in this chapter. Anionic surfactants head-groups are red and nonionic surfactant head-groups are blue. Table modified from Sivak *et al.* (1982) and Maki (1979b). The ethylene oxide group (C_2H_4O) has been abbreviated as EO in the remainder of this thesis.

Surfactant structure	Surfactant name
CH_3—$(CH_2)_n$—CH—CH_3 with benzene ring attached to CH, ring bearing SO_3^-; n = 7-11	Linear alkylbenzene sulphonate (LAS) Branched alkylbenzene sulphonate (ABS) has a highly branched aliphatic tail
CH3—$(CH2)_n$—CH=CH—$CH_2SO_3^-$ 60 to 65% alkene sulphonate CH3—$(CH2)_n$—CH(OH)—$CH_2CH_2SO_3^-$ 35 to 40% hydroxyalkane sulphonate n = 12-16	Alpha olefin sulphonate (AOS)
CH_3—$(CH_2)_n$—CH(SO_3^-)—$(CH_2)_m$—CH_3	Secondary alkane sulphonate (SAS)
CH_3—$(CH_2)_n$—CH2—OSO_3^- n = 10-16	Alkyl sulphate (AS)
CH_3—$(CH2)_n$—$O(C_2H_4O)_x$—OSO_3^- n = 8-16, x = 2-4	Alkyl ethoxylate sulphate (AES)
CH_3—$(CH2)_n$—$(C_2H_4O)_xOH$ n = 6-16, x = 3-20	Alcohol ethoxylate (AE)
CH_3—$(CH_2)_n$—CH—(CH_3) with benzene ring attached to CH, ring bearing $O(C_2H_4O)_xOH$; n = 5-7, x = 4-30	Alkylphenol ethoxylate (APE) n=5 Octylphenol ethoxylate (OPE) n=6 Nonylphenol ethoxylate (NPE)

Surfactant Toxicity in Aquatic Fauna

Surfactants exert toxic effects on aquatic organisms over a very wide range of concentrations. As a general rule, however, the published acute toxicity data for fish, and a relatively narrow suite of invertebrates exposed to surfactants, lie between 0.1 and 100 mg/L with the majority of values lying between 1.0 and 10 mg/L (Fig. 1.2). Most of this data is in the form of LC50 (a statistical estimate of the concentration that will kill 50% of the test animals within a specified time) or EC50 (a statistical estimate of the concentration that will affect 50% of the test animals within a specified time) values generated for exposures ranging in duration from one to 96 hours.

Abel (1974) listed acute toxicity data for branched alkylbenzene sulphonate (ABS), linear alkylbenzene sulphonate (LAS) and nonionic polyethoxylated surfactants exposed to various fish species. The 96-h LC50 data ranged from 1 mg/L for cod (*Gadus morrhua*) exposed to LAS (Swedmark *et al.*, 1971) to 37 mg/L for bluegill sunfish (*Lepomis macrochirus*) exposed to a nonionic polyoxyethylene ester (Henderson *et al.*, 1959). The range of acute toxicity data in fish, however, has been reported to be much wider. Gafa (1974) reported 6-h LC50 values of 60 mg/L and >300 mg/L for a C_{12} alkyl sulphate (AS) and C_{14}AS respectively when exposed to goldfish (*Carassius auraties*). Macek and Krzeminski (1975) reported an 96-h LC50 value of >1000 mg/L for bluegill sunfish (*L. macrochirus*) exposed to a C_9EO_{30} alcohol ethoxylate (AE). Reiff *et al.* (1979) reported a range of low 96-h LC50 values for several surfactants to numerous fish species. A 96-h LC50 value of 0.4 mg/L was reported for brown trout exposed to tallow ethoxylate, while golden orfe (*Idus idus melanotus*) and brown trout (*Salmo trutta*) exposed to $C_{10\text{-}15}$LAS generated 96-h LC50 values of 0.4 mg/L and 0.1 mg/L respectively.

Lewis and Suprenant (1983) listed acute surfactant toxicity data for invertebrates ranging from a 48-h LC50 value of 0.11 mg/L for *Daphnia magna* exposed to C_{16}LAS (Maki and Bishop, 1979), to a 24-h LC50 value of 500 mg/L for mosquito larvae (*Aëdes aegypti*) exposed to nonylphenol ethoxylate (NPE).

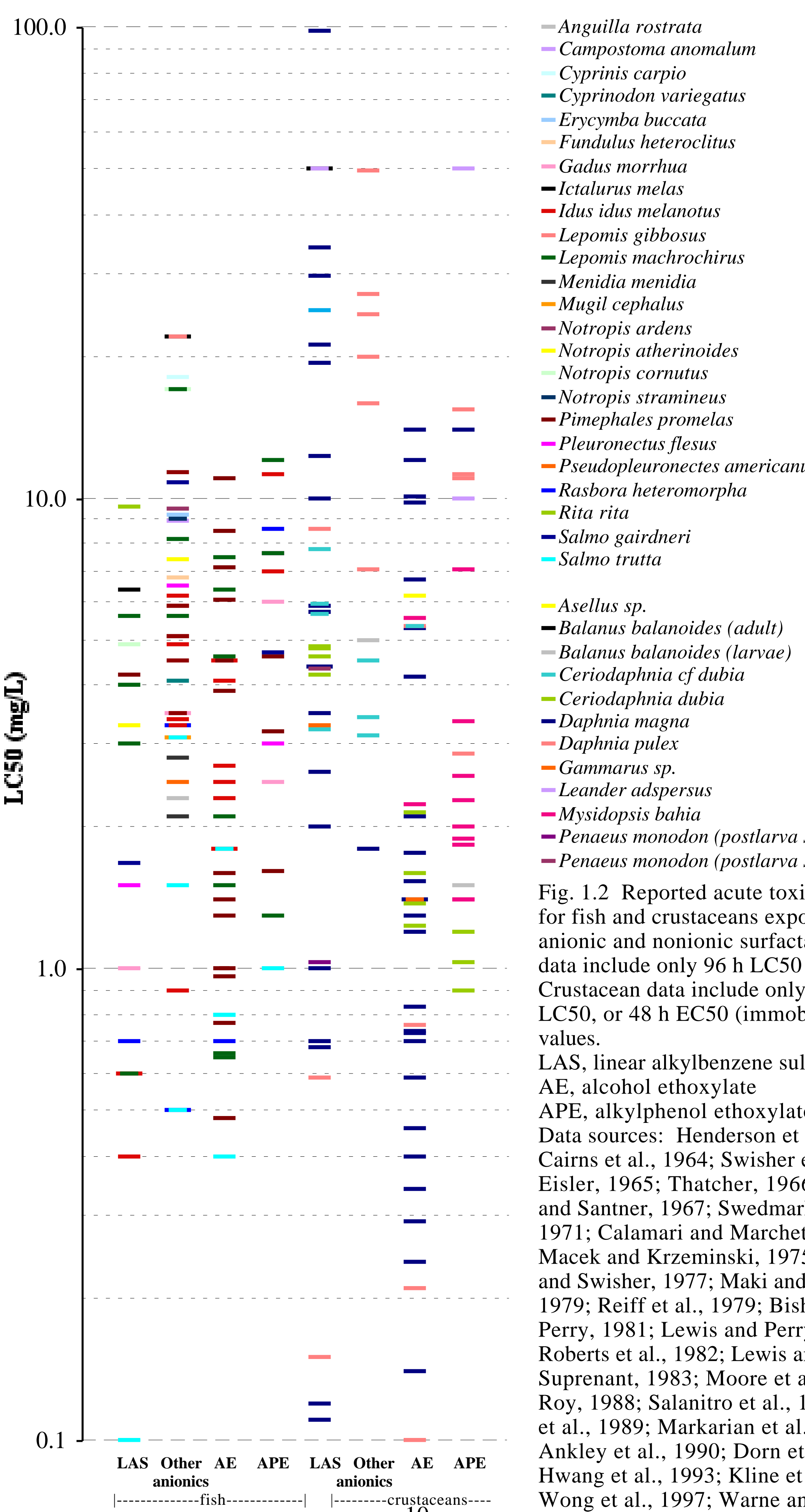

Fig. 1.2 Reported acute toxicity data for fish and crustaceans exposed to anionic and nonionic surfactants. Fish data include only 96 h LC50 values. Crustacean data include only 48 h LC50, or 48 h EC50 (immobilisation) values.
LAS, linear alkylbenzene sulphonate
AE, alcohol ethoxylate
APE, alkylphenol ethoxylate
Data sources: Henderson et al., 1959; Cairns et al., 1964; Swisher et al., 1964; Eisler, 1965; Thatcher, 1966; Thatcher and Santner, 1967; Swedmark et al., 1971; Calamari and Marchetti, 1973; Macek and Krzeminski, 1975; Kimerle and Swisher, 1977; Maki and Bishop, 1979; Reiff et al., 1979; Bishop and Perry, 1981; Lewis and Perry, 1981; Roberts et al., 1982; Lewis and Suprenant, 1983; Moore et al., 1987; Roy, 1988; Salanitro et al., 1988; Hall et al., 1989; Markarian et al., 1989; Ankley et al., 1990; Dorn et al., 1993; Hwang et al., 1993; Kline et al., 1996; Wong et al., 1997; Warne and Shifko, 1999

(van Emden *et al.*, 1974). Once again, however, acute toxicity data outside this range have been reported. Talmage (1994), citing Shell Chemical Co. data, reported a 6-h LC50 value of 20 000 mg/L for adult oysters (*Crassostrea virginica*) exposed to $C_{12-15}AEO_9$ and a 48-h LC50 value of 3300 mg/L for brown shrimp (*Crangon crangon*) exposed to $C_{14-15}AEO_{11}$. Swedmark *et al.* (1971) also reported numerous LC50 values greater than 100 mg/L for various invertebrate species exposed to AEs and alkylphenol ethoxylates (APE) and Hwang *et al.* (1993) reported a 48-h LC50 value of 0.06 mg/L for larval tiger prawns (*Penaeus monodon*) exposed to LAS.

Some surfactant toxicity data for amphibians has been published. Canton and Slooff (1982) reported a 96-h LC50 value of 5.6-10 mg/L for *X. laevis* exposed to an LAS, and the authors ranked its sensitivity as being similar to the guppy (*Poecilia reticulata*). Plotner and Gunther (1987) reported 24-h LC50 values ranging between 16.2 and 49.7 mg/L for embryos and larvae of *Rana* spp. exposed to a detergent containing 10% sodium alkanemonosulphonate (SAS), although no mortality was recorded at equivalent concentrations of technical grade SAS. Finally, Presutti *et al.* (1994) reported a 96-h LC50 value of 31.74 mg/L for *X. laevis* embryos exposed to an AE (Triton DF-16).

To interpret toxicity data, an understanding of the factors that contribute to surfactant toxicity is necessary. Primary amongst these factors are the secondary and tertiary chemical structures of the surfactants concerned.

Surfactant structure as a determinant of toxicity

Surfactant toxicity is predominantly determined by the chemical structure. The size of the alkyl chain, the degree of ethoxylation and the degree of alkyl chain branching will all affect surfactant toxicity.

Toxicity of anionic and nonionic surfactants is a function of alkyl chain length. An increase in the alkyl chain length corresponds to an increase in toxicity of the anionic surfactant LAS (Kimerle and Swisher, 1977; Maki and Bishop, 1979; Moreno-Danvila, 1983) and other anionic surfactants (A D Little Co., 1977;

Lundahl and Cabridenc, 1978). A similar relationship exists between alkyl chain length and toxicity for AE nonionic surfactants (Gillespie *et al.*, 1999; Lizotte *et al.*, 1999) although the range of structurally distinct AE surfactants used in these studies was restricted to three ($C_{9-11}EO_6$, $C_{12-13}EO_{6.5}$ & $C_{14-15}EO_7$).

The toxicity of nonionic surfactants and AES (anionic) is also a function of ethoxylate chain length. A decrease in ethoxylation has been demonstrated to correspond with an increase in toxicity of AES (Painter, 1992), AE (Gloxhuber and Fischer, 1968; Wildish, 1972; Wildish, 1974; Maki and Bishop, 1979; Wong *et al.*, 1997) and APE surfactants (Janicke *et al.*, 1969; Macek and Krzeminski, 1975; Turner *et al.*, 1985; Yoshimura, 1986; Baillie *et al.*, 1989; Guhl and Gode, 1989; Hall *et al.*, 1989).

An increase in the degree of alkyl chain branching corresponds to a decrease in toxicity amongst anionic surfactants (Kimerle and Swisher, 1977) and nonionic (AE) surfactants (Kravetz *et al.*, 1991; Dorn *et al.*, 1993). To what extent branching of APE alkyl chains affects the toxicity of APEs is less clear. Hall *et al.* (1989) compared the toxicity of a linear $APEO_9$ (equivalent to a $NPEO_9$) with that of a highly branched $NPEO_9$ in 48-h acute tests with an estuarine crustacean (*Mysidopsis bahia*). The 48-h LC50 values for the linear compound ranged between 1.23 and 1.89 mg/L (three tests), while the 48-h LC50 values for the branched compound ranged between 0.90 and 2.28 mg/L (three tests), which indicated that extensive branching of the alkyl chain in APEs has little impact on the compounds toxicity. A difference in toxicity as a consequence of differences in alkyl chain structure may have been masked by the choice of test animal. Wong *et al.* (1997) found that the degree of surfactant ethoxylation had a stronger effect than alkyl chain structure on toxicity of AEs to the crustacean, *D. magna* than fathead minnow (*Pimephales promelas*). Different test species may be more sensitive to the degree of alkyl chain branching in APEs.

Alcohol ethoxylate (AE) surfactants belong to a larger group of nonionic surfactants called alcohol alkoxylates. This group of surfactants also includes polyoxyalkylene block copolymer surfactants that incorporate propylene oxide

(PO, C_3H_6O) or butylene oxide (BO, C_4H_8O) as well as ethylene oxide in the hydrophilic alkoxylate chain. The number and isomeric positioning of these monomers within the alkoxylate chain, influence toxicity (Rodriguez and Singer, 1996) and biodegradability of the surfactant (Balson and Felix, 1995).

Any examination of surfactant toxicity ideally requires a description of the structure of the surfactant. Descriptions, of AE and APE surfactants usually state the average number of alkyl carbons and/or ethylene oxide units per oligomer (for example, $C_{12.5}AEO_{6.5}$ or $NPEO_{9.5}$). However, such a description may not be adequate. Most commercial AE and APE surfactants are mixtures of oligomers of varying ethoxylate chain length. Narrow-range mixtures of ethoxylates are less toxic than broad-range mixtures, reflecting the lower proportion of short chain oligomers (Garcia *et al.*, 1996). Therefore, some recent examinations of AE toxicity have restricted experimental exposures to a finite fraction of ethoxylate oligomers of known length (Gillespie *et al.*, 1999; Lizotte *et al.*, 1999).

In this thesis, the toxicities of two commercial pesticide-spray-tank surfactant-additives (Agral®600 and BS1000®) and a Huntsman Corporation teric (Teric GN8) have been assessed. The tradename Agral®600 and Teric GN8 were both NPEs with an average oligomer length of eight ethoxylate units. The description of their structure in Chapters 3 and 6 includes an analysis of oligomer distribution. The tradename BS1000® was described as an alcohol alkoxylate and may have been a mixture of oligomers of different alkyl chain and alkoxylate chain lengths with numerous combinations of EO, PO or BO block copolymers. The financial and time constraints imposed on this thesis prevented a satisfactory structural description of this product. The Acute toxicity data generated for BS1000® (Chapter 3) have, however, been retained in the written thesis since they are likely to be representative of similar commercial products used as pesticide additives.

Surfactant biodegradation as a determinant of surfactant toxicity

The correlation between alkyl or ethoxylate chain length and acute toxicity has implications for the hazard presented by degradation of surfactants. The biodegradability of surfactants, the rate of degradation and the toxicity of degradation products vary depending on the type of surfactant.

Degradation of branched anionics such as ABS is slow. In the late 1950s and early 1960s rivers in industrialised nations were foaming because of the accumulation of "hard" (branched alkyl chain) surfactants like ABS that had poor biodegradation profiles (Malz, 1997). Consequently, "hard" anionic surfactants were largely replaced by biodegradable or "soft" (unbranched alkyl chain) anionic surfactants such as LAS and nonionic surfactants (i.e. AE & APE).

The linear nature of LAS and AE surfactants implies greater toxicity, however, they also biodegrade relatively rapidly. Furthermore, biodegradation of anionic surfactants and AE results in less toxic degradation products (Kimerle and Swisher, 1977; Maki *et al*., 1979; Turner *et al*., 1985; Patoczka and Pulliam, 1990)

Surfactant biodegradation occurs by the metabolic activity of microorganisms such as bacteria that utilise the surfactant as a carbon source. Steber and Berger (1995) and Balson and Felix (1995) discuss the biodegradation pathways of anionic and nonionic surfactants in detail. Briefly, three general mechanistic pathways are involved in biodegradation of anionic and nonionic surfactants:

1. Hydrophile-hydrophobe scission followed by -oxidation of the hydrophobic alkyl chain.
2. -Oxidation of the terminal alkyl group followed by -oxidation of the hydrophobic alkyl chain.
3. Progressive oxidation of the hydrophilic alkoxylate chain by the -hydrophile pathway.

Scission of the hydrophilic and hydrophobic moiety readily occurs by desulphation or desulphonation amongst AS, SAS, and AOS anionics followed by rapid -oxidation of alkyl chain. Stearic hindrance imposed by the sulphophenyl

group prevents scission of the hydrophile in alkylbenzene sulphonates (LAS, ABS). Biodegradation of LAS occurs by -oxidation of the terminal end of the alkyl chain followed by rapid -oxidation of the alkyl chain resulting in short-chain sulphophenyl carboxylic acids. Subsequent ring cleavage results in mineralisation of LAS. Extensive branching within the hydrophobe of ABS prevents -oxidation of the alkyl chain, explaining its persistence in the environment. Alkyl chain shortening is also the main mechanism for biodegradation of AE nonionic surfactants, although all three pathways are likely to occur.

For APEs, biodegradation is problematic because it is relatively slow and results in toxic metabolites. The presence of a highly branched alkyl chain impedes -oxidation of the hydrophobe. Also, stearic hindrance imposed by the phenol ring prevents hydrophobe-hydrophile scission. Consequently, biodegradation of APEs is slow and relies on the stepwise and progressive shortening of the ethoxylate chain by the -hydrophile pathway. Degradation by this pathway results in the accumulation of environmentally persistent metabolites; predominantly alkylphenol (i.e. OP or NP), alkylphenol mono-, di- and triethoxylates (i.e. $NPEO_{1-3}$ or $OPEO_{1-3}$), and alkylphenoxy carboxylates (i.e. $NPE_{1-2}C$ or $OPE_{1-2}C$) (Giger *et al.*, 1984; Yoshimura, 1986; Marcomini *et al.*, 1990; Ahel *et al.*, 1994; Field and Reed, 1996; Hawrelak *et al.*, 1999).

These metabolites are more toxic than the parent surfactant (Yoshimura, 1986), oestrogenic in nature (Jobling and Sumpter, 1993; White *et al.*, 1994; Jobling *et al.*, 1996; Nimrod and Benson, 1996; Kloas *et al.*, 1999) and may interfere with reproduction (Schurin and Dodson, 1997) or reproductive metabolic pathways (Baldwin *et al.*, 1997). The toxicity of NP and OP has recently been reviewed by Staples *et al.* (1998). Acute toxicity values ranged over two orders of magnitude, from a 96-h LC50 value of 0.017 mg/L for winter flounder (*Pleuronectus americanus*) exposed to NP in a flow-through test, to a 144-h LC50

value of 1.7 mg/L for a freshwater clam (*Anadonta cataractae*) exposed to NP in a static renewal test (Staples *et al*., 1998).

Different countries have responded differently to the perceived hazards associated with APE surfactants. As a consequence of concerns about the persistence of toxic metabolites, use of APEs have been largely phased out in Europe (Renner, 1997). However, in the USA, a view persists that wastewater treatment plants in the United States effectively breakdown the otherwise persistent metabolites, and consequently APEs are still in use (Renner, 1997) as they are in Australia.

APE surfactants may be expected to persist longer in the environment when they are applied as adjuvants and dispersants in pesticide formulations. In Chapter 6, the disappearance and formation of individual oligomers has been examined during the biodegradation of $NPEO_8$ in a static die-away test, in order to evaluate the potential persistence of short chain metabolites in a freshwater system.

Biological and environmental determinants of surfactant toxicity

Species differences in surfactant toxicity have been documented. Lewis and Suprenant (1983) generated 48-h LC50 values for six distinct invertebrate species that represented four phyla (Arthropoda, Platyhelminthes, Annelida and Aschelminthes) exposed to three different surfactants including a LAS, an AE and a cationic surfactant. Two crustaceans including an amphipod (*Gammarus* sp.) and an isopod (*Asellus* sp.) represented the arthropods. The acute toxicity data reported for the six species spanned more than three orders of magnitude. For LAS the lowest LC50 value was 1.7 mg/L for an aquatic oligochaete (*Dero* sp.) and the highest was 270 mg/L for the isopod. The range was narrower for the AE (1.0 mg/L for the platyhelminth to 6.2 mg/L for the isopod). Interestingly, the isopod was the least sensitive species in response to exposure to all three surfactant types, while the other crustacean (*Gammarus* sp.) was consistently one of the most sensitive species, indicating variation in sensitivity exists within

phyla as well as between phyla. Swedmark *et al.* (1971) also noted that representatives within the crustacea varied in their susceptibility to specific surfactants. Decapods were more sensitive to LAS, while cirripedians (barnacles) were more sensitive to $NPEO_{10}$. In 96-h LC50 tests, Arthur (1970) reported a threefold difference in sensitivity between two molluscs (*Physa integra* & *Campeloma decism*). Eisler (1965) reported 96-h LC50 values for fish species exposed to ABS that ranged between 2.1 mg/L (*Menidia menidia*) and 6.8 mg/L (*Fundulus heteroclitis*) and Thatcher (1966) reported 96-h LC50 values for fish species exposed to ABS that ranged between 7.4 mg/L (*Notropis atherinoides*) and 22.0 mg/L (*Ictalurus melas*). Finally, Thatcher and Santner (1967) reported 96-h LC50 values for fish species exposed to LAS that ranged between 3.3 mg/L (*N. atherinoides*) and 6.4 mg/L (*I. melas*).

Species comparisons have been conspicuously absent over recent years. Kline *et al.* (1996) reported LC50, LOEC (lowest observable effect concentration) and NOEC (no observable effect concentration) data for juvenile bluegill sunfish (*L. macrochirus*) and adult fathead minnows (*P. promelas*) exposed to $C_{14-15}AEO_7$ but only a minor difference in sensitivity was observed. Dorn *et al.* (1997) and Gillespie *et al.* (1996) also reported differences in population densities between different invertebrate groups at the end of 30-d mesocosm studies with $C_{12-13}AEO_{6.5}$.

The age or developmental stage of the test organism will affect surfactant toxicity (Marchetti, 1965; Pickering and Thatcher, 1970; Swedmark *et al.*, 1971; van Emden *et al.*, 1974; Hwang *et al.*, 1993). Marchetti (1965) described the sensitivity of rainbow trout (*Salmo gairdnerii*) at successive stages of development, from newly hatched alevin through to 210 day old fingerlings, to $NPEO_{10}$. Newly hatched alevin with yolks were the most tolerant (6-h LC50, 42 mg/L), while the transitional stage between alevin and fry, when yolk absorption was nearly complete, but prior to the commencement of feeding, were 20 times more sensitive (6-h LC50, 2.1 mg/L). Larval stages of bluegill sunfish (*L. macrochirus*) and fathead minnow (*P. promelas*) were similarly found to be

most sensitive to LAS in other studies (Pickering and Thatcher, 1970; Hokanson and Smith, 1971; Macek and Sleight, 1977; McKim, 1977; Holman, 1980). In 24/48-h exposures to C_{10-13}LAS, larval stage tiger prawns (*P. monodon*) were up to 60 times more sensitive than later post larval stages (Hwang *et al.*, 1993). In 96-h exposures, embryonic and larval stages of mussels (*Mytilus edulis*) spider crabs (*Hyas araneus*) and barnacles (*Balanus balanoides*) were also more sensitive to $NPEO_{10}$ than adult stages (Swedmark *et al.*, 1971). Larval spider crabs were more than two orders of magnitude more sensitive than adults (Swedmark *et al.*, 1971).

The nutritional status of the test organism may also affect sensitivity to surfactants. Cripe *et al.* (1989) reported that food availability would significantly affect toxicity of octylphenol (non-ethoxylated) to *M. bahia*, with an increased feeding rate resulting in reduced sensitivity. Another interpretation of this result, however, is that bioavailability of the toxicant was reduced because of sorption of the toxicant to the excess food (*Artemia* nauplii). Taylor (1985) reported the toxicity of $C_{11.8}$LAS to *D. magna* to be variously affected by the diet. Specifically, diet type significantly affected toxicity of LAS to daphnids in 21-d chronic tests. Feeding during 48-h acute tests reduced toxicity and high concentrations of food during chronic tests (21-d) also resulted in lower LC50 values. However, the author concluded that the variation in LC50 values as a consequence of diet variants, was no greater than the normal variation expected in toxicity tests with *D. magna*.

Several environmental factors affect toxicity of surfactants to aquatic fauna (Lewis 1992). Water hardness in particular has been the focus of several studies (Table 1.2).

The toxicity of anionic surfactants was generally higher in hard water, although conflicting data exists (see Table 1.2). Bioaccumulation of AS was also higher in goldfish (*C. auraties*) in hard water (Tovell *et al.* 1974). Tovell *et al.* (1975) speculated that the difference in observed toxicity of anionic surfactants in hard water was largely due to interactions between the ionic hydrophile of anionic

surfactants, and cations in solution, thereby modifying either the diffusion characteristics, or solubility of the surfactant at cell membranes (see below- Mode of Action). This may also explain why commercial detergent products containing ABS were conversely found to be less toxic in hard water (Henderson *et al.*, 1959). Other components of the detergents may have altered the electrostatic properties of the hydrophilic moiety.

Table 1.2 Effect of increased water hardness on toxicity of anionic and nonionic surfactants

Surfactant type	Species	Effect on toxicity	Reference
ABS (anionic)	*P. promelas*		Henderson *et al.*, 1959
ABS (anionic)	*Puntius gonionotus*		Eyanoer *et al.*, 1985
LAS (anionic)	*P. promelas*	No effect	Holman, 1980
LAS (anionic)	*C. auraties*		Gafa, 1974
LAS (anionic)	*L. macrochirus*		Hokanson and Smith, 1971
LAS (anionic)	*P. gonionotus*		Eyanoer *et al.*, 1985
LAS (anionic)	*D. magna*		Lewis and Perry, 1981
AS (anionic)	*P. promelas*		Henderson *et al.*, 1959
AS (anionic)	*C. auraties*		Tovell *et al.*, 1974
Polyoxyethylene ester (nonionic)	*P. promelas*	No effect	Henderson *et al.*, 1959
AE (nonionic)	*S. gairdneri*		Tovell *et al.*, 1975
AE (nonionic)	*D. magna*		Maki and Bishop, 1979
AE (nonionic)	*D. magna*	No effect	Lewis and Perry, 1981

The hardness of the water in which test organisms were cultured was reported to alter the effects of water hardness (Maki and Bishop, 1979). Specifically, LAS was found to be more toxic to *D. magna* in soft water when the

test animals had been cultured in hard water. Culture history was not found to be a confounding factor for the toxicity of AE (Tovell *et al.*, 1975).

Although studied to a lesser degree, increases in water temperature have been found to increase the toxicity of both LAS (Marchetti, 1968; Hokanson and Smith, 1971; Swedmark *et al.*, 1971) and NPE (Swedmark *et al.*, 1971). The toxicity of ABS was not similarly affected (Eyanoer *et al.*, 1985).

The effect of decreased dissolved oxygen (DO) has been studied even less, even though one study has found the toxicity of LAS to increase by an order of magnitude under low DO conditions (Hokanson and Smith, 1971). The median lethal toxicity of LAS to bluegill sunfish was 2.2 mg/L at 7.5 mg/L DO and 0.2 mg/L at 1.9 mg/L DO.

It is with all these factors in mind that the experimental aspects of this thesis have been performed using standardised test procedures, so that all the toxicity data reported can be directly comparable to other data produced with other amphibians or other test organisms. In Chapters 2 and 3, a formulated soft water was chosen as having an ionic profile which best matched the habitat conditions for frog fauna in the south-western Australia. In Chapters 4 and 5 a formulated water specific to the Frog Embryo Teratogenesis Assay (FETAX) (ASTM, 1993a) was adopted to promote consistency with other FETAX assays carried out in other laboratories. In comparative tests (Chapter 2, 3 & 4) the age classes of individual species have been carefully matched. In Chapter 2, a series of high temperature and low DO tests were performed in an attempt to replicate some aspects of Australian conditions.

Test duration as a determinant of toxicity

The toxicity of surfactants can be estimated within one to four days in acute toxicity tests using lethality as an endpoint. Both acute and chronic studies (tests which estimate lethal and sublethal endpoints over an extended duration) appear to support the appropriatness of short-term acute tests, although the alternative view has been expressed. Abel (1974) suggested that detergents might

continue to act lethally over long periods of time without reaching a lethal threshold concentration, and cited data from Herbert *et al.* (1957) and Swedmark *et al.* (1971). Herbert *et al.* (1957) indicated that no lethal threshold was reached in rainbow trout (*S.* gairdneri) exposed for twelve weeks to ABS, while the data presented by Swedmark *et al.* (1971) indicated that the lethal action of ABS and LAS on cod (*G. morrhua*) and flounder (*Pleuronectus flesus*) had not ceased after twenty and eight days respectively.

However, the majority of acute and chronic studies have reported cessation of mortality within two days exposure to anionic and nonionic surfactants. Kline *et al.* (1996) reported daily LC50 data for bluegill sunfish (*L. macrochirus*) and fathead minnow (*P. promelas*) exposed to $C_{14-15}AE_7$ for ten days. Although some mortality was reported over the entire ten-day duration of the test, the LC50 values changed little after two days exposure (less than the 95% confidence intervals for individual values). Roy (1988) also reported large decreases in LC50s between two and 24 hours, but little further mortality in the fish, *Rita rita* following 24 hours exposure to LAS. Macek and Krzeminski (1975) stated that the toxic effects of thirteen AE and APE surfactants tested were most pronounced during the first 24 hours of exposure in both static and dynamic (flow-through) bioassays using *L. macrochirus* and Calamari and Marchetti (1973) stated that much of the acute toxicity of LAS, ABS and $NPEO_8$ in rainbow trout (*S. trutta*) occurs in 48 hours or less. Henderson *et al.* (1959) also reported no further mortality between 48 and 96 h in *P. promelas* exposed to ABS, AS or a nonionic polyoxyethylene ester, although the 96-h LC50 for *L. macrochirus* exposed to ABS was somewhat lower than the 48-h LC50.

Observable sublethal effects in test organisms occur at concentrations close to the LC50 concentration. Very small changes in concentration are needed to span the EC0 and LC100 concentrations (van Emden *et al.*, 1974; Dorn *et al.*, 1993; Wong *et al.*, 1997; Staples *et al.*, 1998). Chronic exposures to surfactants, that have measured sublethal effects including growth, reproduction or behaviour, indicated that surfactants do not have sublethal effects at concentrations much

lower than the LC50 concentration. In his review of chronic toxicity studies with surfactants, Lewis (1991) presented a diagram similar to Fig 1.2 using chronic LOEC and NOEC data. The range of surfactant concentrations that encompassed NOEC and LOEC data was very similar to those presented in Fig. 1.2.

A number of studies have presented both LC50 lethality data and chronic sublethal effect data (i.e. NOEC or LOEC). Kline *et al.* (1996) reported 96-h LC50 data as well as 10 and 30-d LOEC/NOEC data for juvenile bluegill sunfish (*L. macrochirus*) and adult fathead minnows (*P. promelas*) exposed to $C_{14-15}AEO_7$. The 96-h LC50 values were 0.650 mg/L and 0.770 mg/L respectively. Ten-day LC50 values in the same tests were 0.560 mg/L and 0.690 mg/L respectively. The 10-d LOEC (swimming performance) value for bluegill sunfish was 0.460 mg/L. In 30-d mesocosm tests, reduced growth and egg-laying was reported for fathead minnow at 0.330 mg/L. The LOEC data for these two fish species were therefore between 1.4 and 2.3 times lower than the 96-h acute data (Kline *et al.*, 1996).

In 30-d mesocosm trials with fathead minnow exposed to $C_{12-13}AEO_{6.5}$, all endpoints were observed in two sequential exposure concentrations (0.88 and 1.99 mg/L) (Dorn *et al.*, 1997). The LOEC (survival) value of 1.99 mg/L was higher than the 30-d LC50 value of 1.27 mg/L. Coloration, and behavioural effects (cleaning, feeding) were observed in bluegill sunfish exposed to 0.88 mg/L, indicating a factor of 1.4 between 30-d LC50 and LOEC values. Similar differences in LOEC (growth) and LC50 data were reported by Lizotte *et al.* (1999) for fathead minnow fry exposed to three AE surfactants in 28-d flow-through bioassays. Similar margins between LC50 and LOEC (reproduction) data were also reported for *D. magna* exposed to three AE surfactants in 21-d mesocosm trials (Gillespie *et al.*, 1999).

For *D. magna* exposed to LAS, AES and AE surfactants, the differences between 96-h LC50 and 21-d EC50 data were smaller than the 95 % confidence intervals for individual values (Maki, 1979a). Masters *et al.* (1991) also concluded that 4-d tests with *Ceriodaphnia dubia* exposed to LAS, AE and a cationic

surfactant were comparable to those of a 7-d test, although the 96-h LC50 for one test with AE differed from the 7-d EC50 (reproduction) by almost an order of magnitude.

Pickering and Thatcher (1970) reported differences between 96-h LC50 values and chronic values in a life cycle test to be in the order of 3.6 to 7.1 times, for fathead minnow (*P. promelas*) exposed to LAS. The disparity between 96-h LC50 and chronic values obtained in this study, however, merely reflects the increased sensitivity of post-hatch fry, rather than a large difference between LC50 and LOEC values, since growth (of adults), egg production and hatchability of eggs were not affected (Pickering and Thatcher, 1970).

Chronic studies employing APE surfactants are scarce. Dorn *et al.* (1993) reported a 96-h LC50 value of 4.6 mg/L for fathead minnow exposed to $NPEO_9$ and although generated in a separate trial, this study also reported a 7-d LC50 value of 2.9 mg/L and an LOEC (growth) of 2.0 mg/L (2.3 times lower than the 96-h LC50) for the same species. There are, however, a few studies which have examined the chronic effects of non-ethoxylated NP (Staples *et al.*, 1998). The reported differences between 96-h LC50 values and chronic LOEC values are somewhat more variable, ranging from a factor of 1.3 for *C. dubia* exposed to NP in a 7-d reproduction test (England, 1995), to a factor of 36.8 for rainbow trout (*Oncorhynchus mykiss*) exposed to NP in a 90-d flow-through test (Brook, 1993).

Two factors were considered when deciding on exposure times for tests described in this thesis. Firstly, the literature is in general agreement that intact nonionic surfactant toxicity can be assessed within two days (see above). Also, starvation was considered an important confounding factor when recently hatched tadpoles are exposed for long periods without food (Tyler, *pers. com.*). An exposure period of 48 h was therefore selected for the majority of tests with feeding stage tadpoles (Chapters 2 & 3). Some 96-h exposures were included (Chapter 3) to validate the reliability of 48-h tests. Also, some 12-h tests were included (Chapter 3) where longer exposures were not logistically possible. Exposure duration in experiments that followed the FETAX procedure with pre-

feeding stage embryos, was dictated by developmental progress of the species involved and ranged between 96 and 140 h.

Mode of Action of Surfactants

The mechanism of toxicity of surfactants is not well understood beyond being an interaction with lipid membranes (Abel, 1974; Talmage, 1994). However, a number of physiological and behavioural endpoints (concentration dependent) have been observed in test organisms that indicate mechanisms of surfactant toxicity.

At high concentrations, surfactants will destroy biological tissue by disrupting biological membrane and protein integrity. This is a physical effect brought about by a reduction of surface tension (Abel, 1974). At concentrations of C_{12}AS that would kill salmon in 4 minutes to 45 hours (560 to 18 mg/L), histological evidence of extensive gill tissue destruction was evident (Abel and Skidmore, 1975; Abel, 1976). At lower but still acutely lethal concentrations of LAS (1.5-20 mg/L), corneal thinning in tiger perch (*Terapon jarbua*) (Huang and Wang, 1995) and gill damage in fish (Schmid and Mann, 1961; Zaccone *et al.*, 1985; Huang and Wang, 1994) has been reported.

Tissue disruption has also been reported at sublethal concentrations, however many of these reports are indicative of a generalised stress response, since similar non-specific pathologies are seen in fish tissue following exposure to other contaminants (Mallatt, 1985). Maintenance of fish fingerlings (*Cirrhina mrigala*) for 30 days in 0.005 mg/L LAS resulted in gill epithelial hyperplasia, distortion of secondary gill lamellae, increases in gill epithelial mucous cells (Misra *et al*., 1985), hyper production of epidermal mucous and hyperplasia of epidermal cells of the skin (Misra *et al.*, 1987). Similarly, hypertrophy of gill epithelia was reported in fish following 54 days exposure to 0.2 mg/L LAS (Hofer *et al.*, 1995) and 30 days exposure to 6-13 mg/L ABS (Lemke and Mount, 1963). In an examination of respiration rate and oxygen consumption in fish fingerlings (*C. mrigala*), Lal *et al.* (1984) reported reduced oxygen consumption concomitant

with an increase in lactate production and increased opercular movement following exposure to 0.015 mg/L LAS, indicating respiratory stress. Respiratory stress and mucous production was also observed in *R. rita* exposed to 0.1 mg/L LAS within 24 hours (Roy, 1988).

Indirect stress responses are, however, likely to reflect a direct chemical toxicity of surfactants at exposed membranes, possibly by interference with membrane permeability (Florence *et al.*, 1984; de la Maza and Parra, 1996) or membrane proteins. Nonionic surfactants have been reported to reduce sodium uptake by crayfish stretch receptor neurones (Ottoson and Rydqvist, 1978), and LAS was reported to interfere with catecholaminergic receptors in fish gills (Bolis and Rankin, 1980). Furthermore, inhibition of avoidance behaviour and olfactory bulbar electrical responses in whitefish (*Coregonus clupeaformis*) exposed to 0.1 mg/L C_{12}AS suggested interference with olfactory receptor proteins (Sutterlin *et al.*, 1971).

Surfactant interactions with membrane proteins may be an indirect effect of changes in membrane fluidity (van Wezel and Opperhuizen, 1995). For example, membrane fluidity changes effect sodium channel function (van Wezel and Opperhuizen, 1995). Changes in membrane fluidity would also explain increased permeability of biological membranes to some pesticides (Solon *et al.*, 1969; Solon and Nair, 1970) and metal ions (Calamari and Marchetti, 1973; Pärt *et al.*, 1985) in the presence of surfactants.

In contrast to the observed effects with anionic surfactants, Sutterlin *et al.* (1971) also reported that nonionic surfactants were unable to block olfactory receptor responses in Atlantic salmon (*S. salar*). The observed differences in toxicity between anionic and nonionic surfactants are likely to lie in chemical specific interactions with cell membranes. While general hydrophobicity explains gross trends in toxicity, there are several other points of interaction between surfactant molecules and biological membranes. There are dispersive interactions involving alkyl chains and electrostatic interactions involving polar head groups (Versteeg *et al.*, 1997). Indeed, the electrostatic properties of the hydrophilic

head-groups of ionic surfactants and their specific interactions with membrane phospholipid head-groups or membrane proteins, may be adequate to explain differences in physiological effects between anionic and nonionic surfactants.

Induction of narcosis by nonionics is one of the most notable differences between anionic and nonionic surfactants. While several authors have reported, or at least commented briefly, on the loss of activity amongst test species exposed to both anionic and nonionic surfactants (Marchetti, 1965; Swedmark *et al.*, 1971; Calamari and Marchetti, 1973; van Emden *et al.*, 1974; Baillie *et al.*, 1989), many of these reports indicated differences in mode of action for anionic and nonionic surfactants. Calamari and Marchetti (1973) reported that NPE induced a "condition of apparent death" which was not seen in tests with anionic surfactants, and Gloxhuber and Fischer (1968) concluded that anionic surfactants act exclusively at the gill surface, while nonionic surfactants actually compromise membrane function throughout the organism in a similar manner to anaesthetics. Swedmark *et al.* (1971) described initial increases of activity followed by immobilisation in numerous invertebrate and fish species exposed to LAS, ABS, AE and NPE. The author also stated that, animals exposed to the anionic surfactants were less likely to recover from the immobile state when placed in clean water than were animals exposed to the nonionic surfactants. van Emden *et al.* (1974) also noted that guppies (*Lebistes reticulatus*) showed irreversible damage of gill epithelium by anionics and long chain nonionics, but reversible narcosis without gill damage by a short chain nonionic surfactant ($NPEO_{11}$).

The narcotic effect of nonionic surfactants was most elegantly demonstrated by Maki (1979b) who monitored ventilation frequency in bluegill sunfish (*L. macrochirus*) exposed to various concentrations of three anionic surfactants ($C_{11.8}$LAS, C_{13}LAS & $C_{17}AEO_3S$) and two nonionic surfactants ($C_{14.5}AEO_7$ & $C_{12\text{-}13}AEO_{6.5}$). Ventilation rate increased in a dose dependant manner in fish exposed to anionic surfactants, which indicated respiratory stress. Conversely, there was a depression of ventilation rate in fish exposed to the nonionic surfactants, which indicated narcosis.

The anaesthetic effects of AE surfactants in mammals are well established (Talmage, 1994). Also, the narcotic effect of AE surfactants has recently been employed as a form of chemical control in its own right. Genapol OXD 080, is used to inhibit digging activity of red swamp crayfish (*Procambarus clarkii*) in newly established rice crops in southern Spain (Cano *et al.*, 1999).

Even mild narcotic effects will limit an organisms ability to avoid predation and obtain food. In chronic studies, growth or behavioural activities such as feeding, cleaning and defence were affected in invertebrates (Hidu, 1965) and fish (Dorn *et al.*, 1993; Dorn *et al.*, 1997) exposed to nonionic surfactants. In all those studies, growth reduction and behavioural effects occurred at concentrations close to those which caused mortality as would be expected from compounds with such steep mortality response curves (Dorn *et al.*, 1993; Wong *et al.*, 1997).

An observable estimation of narcotic effects does, however, provides a closer approximation of effect concentrations for aquatic organisms. Chapter 3 of this thesis showed that narcosis could be distinguished from normal activity at concentrations significantly lower than lethal concentrations, and has therefore been used as an observable endpoint.

Narcotic effects are characteristic of organic chemicals. If a chemical does not otherwise have a specific target action at a particular receptor or target site, then it will exert a non-specific narcotic effect if bioconcentrated in membranes to a sufficient extent (van Wezel and Opperhuizen, 1995). The ability of an organic chemical to bioconcentrate in membranes is a function of its lipophilicity (Tolls and Sijm, 1995) and as noted above, the degree of ethoxylation and alkyl chain length primarily affect hydrophobicity/lipophilicity. An increase in lipophilicity is directly related to toxicity, although toxicity cut-off thresholds are evident (Florence *et al.*, 1984; van Wezel and Opperhuizen, 1995). Both anionic and nonionic surfactants have been shown to bioconcentrate in fish (Tolls *et al.*, 1994). Uptake is via the gills, with subsequent detoxification in the liver and elimination via the gallbladder (Tolls *et al.*, 1994).

In embryonic and larval stage fish, the metabolic capacity to detoxify organic xenobiotics is not fully developed (Binder and Stegeman, 1984; Goksøyr *et al.*, 1991). Furthermore, larval organisms have large exposed surface areas (relative to body volume) and seldom have protective shells, exoskeletons, scales or skin allowing organic compounds to penetrate larval organisms over their entire surface rather than via the gills alone. Consequently the bioconcentration kinetics of organic compounds in adult and larval organisms differ substantially (Petersen and Kristensen, 1998). These factors may be adequate to explain the increased sensitivity of larval organisms.

Chapter 2 and 4 of this thesis also highlight the increased sensitivity of tadpoles over adult frogs and embryos to surfactants. Exposure of tadpoles for the majority of tests rather than other life-stages was therefore considered to be adequate for estimation of surfactant toxicity.

Note: For copyright reasons chapter 2 has not been reproduced.

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Chapter 3

The acute toxicity of agricultural surfactants to the tadpoles of four Australian and two exotic frogs.

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Abstract

Nonionic surfactants are frequently incorporated into pesticide formulations, and are therefore a group of chemicals to which amphibians may be exposed in agricultural or urban landscapes. However, little is known about the effects of surfactant exposure in amphibians. Feeding stage tadpoles of *Bufo marinus*, *Xenopus laevis* and four species of Australian frogs (*Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis* and *Litoria moorei*) were exposed to nonylphenol ethoxylate (NPE) and alcohol alkoxylate in static renewal acute toxicity tests. All species exhibited non-specific narcosis following exposure to both these surfactants. The 48-h EC50 values for NPE ranged between 1.1 mg/L (mild narcosis) and 12.1 mg/L (full narcosis). The 48-h EC50 values for alcohol alkoxylate ranged between 5.3 mg/L (mild narcosis) and 25.4 mg/L (full narcosis). *Xenopus laevis* was the most sensitive species tested. The sensitivity of the other five species was size dependent with larger species displaying greater tolerance. Replicate acute toxicity tests with *B. marinus* exposed to NPE at 30°C over 96 hours indicated that the narcotic effects were not particularly time dependant. The mean 24, 48, 72 and 96-h EC50 (mild narcosis) values were 3.6, 3.7, 3.5 and 3.5 mg/L respectively. The mean 24, 48, 72, and 96-h EC50 (full narcosis) values were 4.0, 4.1, 4.2 and 4.0 respectively. Acute toxicity tests with *B. marinus* exposed to NPE at 30°C under conditions of low dissolved oxygen (0.8-2.3 mg/L) produced a two to threefold increase in toxicity. The 12-h EC50 values ranged from 1.4 to 2.2 mg/L.

Keyword: amphibian-tadpole, nonionic surfactant, acute toxicity, nonylphenol ethoxylate, narcosis

Introduction

Chemical contamination as a consequence of pesticide application continues to be postulated as a contributing factor in the global decline of amphibian populations (Ankley *et al.*, 1998; Berrill *et al.*, 1998; La Clair *et al.*, 1998; Schuytema and Nebeker, 1998; Bishop *et al.*, 1999; Mann and Bidwell, 1999b; Saka, 1999). Indeed, amphibians may be at greater risk from the toxic effects of pesticides than other aquatic vertebrates because their preferred breeding habitats are often shallow, lentic or ephemeral water bodies (Tyler, 1994) where contaminants may accumulate without substantial dilution.

A much-overlooked aspect of pesticide use is the potential toxic hazard posed by surfactant additives in pesticide formulations. Often, where the active constituents of a pesticide are of low toxicity, the additive surfactant components may pose the most significant risk to aquatic fauna, especially when they are applied around or over standing or ephemeral waters with a low capacity for dilution (Mann and Bidwell, 1999b). While a number of studies have assessed surfactant toxicity in a range of aquatic species, they have focused predominantly on fish and crustaceans (for review see Abel, 1974; Lewis, 1991; Talmage, 1994). There is limited data pertaining to the effects of these chemicals on amphibians (Plotner and Gunther, 1987; Presutti *et al.*, 1994, Mann and Bidwell, 2000).

Much of the amphibian toxicological literature describes studies using representatives of the genera- *Bufo*, *Rana*, or *Xenopus* (Power *et al.*, 1989; Mann and Bidwell, 1999a). The Australian amphibian fauna are phylogenetically distinct from these northern hemisphere and African species, prompting concern that the available toxicity data may not predict the hazards posed to Australian taxa. In this study, the toxicity of two commonly used agricultural surfactants was compared in four species of Australian frogs and two exotic species- the African clawed frog, *Xenopus laevis* and the cane toad, *Bufo marinus*. Comparative studies of this kind will not only help ascertain whether existing toxicity data can be applied in Australia, but may also advance the use of exotic species as surrogate

test species, since some non-native species are more amenable to laboratory rearing than Australian frogs.

While the Australian species used in this study were from temperate southwestern Australia, the majority of Australian frogs are tropical or sub-tropical species that live and breed in environments where water temperatures may exceed 40°C (Tyler, 1994). Furthermore, such aquatic environments are often eutrophic and deplete in dissolved oxygen (DO) (Lahr, 1997). Therefore, the established use of 20°C and high DO as standard test parameters may not be appropriate for Australian fauna (Chapman, 1995). A further component of this study was the inclusion of toxicity tests at high temperatures and low DO, using the tropical species, *B. marinus*.

Materials and Methods

In general, toxicity test procedures employed in this study followed those outlined in American Society for Testing and Materials Standard E729-88a^{E1}, Standard practice for conducting acute tests with fishes, macroinvertebrates and amphibians (ASTM, 1993b).

Test Substances

Agral$^{®}$600 (60% NPE and unspecified concentrations of oleic acid and 2-ethyl hexanol) was purchased from a retail outlet. Teric GN8 (100% NPE with an average oligomer length of eight ethoxylate units) was provided by Huntsman Corporation Australia Ltd and BS1000$^{®}$ (100% alcohol alkoxylate) was provided by Crop Care Australasia.

Test Organisms

Two species exotic to Australia (*X. laevis* and *B. marinus*) and four Australian native frog species (*Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis* and *Litoria moorei*) were used in this study. *Xenopus laevis* is a commonly used experimental animal that has been proposed as a model

species for frog research (Cannatella and de Sá, 1993; Bantle, 1995). *Bufo marinus* is an introduced pest in Australia and serves as a representative of a genus frequently used in toxicity testing. The four native species serve as examples of the two major phylogenetic groups of frogs in Australia (Myobatrachidae and Hylidae) and are also representative of large and small frogs with varying habitat requirements (Cogger, 1992). *Crinia insignifera* is a small (14-29 mm s-v, snout-vent length) ground dwelling frog that inhabits areas temporarily inundated by water. *Heleioporus eyrei* is a medium sized (45-66 mm s-v) burrowing frog inhabiting sandy soils in areas prone to temporary inundation. *Limnodynastes dorsalis* is a relatively large (60-73 mm s-v) ground frog which inhabits vegetation close to temporary and permanent water. *Litoria moorei* is also a relatively large (53-74 mm s-v) frog found in permanent waters where it inhabits emergent vegetation.

Xenopus laevis were induced to breed by intralymphatic administration of human chorionic gonadotropin (Profasi®, Serono, Italy) according to the procedure outlined by Mann and Bidwell (2000; Chapter 4). *Bufo marinus* were induced to breed by intralymphatic administration of 10 μg luteinizing hormone-releasing hormone (LH-RH, Sigma, L4513) to both the male and female toads. The toads were introduced to a large breeding tub filled with dechlorinated tap water in a room held at 29°C where egg deposition was completed. *Crinia insignifera* eggs were harvested from the matings of adult animals collected in amplexus from a single location in the Perth metropolitan area in Western Australia. *Limnodynastes dorsalis* and *L. moorei* were collected as egg masses from a single location in the Mandurah district south of Perth. *Heleioporus eyrei* were collected as egg masses from two locations in the Perth metropolitan area. The metropolitan sites were subject to some urban storm-water runoff, however, all collection sites had large healthy populations of frogs.

Tadpole Rearing and Acclimation

Tadpoles of the six species were reared for use in a comparative study performed at 20°C. Tadpoles of *B. marinus* were also reared for two additional series of tests. One series of tests was at 30°C under conditions of oxygen saturation and the second series was at 30°C under conditions of low dissolved oxygen (DO, <20%). All eggs and tadpoles were held in glass tanks fitted with air stones and maintained in soft water (ASTM, 1993b) at approximately the same temperature as that used in subsequent tests.

For the comparative study, 48 h old *B. marinus* embryos, were initially transferred to a climate chamber where the temperature was reduced from 29°C to 20°C over seven days. Similarly, freshly laid *X. laevis* embryos that had been deposited at 24°C were transferred to a room held at 20°C. Eggs of Australian native species were either laid within the laboratory at 20°C, or transferred directly from field sites to the laboratory. Holding/acclimation periods ranged from one to three weeks prior to testing. During the holding and acclimation periods the animals showed no signs of disease or stress. Water quality (pH and ammonia) was monitored daily and maintained by daily water changes. Ammonia concentrations were monitored with a Merck Ammonium Aquaquant test kit. Daily water changes were adequate to maintain ammonium levels below 100 ppb. During the holding period the tadpoles were fed *ad libitum* with commercial fish food and pelletised rabbit chow.

For the high temperature study, 48 h old *B. marinus* embryos were transferred and held as described above at 30°C within a climate control room for approximately one week before use. For the high temperature—low DO study, *B. marinus* tadpoles were obtained as described above and maintained in a climate room at 30°C in a large cylindrical glass holding tank filled with partially deoxygenated soft water. Twenty five-litre carboys of partially deoxygenated soft water were obtained by displacing DO with nitrogen gas. The low DO was sustained in these carboys by applying a head of nitrogen to the carboy. To ensure that the acclimation water exhibited consistently low concentrations of DO,

a flow-through system was employed. Fresh water (from the carboys), partially depleted in oxygen (30% saturation), was continuously introduced at the bottom of the cylindrical acclimation tank at a rate of 30 mL/min. Waste-water was allowed to drain through a tube positioned centrally at the top of the tank. Dissolved oxygen measurements were taken from water as it exited the chamber with an oxygen probe. The metabolic activity of the animals lowered the DO further, such that the tadpoles were constantly maintained at 10 to 20% saturation DO. The observed fluctuation in DO was accounted for by the increased oxygen demand, which occurred twice daily upon feeding. As a consequence of low DO, tadpoles were obliged to either swim constantly near the surface of the water to obtain adequate oxygen, hang at the surface, or rest on pieces of nylon mesh inserted vertically through the water-air interface. In this manner, the tadpoles were acclimated to a low-oxygen environment and maintained for ten days.

Preparation of Test Concentrations

Prior to testing, a primary stock was prepared for each test surfactant as a nominal concentration of 1000 mg/L NPE or alcohol alkoxylate. The diluent used for the stock solutions was soft water with a hardness of 40 to 48 mg/L $CaCO_3$ and a conductivity of approximately 210 µS/cm (ASTM, 1993b). Test concentrations were made up immediately prior to the beginning of the tests using soft water.

Test Procedure: Recorded Endpoints

Two endpoints were recorded- Full Narcosis (FN) or Mild Narcosis (MN). Narcosis is a common means by which organic chemicals elicit effect and is the result of chemicals accumulating in a non-specific manner and resulting in decreased activity and reduced reaction to external stimuli (van Wezel and Opperhuizen, 1995). Where necessary, narcosis was assessed in immobile tadpoles by eliciting a flight response by physical prodding. Immobile tadpoles were tapped on the tail with a blunt glass rod. Tadpoles were prodded only once

during any one observation period. Control tadpoles were rarely immobile for more than a few seconds (except under dark conditions). When prodded, immobile control tadpoles would characteristically respond by swimming away swiftly. This response in control tadpoles was always co-ordinated and persisted for more than one second. If in response to prodding, a tadpole failed to swim strongly for at least one second, or if it swam in an uncoordinated manner, then it was recorded as displaying MN. If a tadpole displayed a total lack of activity and an inability to respond to physical prodding then it was recorded as displaying FN. This category included dead animals, because it was often difficult to distinguish totally inactive animals from those that had recently died. Animals were only removed when tissue necrosis became evident. Because of the relative subjectivity in assigning MN to a tadpole, all observations were made by the same person to avoid inter-observer variation. It was not possible to observe a change in status of individual tadpoles from normal to MN to FN or *vice versa*, and observed endpoint data reflect the proportion of tadpoles displaying narcotic effects.

Test Procedure: Comparative Tests

Where possible, Gosner-stage 25 (Gosner, 1960) tadpoles from a single clutch were used for each test. Gosner-stage 25 was chosen because post-hatch feeding stage tadpoles of the native species remained at this stage for the full duration of the 48-h tests. Other developmental stages were more transient. *Xenopus laevis* were at equivalent development stages 48 to 50 (Nieuwkoop and Faber, 1975). The average mass of at least ten tadpoles (blotted dry) from the same clutch was used as an indication of tadpole weight. Biomass loading (defined as the total wet weight of tadpoles per litre of test water) was maintained below 0.6 g/L as recommended in the ASTM guidelines (ASTM, 1993b). Either 400 ml or 600 ml acid-washed glass beakers with 200 to 500 ml (depending on tadpole weight) of solution were used for all tests. Following range-finding tests, definitive tests incorporated at least five concentrations and a control from which EC50 values were generated. For native species, five tadpoles were impartially

allocated to each of four replicate beakers until there was a total of 20 animals for each test concentration and a control group. For the two exotic species, seven animals were impartially allocated to three replicate beakers until there were 21 animals for each test concentration and a control group. The beakers were arranged randomly on a bench in a climate-controlled room held at 20°C. Animals were not fed for the 48-h duration of the tests. Tests were run for 48 h rather than 96 h because starvation was considered an important factor affecting the survival of young tadpoles. Test solutions were renewed after 24 h. Animal condition was assessed and dead animals were removed at 12-h intervals. At 24-h intervals, animals were recorded as displaying either FN or MN.

Test Procedure: High Temperature trials

Six acute tests were run consecutively over a period of 10 days utilising progeny derived from a single clutch of *B. marinus* tadpoles. All tests exposed tadpoles to Teric GN8. Tadpole development stages ranged from 25 to 30 (Gosner, 1960). Following range-finding tests, definitive tests incorporated at least five concentrations and a control from which EC50 values were generated. Five tadpoles were impartially allocated to each of four replicate beakers until there was a total of 20 animals for each test concentration and a control group. A seventh test was performed using tadpoles with well-developed hind-limbs (Gosner-stages 39 to 40). Restricted animal availability dictated that a total of twelve animals per test-concentration (three tadpoles per replicate) were used for the seventh test rather than the usual 20. The beakers were arranged randomly on a bench in a climate-controlled room held at 30°C. Animals were not fed for the duration of the tests. Animal condition was assessed and dead animals were removed at 12-h intervals. The first six tests were run for 96 h in static-renewal tests. The seventh test was terminated at 48 h since many of the tadpoles had developed fore-limbs (Gosner-stages 41 to 43). At 24-h intervals, animals were recorded as displaying either FN or MN.

Test Procedure: High Temperature—Low DO trials

Three acute tests were conducted in which Gosner-stage 25 tadpoles were exposed to five concentrations of NPE (Teric GN8) and a control under low DO conditions. A fourth test was run at normal DO (>75% saturation) for comparison. All four tests were run at 30°C. A flow through system was employed to maintain low DO in much the same manner as that employed to acclimate stock animals. The exposure chambers were 250 mL integrally moulded polyethylene (LDPE) wide-mouthed Unitary™ washbottles (Nalgene, USA). Toxicant was introduced at the bottom of the washbottle through the spout via 6 mm PVC tubing. Flow rate was maintained at approximately 10 mL/min using plastic aquarium-airline valves. Waste water was removed via a tube inserted just below the 'fill line'. Nylon mesh (2 mm) covering the end of the tube prevented loss of tadpoles through the drain hole. The airspace at the top of the bottle allowed tadpoles to gulp at the surface for air. Just prior to beginning the tests, 25 L reservoirs of test solution were formulated in 25 L polyethylene (HDPE) carboys using oxygen depleted soft water. Low DO was maintained by applying a head of nitrogen to each reservoir.

In trials one, two and three, DO was reduced to 20%, 15% and 10% saturation respectively, while in trial four, fully oxygenated water was used to formulate exposure concentrations. In all four trials, seven tadpoles were impartially allocated to each of three replicate chambers until there was a total of 21 animals for each test concentration and a control group. Apparatus design precluded a random arrangement of exposure chambers within the climate room. Acute toxicity tests were run for 12 h. At 2-h intervals, animals were either recorded as displaying FN or MN as described above. Temperature, pH, conductivity and DO were recorded at 2, 6, and 12 h in at least one replicate test chamber of each concentration.

Environmental Conditions

All tests with native species were performed in a Conviron C10 climate room. Test temperature was maintained at 20±1°C. A 12-h light and 12-h dark photoperiod was programmed. Temperature, DO and pH measurements were taken at the beginning of the test, and after 24 and 48 hours. Temperature and DO were measured using a WTW OXI 320 oxygen meter and pH was measured with a HANNA 8417 pH meter. Tests with *X. laevis* and *B marinus* were conducted in a temperature controlled room at either 20±1°C or 30±1°C with an artificial 12-h light and 12-h dark photoperiod. For all tests with *X. laevis* and *B. marinus*, temperature, DO, pH and conductivity were all measured with a TPS 90FL multimeter.

Analytical Chemistry

The concentration of alcohol alkoxylate was not measured, and all EC50 values generated in these tests were derived from nominal concentrations. For tests with NPE, 200 mL water samples were taken at the beginning of the test and after 24 h (prior to test solution renewal). All water samples were preserved by the addition of 1% formaldehyde to prevent microbial degradation of the surfactant (Szymanski *et al.*, 1995). The method for extraction and quantification of NPE has been described elsewhere (Chapter 6; Mann and Boddy, 2000). Briefly, NPE was extracted from the water samples using solid-phase extraction with 2.8 mL, 500 mg C18 cartridges (Alltech, USA) following a protocol described by Scullion *et al.*(1996). High performance liquid chromatography (HPLC) was used to quantify NPE as described in Scarlett et al. (1994). A Waters™ Resolve CN 8x10 Radial-Pak column was used for normal phase separation of NPE oligomers. UV detection was carried out using a Waters™ 486 tuneable absorbance detector (=225 nm) and data output via a Hewlett-Packard HP3396A integrator. The NPE derived peaks were quantified against Teric GN8. The oligomer distribution of Agral®600 was virtually identical to this standard (Fig. 3.1). Because all oligomers have almost identical molar absorptivities, the

integrated peak area of individual oligomers could be used to determine the mole fraction of each oligomer (Wang and Fingas, 1993). Furthermore, there is a robust linear relationship between concentration and the integrated peak area response (Mann, unpubl. data), therefore, changes in individual peak areas during the trial have been used to represent changes in molar concentration. Initial concentrations of surfactant were established by comparing aggregate integrated peak areas with a standard curve generated with six concentrations of Teric GN8 that had been prepared for analysis in a similar manner.

Data Analysis

Narcotic effect data were used to generate EC50 values by the Spearman-Karber method (Hamilton *et al.*, 1977). Where available, initial measured surfactant concentrations were used to generate EC50 values. Nominal data were used to generate EC50 values for *L. dorsalis,* and *L. moorei* exposed to Agral®600 and all species exposed to BS1000®. Nominal data were also used to generate EC50 values for *B. marinus* exposed to Teric GN8 in replicate tests at 30°C and normal DO. An ANOVA with Fisher's Protected Least Significant Difference posthoc test ($p<0.05$) was also employed to determine if there were any differences between 24, 48, 72 and 96-h EC50 values generated in the six replicate trials with *B. marinus* at 30°C. Percent Coefficients of Variation ($\%CV = 100 \times SD/\mu$) were also determined for 24, 48, 72 and 96-h EC50 values generated in the six replicate trials with *B. marinus* at 30°C to evaluate the reproducibility of individual EC50 values.

Results

Chemical analysis

HPLC analysis of NPE indicated that oligomer distribution did not change over 24 hours (Fig. 3.2) at either 20°C or 30°C. Also, aggregate peak area (as a measure of total surfactant) did not diminish by more than 10% over 24 h.

Therefore, initial NPE measurements have been used to generate EC50 data. Measured concentrations always corresponded well with nominal concentrations.

Water Quality-Comparative Study:

Water temperature, pH, DO, and conductivity were consistent across all tests. Recorded temperatures ranged between 18.9°C and 21.4°C. For any single test the difference between the highest and lowest recorded temperature over 48 h was no greater than 1.3°C. The pH of test solutions ranged between 7.0 and 7.9. For any single test the difference between the highest and lowest pH was no greater than 0.6 of a pH unit. In most cases the DO remained above 80% saturation. Dissolved oxygen occasionally dropped below 80% (but never below 70%) if the presence of dead tadpoles resulted in high oxygen demand.

Water Quality-High Temperature Trials:

Water temperature, pH, DO, and conductivity were also consistent across all tests. Recorded temperatures ranged from 29.0°C to 31.0°C. For any single test the difference between the highest and lowest recorded temperature over 96 h was no greater than 1.6°C. The pH of test solutions ranged between 6.8 and 7.6. In most cases DO remained above 75% (~5.7 mg/L). Dissolved oxygen occasionally dropped below 75% (but never below 65%) if the presence of dead tadpoles resulted in a high oxygen demand.

Water Quality-High Temperature—Low DO Trials:

Water temperature for the three low DO trials and the single high DO trial ranged between 29.0°C and 32.8°C. The pH of test solutions ranged between 7.1 and 7.6. DO varied depending on the level of activity of tadpoles in exposure chambers (Table 3.3).

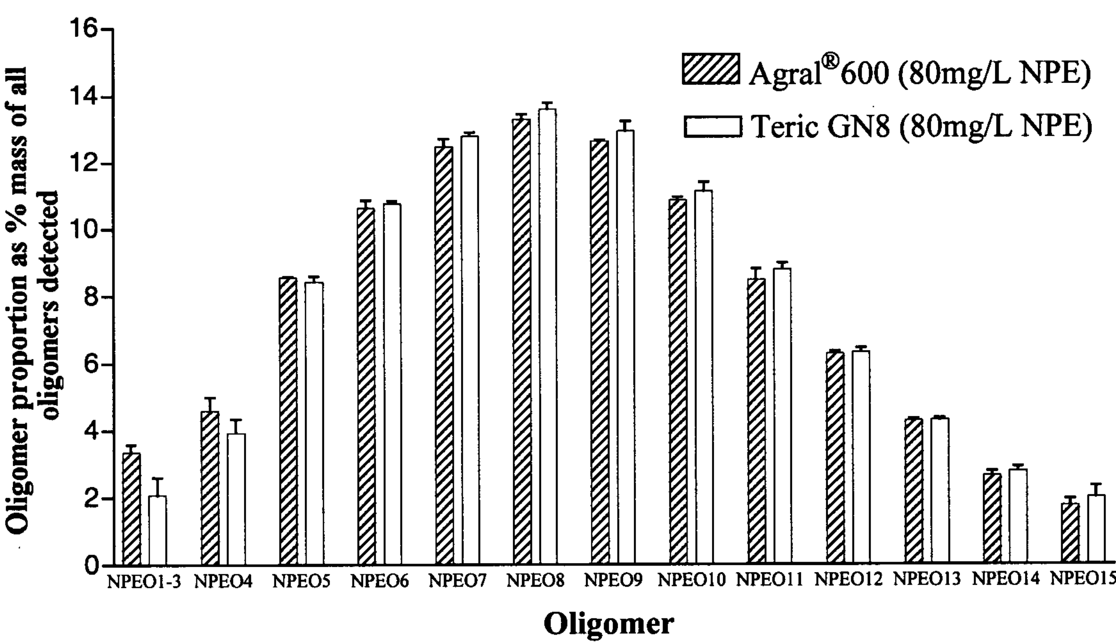

Fig. 3.1 Oligomer distribution for Agral®600 and Teric GN8. Error bars represent standard deviations for 6 measurements of Teric GN8 and 2 measurments of Agral®600.

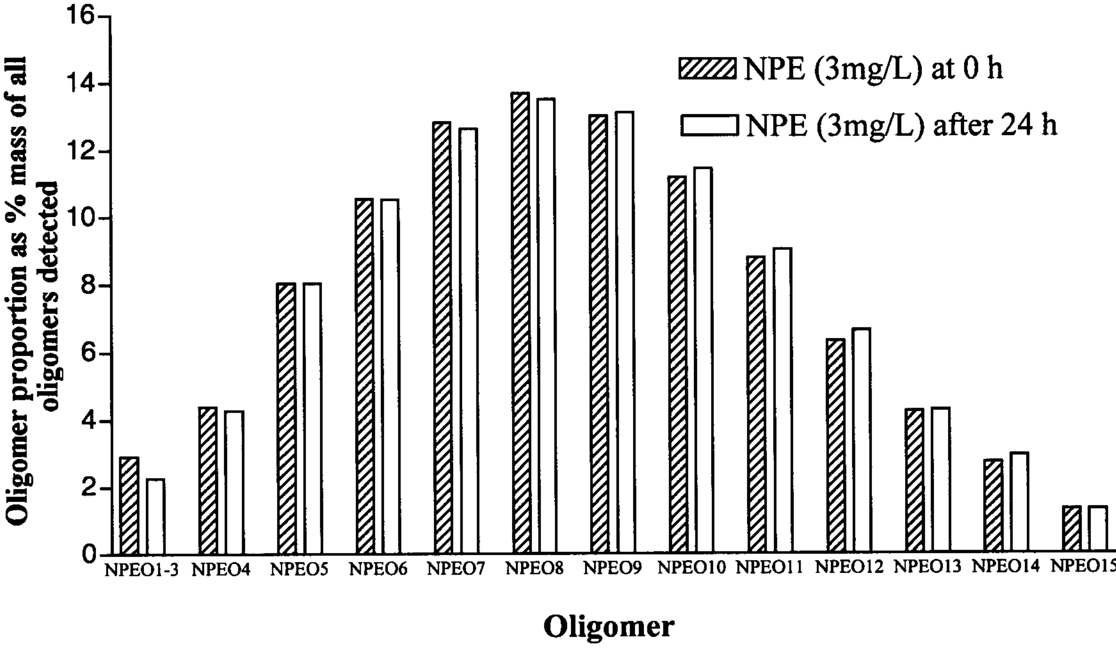

Fig. 3.2 Oligomer distribution for Teric GN8 at 0 and 24 h at 20°C.

Acute Toxicity

The comparative 48-h EC50 values obtained in this study are presented in Table 3.1. All 48-h EC50 values for the six species exposed to Teric GN8, Agral®600 or BS1000®, were within 1.5 orders of magnitude, and ranged between 1.1 mg/L (MN) for *X. laevis* exposed to Teric GN8 to 25.4 mg/L (FN) for *H. eyrei* exposed to BS1000®. Teric GN8 and Agral®600 both produced similar EC50s in the three species for which both compounds were tested- *C. insignifera*, *X. laevis* and *B. marinus*. *Xenopus laevis* was the most sensitive of all the species tested, while *H. eyrei* was the least sensitive. There was a general trend towards lower sensitivity with increased size, although this was not consistent across all six species. Tadpoles of *X. laevis*, though much larger than both *C. insignifera* and *L. dorsalis*, was more sensitive to Agral®600. All species displayed mild narcosis at concentrations lower than that which induced full narcosis or mortality. The range of concentrations over which a narcotic effect was observed was much broader for animals exposed to BS1000® than for Agral®600.

The mean 24, 48, 72, and 96-h EC50 values (MN and FN) for the six *B. marinus* tests conducted at 30°C showed no significant difference (p<0.05) in EC50 values among the four time intervals (Table 3.2). All EC50 values for the six tests were derived from nominal concentrations. There was a slight increase in EC50 values for full narcosis over the first 72 h as some of the animals appeared to recover from the full narcotic condition although this trend was reversed between 72 and 96 h. There was little variation between individual tests. Coefficients of variation (Table 3.2) did not exceed 10%. The mean wet weight of tadpoles in each of the six tests ranged between 25.7 and 32.7 mg (Table 3.2). Tadpoles with hind limbs were no more or less sensitive than less developed tadpoles (Table 3.2). The mean wet weight of tadpoles in this test was 97.8 mg/L (Table 3.2).

Low DO at 30°C produced a pronounced increase in toxicity. The control flow-through test exposing *B. marinus* tadpoles to NPE (Teric GN8) at normal DO (>5.7 mg/L) and 30°C, generated 12-h EC50 (MN and FN) values of 3.6 mg/L

and 4.1 mg/L respectively (Table 3.3). These values are similar to the 24, 48, 72 and 96-h EC50 values generated in static renewal tests at normal DO (Table 3.2). The 12-h EC50 (FN) at low DO and 30°C ranged from 1.4 to 2.2 mg/L (Table 3.3, Fig. 3.3).

At low DO, a maximal narcotic effect was produced after 2 to 6 h (Fig. 3.3). In trial one, DO did not fall below 1.7 mg/L and mild narcosis was noted in tadpoles over the entire 12-h duration of the test (Fig. 3.3a). In trials two and three, DO was consistently lower than 1.5 mg/L and no tadpoles displayed MN after 4 to 6 h (Fig. 3.3b,c). Indeed, most of the animals categorised as displaying FN were dead. Recovery from the initial effects of surfactant was noted in animals if DO was not low enough to cause death in narcotised animals (Fig. 3.3a).

Discussion

The nonionic surfactants used in this study are examples of polyethoxylated derivatives of alkylphenols and polyalkoxylated fatty alcohols. The acute toxicity of these classes of chemicals appears to be substantially related to their ability to induce non-specific narcosis in aquatic organisms (Maki, 1979b; Schüürmann, 1990). Oxyethylene (EO) narcosis syndrome (Schüürmann, 1991) was a salient feature of surfactant exposure in this study.

No attempt was made to distinguish between mortality and full narcosis. The dark pigmentation of several of the species used prohibited observation of heartbeat as an indicator of mortality. Even tissue necrosis was not always a reliable sign of death, since respiratory function was occasionally evident even when peripheral tissues were disintegrating. Calamari and Marchetti (1973) encountered similar difficulties with trout exposed to NPE. The distinction, however, may be unimportant since it is arguable that full narcosis is equivalent to death in an ecosystem where the ability to avoid predation is an essential survival trait (Lawler, 1989).

Table 3.1 48-h EC50 values with 95% confidence intervals (CI) for acute toxicity tests with Gosner-stage 25 tadpoles of six species of frogs exposed to nonylphenol ethoxylate (Agral®600 and Teric GN8) and alcohol alkoxylate (BS1000®) in static-renewal tests at 20 °C

	GN8			Agral®600			BS1000®		
	Avg. wet wt.	EC50 (95% CI) mg/L[a]		Avg. wet	EC50 (95% CI) mg/L[a]		Avg. wet wt.	EC50 (95% CI) mg/L[a]	
Species	mg (SD)	Mild Narcosis	Full Narcosis	wt. mg (SD)	Mild Narcosis	Full Narcosis	mg (SD)	Mild Narcosis	Full Narcosis
Crinia insignifera	4.6 (2.0)	2.7 (2.6-2.9)	3.8 (3.4-4.3)	5.6 (1.4)	2.7 (2.5-3.0)	3.5 (3.2-3.9)	5.6 (0.8)	5.3 (4.9-5.7)*	6.0 (5.5-6.6)*
Limnodynastes dorsalis				8.8 (2.0)		4.1 (3.9-4.4)*	8.8 (2.0)	<6.0[b]*	14.3 (13.8-14.8)*
Xenopus laevis	12.3 (1.9)	1.1 (0.9-1.2)	2.8 (2.7-2.9)	12.3 (1.9)	1.2 (1.0-1.4)	2.3 (1.9-2.7)			
Bufo marinus	16.2 (3.2)	2.8 (2.0-4.0)	5.1 (4.8-5.4)	16.2 (3.2)	2.9 (2.7-3.0)	5.4 (5.1-5.8)			
Litoria moorei				17.2 (3.0)		4.6 (4.2-5.0)*	17.2 (3.0)	<11.0[b]*	<11.0[b]*
Heleioporus eyrei				32.6 (7.4)	<10.6[b]	12.1 (11.8-12.5)	41.6 (11.0)	<10.0[b]*	25.4 (24.2-26.6)*

[a] EC50 values are expressed as mg/L nonylphenol ethoxylate (GN8 & Agral®600) or mg/L alcohol alkoxylate (BS1000®)

[b] Data for which there were insufficient lower concentrations to generate an EC50

* Data for which nominal concentrations were used to calculate an EC50

Table 3.2 24, 48, 72 and 96-h EC50 values with 95% confidence intervals (CI) for acute toxicity tests with feeding stage (Gosner stage 25-30) and hindlimb stage (Gosner stage 39-40) *Bufo marinus* tadpoles exposed to Teric GN8 (100% nonylphenol ethoxylate) in static-renewal tests at 30°C. All EC50 values are based on nominal concentrations.

	Avg. wet wt. mg (SD)	EC50 Mild Narcosis (95% CI) mg/L				EC50 Full Narcosis (95% CI) mg/L			
		24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
B. marinus (30°C) Trial 1	31.3 (9.4)	3.5*	3.5 (3.4-3.6)	3.5*	3.5*	3.8 (3.6-4.1)	3.9 (3.7-4.2)	4.0 (3.8-4.3)	4.0 (3.8-4.2)
B. marinus (30°C) Trial 2	32.5 (10.1)	3.9 (3.7-4.1)	3.5 (3.5-3.7)	4.0 (3.8-4.2)	3.7 (3.5-3.9)	4.4 (4.2-4.5)	4.4 (4.3-4.5)	4.4 (4.2-4.5)	4.4 (4.2-4.5)
B. marinus (30°C) Trial 3	31.4 (11.1)	3.3 (3.2-3.4)	3.4 (3.3-3.5)	3.3 (3.2-3.4)	3.3 (3.2-3.4)	3.4 (3.3-3.5)	3.5 (3.4-3.6)	3.4 (3.3-3.5)	3.4 (3.3-3.5)
B. marinus (30°C) Trial 4	25.7 (8.3)	4.1 (4.0-4.3)	4.1 (3.9-4.3)	3.9 (3.8-4.1)	3.7 (3.5-3.8)	4.3 (4.2-4.4)	4.5 (4.4-4.6)	4.5 (4.4-4.6)	4.2 (4.1-4.3)
B. marinus (30°C) Trial 5	28.5 (6.2)	3.9 (3.9-4.0)	3.7 (3.6-3.9)	3.8 (3.6-4.0)	3.5 (3.3-3.7)	4.1 (4.0-4.2)	4.0 (3.9-4.2)	4.3 (4.2-4.4)	4.1 (3.9-4.2)
B. marinus (30°C) Trial 6	32.7 (10.5)	3.2 (3.1-3.3)	3.7 (3.6-3.8)	3.5 (3.4-3.7)	3.5 (3.3-3.6)	4.1 (3.9-4.3)	4.4 (4.2-4.6)	4.5 (4.3-4.6)	4.2 (4.0-4.4)
Mean		3.6	3.7	3.7	3.5	4.0	4.1	4.2	4.0
Standard Deviation (SD)		0.36	0.25	0.27	0.15	0.38	0.38	0.41	0.34
Coefficient of variation		10%	6.75%	7.3%	4.29%	9.5%	9.27%	9.76%	8.5%
B. marinus (30°C) Hind-limbs	97.8 (9.8)	3.7 (3.5-4.0)	4.1 (3.8-4.4)			4.0 (3.7-4.3)	4.1 (3.8-4.4)		

* Insufficient data to calculate 95% confidence interval

Table 3.3. Tadpole wet weights, DO ranges and 12-h EC50 data for *Bufo marinus* tadpoles exposed to Teric GN8 (100% nonylphenol ethoxylate) in flow-through tests at 30°C and under low DO conditions. All EC50 values are based on initial measured concentrations.

		12-h EC50 (95% CI) mg/L		DO (mg/L)[a]	
Species/Test #	Avg. wet wt. mg (SD)	Mild Narcosis	Full Narcosis	Control	Teric GN8 (0.5-4.0 mg/L)
B. marinus (30°C, Normal DO)	20.8 (5.6)	3.6 (3.4-3.7)	4.1 (4.0-4.2)	>5.7	>5.7
B. marinus (30°C, Low DO #1)	25.5 (5.1)	1.8 (1.6-2.1)	2.2 (2.0-2.4)	2.3-2.7	1.7-2.3
B. marinus (30°C, Low DO #2)	34.1 (8.0)	-	1.8 (1.7-1.9)	1.3-2.4	1.2-1.7
B. marinus (30°C, Low DO #3)	43.0 (9.3)	-	1.4 (1.3-1.5)	0.7-1.7	0.8-1.3

[a] DO was measured at 2,6 and 12 hours, in at least one replicate chamber for each exposure concentration.

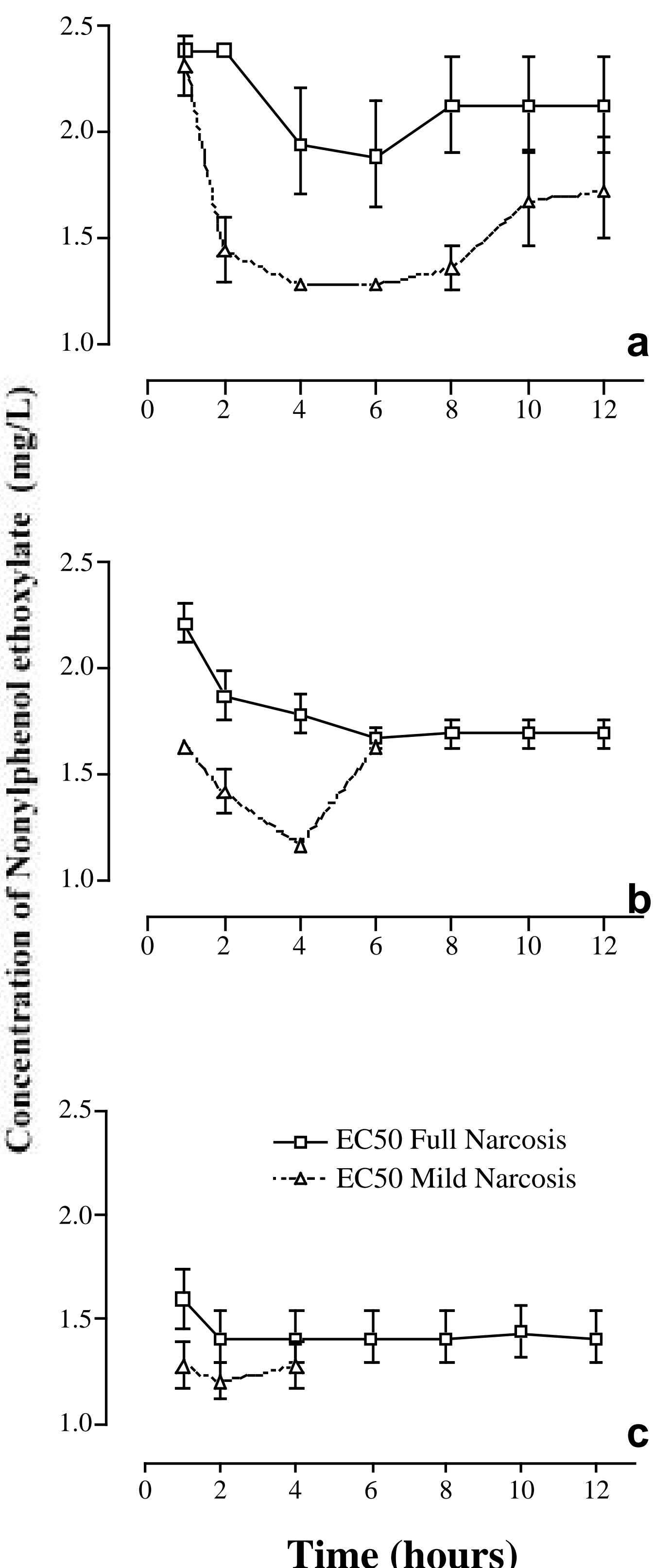

Fig. 3.3 EC50 values for *Bufo marinus* exposed to Teric GN8 (100% nonylphenol ethoxylate) in 12–h flow-through acute toxicity tests at 30°C and 1.7 to 2.3 mg/L DO (**a**), 1.2 to 1.7 mg/L DO (**b**), 0.8 to 1.3 mg/L DO (**c**). Error bars represent 95% confidence intervals. Absence of error bars indicates insufficient data for the calculation of a 95% confidence interval.

At 20°C, mild narcosis often occurred at less than half the concentration required to induce full narcosis. Mild narcosis can therefore be considered an imprecise indicator of those surfactant concentrations that will elicit an observable effect in an acute test. A swimming performance assay (Bridges, 1997) may have discerned an observable effect at an even lower concentration. However, the inherent steepness of surfactant toxicity curves (Dorn *et al.*, 1993; Wong *et al.*, 1997) suggests data obtained in performance tests would not be much lower than the EC50 values presented in this study.

Another notable observation in this study was the tendency for tadpoles to recover from narcosis. EC50 values for 48 and 72 h, in trials with *B. marinus* at 30°C were slightly higher than preceding intervals (Table 3.2). This phenomenon was also distinct over the 2-h intervals in the low DO trials (Fig. 3.3a). None of the observed recoveries were statistically significant since EC50 values varied less than the 95% confidence intervals of individual values. However, the observation remains noteworthy because tadpoles, which in a previous interval had been totally non-reactive to stimuli, were subsequently active or at least reactive to prodding. Recovery from the narcotic effects of nonylphenol has also been noted in fish following 36 h exposure and snails following 8 h exposure (Talmage, 1994).

There are two possible explanations for this recovery. Initiation of metabolism of NPE in the liver is likely (Granmo and Kollberg, 1976), however daily renewal of surfactant did not appear to result in even temporary recurrence of the narcotic state in those animals that had recovered. The alternative explanation lies in a theoretical mechanism for the narcotic action of organic chemicals at biological membranes (van Wezel and Opperhuizen, 1995). Biological membranes are in a constant state of flux between an ordered gel phase and a disordered liquid-crystalline phase. The transition between the two states occurs at the phase-transition temperature (T_{tr}). Poikilothermic organisms are able to change the composition of their membranes so as to adapt to varying environmental temperatures, but this adaptation takes time and rapid temperature changes will result in narcosis-like symptoms (Cossins *et al.*, 1977). Most

narcotic chemicals reduce the T_{tr} of biological membranes, and it is possible that a poikilothermic animals such as fish and tadpoles are able to slowly respond to this in the same way that they respond to changes in temperature by adjusting their lipid membrane composition. Furthermore, the observed recovery after only eight hours exposure is not surprising, since changes in membrane fluidity and phospholipid composition were observed in trout following six hours exposure to cold water (Williams and Hazel, 1994; 1995).

Only minor differences in sensitivity among species were evident. Comparing EC50 values, *X. laevis* was more sensitive than the other five species. In the absence of the *X. laevis* result, the ranking of toxicity was consistent with a size-sensitivity relationship as the smallest species, *C. insignifera* displayed the greatest sensitivity and the largest species, *H. eyrei*, the lowest. The reason the differences in sensitivty is not clear. The acute toxicity of narcotic chemicals is primarily a function of body burden, which is dependent on the compounds lipophilicity. Species differences might be due to species-specific bioaccumulation rates as a consequence of different rates of biotranformation and elimination (Arukwe *et al.*, 2000).

The higher sensitivity of *X. laevis* is, however, consistent with results obtained in previous studies. For example, larval stages of *X. laevis* were more sensitive to the herbicide, diuron, and the organophosphate insecticide, azinphosmethyl, than those of *Pseudacris regilla* (Schuytema *et al.*, 1995; Schuytema and Nebeker, 1998). *Xenopus laevis* was also more sensitive than *Rana pipiens* and the salamander *Ambystoma maculatum* to UV radiation and photoinduced degradation products of a polyaromatic hydrocarbon (Hatch and Burton, 1998), and in developmental toxicity tests and chronic survival tests, *X. laevis* was more sensitive than *Rana catesbeiana* or *R. pipiens* when exposed to the organochlorine, dieldrin (Schuytema *et al.*, 1991). However, the relative sensitivities of test organisms used in comparative studies are dependant upon the chemical to which test species are exposed (Slooff and Baerselman, 1983; Holcombe *et al.*, 1987). In other studies involving exposures to both organic and

inorganic chemicals, *X. laevis* was of similar sensitivity or less sensitive than other amphibian species (Rzehak *et al.*, 1977; Slooff and Baerselman, 1980; Schuytema *et al.*, 1991; Schuytema and Nebeker, 1998).

Additional comparative studies with a broader range of chemical types, species and endpoints will be useful to further assess how well *X. laevis* represents the various amphibian taxa. Should *X. laevis* be found to be consistently of greater or similar sensitivity to environmental contaminants at various developmental stages, compared to other amphibians, then it may provide a useful test species for the purpose of acute toxicity testing. Certainly, the ease with which these animals can be maintained and bred in captivity makes them an ideal surrogate test species if wild species are otherwise difficult to obtain or are highly seasonal in their availability.

In trials with *B. marinus* at 30°C, tadpoles with hind limbs displayed similar sensitivity as younger feeding-stage tadpoles. The lack of difference between the sensitivity of early stage and late stage *B. marinus* tadpoles is notable as it is contrary to studies with other organic contaminants, which indicate that late stage, premetamorphic tadpoles are more sensitive than early stage tadpoles (Wohlgemuth, 1977; Howe *et al.*, 1998).

The trials at 30°C with *B. marinus* were replicated six times. This was possible because *B. marinus* could be induced to mate in the laboratory and could produce between 8000 and 25 000 eggs, while the majority of Australian species will typically produce egg clutches ranging in size from ten to several hundreds of eggs (Tyler, 1994). The difficulty in obtaining large numbers of native frog eggs from field collection sites during short breeding seasons, precluded replication of data. This was seen as a flaw in the native species data, since EC50 data can be notoriously variable even when following standardised protocols (Warren-Hicks and Parkhurst, 1992). The availability of *B. marinus*, however, provided an opportunity to assess the reproducibility of an individual response.

There was a consistently low level of variation between EC50 (MN and FN) values generated for the six trials at 30°C. The highest level of variation was

10% for MN at 24 h. This would be considered low even for repeat test with reference toxicants (Environment Canada, 1990). Although performed at a higher temperature than the test performed as part of the comparative study, the low level of variation is expected to also apply to the test with *B. marinus* in the comparative study. It is not possible to attribute with any certainty, the same low level of variation to other species used in the comparative test, however, the EC50 data generated for the other five species are likely to be representative of species sensitivity in acute toxicity tests under the conditions described.

The precise mode of toxicity of nonionic surfactants remains unclear. The observed acute toxicity may be entirely a function of membrane narcosis (Schüürmann, 1990; van Wezel and Opperhuizen, 1995), with mortality resulting as a consequence of general loss of cellular function. Exposure to relatively high concentrations of surfactant will disrupt gill epithelial membranes (Abel, 1976; Mallatt, 1985), resulting in either asphyxiation or osmoregulatory failure. On the other hand, chronic exposures to sub-acute concentrations induce a pathological response to the toxicant. A histological examination of tadpole gill tissue following a 96-h exposure to 2 mg/L Agral®600 induced epithelial hyperplasia (Kirkpatrick *et al.*, 1999). Similar non-specific pathology is seen in fish gill tissue following exposure to anionic surfactants as well as other contaminants (Mallatt, 1985; Misra *et al.*, 1985; Hofer *et al.*, 1995), and is likely to be a generalised stress response. Gill epithelial hyperplasia increases the diffusion distance for oxygen and may result in respiratory stress. The slight increase in incidence of FN between 72 and 94 h (Table 3.2) may therefore reflect the detrimental effects of cumulative pathology as much as the nutritional status of the animals.

Narcosis appears to be an indirect factor involved in tadpole mortality under low DO conditions. Animals that were unable to swim to the surface to obtain oxygen in a low DO environment asphyxiated. The elimination of differences between EC50 (MN) and EC50 (FN) under low DO conditions (Fig. 3.3b,c) reflects the rapid mortality of tadpoles that were unable to obtain adequate oxygen. Interestingly, tadpoles were able to persist in a narcotised state at

approximately 2.0 mg/L (26% saturation) DO (Fig. 3.3a) for the 12-h duration of the test and indicates a high degree of tolerance to relatively low DO. The low DO condition had a more pronounced effect on toxicity of NPE than did increased temperature alone. This is consistent with results obtained by Hokanson and Smith (1971) in their examination of the toxicity of the anionic surfactant, linear alkylbenzene sulphonate (LAS) to *Lepomis macrochirus*.

High temperature—low DO conditions are characteristic of tropical aquatic environments. Extremely low DO, however, is usually associated with a high oxygen demand created by the presence of high levels of organic matter. This study has not attempted to replicate eutrophic conditions. It is likely that surfactants will be rapidly eliminated from eutrophic waters either by bio-degradation or sorption to organic substrates. Evaluation of the hazard presented by surfactant compounds to amphibians under such conditions may require the use of mesocosms that can accommodate this increased environmental complexity.

Conclusion

Acute toxicity tests with tadpoles of all six species produced EC50 (narcosis) values between 1.1 and 12.1 mg/L following exposure to nonylphenol ethoxylate and between 5.3 and 25.4 mg/L following exposure to alcohol alkoxylate. *Xenopus laevis* was the most sensitive species tested, however the difference was not greater than the variation amongst native species. Rearing and exposure of *Bufo marinus* tadpoles at higher temperatures had little effect on EC50. Rearing and exposure of *Bufo marinus* tadpoles at high temperatures and under conditions of low DO resulted in a two to threefold increase in toxicity. Toxicity testing under conditions that more closely resemble those of shallow, tropical, lentic ponds may provide a more realistic indication of surfactant toxicity to amphibians.

Chapter 4

Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs.

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Abstract

The FETAX protocol has recently been adopted as a valuable tool for evaluating the embryotoxicity of environmental contaminants in amphibians. The bioassay utilises *Xenopus laevis* as a test species, but there are few comparative studies to evaluate whether data collected in this species is applicable to other amphibians. In this study the embryotoxicity of the nonionic surfactant, nonylphenol ethoxylate was determined in *X. laevis* and the Australian frogs, *Litoria adelaidensis* and *Crinia insignifera* using the FETAX protocol. The 96-h LC50, EC50 and MCIG values for *X. laevis* were 3.9 to 5.4 mg/L, 2.8 to 4.6 mg/L and 1.0 to 3.0 mg/L respectively. The 140-h LC50, EC50 and MCIG values for *L. adelaidensis* were 9.2 mg/L, 8.8 mg/L and 5.1 to 6.0 mg/L respectively. The 134-h LC50, EC50 and MCIG values for *C. insignifera* were 6.4 mg/L, 4.5 mg/L and 4.0 mg/L respectively. Teratogenicity indices for the three species ranged between 1.0 and 1.6 indicating either no or low teratogenicity. Growth inhibition as assessed by embryo length was the most sensitive indicator of effect in all three species. *Xenopus laevis* was the more sensitive of the three species and the only species that displayed indisputable terata.

Key Words

Amphibian, FETAX, Nonionic surfactant, Nonylphenol ethoxylate, Developmental toxicant

Introduction

Because of their widespread industrial, domestic and agricultural use, synthetic surfactants are among the more ubiquitous contaminants in aquatic systems. Nonionic surfactants are routinely included as wetting agents and dispersants in pesticide formulations. Often, where the active constituents of a pesticide are of low toxicity, the additive surfactant components may pose the most significant risk to aquatic fauna, especially when they are applied around or over standing or ephemeral waters with a low capacity for dilution (Mann and Bidwell, 1999b).

Amphibians are often the main vertebrate group at risk of exposure to contaminants in ephemeral systems (Lahr, 1997). In Australia, approximately half of more than 200 species of frogs are reliant on temporary waters in order to complete their breeding cycle (Cogger, 1992). While a number of studies have assessed surfactant toxicity in a range of aquatic species (Abel, 1974; Lewis, 1991; Talmage, 1994), there is limited data pertaining to the effects of these chemicals on amphibians (Plotner and Gunther, 1987; Presutti *et al.*, 1994).

The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) is a highly reproducible assay for evaluating embryotoxic potential of environmental contaminants. The assay has been used in recent years to assess the embryotoxicity of various chemicals including the herbicides atrazine (Morgan *et al.*, 1996), diuron (Schuytema and Nebeker, 1998) and phenoxy-acid herbicides (Morgan *et al.*, 1996; Vismara and Garavaglia, 1997). Organophosphate and organochlorine insecticides have also been the focus of FETAX type assays (Schuytema *et al.*, 1991; 1994; Snawder and Chambers, 1993). Other studies have focused on surfactants (Presutti *et al.*, 1994), nitrogenous compounds (Schuytema and Nebeker, 1999), metals (Herkovits *et al.*, 1997; Sunderman, 1998) sediment extracts (Dawson *et al.*, 1988; Fort *et al.*, 1995; Burkhart *et al.*, 1998), surface waters (Dawson *et al.*, 1984; Bruner *et al.*, 1998; Burkhart *et al.*, 1998) and the photoinduced degradation products of the insect growth inhibitor, methoprene

(La Clair *et al.*, 1998) and the aromatic hydrocarbon, fluoranthene (Hatch and Burton, 1998).

As a bioassay that utilises an amphibian as a test species, FETAX is particularly useful for evaluating the importance of environmental contaminants in the phenomenon of global amphibian decline (Blaustein and Wake, 1995; Carey and Bryant, 1995). The use of *X. laevis* as a test organism, however, assumes that this species is representative of amphibians in general. As an assay for teratogenesis, the use of *X. laevis* is justified on the basis that the genetic blueprint that controls embryonic development has been highly conserved over evolutionary time (Bantle, 1995). However, relatively few studies have compared the sensitivity of *X. laevis* with other amphibian species. The US EPA have employed the FETAX protocol to examine the developmental toxicity of a herbicide (diuron) to Pacific tree frog (*Pseudacris regilla*) embryos, an organochlorine insecticide (dieldrin) to bullfrog (*Rana catesbeiana*) and leopard frog (*Rana pipiens*) embryos and fertiliser components (ammonium and nitrate) to *P. regilla* embryos (Schuytema *et al.*, 1991; Schuytema and Nebeker, 1998; 1999). In all these studies *X. laevis* embryos were also tested and were often (but not always) found to be more sensitive to the detrimental effects of the chemicals tested.

Much of the amphibian toxicological literature describes studies using representatives of the genera *Rana*, *Bufo* or *Xenopus* (Power *et al.*, 1989; Mann and Bidwell, 1999a). The Australian amphibian fauna are phylogenetically distinct from these northern hemisphere and African species, prompting concern that the available toxicity data may not predict the hazards posed to Australian taxa. As part of an ongoing evaluation of the toxicity of agricultural surfactants to Australian frogs (Mann and Bidwell, 1999b), the embryotoxicity of nonylphenol ethoxylate (NPE) to two Australian species, *Crinia insignifera* and *Litoria adelaidensis* was compared to that of *X. laevis* using a modified FETAX protocol.

Materials and Methods

In general, toxicity test procedures employed in this study follow those outlined in ASTM Standard E1439-91, Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-*Xenopus* (ASTM, 1993a). Specific details and departures from ASTM guidelines are detailed below.

Test Substances

Teric GN8 (100% NPE with an average oligomer length of eight ethoxylate units) was provided by Huntsman Corporation Australia Ltd. This surfactant is similar to surfactant species used in agricultural wetting agents (Dodd *et al.*, 1993). The teratogen, 6-Aminonicotinamide (6-ANA) (6-aminopyridine-3-carboxamide, Sigma®, USA) has become established as the standard reference toxicant for FETAX (ASTM, 1993a) and was used in this study to further compare the sensitivities of the native species with *X. laevis.*

Test organisms

The African clawed frog (*X. laevis*) is a commonly used frog that has been proposed as a model species for frog research (Cannatella and de Sá, 1993; Bantle, 1995). The native species were *C. insignifera* and *L. adelaidensis.* They serve as examples of the two major phylogenetic groups of frogs in Australia (Myobatrachidae and Hylidae) and are also representative of temperate frogs with varying habitat requirements (Cogger, 1992; Tyler *et al.*, 1994). The myobatrachid frog, *C. insignifera*, is a small (14-29 mm s-v, snout-vent length) ground dwelling frog that inhabits areas temporarily inundated by water. The hylid frog, *L. adelaidensis*, is a larger (34-47 mm s-v) frog found in either temporary or permanent waters where it inhabits dense emergent vegetation.

Male and female *X. laevis* adults were maintained in dechlorinated tap-water at 24°C in plastic tubs with metal-mesh covers. The adults were fed twice weekly with diced beef-liver. A powdered vitamin supplement (Reptivite™, Zoo Medsan, USA) was incorporated into the liver.

Mating in *X. laevis* was induced by intralymphatic administration of human chorionic gonadotropin (HCG) (Profasi®, Serono, Italy). Thirty six hours prior to the commencement of mating, both males and females were transferred to individual tubs half filled with FETAX solution (ASTM, 1993a). Males were injected with 150 international units (IU) of HCG using a 27-gauge hypodermic needle. Twelve hours prior to the commencement of mating, males and females were similarly administered 150 IU and 250 IU HCG, respectively. Just prior to mating, 500 IU HCG was administered to females. The adults were subsequently placed together in a single tub positioned in a quiet dark location. Eggs were deposited 6 to 12 hours after the commencement of mating.

Fertilised eggs of both *C. insignifera* and *L. adelaidensis* were harvested from the matings of adult animals collected in amplexus from a single location in the Perth metropolitan area. For *X. laevis* and *L. adelaidensis*, embryos derived from single clutches were used for each individual test. Because *C. insignifera* produce only 70-200 eggs per clutch, eggs from up to three clutches were required for each test using this species. These clutches were pooled and mixed prior to testing.

All FETAX assays were carried out using embryos with jelly coats removed. Jelly coats were removed by gentle swirling for 2-3 minutes in 2% cysteine (Sigma®) prepared in FETAX solution, and adjusted to pH 8.1 with NaOH. Normally cleaving embryos were sorted from infertile or abnormal embryos with the aid of a stereo microscope (Bantle *et al.*, 1991). Only mid-blastula (stage 8) to early gastrula (stage 11) embryos (Nieuwkoop and Faber, 1975) were selected for testing.

Test procedure

Prior to testing, a primary stock was prepared as a nominal concentration of 1000 mg/L NPE (Teric GN8). The diluent used for the stock solutions was FETAX solution (ASTM, 1993a). Test concentrations were made up just prior to the beginning of the tests using FETAX solution.

Embryos were impartially allocated to 80 mm acid-washed glass petri dishes (25 embryos per petri dish) with large bore plastic or glass pipettes. Embryos were exposed to 10 ml of NPE solutions ranging from 0.5 to 10.0 mg/L and the reference toxicant 6-aminonicotinamide (6-ANA) solutions ranging from 5.5 to 4000 mg/L in either duplicate or triplicate petri dishes (Table 4.1) in static-renewal tests at 24±1°C with a 12 h light, 12 h dark photoperiod ($L_{12}D_{12}$) in a climate control cabinet. Duplicate, triplicate or quadruplicate petri dishes with embryos in FETAX solution and no toxicant were included for each test as controls (Table 4.1). FETAX tests with *X. laevis* were run for 96 h or until control embryos developed to stage 46 (Nieuwkoop and Faber, 1975). Tests with *C. insignifera* and *L. adelaidensis* were run until control embryos had developed to a stage of development analogous to stage 46 *Xenopus* (i.e. Gosner (1960) stage 24).

At 12-h intervals, dead animals were removed. Temperature, DO and pH measurements were taken at the beginning of the test, and at 24 h intervals. For trials with *X. laevis*, temperature, DO, and pH were all measured with a TPS 90FL multimeter. For trials with native species, temperature and DO were measured using a WTW OXI 320 oxygen meter and pH was measured with a HANNA 8417 pH meter.

At the end of the assay, all surviving embryos were fixed in 3% formalin and assessed for abnormalities. Images of fixed embryos were digitally recorded with a Sony Hyper HAD colour video camera mounted on an Olympus SZH10 stereo microscope. The video images were processed with NIH Image (National Institutes of Health). The lengths of those embryos not displaying abnormalities were measured with the ImageTool (University of Texas Health Science Centre) image analysis package.

Table 4.1 Exposure concentration of Teric GN8 (nonylphenol ethoxylate) and 6- aminonicotinamide

	Replicates Exposed (Control)	Nonylphenol ethoxylate (Teric GN8) concentration series (mg/L)	6-aminonicotinamide concentration series (mg/L)
Xenopus laevis			
trial 1	2 (2)	0.1, 0.5, 1.0, 5.0, 10.0	
trial 2	3 (3)	1.0, 2.0, 4.0, 8.0	
trial 3	2 (2)	1.25, 2.0, 3.2, 5.12, 8.19	5.5, 2500
trial 4	2 (3)	2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0	5.5, 2500
Litoria adelaidensis			
trial 1	2 (2)	1.25, 2.0, 3.2, 5.12, 8.19	
trial 2	2 (4)	1.25, 2.0, 3.2, 5.12, 8.19	
trial 3	2 (3)	5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0	
trial 4	2 (4)	6.0, 6.4, 6.8, 7.2, 7.6, 8.0, 8.4, 8.8, 9.2, 9.6	25, 50, 75, 100, 125, 2000, 2500, 3000, 3500, 4000
Crinia insignifera			
trial 1	2 (2)	2.0, 4.0, 5.0, 6.0, 7.0, 8.0	

Analytical Chemistry

Stock solutions were analysed for NPE as described by (Mann and Boddy, 2000). This was done to avoid inaccuracies in the nominal concentrations reported in this study. Briefly, NPE was extracted from the aqueous solution by solid phase extraction (Scullion *et al.*, 1996) and quantified against a standard curve (generated with six concentrations of Teric GN8) by high performance liquid chromatography employing normal phase isocratic separation of NPE oligomers (Scarlett *et al.*, 1994). The low test volumes used in these tests, and the frequent renewals of test solution precluded analysis of individual test solutions. Nominal concentrations have therefore been used to calculate all endpoint data. Surfactant loss as a consequence of degradation or surface adsorption is always less than 10% over 24 h (Chapter 3).

Data Analysis

Mortality and abnormality data were used to generate LC50 and EC50 (malformation) values by the Spearman-Karber method (Hamilton *et al.*, 1977). Teratogenicity Indices (TI) were derived from LC50 and EC50 values (ASTM, 1993a). Embryo length data were used to obtain a minimum concentration to inhibit growth (MCIG). Homogeneity between replicate embryo length data was tested with the Levene test (Levene, 1960). Those replicates that displayed homogeneity of variance were pooled. Pooled embryo length data was tested for normality with the Shapiro-Wilk test (Zar, 1996), and then tested in Student t-tests (Zar, 1996) for unpaired data to determine if there were significant (= 0.05) differences between the lengths of control embryos and treated embryos.

Results

Water temperature, pH and DO were consistent across all tests. Recorded temperatures ranged between 23.6°C and 24.1°C. The pH of test solutions ranged between 7.0 and 7.6. In most cases the DO remained above 80% saturation.

Dissolved oxygen occasionally dropped below 80% (but never below 60%) if the presence of dead tadpoles resulted in a high oxygen demand.

The LC50, EC50, TI, and MCIG values generated in this study are presented in Table 4.2. The LC50, EC50 and MCIG for all trials with the three species exposed to NPE (Teric GN8) are all within a single order of magnitude. The trends in embryo length following exposure to NPE are presented in Fig. 4.1.

The LC50 values for *X. laevis* exposed to NPE ranged between 3.9 and 5.4 mg/L. The EC50 (malformation) values for *X. laevis* exposed to NPE ranged between 2.8 and 4.6 mg/L producing TI values ranging between 1.0 and 1.6. Malformations included cardiac oedema, microphthalmia and improper gut coiling (Fig. 4.2a). These malformations always occurred simultaneously in each individual. Intermediate gradations of malformation were not observed. In the higher concentrations (6.0-10.0 mg/L), mortality consistently occurred when the embryos attained stages 39 to 40 (Nieuwkoop and Faber, 1975). Trials 3 and 4 included exposures to the reference toxicant 6-ANA at EC50 and LC50 concentrations. In trial 3 and 4 exposure to 5.5 mg/L 6-ANA induced malformations in 29.7% and 63% of embryos respectively while exposure to 2500 mg/L 6-ANA induced mortality in 44% and 52% of embryos respectively.

The LC50 and EC50 values for *L. adelaidensis* exposed to NPE (trial 3 and 4) were 9.2 mg/L and 8.8 mg/L respectively, producing a TI value of 1.0. The LC50 and EC50 values generated following exposure to 6-ANA were 3742 and 37.6 mg/L respectively producing a TI value of 99.6. The LC50 and EC50 values for *C. insignifera* exposed to NPE were 6.4 mg/L and 4.5 mg/L respectively, producing a TI value of 1.4. Observed malformations that contributed to the EC50 were generalised oedema, failure of the tail to straighten and severe stunting. Malformations were not as prevalent in higher concentrations, as they were in *X. laevis*.

Exposure to NPE resulted in a dose-dependent inhibition in growth in the three species. In *X. laevis*, this inhibition in growth was evident up to 4.5 mg/L NPE (Fig. 4.1). At this point developmental malformations became predominant.

In *L. adelaidensis* and *C. insignifera* growth retardation became more pronounced with increased NPE concentrations (Fig. 4.1, Fig. 4.2b,c).

For *X. laevis*, the lowest NPE concentration that significantly (t_{82}= 3.397; $p = 0.011$) inhibited growth (MCIG) was 1.0 mg/L in trial 2. In this trial, 1.0 mg/L was the lowest dose in the NPE concentration series. In trials 3 and 4 the MCIG values were 2 mg/L (t_{56}= 3.721; $p = 0.0005$) and 3 mg/L (t_{81}= 5.092; $p < 0.0001$) respectively with lower NPE concentrations having no significant ($p < 0.05$) effect on embryo length.

The MCIG values for trials 1 to 3 with *L. adelaidensis* exposed to NPE ranged between 2.0 and 6.0 mg/L. In trial 1, the lengths of embryos exposed to 2.0 mg/L NPE were significantly (t_{96}= 4.585 $p < 0.0001$) lower than control animals. However, in this same trial, there were no significant ($p < 0.05$) differences between the lengths of embryos exposed to 2.0, 3.2 and 5.12 mg/L NPE (Fig. 4.1). Furthermore, embryos exposed to NPE between 1.25 and 3.2 mg/L showed no retardation in gut development, which was evident in those, exposed to 5.12mg/L. There was a significant (t_{96}= 9.096; $p < 0.0001$) difference in embryo length between those animals exposed to 5.12 and 8.19 mg/L NPE. In trials 2 and 3, MCIG values were 5.12 mg/L (t_{141}= 10.239; p<0.0001) and 6.0 mg/L (t_{95}= 3.890, $p = 0.002$) NPE respectively. These same animals also displayed obvious retardation in gut development. The MCIG value for the single trial with *C. insignifera* exposed to NPE was 4.0 mg/L (t_{78}= 3.668; $p = 0.0004$).

Table 4.2 LC50, EC50 (malformation), TI and MCIG data for nonylphenol ethoxylate (Teric GN8) and 6-aminonicotinamide (6-ANA) for *Xenopus laevis*, *Litoria adelaidensis*, and *Crinia insignifera.*

Species and trial #	Exposure time (h)	LC50 (95% CI) mg/L	EC50 (95% CI) mg/L	TI (LC50/EC50)	MCIG (mg/L)
Xenopus laevis					
trial 1	96	4.8 (4.1-5.7)	*	*	*
trial 2	96	5.4 (5.2-5.7)	3.3 (2.7-4.2)	1.6	1.0
trial 3	96	3.9 (3.6-4.1)	2.8 (2.4-3.3)	1.4	2.0
trial 4	96	4.6 (4.5-4.7)	4.6 (4.5-4.7)	1.0	3.0
Litoria adelaidensis					
trial 1	140	*	*	*	2.0[a]
trial 2	140	*	*	*	5.1
trial 3	140	9.2 (9.1-9.4)	8.8 (8.7-8.9)	1.0	6.0
trial 4	140	9.2 (9.17-9.3)	8.8 (8.7-8.9)	1.0	*
trial 4 (6-ANA)	140	3742 (3534-3962)	37.6 (35.5-39.7)	99.6	*
Crinia insignifera					
trial 1	134	6.4 (6.1-6.6)	4.5 (4.2-4.9)	1.4	4.0

[a] This is likely to be an underestimate since there were no other indications of growth inhibition between 2.0 and 5.12 mg/L

* Insufficient data to calculate endpoint

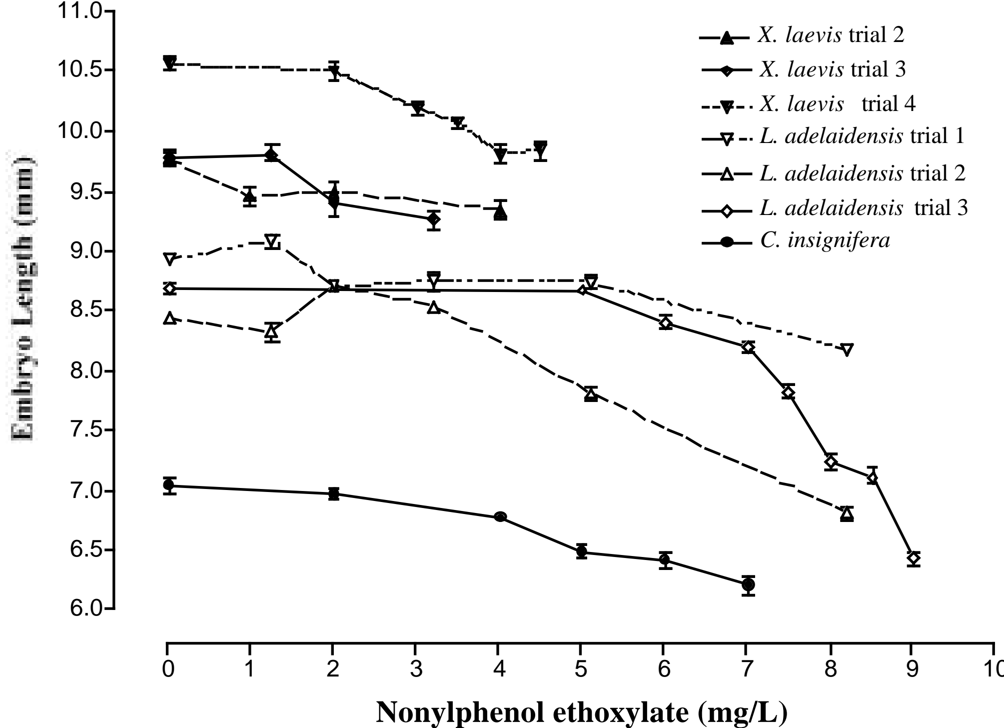

Fig. 4.1 Mean embryo lengths as an indicator of growth inhibition following exposure to nonylphenol ethoxylate (Teric GN8). Error bars represent standard errors.

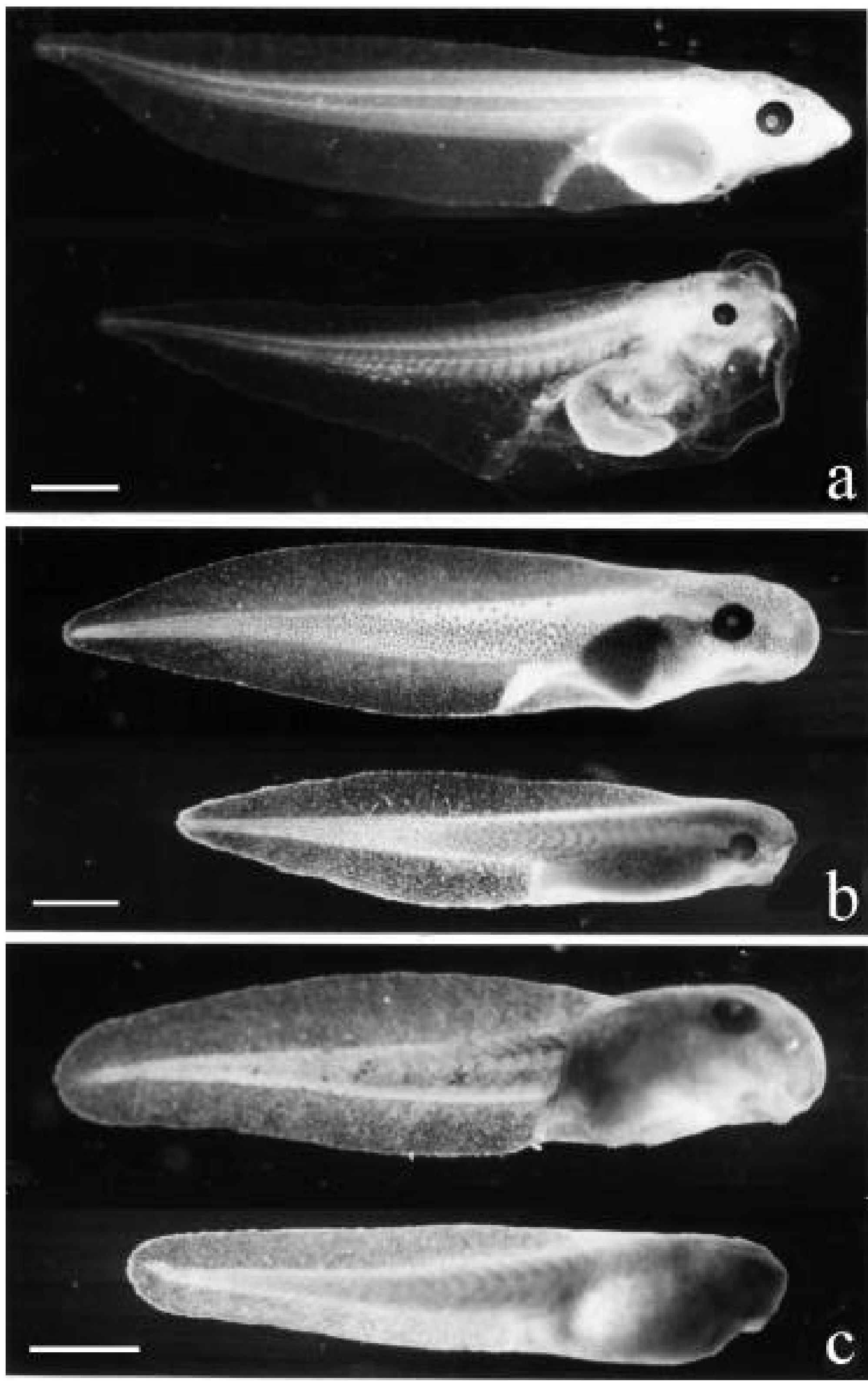

Fig. 4.2 Effects of exposure to nonylphenol ethoxylate (NPE) on the embryos of three species of anuran. The upper and lower animals in each couplet represent control and treated animals respectively. **a** *Xenopus laevis* after 96 h exposure to 4.0 mg/L NPE displaying cardiac oedema, microphthalmia and improper gut coiling. **b** *Litoria adelaidensis* after 140 h exposure to 8.0 mg/L NPE. **c** *Crinia insignifera* after 134 h exposure to 7.0 mg/L NPE. Bar represents 1 mm.

Discussion

In the current study the LC50, EC50 (malformation) and MCIG values for *X. laevis* exposed to NPE (Teric GN8), ranged between 1.0 and 5.4 mg/L. The same endpoints for *C. insignifera* ranged between 4.0 and 6.4 mg/L. In a previous examination of the acute toxicity of NPE (Teric GN8), Mann and Bidwell (in press; Chapter 3) observed narcotic effects in feeding stage *X. laevis* tadpoles (48-h EC50, 1.1 mg/L) and feeding stage *C. insignifera* tadpoles (48-h EC50, 2.7 mg/L). Narcosis was also observed in embryos during this study, but was not recorded as an endpoint. The effect concentrations generated in this study, however, do not differ greatly from those generated in the tadpole study.

This apparent conformity of sensitivity amongst different stages of development is notable. Embryonic developmental stages of both invertebrates and fish are more sensitive to nonylphenol ethoxylates than later stages (Marchetti, 1965; Swedmark *et al.*, 1971). Marchetti (1965) described the sensitivity of rainbow trout (*S. gairdnerii*) alevin and fry at successive developmental stages to a nonylphenol ethoxylate. Newly hatched alevin with egg yolks were the most tolerant (6-h LC50, 42 mg/L), while the transition stage between alevin and fry, when yolk absorption was nearly complete, but prior to the commencement of feeding, was 20 times more sensitive (6-h LC50, 2.1 mg/L).

A stage-dependent response to xenobiotics has also been reported in amphibians, although a consistent trend is not apparent. For example, mid-blastula and swimming *X. laevis* larvae were more sensitive to aromatic amines than tailbud embryos (Davis *et al.*, 1981). Conversely, however, the sensitivity of *X. laevis* to the toxic effects of cadmium was found to increase from the two blastomere stage up until stage 40, after which sensitivity decreased again (Herkovits *et al.*, 1997). In another study the gastrula stage of *Microhyla ornata* was less sensitive to mercuric chloride than tadpole stages (Ghate and Mulherkar, 1980), while Dial (1976) found the gastrula stage of *R. pipiens* to be particularly sensitive to methylmercury exposure. Schuytema *et al.* (1991), in a study comparing the sensitivity of tadpoles and embryos of *X. laevis* to dieldrin,

generated 96-h LC50 values of 40.4 to 49.5 µg/L for tadpoles and also reported gross deformities in animals exposed as embryos to concentrations as low as 1.3 µg/L.

There was, however, some indication of developmental variation in tolerance to NPE in the present study. In *X. laevis* embryos exposed to concentrations above the LC50, mortality consistently occurred at stage 39-40 indicating that earlier developmental stages were relatively tolerant to this compound. The increased sensitivity of *X. laevis* embryos at stages 39-40 coincides with rupture of the oral plate leaving the mouth open (Nieuwkoop and Faber, 1975). It is possible that the increased exposure of internal structures leads to damage that results in mortality. Similarly, in the two native species, mortality occurred quite late in the trials, but did not coincide so consistently with a specific developmental stage.

The MCIG provided an indicator of the lowest observable effect concentration (LOEC). Growth inhibition was more pronounced in *L. adelaidensis* and *C. insignifera* than it was in *X. laevis* (Fig. 4.1) but this is primarily because no *X. laevis* embryos survived beyond 72 h at concentrations above 5.12 mg/L. The mechanism underlying growth inhibition is unknown. Generalised narcosis is a recognised effect of exposure to nonionic surfactants (Schüürmann, 1990) which is likely to retard cellular processes, and thereby result in the observed growth inhibition.

Nonylphenol ethoxylate could not be considered to be teratogenic on the basis of the results presented here. Teratogenicity indices for the three species ranged between 1.0 and 1.6. Teratogenicity indices less than 1.5 indicate little or no teratogenic hazard (Bantle, 1995). These results are consistent with a similar study that examined the developmental toxicity of an alcohol ethoxylate based nonionic surfactant (Triton®DF-16) using a modified FETAX protocol (Presutti *et al.*, 1994). Presutti et al. (1994) reported a TI value of 1.98. A compound is also considered to be teratogenic if the MCIG is less than 30% of the 96-h LC50 (Bantle, 1995). The MCIG for *X. laevis*, trial 2 was less than 30% of the LC50

for the same trial, however this was not the case for trials 3 and 4 or for any of the trials with native species.

For the native species, the observed malformations included generalised oedema, failure of the tail to straighten, and severe stunting. However, the oedemas observed in *C. insignifera* and *L. adelaidensis* embryos were not the pronounced cardiac protrusions observed in *X. laevis* and were not accompanied by microphthalmia and improper gut coiling (Fig. 4.2a). Also, failure of the tail to straighten and the severe stunting may be explained as developmental retardation. These distinctions however may not be particularly important, since the generated TIs for all three species were low.

In this study the LC50 (3742 mg/L) and EC50 (37.6 mg/L) values generated by exposing *L. adelaidensis* to 6-ANA were very different from the LC50 (2500 mg/L) and EC50 (5.5 mg/L) values expected following exposure of *X. laevis* to 6-ANA (ASTM, 1993a). Furthermore, the derived TI (99.6) was 4.5 times lower than the expected TI (454) for *X. laevis*. These results suggest that *L. adelaidensis* is less sensitive to 6-ANA, and less prone to its teratogenic effects.

The lower sensitivity of *L. adelaidensis* is also reflected in the endpoints generated following exposure to nonylphenol ethoxylate. The LC50, EC50 and MCIG values for *L. adelaidensis* are approximately twofold higher than those generated for *X. laevis*. The disproportionately high sensitivity of *X. laevis* is however consistent with the findings of other comparative studies. In acute tests with newly hatched tadpoles and embryos, *X. laevis* was found to be more sensitive than *R. pipiens* and the salamander, *Ambystoma maculatum* when exposed to either UV light or the photoinduced degradation products of a polyaromatic hydrocarbon (Hatch and Burton, 1998). Larval stages of *X. laevis* were also more sensitive to the organophosphate insecticide, azinphosmethyl, than those of *P. regilla* (Schuytema *et al.*, 1995). In embryo-larval tests *X. laevis* was more sensitive than *R. catesbeiana* or *R. pipiens* when exposed to the organochlorine, dieldrin (Schuytema *et al.*, 1991). Notwithstanding these

instances, care must be exercised in imputing *X. laevis* with a heightened level of sensitivity to xenobiotics since a few studies have also found *X. laevis* to be less sensitive than other amphibians (Rzehak *et al.*, 1977; Slooff and Baerselman, 1980; Schuytema *et al.*, 1991; Schuytema and Nebeker, 1998). The relative sensitivities of test organisms used in comparative studies are also dependent upon the chemical to which test species are exposed (Slooff and Baerselman, 1983; Holcombe *et al.*, 1987; Deyoung *et al.*, 1996).

Xenopus laevis has a number of advantages over native species for toxicity testing. Its developmental biology is well understood, it is easily reared and maintained in captivity and can be induced to breed as required. For these reasons *X. laevis* is well placed to serve as a surrogate test species in toxicity testing. However, additional comparative studies with a broader range of chemical types, species and endpoints will be useful to further assess how well *X. laevis* represents the various amphibian taxa. In general, the comparative studies cited above report mortality, malformation, or developmental endpoints similar to those presented in this study. While acute studies such as these will continue to provide useful comparative data, the inclusion of other endpoints may highlight species differences. Zaga *et al.* (1998) exposed embryos and tadpoles of *X. laevis* and *Hyla versicolor* to carbaryl and reported LC50 values that indicated *X. laevis* was slightly more sensitive than *H. versicolor*. In contrast, a behavioural assay indicated that *H. versicolor* tadpoles were more sensitive to carbaryl exposure than *X. laevis* (Zaga *et al.*, 1998).

Conclusion

The embryological development of *X. laevis*, *C. insignifera* and *L. adelaidensis* is acutely affected by the nonionic surfactant, NPE within a narrow range of concentrations. Concentrations between 1.0 and 10.0 mg/L will inhibit embryo growth, cause developmental malformations or cause mortality in the three species. *Xenopus laevis* is more sensitive to NPE and more likely to exhibit indisputable terata than the two native species.

Chapter 5

Embryonic exposure to nonylphenol ethoxylate delays metamorphosis and alters sex ratios in the Australian frog, *Crinia insignifera*

Abstract

Nonionic surfactants are one of the many types of chemicals to which amphibians may be exposed in an agricultural landscape. However, little is known about the effects of surfactant exposure in amphibians. This study has examined the enduring effects of limited embryonic exposure to a nonylphenol ethoxylate (NPE). *Crinia insignifera* embryos were exposed during early embryogenesis to sublethal concentrations of NPE. Exposure to NPE did not affect either weight nor size (snout-vent length) at metamorphosis. Exposure to 5.0 mg/L NPE resulted in a significant delay in the time required to reach metamorphosis. Also, exposure to 3.0 mg/L NPE for the first 144 h of embryonic development or exposure to 5.0 mg/L NPE from 48 h to 144 h, resulted in a statistically significant predominance in the female phenotype amongst metamorphosing froglets. Exposure for the first five days to 1.5 mg/L or 3.0 mg/L NPE had no effect on sex ratio. The results indicated that exposure to NPEs has endocrine disruptive effects in this species and that a narrow window of susceptibility exists for the induction of predominantly female phenotype.

Keywords

Endocrine disruptors, Nonionic surfactant, Toxicity, Sublethal, Amphibian

Introduction

Nonionic surfactants are routinely included as wetting agents, emulsifiers or adjuvants in pesticide formulations. Often, where the active constituents of a pesticide are of low toxicity, the surfactant additives may pose the most significant risk to aquatic fauna, especially when they are applied around or over standing or ephemeral waters with a low capacity for dilution (Mann and Bidwell, 1999b). Amphibians are often the main vertebrate group at risk of exposure to contaminants in ephemeral systems (Lahr, 1997).

While a number of studies have assessed the toxicity of nonionic surfactants in a range of aquatic species (Abel, 1974; Lewis, 1991; Talmage, 1994), there is limited data pertaining to sublethal effects on amphibians (Presutti *et al.*, 1994; Mann and Bidwell, 2000). One class of nonionic surfactants that has raised particular concern are the alkylphenol ethoxylates, primarily because their degradation proceeds by stepwise shortening of the hydrophilic chain to short chain ethoxylates and alkylphenol derivatives that exhibit high toxicity (Yoshimura, 1986; Talmage, 1994) and oestrogenic properties (White *et al.*, 1994; Jobling *et al.*, 1996; Nimrod and Benson, 1996; Renner, 1997; Gray *et al.*, 1999).

Short term exposure to sublethal concentrations of nonylphenol ethoxylates (NPE) has induced narcosis in feeding stage amphibian tadpoles (Mann and Bidwell, in press; Chapter 3) and growth inhibition in embryos (Mann and Bidwell, 2000; Chapter 4). However, the enduring effects of short-term exposure have yet to be assessed in an amphibian. As part of an ongoing evaluation of the effects of agricultural surfactants in Australian frogs (Mann and Bidwell, 1999b; 2000; in press) this study assessed the survival, growth and sexual differentiation at metamorphosis of the Australian froglet *Crinia insignifera* following embryonic exposure to a NPE.

Materials and Methods

Test Substance

Teric GN8 (100% NPE with an average oligomer length of eight ethoxylate units) was provided by Huntsman Corporation of Australia Ltd. This surfactant is similar to surfactant types incorporated into agricultural wetting agents (Dodd *et al.*, 1993).

Test Organism

The myobatrachid frog, *Crinia insignifera*, is a small (14-29 mm, snout-vent length) ground dwelling frog which inhabits areas temporarily inundated by water on the Swan Coastal Plain surrounding Perth (24°59 S, 128°59 E), Western Australia (Cogger, 1992). Fertilised eggs of *C. insignifera* were harvested from matings of adult pairs collected in amplexus from a single location in the Perth metropolitan area in Western Australia. Eggs from three combined clutches were used for each of the exposures in this study.

Test Procedure

Exposure of embryos was carried out according to procedures outlined in ASTM Standard E1439-91, Standard guide for conducting the frog embryo teratogenesis assay-*Xenopus* (ASTM, 1993a) and with approval from the Curtin University Animal Ethics Committee. All exposures were carried out using eggs with jelly coats removed. Jelly coats were removed by gentle swirling for 2-3 minutes in 2% cysteine (Sigma, USA) prepared in FETAX solution (ASTM, 1993a), and adjusted to pH 8.1 with NaOH. Normally cleaving embryos were sorted from infertile or abnormal embryos with the aid of a stereo microscope. Mid-blastula (stage 8/9) embryos (Gosner, 1960) were selected for exposure. Embryos were impartially assigned to 60 mm acid-washed glass petri dishes with large-bore glass pipettes. Twenty five embryos were allocated to each petri dish. Embryos were exposed to 10 ml of NPE solutions ranging in concentration from 1.5 to 5.0 mg/L in either duplicate or triplicate petri dishes (Table 5.1, 5.2), in

static-renewal tests at 24±1°C with a 12 h light, 12 h dark photoperiod in a climate control cabinet. Solutions were renewed daily. The highest exposure concentration (5.0 mg/L) was selected as a concentration close to the LC50 for *C. insignifera* exposed to Teric GN8 (Mann and Bidwell, 2000; Chapter 4). Because a high degree of mortality was expected amongst animals exposed to 5.0 mg/L NPE for the full 144 h, a second treatment group was exposed for a shorter duration at a concentration of 5.0 mg/L NPE (Table 5.1). In trial two, embryos were exposed to 1.5 mg/L and 3.0 mg/L for 130 h (Table 5.2). Samples (200 mL) of stock solutions of Teric GN8 were preserved by the addition of 1% formaldehyde (Szymanski *et al.*, 1995) and analysed for nonylphenol ethoxylate as described by Mann and Boddy (2000) (Chapter 6). This was done to ensure that nominal exposure concentrations were as accurate as possible. The low test-volumes used in these tests, and the frequent renewals of test solution precluded analysis of individual test solutions. Nominal concentrations are therefore presented (Table 5.1 & 5.2). Previous studies (Mann and Bidwell, in press; Chapter 3) indicate that surfactant loss, as a consequence of degradation or surface adsorption, is always less than 10% over 24 h. Temperature and pH were monitored daily.

The stage of development attained by the embryos at the end of the exposure period was noted (this was a qualitative assessment because not all embryos were examined). At the end of the exposure period, surviving embryos were washed in fresh FETAX solution and transferred to a 120 L aquarium filled with FETAX solution and maintained at 24±1°C. Animals from individual treatment replicates were segregated within 2500 cm^3 nylon-mesh nursery-nets suspended at the top of the aquarium. Water quality was maintained by use of undergravel filters. Ammonium, nitrite, pH, temperature and dissolved oxygen (DO) were monitored daily. Tadpoles were fed dried fish food (Sera granumix, Germany) daily and grown to metamorphosis.

In the first trial, growth was terminated at tail-stub stage (Gosner stage 45). Several developmental parameters were measured, including wet weight,

snout-vent length and time to metamorphosis. Sex was determined by microscopic examination of gonads. In the second trial, growth was terminated as fore-limbs broke through (Gosner stage 42) and time to metamorphosis, and sex were recorded. Prior to dissections, all metamorphs were euthenased by immersion in MS222 (Sigma®, USA) or halothane inhalation (Fluothane™, ICI Pharmaceuticals).

Statistical Analysis

Data analyses were performed using the JMP® statistical package. The Pearson ^{2} test was used to determine homogeneity of replicate data, so that numerical results could be pooled. Comparison of mean time to metamorphosis between control animals and those exposed to 5.0 mg/L were analysed using Student t-test (= 0.05). Animal weight and length at metamorphosis, using concentration as the explanatory variable were analysed using one-way analysis of variance (ANOVA) with the Dunnett test to detect significant differences between controls and exposed animals (= 0.05). A logistic regression model utilized sex (male; female) as the response variable and exposure as the explanatory variable to determine if a relationship existed between the sex at metamorphosis and exposure to NPE. Statistical significance was determined at = 0.05.

Results

Temperature during exposure, ranged between 24.0°C and 24.9°C. The pH ranged between 7.3 and 7.6. Dissolved oxygen was not monitored. During the grow-out phase, ammonium concentrations in rearing aquariums did not rise above 75 µg/L and nitrite was not detected. The pH during rearing ranged between 7.1 and 7.4. Temperature ranged between 22.8°C and 24.1°C and DO ranged between 6.7 and 7.8 mg/L

In trial one, all embryos survived exposure but differed in their stage of development at the end of the exposure period. Control embryos were at stage 24 while those embryos exposed to 5.0 mg/L for 144 h were at Gosner stage 22. All

other embryos were at Gosner stage 23 at the end of the exposure period (Table 5.1). With the exception of those animals exposed to 5.0 mg/L for the full 144 h, survivorship at metamorphosis was similar to that of controls (Table 5.1). Of those animals exposed to 5.0 mg/L for the full 144 h, only 58.0% survived to metamorphosis (Table 5.1). All but one of these animals died within 1 day after exposure.

The first metamorphs (Gosner stage 45) emerged after 27 days development (Fig. 5.1a). Animals continued to emerge up until day 72. Exposure to 5.0 mg/L nonylphenol ethoxylate for either 144 or 96 h resulted in a significant delay in metamorphosis ($t_{101(1)} = -2.845$; $p = 0.0054$). Exposure to 3.0 mg/L for 144 h resulted in an initial delay in metamorphosis time compared to controls. After approximately 37 days into the grow-out phase, however, there was no longer any difference in the proportion of animals that had reached metamorphosis (Fig. 5.1a).

Exposure to NPE irrespective of concentration had no detectable effect on animal weights ($F_{3,174}= 0.9607$; $p = 0.4126$) or lengths ($F_{3,174}= 0.2380$; $p = 0.8698$) at metamorphosis. At metamorphosis, 41.5% of control animals that survived to metamorphosis were female. Of those animals exposed to 3.0 mg/L for 144 h, 69.0% were females. Of those animals exposed to 5.0 mg/L for 144 h, 46.4% were females. Exposure to 5.0 mg/L from 48 to 144 h resulted in 61% females amongst surviving animals. Animals exposed to either 3 mg/L NPE for 144 h or 5 mg/L NPE from 48 h to 144 h were 2.6 times more likely to be phenotypically female than control animals (Logistic regression, $^{2}{}_{1}= 6.3516$; $p = 0.0117$). Only those animals that had reached at least stage 23 exhibited an alteration in sex ratio.

In trial two, all embryos survived the exposure period. Embryos also differed in their stage of development at the end of the exposure period. Control embryos were at stage 24 while those embryos exposed to 3.0 mg/L for 130 h were at Gosner stage 21/22 and those exposed to 1.5 mg/L for 130 h were at Gosner stage 23 (Table 5.2). Survivorship at metamorphosis was lower than trial

one with 72.0% of controls, 74.7% of animals exposed to 1.5 mg/L and 69.3% of animals exposed to 3.0 mg/L surviving to metamorphosis (Table 5.2).

The first metamorphs (Gosner stage 42) emerged on day 27 and continued to emerge up until day 68 (Fig. 5.1b). Control animals were the first to emerge. There was no observable difference (not tested statistically) in time to metamorphosis between control animals and those exposed to either 1.5 or 3.0 mg/L for 130 h (Fig. 5.1b).

In trial two, the percentage of females in controls, 1.5 mg/L treatments and 3.0 mg/L treatments were 53.7%, 48.2% and 51.9% respectively. No sex ratio bias was evident.

Table 5.1. Endpoint data at metamorphosis for *Crinia insignifera* exposed in trial one to nonylphenol ethoxylate (NPE) during embryonic development

Treatment mg/L NPE	Exposure interval (h)	Exposure interval (Gosner stage)	% Survival at metamorph.	Sex ratio males:females
Control (rep. A & B pooled)	0 144	8/9 24	82.0	24:17 (1:0.7)
3.0 (rep. A & B pooled)	0 144	8/9 23	84.0	13:29 (1:2.2)
5.0 (rep. A & B pooled)	0 144	8/9 22	58.0	15:13 (1:0.7)
$5.0_{48\text{-}144}$ (rep. A & B pooled)	48 144	19 23	88.0	17:27 (1:1.5)

Table 5.2. Endpoint data at metamorphosis for *Crinia insignifera* exposed in trial two to nonylphenol ethoxylate (NPE) during embryonic development

Treatment mg/L NPE	Exposure interval (h)	Exposure interval (Gosner stage)	% Survival at metamorph.	Sex ratio males:females
Cont. (rep. A, B & C pooled)	0 130	8/9 24	72.0	25:29 (1:1.6)
1.5 (rep. A, B & C pooled)	0 130	8/9 23	74.7	29:27 (1:0.9)
3.0 (rep. A, B & C pooled)	0 130	8/9 21/22	69.3	25:27 (1:1.1)

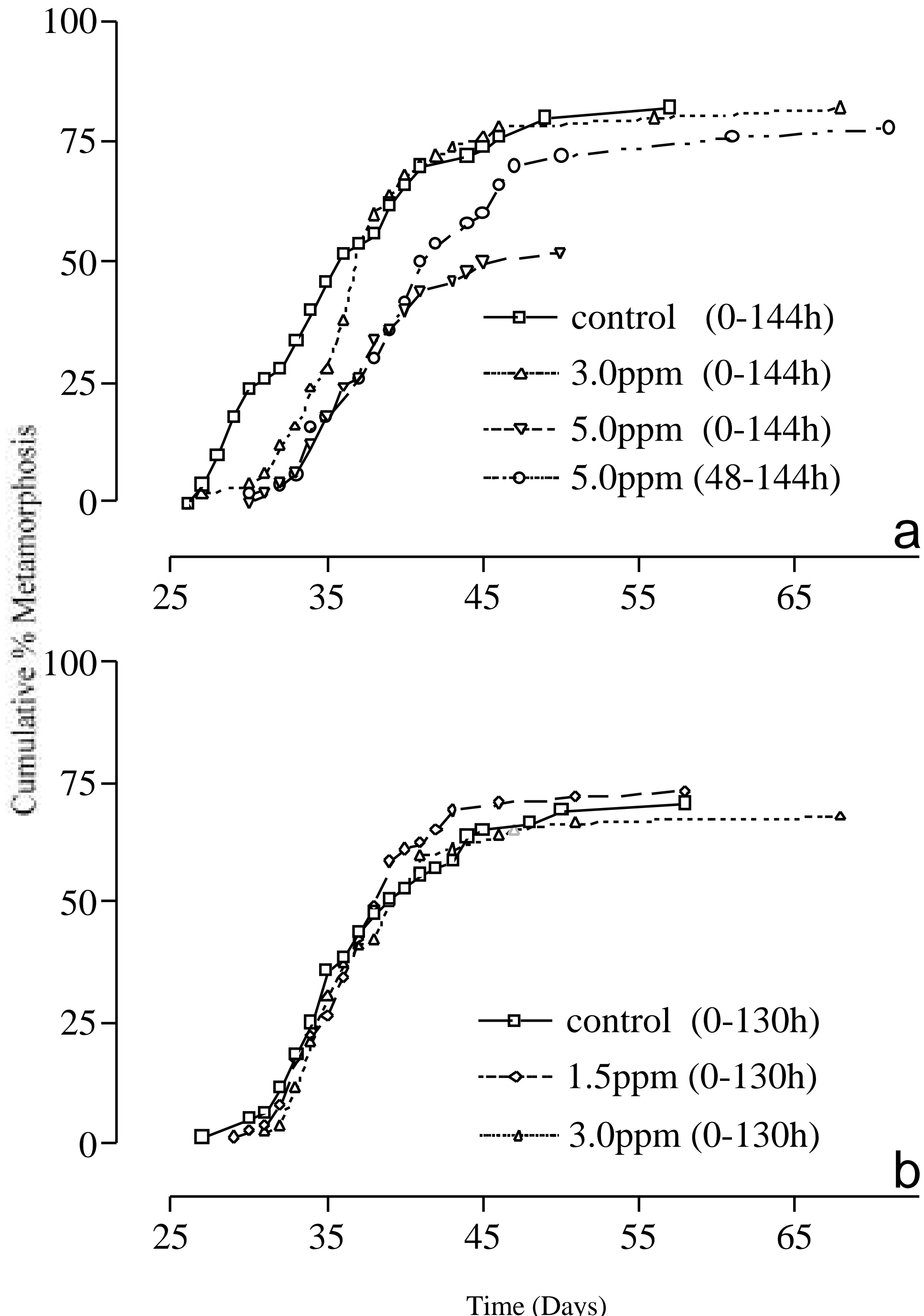

Fig. 5.1 Cumulative % metamorphosis of *Crinia insignifera* exposed to nonylphenol ethoxylate during embryological development. **a** Trial one. **b.** Trial two.

Discussion

There was a significant delay in development of animals exposed to 5.0 mg/L for the full 144 h. This was expected as a previous examination of the developmental toxicity of nonylphenol ethoxylate, (Chapter 4; Mann and Bidwell, 2000), observed significant growth inhibition in *Xenopus laevis*, and the Australian native frogs, *Litoria adelaidensis* and *C. insignifera* when exposed to Teric GN8 during the first four to six days of development. In the current study, metamorphosis was delayed by five to six days in animals exposed to 5.0 mg/L NPE (Fig. 5.1a). However, at the end of the exposure period, the difference in developmental stage displayed by embryos exposed to 5.0 mg/l represented only one to two days delay in normal development compared to controls, which indicated that recovery from the effects of exposure is also delayed. At the end of the 144 h exposure period these same animals were at Gosner stage 22. Those embryos exposed to 5.0 mg/L from 48 to 144 hours were slightly more advanced in their development (Gosner stage 23), even though time to metamorphosis was delayed to the same extent as those exposed for the longer period. It is likely that greater experimental replication, with larger numbers of animals, would more accurately resolve the differences in time to metamorphosis between treatments.

The 144-h LC50 for *C. insignifera* embryos was 6.4 (95% confidence interval, 6.1-6.6) mg/L (Chapter 4; Mann and Bidwell, 2000). It is not surprising therefore, that exposure to 5.0 mg/L for the full six days resulted in significant mortality of embryos subsequent to exposure. For all other treatments, post-exposure mortality was similar to control mortality.

In trial 1, weights and lengths of treated animals did not differ from those of controls, indicating that growth, although delayed, was otherwise unaffected by embryonic exposure to sublethal concentrations of NPE.

There was a significant predominance of females amongst metamorphs in the 3.0 mg/L treatment. A similar sex bias was observed in animals exposed to 5.0 mg/L from 48 to 144 h. The complete reversal of sex following developmental exposure to environmental sex steroids is a characteristic of amphibian

development which has been documented many times (for review see Hayes, 1998). While sex determination in amphibians appears to be under genetic control, sexual differentiation can be influenced by environmental factors such as temperature and exogenous hormones (Hayes, 1998). Complete sex reversal has been demonstrated in males or females of several species reared in the presence of oestrogens or androgens (Hayes, 1998).

The NPE used in this study was a mixture of ethoxylates of varying chain length. The shorter mono- and di-ethoxylates (i.e. $NPEO_1$, $NPEO_2$) and nonylphenol (NP), are known to be oestrogenic in nature, with oestrogenic activity decreasing with increased ethoxylate chain length (Jobling and Sumpter, 1993). In an examination of the oestrogenic effects of alkylphenolic compounds, Jobling *et al.* (1996) described induction of vitellogenesis (a process normally dependent on endogenous oestrogens) and concomitant inhibition of testicular growth in male rainbow trout following exposure to 30 μg/L of NP, $NPEO_1$, $NPEO_2$ or octylphenol (OP). In another study, vitellogenin-mRNA has been induced in male *X. laevis* cultured hepatocytes following incubation with 10^{-7} M NP (Kloas *et al.*, 1999). Of greater relevance to this study is the induction of female phenotype in *X. laevis* when embryos were reared to metamorphosis in water containing 10^{-7} M NP or OP (Kloas *et al.*, 1999). In this study, only the short chain ethoxylates (i.e. $NPEO_1$, $NPEO_2$) which make up 1 to 3% of the surfactant (Chapter 6; Mann and Boddy, 2000) were likely to be responsible for the observed sex bias. At 3 to 5 mg/L (total surfactant) these components were at concentrations (approximately 30 to 100 μg/L) similar to those reported by Jobling *et al.* (1996).

The developmental stage of exposure is critical. In *X. laevis*, sexual differentiation of the gonads begins at developmental stage 52 (Nieuwkoop and Faber, 1975), and administration of estradiol from stages 50 to 52 will completely feminise the developing gonads (Miyata *et al.*, 1999). Studies with other amphibian species, however, indicate that the timing of gonadal differentiation and maturation is species specific and can be highly variable (Hayes, 1998). The

results presented here indicate that exposure of *C. insignifera* embryos up until Gosner stage 24 to Teric GN8 at 3.0 to 5.0 mg/L, may effect sex ratios in this species. Furthermore, only those animals that had reached at least stage 23 exhibited an alteration in sex ratio, indicating that Gosner stage 23 is the critical stage in *C. insignifera*. In trial two, those animals exposed to 3.0 mg/L for 130 h did not display a bias toward female phenotype, but also, had not reached development stage 23 by the end of the exposure period. On the other hand, those animals exposed to 1.5 mg/L for 130 h were at stage 23 at the end of the exposure period but no sex-ratio bias was observed. It is possible that the exposure period was still short of the critical developmental stage or alternatively, the lower concentration was insufficient to induce a developmental change.

Another aspect of this study needs to be highlighted. In trial 1, 41.5% of control animals were female, while in trial 2, 53.7% of animals were female, indicating that there is likely to be a large degree of variability in non-manipulated sex ratios. In this study, statistical analyses have compared sex ratios in treated animals with control animals. If the sex ratios within this species are naturally variable then the results presented here remain somewhat speculative. Further investigations with larger sample sizes will be useful to validate the results presented here.

Chapter 6

Biodegradation of a nonylphenol ethoxylate by the autochthonous microflora in lake water, with observations on the influence of light.

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Abstract

Alkylphenol polyethoxylates (APE) are routinely used as additives in pesticide formulations. Biodegradation of APEs results in the accumulation of persistent short chain mono-, di- and tri-ethoxylates ($APEO_1$, $APEO_2$ $APEO_3$) that are more toxic than the parent compounds and potentially oestrogenic. Accumulation of persistent APE metabolites in shallow or ephemeral waters may pose a hazard to aquatic fauna. This study has followed the degradation of a nonylphenol ethoxylate (NPE) in freshwater, in static die-away tests, with and without illumination. Over 33 days in darkness there was a progressive and complete loss of long chain oligomers ($NPEO_{8-17}$), transient increases and subsequent loss of short to medium chain oligomers ($NPEO_{4-7}$), and large persistent increases (~1000%) in short chain oligomers ($NPEO_{1-3}$). In the presence of illumination, biodegradation was retarded and heterotrophic bacterial proliferation was inhibited. After 33 days there was complete loss of long chain oligomers ($NPEO_{9-17}$), incomplete loss of medium chain oligomers ($NPEO_{6-8}$) and increases in short chain oligomers ($NPEO_{1-5}$).

Keywords

Surfactant, Alkylphenol polyethoxylate, Bacterial degradation, HPLC, Static die-away

Introduction

Alkylphenol polyethoxylates (APE) constitute a large portion of the nonionic surfactant market. In 1997, the worldwide production of APEs was estimated at 500 000 tonnes (Renner, 1997). Industrial applications (plastics and elastomers, textiles, agricultural chemicals and paper products) accounted for 55% of the market, institutional cleaning products- 30% and household cleaning and personal care products- 15% (Talmage, 1994). The principal points of entry into the natural environment are sewage treatment plants and to a lesser extent, industrial sources such as paper pulp mills and wool scouring plants (Talmage, 1994; Field and Reed, 1996).

A more diffuse source of APEs is through their use as wetting agents, emulsifiers or adjuvants in agricultural products (Sundaram and Szeto, 1981; Dodd *et al.*, 1993). Often, where the active constituents of a pesticide are of low toxicity, the additive surfactant components may pose the most significant risk to aquatic fauna, especially when they are applied around or over standing or ephemeral waters. Herbicide formulations containing surfactants, are frequently used directly in water-bodies to control nuisance aquatic macrophytes (Mann and Bidwell, 1999b). Ephemeral waters are by nature, low dilution environments where environmental contaminants may accumulate to levels which may pose a threat to aquatic species that use these environments (Lahr, 1997).

Primary biodegradation of APEs is by stepwise removal of ethylene oxide monomers and is understood to be facilitated by bacterial metabolism of the hydrophilic ethoxylate chain (Swisher, 1987). Numerous studies have investigated the biodegradation of APEs and reported a wide range of degradation rates depending on the kind of test systems and conditions employed (for review see Talmage, 1994). While ultimate biodegradation (Swisher, 1987) results in the complete mineralisation of the aromatic structure of APE, most studies indicated that only primary biodegradation is particularly rapid, resulting in an accumulation of persistent metabolites such as nonylphenol (NP) or octylphenol (OP), short chain mono-, di- and tri-ethoxylates (i.e. $NPEO_1$, $NPEO_2$, $NPEO_3$) and phenoxy

carboxylates (i.e. NPE_1C, NPE_2C) (Talmage, 1994; Field and Reed, 1996; Potter *et al.*, 1999; Staples *et al.*, 1999). These metabolites are more toxic to aquatic fauna than long chain ethoxylates (Yoshimura, 1986) and possess oestrogenic properties (Jobling and Sumpter, 1993; Kloas *et al.*, 1999).

Few studies have followed the biodegradation of individual APE oligomers over time. While there were many early studies that examined the biodegradation of APEs, most of these used indirect measurements of surfactant activity that were unable to discriminate between individual oligomers and underestimated the levels of short chain ethoxylates (Talmage, 1994). More recently, high performance liquid chromatography (HPLC) has been adopted for the routine quantitative detection of individual APE oligomers (Kiewiet and de Voogt, 1996; Miszkiewicz and Szymanowski, 1996), including short chain ethoxylates.

Biodegradation of nonionic surfactants that enter the environment via routes other than sewage treatment plants, is best studied in static die-away tests (Swisher, 1987). This test has recently been used to follow the biodegradation of nonylphenol ethoxylate in estuarine (Kvestak and Ahel, 1995; Potter *et al.*, 1999) and river water (Manzano *et al.*, 1998). These tests were performed in the dark to avoid interference from algal proliferation. From an ecological viewpoint, the elimination of an algal element in the biodegradation process is likely to misrepresent realism. Microalgae have been shown to be capable of degrading organic pollutants, including aromatic compounds (Kuritz and Wolk, 1995; Semple *et al.*, 1999). The proliferation of an algal component may therefore enhance the degradation process. Alternatively, competition for resources, or other trophic relationships between algae and bacteria may inhibit the proliferation of those bacteria involved in the degradation process (Currie, 1990; Legrand *et al.*, 1998).

As part of an on-going evaluation of the toxicity of agricultural surfactants (Mann and Bidwell, 1999b; 2000; in press), this study has re-examined the biodegradation of a nonylphenol ethoxylate in a freshwater sample under conditions of complete darkness and a 12 h light, 12 h dark photoperiod.

Materials and Methods

Static Die-Away Test

The water used in this degradation study was sourced from a small lake within the grounds of Curtin University of Technology in Perth, Western Australia. The lake receives rainwater run-off from the university grounds during winter. During summer, a minimum level is maintained with water pumped from a subterranean aquifer. The water quality parameters at the time of the test are given in Table 6.1. Water for the trial was collected on the day the trial was initiated.

Table 6.1. Curtin Lake-water characteristics

Parameter	Value
Colony-forming Units	1.48×10^5
Total Suspended Solids	2.2 mg/L
Conductivity	406 µS/cm
pH	6.6
$N0_2^-$	< 0.01 mg/L
NH_4^+	0.05 mg/L
SO_4^{2-}	7.0 mg/L

The surfactant used in this study was a nonylphenol ethoxylate with an average oligomer length of eight ethoxylate units (Teric GN8) supplied by Huntsman Corporation Australia Ltd.

Twelve 45-L polyethylene tubs with tight fitting lids were filled with 40 L of lake-water. Prior to filling, the tubs were swabbed with 70% ethanol to remove residual bacteria. Six tubs were dosed with enough surfactant to obtain nominal concentrations of 5 mg/L. The remaining six tubs were designated as controls with no added surfactant. Six tubs (3 treated & 3 controls) were placed in a dark room at 22±1°C. The remaining six tubs (3 treated & 3 controls) were placed in a room

at 22±1°C with a 12 h light, 12 h dark photoperiod ($L_{12}D_{12}$). The light source was two 58W, white fluorescent tubes. Light intensity at the test water surface was measured with a digital light meter (TPS Pty Ltd, Brisbane, Australia). Ultraviolet radiation at the water surface was measured with a UV sensor with a peak sensitivity at 360±4 nm and a band-width of 72±5 nm (Delta-T Devices Ltd, Cambridge, England). Dissolved oxygen was measured at sporadic intervals with an Oxi 320 oxygen meter fitted with an OxiCal®-SL oxygen probe (WTW, Weilheim, Germany). Surfactant oligomer distribution and heterotrophic bacterial complement was monitored for 33 days.

Surfactant Analysis

Water samples (500 mL) were taken at the beginning of the trial from the middle (15 cm below the surface) of each tank. Subsequent water samples (500 or 800 mL) were similarly taken after 1, 2, 6, 9, 14, 19, 24 and 33 days. Nonylphenol ethoxylate was immediately extracted from the water samples using solid-phase extraction with 2.8 mL, 500 mg C_{18} cartridges (Alltech, USA) following a protocol described by Scullion *et al.* (1996). The cartridges were preconditioned with 7 mL methanol followed by 7 mL Milli-Q™ water. Water samples were drawn through the cartridges under vacuum. The cartridges were washed with 12 mL of water-methanol (70:30) and air-dried. Elution of surfactants was achieved using 5 mL of 100% methanol. Finally, the extracts were blown dry with nitrogen and re-suspended in 5 mL of 100% acetonitrile.

High performance liquid chromatography was used to quantify nonylphenol ethoxylate as described by Scarlett *et al.* (1994). The procedure utilised isocratic separation with an acetonitrile-water (95:5) mobile phase. A Waters™ Resolve CN 8x10 Radial-Pak column was used for normal phase separation of nonylphenol ethoxylate oligomers. Ultraviolet (UV) detection was carried out using a Waters™ 486 tuneable absorbance detector (=225 nm) and data output via a Hewlett-Packard HP3396A integrator. A typical trace and oligomer distribution is presented in Fig. 6.1. Allocation of oligomer identity was

confirmed by LSMIS mass spectrometry following elution of individual oligomers. Because all oligomers have almost identical molar absorptivities, the integrated peak area of individual oligomers could be used to determine the mole fraction of each oligomer (Wang and Fingas, 1993). Furthermore, there is a robust linear relationship between concentration and the integrated peak area response (Mann, unpubl. data), therefore, changes in individual peak areas during the trial have been used to represent changes in molar concentration.

Initial concentrations of surfactant were established by comparing aggregate integrated peak areas with a standard curve generated with six concentrations of Teric GN8.

Heterotrophic Bacteria Counts

Water samples (5 mL) were taken with a sterile pipette at the beginning of the trial from the middle of each of the twelve tanks. Subsequent water samples were similarly taken after 1, 3, 6, 9, 14, 19, 24 and 33 days. The samples were diluted in 1:10 serial dilutions with 0.1% tryptone water. 1 mL of each dilution was added to each of three replicate sterile petri dishes. R2A Agar (15 mL) (Becton Dickinson and Company, USA) at 48°C was added to each of the petri dishes, mixed and incubated at room temperature for 72 h. At the end of the incubation period colonies were counted to obtain a measure of colony forming units (CFU) per millilitre.

Results

The initial measured concentration of nonylphenol ethoxylate in the six treatment tubs was 4.49 mg/L. The standard deviation (SD) around this mean was 0.06 (1.3%). In $L_{12}D_{12}$ light intensity at the water surface was 465 Lux. Ultraviolet radiation was undetectable at the water surface. Dissolved oxygen did not fall below 60% for the duration of the test tanks kept in darkness or $L_{12}D_{12}$.

HPLC analysis of lake-water with and without surfactant produced an early peak indicating the pre-existence or an organic compound in the water

sample (Fig 6.1a). This peak partially masked the first surfactant peak ($NPEO_{1-3}$), therefore, the proportion of total surfactant constituted by these short oligomers is likely to be underestimated in Fig. 6.1b. At later sampling times, this pre-existent peak is almost entirely masked by a large peak representing $NPEO_{1-3}$ (Fig. 6.2). The percentage change in oligomer molar concentration of $NPEO_{1-3}$ (Fig. 6.3c,d) is therefore likely to be slightly overestimated.

Surfactant degradation proceeded more rapidly in darkness than in $L_{12}D_{12}$. In both darkness and $L_{12}D_{12}$, there was a progressive loss of heavier oligomers ($NPEO_{6-13}$) (Fig. 6.3c,d,e,f). Early increases in areas of those peaks corresponding to high molecular weight oligomers ($NPEO_{14-17}$) were subsequently found to be caused by interference from the formation of polar degradation products (Fig. 6.2). In both darkness and $L_{12}D_{12}$ short chain oligomers increased after initial decreases (Fig. 6.3c,d).

Heterotrophic bacterial counts indicated a pronounced increase in bacterial growth in treated tanks kept in darkness and $L_{12}D_{12}$ (Fig. 6.3a.b). Those treated tanks kept in darkness sustained high numbers of heterotrophs between 6 and 24 days (Fig. 6.3a). The counts for individual replicate tanks varied greatly reflecting an out-of-phase oscillation in heterotroph numbers. This oscillation was very pronounced in replicate tanks A and C. The oscillation in heterotroph numbers was not pronounced in replicate tank B. In treated tanks kept in $L_{12}D_{12}$, an initial peak in heterotroph numbers at 6 days was not sustained, dropping dramatically in all replicate tanks at day 9 (Fig. 6.3b). The observed decrease in heterotroph counts coincided with algal growth on the sides of the degradation tanks in $L_{12}D_{12}$.

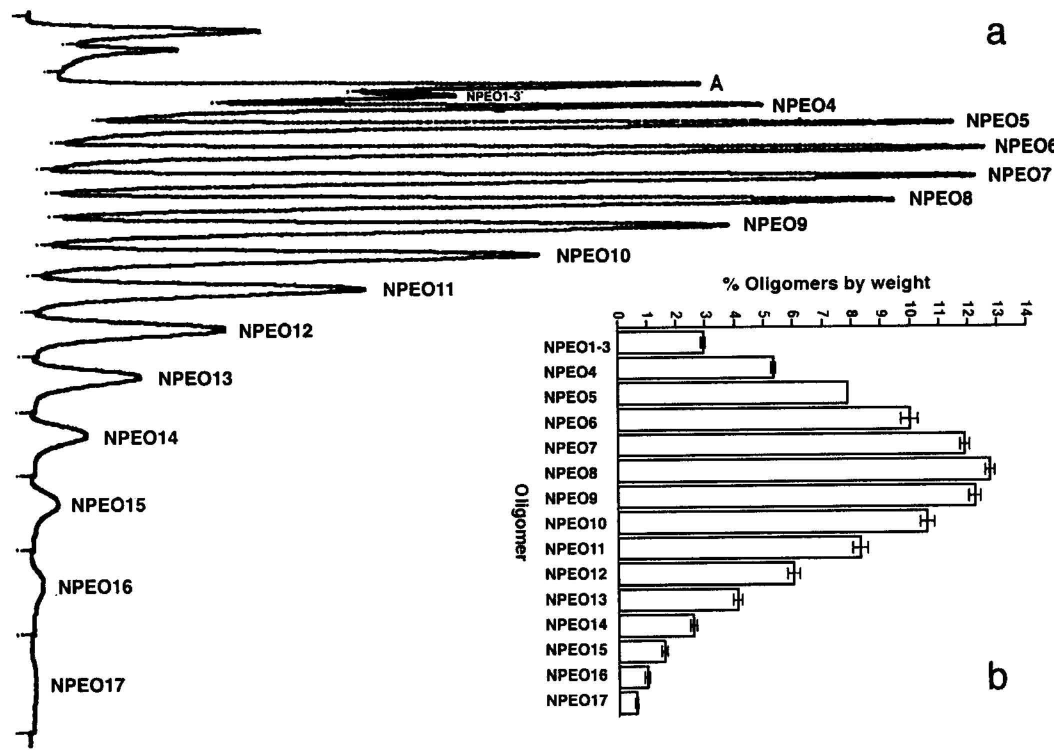

Fig. 6.1 **a** HPLC trace of Teric GN8 sampled on day one. A pre-existing compound A, was present in the lake water source and persisted throughout the trial. **b** Proportion of individual oligomers on day one of the test. Error bars represent standard deviations (n=6).

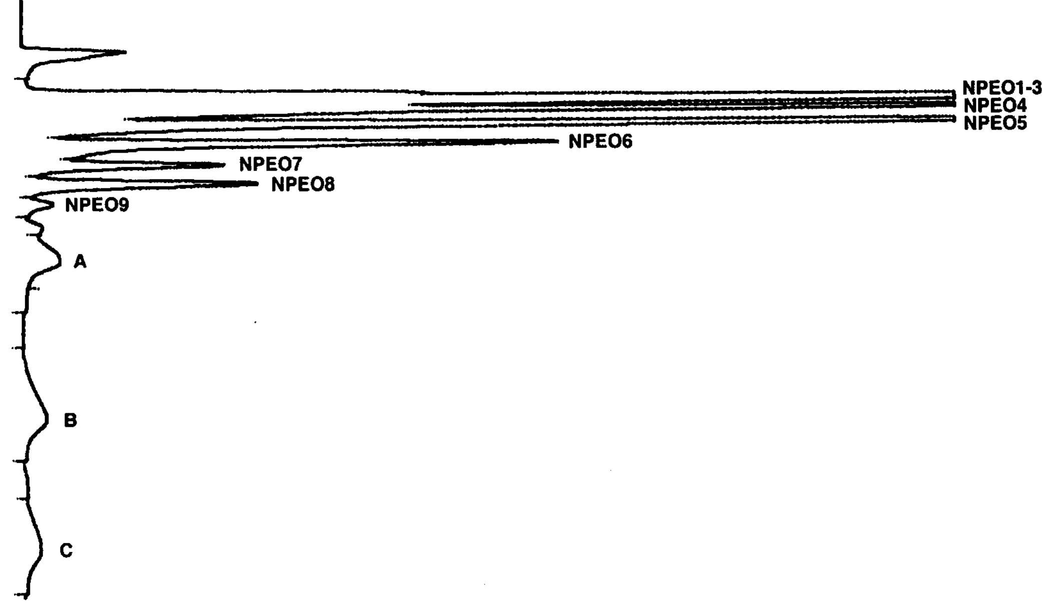

Fig. 6.2 HPLC trace of NPEs after 33 days biodegradation at 22±1°C, with a 12 h light and 12 h dark photoperiod. Peaks A, B & C represent polar degradation products.

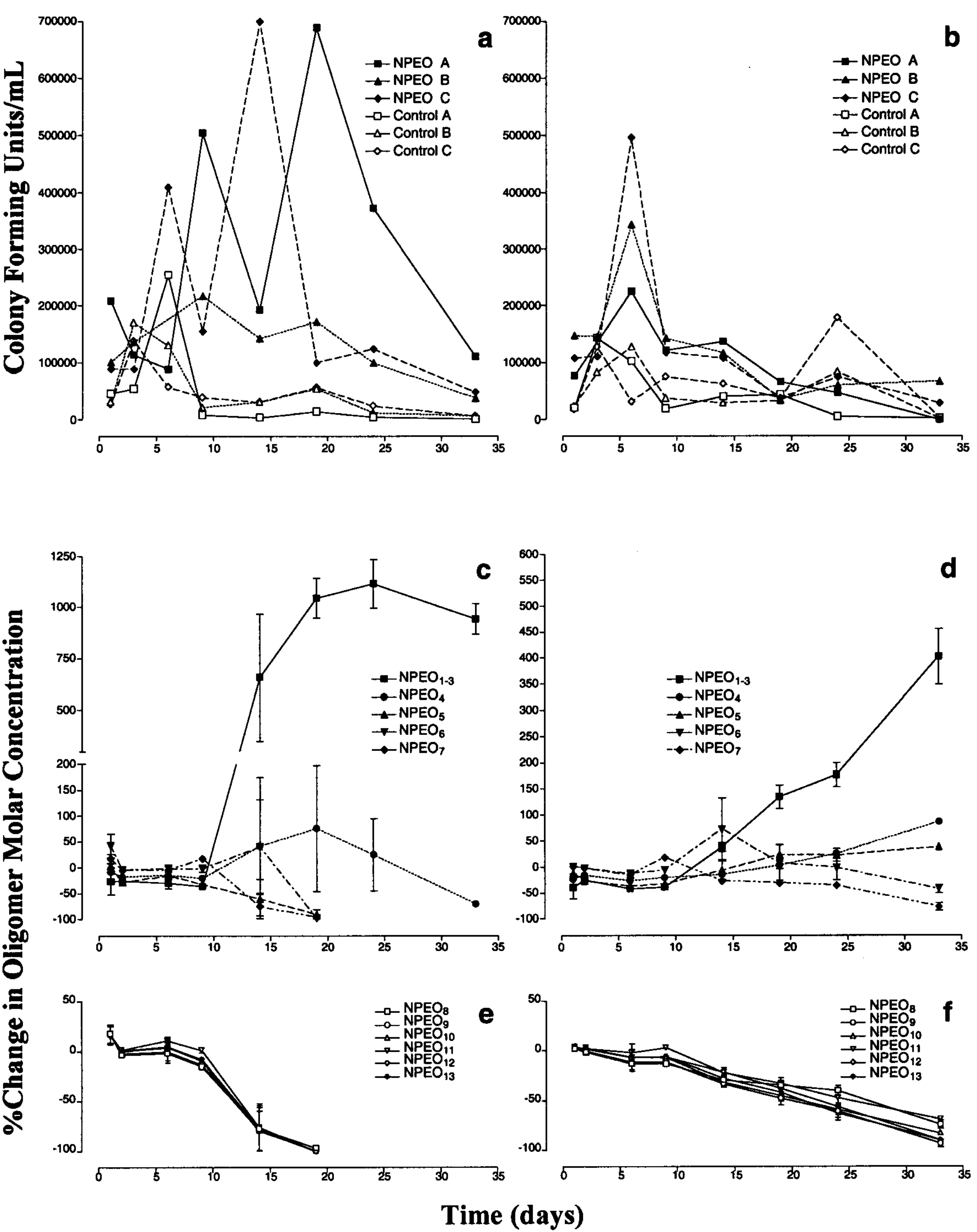

Fig. 6.3 Time course of heterotrophic bacterial growth and biodegradation of Teric GN8. **a** Heterotrophic bacteria population in replicate tanks kept in darkness. **b** Heterotrophic bacteria population in replicate tanks kept in a 12 h light and 12 h dark photoperiod. **c-f** Change in oligomer concentration over time in darkness (**c, e**) and in a 12 h light and 12 h dark photoperiod (**d, f**). Note that the Y-axis in **c** is split into a high and low range. Error bars represent standard error.

In darkness, the degradation of heavier oligomers accelerated after a 7-day lag phase and was virtually complete after 19 days (Fig. 6.3e). Slight increases in $NPEO_7$ and $NPEO_6$ (replicate tank A only) were recorded at 9 and 14 days respectively, before decreasing (Fig. 6.3c). At 33 days, $NPEO_4$ remained at approximately 30% of the original concentration after increasing to 198% (replicate tank A) at 19 days and 217% (replicate tank C) at 24 days (Fig. 6.3c). An increase in $NPEO_4$ was not recorded in replicate tank B. Maximum $NPEO_{1-3}$ peak area was recorded in replicate tanks A (~1290%), B (~1230%) and C (~1090%) at 24, 19 and 33 days respectively (Fig. 6.3c).

In $L_{12}D_{12}$ the loss of heavier oligomers accelerated after an initial lag phase of 7 to 9 days, with complete loss of $NPEO_9$ to $NPEO_{13}$ (and presumably all high molecular weight oligomers) after 33 days (Fig. 6.3d). At 33 days $NPEO_8$, $NPEO_7$ each remained at approximately 30% of their original concentrations (Fig. 6.3d,f). There was a slight increase in $NPEO_7$ recorded at day 9 (Fig. 6.3d). At 33 days $NPEO_6$ remained at approximately 58% of original concentrations after increasing to 174% (replicate tank A) and 76% (replicate tank B) of their original concentrations (Fig. 6.3d). An increase in $NPEO_6$ was not recorded in replicate tank C. After 33 days $NPEO_5$, $NPEO_4$ and $NPEO_{1-3}$ were at 40%, 88% and 403% of their original concentrations respectively (Fig. 6.3d).

Discussion

The measured concentrations of NPE at the start of the test were 10% lower than expected. The discrepancy is likely to be a consequence of adsorption of NPE to the test chambers, volume measurement errors or losses during analysis.

No attempt was made to resolve the first eluted peak into constituent oligomers (i.e. $NPEO_1$, $NPEO_2$, $NPEO_3$). Nonylphenol di-ethoxylate ($NPEO_2$) has been reported as the major primary metabolite of degradation (Kvestak and Ahel, 1995; Potter *et al.*, 1999) with $NPEO_1$ and $NPEO_3$ as minor metabolites. Further degradation to NP requires anaerobic conditions (Giger *et al.*, 1984) and would not be expected to be a major degradation product in this study since

aerobic conditions prevailed. The transformed degradation products NPE_1C NPE_2C and NPE_3C are also likely to be present and may be represented by the integrated peaks A, B and C in Fig. 6.2.

Primary biodegradation of Teric GN8, in darkness proceeded as reported previously for NPEs in static die-away tests in the presence of mixed bacterial cultures (Kvestak and Ahel, 1995; Manzano *et al.*, 1998; Potter *et al.*, 1999). There was a nett loss of long chain oligomers and an accumulation of short chain oligomers and biodegradation products. Those factors affecting the rate of degradation in these previous studies included temperature, salinity and bacteria exposure history (Kvestak and Ahel, 1995; Potter *et al.*, 1999). Kvestak and Ahel (1995) also cited initial surfactant concentration as a factor affecting rate of degradation, although Manzano *et al.* (1998) reported initial concentration as a factor affecting only the length of the initial lag phase prior to commencement of degradation.

A lag phase was also observed in this study. Pronounced decreases in long chain oligomers and concomitant increases in short chain oligomers were recorded only after approximately 7 to 9 days. This was longer than the lag phase (2 to 3 days) reported by Manzano *et al.* (1998) for the degradation of Empilan NP15 (NPE with an average oligomer length of 15 ethoxylated units) at an initial concentration of 5 mg/L in fresh water at 21°C with an initial heterotrophic bacterial count of 84 000 CFU/mL. The longer lag phase reported in this study is consistent with the range reported in a static die-away test in estuarine waters, at 28°C for the degradation of Intan-100 (NPE with an average oligomer length of 18 ethoxylated units) at an initial concentration of 4 mg/L (Potter *et al.*, 1999). The lag phase is attributable to bacterial acclimation (Talmage, 1994). When a novel compound is introduced into the microbial environment, time is required for either the induction of specific metabolic enzymes or for growth of those species capable of metabolising the surfactant (Swisher, 1987; Terzic *et al.*, 1992). In previous studies, the presumption of prior exposure of endogenous bacteria to NPE was

offered as an explanation for reduced degradation times in some estuarine water samples (Kvestak and Ahel, 1995; Potter *et al.*, 1999).

In darkness, the biodegradation of long chain oligomers was virtually complete within 19 days. The formation of short chain oligomers (i.e $NPEO_{1-3}$, $NPEO_4$) peaked between 19 and 24 days. After 33 days $NPEO_{1-3}$ was present at 900% of their original concentration having peaked at more than 1000% between 19 and 33 days. The $NPEO_{1-3}$ components constituted 3% of the original formulation (~0.14 mg/L) (Fig. 6.1b). At their highest levels they would have been at the approximate concentration of 1.6 mg/L prior to their relatively slow degradation (Potter *et al.*, 1999). These concentration increases are similar to those obtained by Kvestak *et al.* (1995) in their studies in estuarine waters.

Biodegradation in tanks subject to a 12 h light, 12 h dark photoperiod, was retarded considerably. After 33 days loss of $NPEO_8$, NP_7EO_8, $NPEO_6$ $NPEO_5$, and $NPEO_4$ was incomplete. Oligomers $NPEO_5$, $NPEO_4$ and $NPEO_{1-3}$ were still increasing and $NPEO_{1-3}$ had reached only 400% of their original concentration. Although the trial was terminated at this time, it is presumed that the degradation profile would follow the same pattern, with a continued loss of long chain oligomers and a continued increase in short chain oligomers.

Explaining the retardation in biodegradation is problematic. Swisher (1987) indicated that static die-away tests should be performed in the dark to avoid algal growth but also stated that diffuse light had negligible effects on surfactant biodegradation. In this study, algal proliferation in $L_{12}D_{12}$ was concomitant with a reciprocal decrease in heterotrophic bacteria in those tanks with added surfactant, indicating either a direct effect of illumination on bacterial proliferation or an interaction between algae and bacteria. Ultra violet radiation is known to inhibit bacterial abundance in surface waters (Lindell and Edling, 1996). Bright sunlight has also been reported to retard biodegradation of nonionic surfactants (Dobarganes Garcia and Cruz, 1977). In this study, however, no UV was detected and is unlikely to be a factor in bacterial growth inhibition. Visible light can also inhibit bacterial growth (Brock, 1974). Possibly those species capable of

biodegrading NPE were relatively intolerant to the low levels of visible light employed in this study. However, the initial increase in CFUs recorded at 6 days and the subsequent reduction in CFUs, indicates that another factor inhibited bacterial proliferation after bacterial growth was initiated.

Competition with algae for phosphorus and nitrogen would explain the bacterial inhibition. In a closed system such as that employed in this study, nutrients are limited. Bacteria are generally considered to be more efficient at assimilating resources (Currie, 1990), and the presence of algae would not be expected to have a pronounced effect on bacterial growth. However, the interactions are complex (Currie, 1990) and competition for resources can not be ruled out. Also, phagotrophic algae are capable of removing bacteria from a water column, especially under low light or nutrient limited conditions (Legrand *et al.*, 1998). The importance of phagotrophs in this study or in local surface waters is not known.

The results presented here raise important questions regarding appropriate test conditions for assessing the biodegradability of organic pollutants. The desire for standardised test conditions has seen the adoption of restrictive test protocols. The variation that occurs in natural systems, however, needs to be highlighted. In shallow ephemeral aquatic systems, dilution factors are low, light levels are often high, and temperature can fluctuate substantially. In a shallow natural water column the concentration of degradation products are likely to rise significantly, as observed in this study. The degree to which these compounds accumulate or persist will depend on the field conditions, however, the results presented here and previous studies (Marcomini *et al.*, 1990; Kvestak and Ahel, 1995; Bennett and Metcalf, 1998; Potter *et al.*, 1999; Staples *et al.*, 1999) indicate that persistence may be prolonged.

Because of the potential for accumulation and persistence of biodegradation metabolites, the introduction of a NPE into an aquatic system even at relatively low concentrations is likely to have an effect on the aquatic fauna and flora. Exposure of rainbow trout (*Oncorhynchus mykiss*) to 30 μg/L of NP,

$NPEO_1$ or $NPEO_2$ resulted in inhibition of testicular growth (Jobling *et al.*, 1996). Also, the life cycles of many aquatic organisms may be affected by extremely short exposures. Kloas *et al.* (1999) reported feminisation of *Xenopus laevis* embryos following embryonic exposure to 100 nM (22 µg/L) NP. Furthermore, Miyata *et al.* (1999) described sex reversal in *X. laevis* following exposure to estradiol during embryological stages 50 to 52 (1 to 2 days exposure). Therefore, even short-term persistence of toxic or oestrogenic metabolites may present a hazard.

Further work is required to evaluate the hazard posed by the introduction of NPE through pesticide application near or over freshwater systems. Mesocosm studies that incorporate greater environmental complexity would be useful for evaluating the extent of accumulation and persistence of toxic metabolites under natural conditions and would help to establish the validity of standardised static die-away tests.

Chapter 7
General Discussion

Test replication and reproducibility

Discrepancies exist between standard test protocols with regard to the degree of replication required within tests. Two test protocols have formed the basis of the toxicity tests described in this thesis. Acute toxicity tests with Gosner-stage 25 tadpoles (Gosner, 1960) (Chapters 2 & 3) included either three or four replicates in each test as specified by the American Society for Testing and Materials, Standard practice for conducting acute tests with fishes, macroinvertebrates and amphibians (ASTM, 1993b). Embryotoxicity tests with early stage embryos (Chapters 4 & 5), followed a protocol specified by the American Society for Testing and Materials, Standard guide for conducting the Frog Embryo Teratogenesis Assay-Xenopus (Fetax) (ASTM, 1993a), which only requires two replicates for each treatment. The role of replication within tests and the differences in protocol are notable and worthy of some discussion.

Replication within a test, where a cohort of test animals is split up into two or more replicates is pseudoreplication with little statistical validity. Dividing animals into replicate test chambers, in combination with a random positioning within the testing facility does, however, ensure that test animals for all treatments experience similar testing conditions. The statistical integrity of acute toxicity and FETAX tests lies in: 1. Ensuring that as large a number of animals as possible are used per treatment, and 2. Repeat testing to provide statistically valid replicates. In acute tests, 10 to 20 animals, are considered adequate (ASTM, 1993b). In FETAX tests, 50 animals per treatment and 100 control animals are considered adequate (ASTM, 1993a). This level of animal replication has been consistently adhered to throughout this thesis.

As indicated in Chapter 3, test replication with native species was not logistically possible, and the six repeat tests with *B. marinus* were performed to assess the reliability of individual tests. While these tests did provide an insight

into the consistency of effect data between tests, they were somewhat flawed. As recommended by ASTM guidelines, animals from a single clutch were used for each test where possible. This ensures that LC50 and EC50 data are not confounded by the presence of unhealthy clutches. This provision is only useful, however, if the test is repeated several times with separate clutches of animals. In the case of the repeat tests with *B. marinus*, all six tests were performed with animals from a single clutch. While the information gained from those repeat tests is useful to confirm the low of variability within the test protocol itself using that specific species and that specific chemical, it sheds little light on genetic or regional variation within the species. It is conceded therefore that further repeat testing with separate clutches would be useful for all species and all test chemicals.

In the case of FETAX exposures with *C. insignifera*, up to three egg clutches were required to obtain the necessary number of animals for a test (Chapters 4 & 5). No attempt was made to mix equal numbers of eggs from individual clutches. It is possible therefore that the variation in results between the two trials in Chapter 5 reflect genetic variability among egg clutches.

Australian native species versus non-native surrogate species

In Australia, much emphasis has been placed on the need to generate toxicity data for indigenous species (ANZECC, 1992; Norris and Norris, 1995). Certainly there are data indicating some Australian species to be either more sensitive (Davies *et al.*, 1994) or less sensitive (Sunderam *et al.*, 1992) than standard North American test species. However, there is also a growing body of data, which indicate no or few differences between equivalent indigenous and non-indigenous species under standardised conditions (Skidmore and firth, 1983; Johnston *et al.*, 1990; Chapman, 1995; Markich and Camilleri, 1997; Bailey *et al.*, 2000). Chapters 3 and 4 raise the possibility that data generated for exotic species such as *X. laevis* or *B. marinus* may provide reasonable estimates of chemical toxicity to Australian species. Although *X. laevis* was found to be consistently more sensitive to surfactants than the Australian species in larval and embryo-

larval tests (Chapters 3 & 4), the differences were not great, and certainly no greater than the variation seen amongst the Australian species alone.

As is the case with other Australian native fauna, for which toxicity data is far from comprehensive, care must still be exercised in allocating uniform sensitivity amongst all Australian frogs. While the Australian species tested in this study are representative of the two major families of Australian anurans, none of them were specialist or rare species, and it is still possible that a great deal of variation in sensitivity occurs amongst Australian frogs. Also, surfactants are somewhat of a blunt instrument with regard to toxicity, having a generalised effect at membranes (see introduction). Toxicants with a more specific toxicity may elicit greater differences between species.

Ecologically relevant testing

With a realisation that Australian fauna may not exhibit large differences in sensitivity from those species used in the northern hemisphere, the emphasis has shifted to testing under ecologically relevant conditions (Chapman, 1995; Lahr, 1997; Markich and Camilleri, 1997; Patra *et al.*, 1999). Any toxicological assessment of chemicals with regard to amphibians needs to accommodate the characteristics of amphibian habitat. All the previous Chapters have emphasised the shallow, lentic or ephemeral nature of breeding habitat as required by many of the world's amphibians. One of the reasons that amphibians have been so successful for over 300 million years, and one of the characteristics of the Australian frog fauna in particular, is their ability to exploit temporary environmental water sources for the purpose of reproduction (Tyler, 1994).

Temporary freshwater habitats are highly variable in chemical and physical characteristics, often displaying large fluctuations in temperature, dissolved oxygen, pH, and light intensity (Lahr, 1997). It has been suggested that the inhabitants of such environments are adapted to fluctuating and extreme conditions and are possibly more resilient to environmental stresses such as pollutants (Koivisto, 1995). The alternative view, however, is that the introduction of a

novel chemical stressor will provoke deleterious effects in organism that are already living close to their biological limits (Lahr, 1997).

In Chapter 3 acute tests with high temperatures and low DO, EC50 values for tadpoles (*Bufo marinus*) exposed to NPE were up to 3.6 times lower than those generated with the same species under standard test conditions, even though the test animals had been reared under conditions of low DO and high temperature. As discussed in Chapter 3, the increased toxicity appeared to be an indirect consequence of narcosis, but the result still highlights the necessity of accommodating habitat characteristics of amphibians in toxicity assessments.

Bioavailability of surfactants and surfactant metabolites to amphibians

The tendancy for surfactants to sorb to non-aqueous substrates will affect surfactant bioavailability to amphibians. The surface active nature of surfactants implies that a proportion of the chemical will adsorb to suspended organic and inorganic particulate matter and benthic sediments (Maki and Bishop, 1979; Pittinger *et al.*, 1989; Cano and Dorn, 1996), or be absorbed by a range of organisms including zooplankton, phytoplankton, and macrophytes. In effect, competition for surfactant can be expected. It is interesting to note therefore, that in a series of artificial stream mesocosm experiments (Dorn *et al.*, 1996a; 1996b; 1997; Lizotte *et al.*, 1999) there was no loss of surfactant, nor was there any significant changes in surfactant oligomer distribution for AE surfactants over the 17 minute flow-through duration. Because a flow-through system was employed in these studies, it is likely that the rapid replacement of surfactant masked any loss due to sorption. Also the sorption process is likely to have been rapid and equilibrium reached in a matter of hours (Cano and Dorn, 1996). These studies did not report concentrations of surfactant or surfactant metabolites in sediment, detritus or resident organisms.

Because of the nature of amphibian habitat, the use of flow-through stream mesocosms may not address the risk to amphibians when contaminants are introduced to temporary ponds. Static systems may be more appropriate. The

use of static artificial pond mesocosms for amphibian studies was pioneered by (Morin, 1981) who employed 1000 litre cattle watering tanks. Such tanks have been used extensively since then for the study of amphibian community dynamics (for review see Rowe and Dunson, 1994). More recently, similar tanks have been used to examine the effects of acidity (Clark and Hall, 1985; Warner *et al.*, 1991; 1993; Rowe *et al.*, 1992; Sadinski and Dunson, 1992; Horne and Dunson, 1995a; 1995b), pyrethroid contamination (Materna *et al.*, 1995), hydrocarbon contamination (Mahaney, 1994), fertilisers (de Wijer *et al.*, 1997) and heavy metals (Lefcort *et al.*, 1998) on amphibian assemblages.

Chapters 5 and 6 of this thesis raise valid concerns about the length of exposure to intact surfactants required to produce deleterious effects in amphibian. The results presented in Chapter 5 suggest that the endocrine effects of a relatively small proportion of the intact $NPEO_8$ surfactant can be expressed after a relatively short (though critical) exposure duration, while the results presented in Chapter 6 indicate that the biodegradation of $NPEO_8$ may occur so slowly as to span the entire embryonic and larval development time of many amphibians. Also, the shorter, oestrogenic and more toxic metabolites of NPE biodegradation have the potential to accumulate and persist for extended periods (Chapter 6).

The results presented in Chapters 5 and 6 raise the question: To what extent are particulate-sorbed NPE or the biodegradative metabolites of NPE bioavailable to amphibian fauna? The surface-active nature of NPEs and the hydrophobic nature of short chain NPE metabolites will enhance sorption to particulate matter. Larval amphibians are opportunistic herbivores and detritivores and can be expected to consume surfactant and surfactant metabolites that are sorbed to particulate matter. To what extent this presents a hazard is not known. In an examination of the dietary uptake of a quaternary ammonium surfactant (cationic) in tadpoles (*R. catesbeiana*), uptake across the gastrointestinal epithelium was slow (Knezovich and Inouye, 1993). However, unlike short chain NPEs, the surfactants employed in those studies were not particularly lipophilic. In another study, uptake via the gastrointestinal tract

contributed significantly to the bioaccumulation of $NPEO_{10}$ in cod (*Gadus morrhua*) (Granmo and Kollberg, 1976). While surfactant contaminants may be removed from the water column rapidly through sorption and degradation processes, they may still be bioavailable to larval amphibians.

I propose that the best way to address this question be through the use of static mesocosms which incorporate several levels of environmental complexity. Regular analysis for individual surfactant oligomers (as in Chapter 6) and other metabolites (i.e. NP, $NPEO_1$, $NPEO_2$ NPE_1C, NPE_2C) in water, sediment and tadpole tissue would be required to follow partitioning pathways for various surfactant components and metabolites. Various effect data could be collected for tadpoles including survival, growth, swimming performance, capacity for predator avoidance, hatching and metamorphosis success or delay and sexual phenotype at metamorphosis. Throughout the duration of my studies this was considered ultimately to be a necessary and logical progression for this project. Unfortunately, the requirement of extensive tissue, sediment and water chemical analysis was incompatible with the time and budgetary constraints. It remains however a desirable further step in assessing the toxic hazard presented to amphibians by surfactant compounds such as NPE.

Conclusion

This study has reported acute toxicity data for four Australian and two exotic anurans exposed to commercial glyphosate formulations and nonionic surfactants. The surfactants tested are routinely included as either pesticide formulation additives or as tank mixture additives. It was inferred that formulation surfactants were responsible for the majority of the toxicity of glyphosate formulations. Under standard conditions, the sensitivities of amphibians to the surfactants tested were similar to that of fish and invertebrates. Furthermore, the larval (tadpole) stages appear to be the most sensitive developmental stage when exposed to these surfactants with no apparent variation amongst different tadpole developmental stages in acute tests. Embryonic exposure to NPE resulted in developmental retardation, and sex ratio alterations amongst metamorphosing individuals.

Distinct species differences were evident although some of these differences might have been related to tadpole size. *Xenopus laevis* was consistently more sensitive than *B. marinus* and native species to the toxic effects of nonylphenol ethoxylate. However, the differences were not so great as to preclude the use of *X. laevis* or *B. marinus* as surrogate test species for acute toxicity testing.

Exposure of tadploles of the tropical species, *B. marinus,* to nonylphenol ethoxylate under conditions of low dissolved oxygen and high temperatures, resulted in lower LC50 values than those generated under standard conditions. This result highlighted the need to test the toxicity of environmental pollutants to amphibians under environmentally relevant conditions.

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Appendix 1

Reported numerical toxicity data for anionic and nonionic surfactants for fish and invertebrates

Table A1 Reported numeric toxicity data for anionic and nonionic surfactants in fish and invertebrates

Class	Subclass	Alkyl C#	EO #	Phylum	Species	Test	Exp. time	Enpoint	Conc. mg/L	Other Comments	Fig.	Reference
anionic	LAS	11 to 12		fish	*Salmo gairdneri*	acute	24	LC50	2.04			(Calamari and Marchetti, 1973)
anionic	LAS	11 to 12		fish	*Salmo gairdneri*	acute	96	LC50	1.68		Fig. 1	"
anionic	LAS	11 to 12		fish	*Salmo gairdneri*	chronic	14 d	LC50	1.66			"
anionic	LAS			fish	*Poecilia reticulata*	chronic	28 d	NOEC	3.20	mortality in 2d		(Canton and Slooff, 1982)
anionic	LAS	10		fish	*Pimephales promelas*	acute	24	LC50	48.00			(Kimerle and Swisher, 1977)
anionic	LAS	11		fish	*Pimephales promelas*	acute	24	LC50	17.00			"
anionic	LAS	12		fish	*Pimephales promelas*	acute	24	LC50	4.70			"
anionic	LAS	13		fish	*Pimephales promelas*	acute	24	LC50	1.70			"
anionic	LAS	14		fish	*Pimephales promelas*	acute	24	LC50	0.60			"
anionic	LAS branched	10		fish	*Pimephales promelas*	acute	24	LC50	87.00			"
anionic	LAS branched	12		fish	*Pimephales promelas*	acute	24	LC50	24.80			"
anionic	LAS branched	14		fish	*Pimephales promelas*	acute	24	LC50	8.10			"
anionic	LAS	10		fish	*Pimephales promelas*	acute	48	LC50	43.00			"
anionic	LAS	11		fish	*Pimephales promelas*	acute	48	LC50	16.00			"
anionic	LAS	12		fish	*Pimephales promelas*	acute	48	LC50	4.70			"
anionic	LAS	13		fish	*Pimephales promelas*	acute	48	LC50	0.40			"
anionic	LAS	14		fish	*Pimephales promelas*	acute	48	LC50	0.40			"
anionic	LAS branched	10		fish	*Pimephales promelas*	acute	48	LC50	86.10			"
anionic	LAS branched	12		fish	*Pimephales promelas*	acute	48	LC50	21.50			"
anionic	LAS branched	14		fish	*Pimephales promelas*	acute	48	LC50	5.30			"
anionic	LAS	11.8		fish	*Lepomis machrochirus*	acute	96	LC50	5.63	hardness 137	Fig. 1	(Lewis and Perry, 1981)
anionic	LAS	10 to 15		fish	*Carassius auratus*	acute	6	LC50	4.30			(Reiff *et al.*, 1979)
anionic	LAS	10 to 15		fish	*Idus idus*	acute	48	LC50	0.90			"
anionic	LAS	10 to 15		fish	*Idus idus*	acute	48	LC50	0.80			"

anionic	LAS	10 to 15	fish	*Idus idus*	acute	48	LC50	1.20			"
anionic	LAS	10 to 15	fish	*Idus idus melanotus*	acute	96	LC50	0.40		Fig. 1	"
anionic	LAS	10 to 15	fish	*Idus idus melanotus*	acute	96	LC50	0.60		Fig. 1	"
anionic	LAS	10 to 15	fish	*Rasbora heteromorpha*	acute	48	LC50	0.90			"
anionic	LAS	10 to 15	fish	*Rasbora heteromorpha*	acute	96	LC50	0.70		Fig. 1	"
anionic	LAS	10 to 15	fish	*Salmo trutta*	acute	48	LC50	0.20			"
anionic	LAS	10 to 15	fish	*Salmo trutta*	acute	48	LC50	0.40			"
anionic	LAS	10 to 15	fish	*Salmo trutta*	acute	96	LC50	0.10		Fig. 1	"
anionic	LAS	10 to 15	fish	*Salmo trutta*	acute	96	LC50	0.25 to 0.5			"
anionic	LAS	12	fish	*Rita rita*	acute	6	LC50	9.6			(Roy, 1988)
anionic	LAS	12	fish	*Rita rita*	acute	8	LC50	8.9			"
anionic	LAS	12	fish	*Rita rita*	acute	10	LC50	8.8			"
anionic	LAS	12	fish	*Rita rita*	acute	12	LC50	8.4			"
anionic	LAS	12	fish	*Rita rita*	acute	24	LC50	7.4			"
anionic	LAS	12	fish	*Rita rita*	acute	48	LC50	7.3			"
anionic	LAS	12	fish	*Rita rita*	acute	72	LC50	7.0			"
anionic	LAS	12	fish	*Rita rita*	acute	96	LC50	6.9		Fig. 1	"
anionic	LAS		fish	*Pleuronectes platessa*	acute	96	LC50	>1, <5	6-8˚C		(Swedmark *et al.*, 1971)
anionic	LAS		fish	*Gadus morrhua*	acute	96	LC50	<1.0	15-17˚C		"
anionic	LAS		fish	*Pleuronectes flesus*	acute	96	LC50	<1.0	15-17˚C		"
anionic	LAS	12	fish	*Lepomis machrochirus*	acute	96	LC50	3.00		Fig. 1	(Swisher *et al.*, 1964)
anionic	LAS	14	fish	*Lepomis machrochirus*	acute	96	LC50	0.60		Fig. 1	"
anionic	LAS		fish	*Notropis atherinoides*	acute	96	LC50	3.30		Fig. 1	(Thatcher and Santner, 1967)
anionic	LAS		fish	*Lepomis machrochirus*	acute	96	LC50	4.00		Fig. 1	"
anionic	LAS		fish	*Pimephales promelas*	acute	96	LC50	4.20		Fig. 1	"
anionic	LAS		fish	*Notropis cornutus*	acute	96	LC50	4.90		Fig. 1	"
anionic	LAS		fish	*Ictalurus melas*	acute	96	LC50	6.40		Fig. 1	"
anionic	ABS		fish	*Lepomis gibbosus*	acute	96	LC50	22.00	soft water	Fig. 1	(Cairns *et al.*, 1964)
anionic	ABS		fish	*Lepomis machrochirus*	acute	96	LC50	17.00	soft water	Fig. 1	"
anionic	ABS	11 to 12	fish	*Salmo gairdneri*	acute	24	LC50	11.00			(Calamari and Marchetti, 1973)
anionic	ABS	11 to 12	fish	*Salmo gairdneri*	acute	96	LC50	10.80		Fig. 1	"
anionic	ABS	11 to 12	fish	*Salmo gairdneri*	chronic	14 d	LC50	10.70			"
anionic	ABS		fish	*Anguilla rostrata*	acute	96	LC50	2.30		Fig. 1	(Eisler, 1965)

anionic	ABS			fish	*Fundulus heteroclitus*	acute	96	LC50	6.80		Fig. 1	"
anionic	ABS			fish	*Menidia menidia*	acute	96	LC50	2.10		Fig. 1	"
anionic	ABS			fish	*Mugil cephalus*	acute	96	LC50	3.10		Fig. 1	"
anionic	ABS			fish	*Pseudopleuronectes americanus*	acute	96	LC50	2.50		Fig. 1	"
anionic	ABS			fish	*Lepomis machrochirus*	acute	24	LC50	8.20	soft water		(Henderson *et al.*, 1959)
anionic	ABS			fish	*Lepomis machrochirus*	acute	48	LC50	7.50	soft water		"
anionic	ABS			fish	*Lepomis machrochirus*	acute	96	LC50	5.60	soft water	Fig. 1	"
anionic	ABS			fish	*Pimephales promelas*	acute	24	LC50	4.80	soft water		"
anionic	ABS			fish	*Pimephales promelas*	acute	24	LC50	4.00	hard water		"
anionic	ABS			fish	*Pimephales promelas*	acute	48	LC50	4.50	soft water		"
anionic	ABS			fish	*Pimephales promelas*	acute	48	LC50	3.50	hard water		"
anionic	ABS			fish	*Pimephales promelas*	acute	96	LC50	4.50	soft water	Fig. 1	"
anionic	ABS			fish	*Pimephales promelas*	acute	96	LC50	3.50	hard water	Fig. 1	"
anionic	ABS			fish	*Gadus morrhua*	acute	96	LC50	3.50		Fig. 1	(Swedmark *et al.*, 1971)
anionic	ABS			fish	*Gadus morrhua*	acute	96	LC50	<1.0	15-17°C		"
anionic	ABS			fish	*Pleuronectes flesus*	acute	96	LC50	<1.0	15-17°C		"
anionic	ABS			fish	*Pleuronectus flesus*	acute	96	LC50	6.50		Fig. 1	"
anionic	ABS			fish	*Notropis atherinoides*	acute	96	LC50	7.40		Fig. 1	(Thatcher, 1966)
anionic	ABS			fish	*Lepomis machrochirus*	acute	96	LC50	8.20		Fig. 1	"
anionic	ABS			fish	*Campostoma anomalum*	acute	96	LC50	8.90		Fig. 1	"
anionic	ABS			fish	*Notropis stramineus*	acute	96	LC50	9.00		Fig. 1	"
anionic	ABS			fish	*Erycymba buccata*	acute	96	LC50	9.20		Fig. 1	"
anionic	ABS			fish	*Notropis ardens*	acute	96	LC50	9.50		Fig. 1	"
anionic	ABS			fish	*Pimephales promelas*	acute	96	LC50	11.30		Fig. 1	"
anionic	ABS			fish	*Notropis cornutus*	acute	96	LC50	17.00		Fig. 1	"
anionic	ABS			fish	*Cyprinis carpio*	acute	96	LC50	18.00		Fig. 1	"
anionic	ABS			fish	*Ictalurus melas*	acute	96	LC50	22.00		Fig. 1	"
anionic	AES	12 to 15	3	fish	*Carassius auratus*	acute	6	LC50	7.90			(Reiff *et al.*, 1979)
anionic	AES	12 to 15	3	fish	*Idus idus*	acute	48	LC50	7.20			"
anionic	AES	12 to 15	3	fish	*Idus idus*	acute	48	LC50	3.40			"
anionic	AES	12 to 15	3	fish	*Idus idus*	acute	48	LC50	5.70			"
anionic	AES	12 to 15	3	fish	*Rasbora heteromorpha*	acute	48	LC50	3.90			"
anionic	AES	12 to 15	3	fish	*Salmo trutta*	acute	48	LC50	1.4 to 2.1			"
anionic	AES	12 to 15	3	fish	*Salmo trutta*	acute	48	LC50	2.60			"

anionic	AES	12 to 15	3	fish	*Idus idus*	acute	96	LC50	6.20		Fig. 1	"
anionic	AES	12 to 15	3	fish	*Idus idus*	acute	96	LC50	3.30		Fig. 1	"
anionic	AES	12 to 15	3	fish	*Salmo trutta*	acute	96	LC50	1.50		Fig. 1	"
anionic	AES	12 to 15	3	fish	*Salmo trutta*	acute	96	LC50				"
anionic	AES	12	3	fish	*Gadus morrhua*	acute	96	LC50	<5	6-8˚C		(Swedmark *et al.*, 1971)
anionic	AES	12	3	fish	*Pleuronectes flesus*	acute	96	LC50	<5	6-8˚C		"
anionic	AOS	14 to 16		fish	*Carassius auratus*	acute	6	LC50	9.90			(Reiff *et al.*, 1979)
anionic	AOS	16 to 18		fish	*Carassius auratus*	acute	6	LC50	3.30			"
anionic	AOS	14 to 16		fish	*Idus idus*	acute	48	LC50	6.80			"
anionic	AOS	16 to 18		fish	*Idus idus*	acute	48	LC50	1.00			"
anionic	AOS	14 to 16		fish	*Idus idus*	acute	48	LC50	3.70			"
anionic	AOS	16 to 18		fish	*Idus idus*	acute	48	LC50	1.00			"
anionic	AOS	14 to 16		fish	*Idus idus*	acute	48	LC50	5.70			"
anionic	AOS	16 to 18		fish	*Idus idus*	acute	48	LC50	1.90			"
anionic	AOS	14 to 16		fish	*Rasbora heteromorpha*	acute	48	LC50	4.80			"
anionic	AOS	16 to 18		fish	*Rasbora heteromorpha*	acute	48	LC50	0.90			"
anionic	AOS	14 to 16		fish	*Salmo trutta*	acute	48	LC50	3.50			"
anionic	AOS	16 to 18		fish	*Salmo trutta*	acute	48	LC50	0.3 to 0.5			"
anionic	AOS	14 to 16		fish	*Salmo trutta*	acute	48	LC50	2.5 to 5.0			"
anionic	AOS	16 to 18		fish	*Salmo trutta*	acute	48	LC50	0.60			"
anionic	AOS	14 to 16		fish	*Idus idus*	acute	96	LC50	4.90		Fig. 1	"
anionic	AOS	14 to 16		fish	*Idus idus*	acute	96	LC50	3.40		Fig. 1	"
anionic	AOS	16 to 18		fish	*Idus idus*	acute	96	LC50	0.90		Fig. 1	"
anionic	AOS	14 to 16		fish	*Rasbora heteromorpha*	acute	96	LC50	3.30		Fig. 1	"
anionic	AOS	16 to 18		fish	*Rasbora heteromorpha*	acute	96	LC50	0.50		Fig. 1	"
anionic	AOS	14 to 16		fish	*Salmo trutta*	acute	96	LC50	3.10		Fig. 1	"
anionic	AOS	14 to 16		fish	*Salmo trutta*	acute	96	LC50	2.5 to 5.0			"
anionic	AOS	16 to 18		fish	*Salmo trutta*	acute	96	LC50	0.50		Fig. 1	"
anionic	AS			fish	*Pimephales promelas*	acute	24	LC50	5.90	soft water		(Henderson *et al.*, 1959)
anionic	AS			fish	*Pimephales promelas*	acute	24	LC50	6.10	hard water		"
anionic	AS			fish	*Pimephales promelas*	acute	48	LC50	5.10	soft water		"
anionic	AS			fish	*Pimephales promelas*	acute	48	LC50	5.90	hard water		"
anionic	AS			fish	*Pimephales promelas*	acute	96	LC50	5.10	soft water	Fig. 1	"
anionic	AS			fish	*Pimephales promelas*	acute	96	LC50	5.90	hard water	Fig. 1	"
anionic	AS			fish	*Pimephales promelas*	acute	96	LC50	5.10	soft water	Fig. 1	"

anionic	AS			fish	*Pimephales promelas*	acute	96	LC50	5.90	hard water	Fig. 1	"
anionic	AS	12		fish	*Cyprinodon variegatus*	acute	96	LC50	4.10		Fig. 1	(Roberts *et al.*, 1982)
anionic	AS	12		fish	*Menidia menidia*	acute	96	LC50	2.80		Fig. 1	"
nonionic	AE branched (4 methyls)	13	7	fish	*Pimephales promelas*	acute	96	LC50	6.10		Fig. 1	(Dorn *et al.*, 1993)
nonionic	AE branched (2 methyls)	13	7	fish	*Pimephales promelas*	acute	96	LC50	4.50		Fig. 1	"
nonionic	AE linear	12 to 15	9	fish	*Pimephales promelas*	acute	96	LC50	1.60		Fig. 1	"
nonionic	AE branched (4 methyls)	13	7	fish	*Pimephales promelas*	chronic	7 d	LC50	4.60			"
nonionic	AE branched (2 methyls)	13	7	fish	*Pimephales promelas*	chronic	7 d	LC50	1.80			"
nonionic	AE branched (4 methyls)	13	7	fish	*Pimephales promelas*	chronic	7 d	LOEC	2.00	growth		"
nonionic	AE branched (2 methyls)	13	7	fish	*Pimephales promelas*	chronic	7 d	LOEC	>1.0	growth		"
nonionic	AE branched (4 methyls)	13	7	fish	*Pimephales promelas*	chronic	7 d	NOEC	1.00	growth		"
nonionic	AE branched (2 methyls)	13	7	fish	*Pimephales promelas*	chronic	7 d	NOEC	1.00	growth		"
nonionic	AE linear	12 to 15	9	fish	*Pimephales promelas*	chronic	7 d	LC50	1.30			(Kravetz *et al.*, 1991; Dorn *et al.*, 1993)
nonionic	AE linear	12 to 15	9	fish	*Pimephales promelas*	chronic	7 d	LOEC	1.00	growth		"
nonionic	AE linear	12 to 15	9	fish	*Pimephales promelas*	chronic	7 d	NOEC	0.40	growth		"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	LC50	1.27	survival-mesocosm		(Dorn *et al.*, 1997)
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	NOEC	0.88	survival-mesocosm		"

nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	LOEC	1.99	survival-mesocosm	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	NOEC	0.88	coloration	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	NOEC	0.32	cleaning	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	NOEC	0.88	defense	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	NOEC	0.32	feeding	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	LOEC	>0.88	colouration	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	LOEC	0.88	cleaning	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	LOEC	>0.88	defense	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-cultured	chronic	30 d	LOEC	0.88	feeding	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	LC50	1.27	survival-mesocosm	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	NOEC	0.88	survival-mesocosm	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	LOEC	1.99	survival-mesocosm	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	NOEC	0.32	colouration	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	NOEC	0.88	cleaning	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	NOEC	0.88	defense	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	NOEC	0.32	feeding	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	LOEC	0.88	colouration	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	LOEC	>0.88	cleaning	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-	chronic	30 d	LOEC	>0.88	defense	"

					farmed							
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*-farmed	chronic	30 d	LOEC	0.88	feeding		"
nonionic	AE	12	2	fish	*Idus melanotus*	acute	1	LC50	1.90			(Gloxhuber and Fischer, 1968)
nonionic	AE	12	4	fish	*Idus melanotus*	acute	1	LC50	4.00			"
nonionic	AE	12	6	fish	*Idus melanotus*	acute	1	LC50	5.00			"
nonionic	AE	12	8	fish	*Idus melanotus*	acute	1	LC50	7.00			"
nonionic	AE	12	10	fish	*Idus melanotus*	acute	1	LC50	10.00			"
nonionic	AE	12	12	fish	*Idus melanotus*	acute	1	LC50	20.00			"
nonionic	AE	12	14	fish	*Idus melanotus*	acute	1	LC50	30.00			"
nonionic	AE	12	16	fish	*Idus melanotus*	acute	1	LC50	40.00			"
nonionic	AE	12	18	fish	*Idus melanotus*	acute	1	LC50	100.00			"
nonionic	AE	12	20	fish	*Idus melanotus*	acute	1	LC50	150.00			"
nonionic	AE	12 to 15	60% by weight	fish	*Lepomis machrochirus*	acute	24	LC50	2.80			(Hendricks *et al.*, 1974)
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	1 d	LC50	1.340	mortality		(Kline *et al.*, 1996)
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	2 d	LC50	0.720	mortality		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	3 d	LC50	0.660	mortality		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	4 d	LC50	0.650	mortality	Fig. 1	"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	5 d	LC50	0.650	mortality		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	6 d	LC50	0.650	mortality		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	7 d	LC50	0.590	mortality		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	8 d	LC50	0.590	mortality		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	9 d	LC50	0.590	mortality		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	10 d	LC50	0.590	mortality		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	10 d	NOEC	0.160	mortality &swimming performance		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	10 d	LOEC	0.460	mortality & swimming performance		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	30 d	NOEC	>0.330	survival-mesocosm		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	chronic	30 d	NOEC	>0.330	growth-mesocosm		"

nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	1 d	LC50	1.150	mortality		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	2 d	LC50	0.830	mortality		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	3 d	LC50	0.800	mortality		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	4 d	LC50	0.770	mortality	Fig. 1	"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	5 d	LC50	0.740	mortality		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	6 d	LC50	0.740	mortality		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	7 d	LC50	0.740	mortality		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	8 d	LC50	0.740	mortality		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	9 d	LC50	0.710	mortality		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	10 d	LC50	0.690	mortality		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	10 d	NOEC	0.160	mortality & swimming performance		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	10 d	LOEC	0.460	mortality & swimming performance		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	30 d	NOEC	0.280	survival-mesocosm		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	30 d	LOEC	0.330	survival-mesocosm		"
nonionic	AE	14 to 15	7	fish	*Pimephales promelas*	chronic	30 d	NOEC	>0.330	growth-mesocosm		"
nonionic	AE	14 to 15	7	fish	*Lepomis machrochirus*	acute	96	LC50	0.66	hardness 137	Fig. 1	(Lewis and Perry, 1981)
nonionic	AE	12 to 15	3	fish	*Lepomis machrochirus*	acute	24	LC50	1.80			(Macek and Krzeminski, 1975)
nonionic	AE	12 to 15	9	fish	*Lepomis machrochirus*	acute	24	LC50	2.10			"
nonionic	AE	10 to 12	6	fish	*Lepomis machrochirus*	acute	24	LC50	6.40			"
nonionic	AE	13	9	fish	*Lepomis machrochirus*	acute	24	LC50	7.80			"
nonionic	AE	11 to 15	9	fish	*Lepomis machrochirus*	acute	24	LC50	4.70			"
nonionic	AE	12 to 15	3	fish	*Lepomis machrochirus*	acute	96	LC50	1.50		Fig. 1	"
nonionic	AE	12 to 15	9	fish	*Lepomis machrochirus*	acute	96	LC50	2.10		Fig. 1	"
nonionic	AE	10 to 12	6	fish	*Lepomis machrochirus*	acute	96	LC50	6.40		Fig. 1	"
nonionic	AE	13	9	fish	*Lepomis machrochirus*	acute	96	LC50	7.50		Fig. 1	"
nonionic	AE	11 to 15	9	fish	*Lepomis machrochirus*	acute	96	LC50	4.60		Fig. 1	"
nonionic	AE	12	4	fish	*Carassius auratus*	acute	6	LC50	42.00	Alevin 0d		(Marchetti, 1965)
nonionic	AE	12	4	fish	*Carassius auratus*	acute	6	LC50	22.00	Alevin 6d		"

nonionic	AE	12	4	fish	*Carassius auratus*	acute	6	LC50	5.20	Alevin 12d		"
nonionic	AE	12	4	fish	*Carassius auratus*	acute	6	LC50	5.20	Alevin 19d		"
nonionic	AE	12	4	fish	*Carassius auratus*	acute	6	LC50	2.10	Transition stage 23d		"
nonionic	AE	12	4	fish	*Carassius auratus*	acute	6	LC50	2.30	Fry 25d		"
nonionic	AE	12	4	fish	*Carassius auratus*	acute	6	LC50	5.20	Fry 40d		"
nonionic	AE	12	4	fish	*Carassius auratus*	acute	6	LC50	5.20	Fingerling 210d		"
nonionic	AE tallow	16, 18	14	fish	*Carassius auratus*	acute	6	LC50	7.90			(Reiff *et al.*, 1979)
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Carassius auratus*	acute	6	LC50	4.30			"
nonionic	AE ziegler	12 to 14	8	fish	*Carassius auratus*	acute	6	LC50	1.80			"
nonionic	AE tallow	16, 18	14	fish	*Idus idus*	acute	48	LC50	2.60			"
nonionic	AE tallow	16, 18	14	fish	*Idus idus*	acute	48	LC50	2.70			"
nonionic	AE tallow	16, 18	14	fish	*Idus idus*	acute	48	LC50	2.10			"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Idus idus*	acute	48	LC50	4.50			"
nonionic	AE ziegler	12 to 14	8	fish	*Idus idus*	acute	48	LC50	2.70			"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Idus idus*	acute	48	LC50	4.60			"
nonionic	AE ziegler	12 to 14	8	fish	*Idus idus*	acute	48	LC50	1.80			"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Idus idus*	acute	48	LC50	3.00			"
nonionic	AE ziegler	12 to 14	8	fish	*Idus idus*	acute	48	LC50	1.40			"
nonionic	AE tallow	16, 18	14	fish	*Idus idus*	acute	96	LC50	2.30		Fig. 1	"
nonionic	AE tallow	16, 18	14	fish	*Idus idus*	acute	96	LC50	2.50		Fig. 1	"

nonionic	AE ziegler	12 to 14	10 to 11	fish	*Idus idus*	acute	96	LC50	4.10	Fig. 1	"
nonionic	AE ziegler	12 to 14	8	fish	*Idus idus*	acute	96	LC50	2.70	Fig. 1	"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Idus idus*	acute	96	LC50	4.50	Fig. 1	"
nonionic	AE ziegler	12 to 14	8	fish	*Idus idus*	acute	96	LC50	1.80	Fig. 1	"
nonionic	AE tallow	16, 18	14	fish	*Rasbora heteromorpha*	acute	48	LC50	0.80		"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Rasbora heteromorpha*	acute	48	LC50	1.6 to 2.8		"
nonionic	AE ziegler	12 to 14	8	fish	*Rasbora heteromorpha*	acute	48	LC50	1.20		"
nonionic	AE tallow	16, 18	14	fish	*Rasbora heteromorpha*	acute	96	LC50	0.70	Fig. 1	"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Rasbora heteromorpha*	acute	96	LC50	1.6 to 2.8		"
nonionic	AE tallow	16, 18	14	fish	*Salmo trutta*	acute	48	LC50	0.70		"
nonionic	AE tallow	16, 18	14	fish	*Salmo trutta*	acute	48	LC50	0.40		"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Salmo trutta*	acute	48	LC50	2.50		"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Salmo trutta*	acute	48	LC50	1.20		"
nonionic	AE ziegler	12 to 14	8	fish	*Salmo trutta*	acute	48	LC50	0.25 to 1.0		"
nonionic	AE tallow	16, 18	14	fish	*Salmo trutta*	acute	96	LC50	0.40	Fig. 1	"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Salmo trutta*	acute	96	LC50	1.80	Fig. 1	"
nonionic	AE ziegler	12 to 14	10 to 11	fish	*Salmo trutta*	acute	96	LC50	0.80	Fig. 1	"
nonionic	AE ziegler	12 to 14	8	fish	*Salmo trutta*	acute	96	LC50	0.80	Fig. 1	"
nonionic	AE	12 to 15	7	fish	*Pimephales promelas*	acute	96	LC50	0.48	Fig. 1	(Salanitro *et al.*, 1988)

nonionic	AE tallow		10	fish	*Gadus morrhua*	acute	96	LC50	0.5 to 1.0	6-8˚C		(Swedmark *et al.*, 1971)
nonionic	AE tallow		10	fish	*Pleuronectes flesus*	acute	96	LC50	0.5 to 1.0	6-8˚C		"
nonionic	AE	14 to 15	7	fish	*Brachydanio rerio*	acute	96	LC50	1.30			(Talmage, 1994)
nonionic	AE	12 to 13	5	fish	*Pimephales promelas*	acute	96	LC50	1.00		Fig. 1	(Wong *et al.*, 1997)
nonionic	AE	12 to 13	4.5 to 6	fish	*Pimephales promelas*	acute	96	LC50	0.96		Fig. 1	"
nonionic	AE	12 to 13	6.5	fish	*Pimephales promelas*	acute	96	LC50	1.30		Fig. 1	"
nonionic	AE	9 to 11	6	fish	*Pimephales promelas*	acute	96	LC50	8.50		Fig. 1	"
nonionic	AE	9 to 11	8	fish	*Pimephales promelas*	acute	96	LC50	11.00		Fig. 1	"
nonionic	AE	11	7	fish	*Pimephales promelas*	acute	96	LC50	3.90		Fig. 1	"
nonionic	AE	11	9	fish	*Pimephales promelas*	acute	96	LC50	7.10		Fig. 1	"
nonionic	AE	12 to 15	12	fish	*Pimephales promelas*	acute	96	LC50	1.40		Fig. 1	"
nonionic	AE	14 to 15	13	fish	*Pimephales promelas*	acute	96	LC50	1.00		Fig. 1	"
nonionic	NPE		8	fish	*Salmo gairdneri*	acute	24	LC50	5.50			(Calamari and Marchetti, 1973)
nonionic	NPE		8	fish	*Salmo gairdneri*	acute	96	LC50	4.70		Fig. 1	"
nonionic	NPE		8	fish	*Salmo gairdneri*	chronic	14 d	LC50	4.25			"
nonionic	NPE branched		9	fish	*Pimephales promelas*	acute	96	LC50	4.60		Fig. 1	(Kravetz *et al.*, 1991; Dorn *et al.*, 1993)
nonionic	NPE branched		9	fish	*Pimephales promelas*	chronic	7 d	LC50	2.90			"
nonionic	NPE branched		9	fish	*Pimephales promelas*	chronic	7 d	LOEC	2.00	growth		"
nonionic	NPE branched		9	fish	*Pimephales promelas*	chronic	7 d	NOEC	1.00	growth		"
nonionic	NPE		4	fish	*Lepomis machrochirus*	acute	24	LC50	1.50			(Macek and Krzeminski, 1975)
nonionic	NPE		5	fish	*Lepomis machrochirus*	acute	24	LC50	2.80			"
nonionic	NPE		9	fish	*Lepomis machrochirus*	acute	24	LC50	7.80			"
nonionic	NPE		9	fish	*Lepomis machrochirus*	acute	24	LC50	8.90			"
nonionic	NPE		30	fish	*Lepomis machrochirus*	acute	24	LC50	>1000			"
nonionic	OPE		4 to 5	fish	*Lepomis machrochirus*	acute	24	LC50	3.50			"
nonionic	OPE		10	fish	*Lepomis machrochirus*	acute	24	LC50	16.20			"
nonionic	OPE		30	fish	*Lepomis machrochirus*	acute	24	LC50	1080.00			"
nonionic	NPE		4	fish	*Lepomis machrochirus*	acute	96	LC50	1.30		Fig. 1	"

nonionic	NPE	9	fish	*Lepomis machrochirus*	acute	96	LC50	7.60		Fig. 1	"
nonionic	NPE	9	fish	*Lepomis machrochirus*	acute	96	LC50	7.60		Fig. 1	"
nonionic	NPE	30	fish	*Lepomis machrochirus*	acute	96	LC50	>1000			"
nonionic	OPE	10	fish	*Lepomis machrochirus*	acute	96	LC50	12.00		Fig. 1	"
nonionic	OPE	30	fish	*Lepomis machrochirus*	acute	96	LC50	531.00			"
nonionic	NPE	7	fish	*Pimephales promelas*	acute	96	LC50	3.20		Fig. 1	(Markarian *et al.*, 1989)
nonionic	NPE	9 to 10	fish	*Carassius auratus*	acute	6	LC50	6.90			(Reiff *et al.*, 1979)
nonionic	NPE	9 to 10	fish	*Idus idus*	acute	48	LC50	7.40			"
nonionic	NPE	9 to 10	fish	*Idus idus*	acute	48	LC50	11.30			"
nonionic	NPE	9 to 10	fish	*Idus idus*	acute	48	LC50	4.90			"
nonionic	NPE	9 to 10	fish	*Idus idus*	acute	96	LC50	7.00		Fig. 1	"
nonionic	NPE	9 to 10	fish	*Idus idus*	acute	96	LC50	11.20		Fig. 1	"
nonionic	NPE	9 to 10	fish	*Rasbora heteromorpha*	acute	48	LC50	11.30			"
nonionic	NPE	9 to 10	fish	*Rasbora heteromorpha*	acute	96	LC50	8.60		Fig. 1	"
nonionic	NPE	9 to 10	fish	*Salmo trutta*	acute	48	LC50	2.70			"
nonionic	NPE	9 to 10	fish	*Salmo trutta*	acute	96	LC50	1.00		Fig. 1	"
nonionic	NPE	7	fish	*Pimephales promelas*	acute	96	LC50	1.62		Fig. 1	(Salanitro *et al.*, 1988)
	branched										
nonionic	NPE	10	fish	*Gadus morrhua*	acute	96	LC50	6.00	6-8˚C	Fig. 1	(Swedmark *et al.*, 1971)
nonionic	NPE	10	fish	*Gadus morrhua*	acute	96	LC50	2.50	15-17˚C	Fig. 1	"
nonionic	NPE	10	fish	*Pleuronectes flesus*	acute	96	LC50	3.00	15-17˚C	Fig. 1	"
nonionic	NPE	1	fish	*Orizias latipse*	acute	48	LC50	3.00			(Yoshimura, 1986)
nonionic	NPE	3.3	fish	*Orizias latipse*	acute	48	LC50	2.50			"
nonionic	NPE	5	fish	*Orizias latipse*	acute	48	LC50	3.60			"
nonionic	NPE	6.4	fish	*Orizias latipse*	acute	48	LC50	5.40			"
nonionic	NPE	8.4	fish	*Orizias latipse*	acute	48	LC50	11.60			"
nonionic	NPE	8.9	fish	*Orizias latipse*	acute	48	LC50	11.20			"
nonionic	NPE	13.1	fish	*Orizias latipse*	acute	48	LC50	48.00			"
nonionic	NPE	16.6	fish	*Orizias latipse*	acute	48	LC50	110.00			"
anionic	LAS		invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	4.81		Fig. 1	(Ankley *et al.*, 1990)
anionic	LAS		invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	4.83		Fig. 1	"
anionic	LAS		invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	4.61		Fig. 1	"
anionic	LAS		invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	4.21		Fig. 1	"
anionic	LAS		invertebrate	*Campeloma decisum*	acute	96	LC50	27.00			(Arthur, 1970)
anionic	LAS		invertebrate	*Gammarus pseudolimnaes*	acute	96	LC50	7.00			"

anionic	LAS		invertebrate	*Physa integra*	acute	96	LC50	9.00			"
anionic	LAS	12	invertebrate	*Daphnia magna*	acute	48	LC50	4.40		Fig. 1	(Bishop and Perry, 1981)
anionic	LAS		invertebrate	*Daphnia magna*	chronic	21 d	LC50	18.00	mortality		(Canton and Slooff, 1982)
anionic	LAS		invertebrate	*Daphnia magna*	chronic	21 d	LC50	>10	reproduction		"
anionic	LAS		invertebrate	*Daphnia magna*	chronic	21 d	NOEC	10.00	mortality		"
anionic	LAS		invertebrate	*Daphnia magna*	chronic	21 d	NOEC	>10	reproduction		"
anionic	LAS		invertebrate	*Tisbe bulbisetosa*	acute	48	LC50	5.12	36 ppt salinity		(Dalla Venezia *et al.*, 1980)
anionic	LAS		invertebrate	*Tisbe bulbisetosa*	acute	48	LC50	4.58	18 ppt salinity		"
anionic	LAS	12	invertebrate	*Isonychia sp.*	acute	96	LC50	5.30			(Dolan *et al.*, 1974)
anionic	LAS	12	invertebrate	*Goniobasis sp.*	acute	24	LC50	19.40			(Hendricks *et al.*, 1974)
anionic	LAS	13	invertebrate	*Goniobasis sp.*	acute	24	LC50	92.00			"
anionic	LAS		invertebrate	*Penaeus monodon* (larvae, zoea 2nd substage)	acute	24	LC50	0.06			(Hwang *et al.*, 1993)
anionic	LAS		invertebrate	*Penaeus monodon* (larvae, mysis 2nd substage)	acute	24	LC50	0.10			"
anionic	LAS		invertebrate	*Penaeus monodon* (postlarva, 12th substage)	acute	24	LC50	3.11			"
anionic	LAS		invertebrate	*Penaeus monodon* (larvae mysis 2nd substage)	acute	48	LC50	0.07			"
anionic	LAS		invertebrate	*Penaeus monodon* (postlarva substage 12)	acute	48	LC50	1.03		Fig. 1	"
anionic	LAS		invertebrate	*Penaeus monodon* (postlarva substage 15)	acute	48	LC50	4.36		Fig. 1	"
anionic	LAS	10	invertebrate	*Daphnia magna*	acute	24	LC50	53.10			(Kimerle and Swisher, 1977)
anionic	LAS	11	invertebrate	*Daphnia magna*	acute	24	LC50	15.80			"
anionic	LAS	12	invertebrate	*Daphnia magna*	acute	24	LC50	10.70			"
anionic	LAS	13	invertebrate	*Daphnia magna*	acute	24	LC50	2.70			"
anionic	LAS	14	invertebrate	*Daphnia magna*	acute	24	LC50	1.20			"
anionic	LAS branched	10	invertebrate	*Daphnia magna*	acute	24	LC50	106.00			"
anionic	LAS branched	12	invertebrate	*Daphnia magna*	acute	24	LC50	55.10			"
anionic	LAS branched	14	invertebrate	*Daphnia magna*	acute	24	LC50	12.40			"

anionic	LAS	10	invertebrate	*Daphnia magna*	acute	48	LC50	12.30		Fig. 1	"
anionic	LAS	11	invertebrate	*Daphnia magna*	acute	48	LC50	5.70		Fig. 1	"
anionic	LAS	12	invertebrate	*Daphnia magna*	acute	48	LC50	3.50		Fig. 1	"
anionic	LAS	13	invertebrate	*Daphnia magna*	acute	48	LC50	2.00		Fig. 1	"
anionic	LAS	14	invertebrate	*Daphnia magna*	acute	48	LC50	0.70		Fig. 1	"
anionic	LAS branched	10	invertebrate	*Daphnia magna*	acute	48	LC50	98.00		Fig. 1	"
anionic	LAS branched	12	invertebrate	*Daphnia magna*	acute	48	LC50	34.10		Fig. 1	"
anionic	LAS branched	14	invertebrate	*Daphnia magna*	acute	48	LC50	10.00		Fig. 1	"
anionic	LAS	11.8	invertebrate	*Daphnia magna*	acute	48	LC50	5.63	hardness 35	Fig. 1	(Lewis and Perry, 1981)
anionic	LAS	11.8	invertebrate	*Daphnia magna*	acute	48	LC50	2.97	hardness 181	Fig. 1	"
anionic	LAS	11.8	invertebrate	*Daphnia magna*	acute	48	LC50	2.71	hardness 340	Fig. 1	"
anionic	LAS	11.8	invertebrate	*Asellus sp.*	acute	48	LC50	270.00			(Lewis and Suprenant, 1983)
anionic	LAS	11.8	invertebrate	*Dero sp.*	acute	48	LC50	1.70			"
anionic	LAS	11.8	invertebrate	*Dugesia sp.*	acute	48	LC50	1.80			"
anionic	LAS	11.8	invertebrate	*Gammarus sp.*	acute	48	LC50	3.30		Fig. 1	"
anionic	LAS	11.8	invertebrate	*Paratanytarsus parthenogenica*	acute	48	LC50	23.00			"
anionic	LAS	11.8	invertebrate	*Rhabditis sp*	acute	48	LC50	16.00			"
anionic	LAS	10	invertebrate	*Daphnia magna*	acute	48	LC50	29.50		Fig. 1	(Maki and Bishop, 1979)
anionic	LAS	11	invertebrate	*Daphnia magna*	acute	48	LC50	21.10		Fig. 1	"
anionic	LAS	12	invertebrate	*Daphnia magna*	acute	48	LC50	5.88		Fig. 1	"
anionic	LAS	13	invertebrate	*Daphnia magna*	acute	48	LC50	2.63		Fig. 1	"
anionic	LAS	14	invertebrate	*Daphnia magna*	acute	48	LC50	0.68		Fig. 1	"
anionic	LAS	16	invertebrate	*Daphnia magna*	acute	48	LC50	0.11		Fig. 1	"
anionic	LAS	18	invertebrate	*Daphnia magna*	acute	48	LC50	0.12		Fig. 1	"
anionic	LAS	11	invertebrate	*Daphnia magna*	acute	48	LC50	19.30	no sediment	Fig. 1	"
anionic	LAS	14	invertebrate	*Daphnia magna*	acute	48	LC50	1.00	no sediment	Fig. 1	"
anionic	LAS	18	invertebrate	*Daphnia magna*	acute	48	LC50	0.09	no sediment	Fig. 1	"
anionic	LAS	11	invertebrate	*Daphnia magna*	acute	48	LC50	13.00	sediment		"
anionic	LAS	14	invertebrate	*Daphnia magna*	acute	48	LC50	1.40	sediment		"
anionic	LAS	18	invertebrate	*Daphnia magna*	acute	48	LC50	0.18	sediment		"

anionic	LAS	12	invertebrate	*Daphnia pulex*	acute	48	LC50	8.62		Fig. 1	"
anionic	LAS	14	invertebrate	*Daphnia pulex*	acute	48	LC50	0.59		Fig. 1	"
anionic	LAS	16	invertebrate	*Daphnia pulex*	acute	48	LC50	0.15		Fig. 1	"
anionic	LAS	10	invertebrate	*Daphnia magna*	acute	24	LC50	55.00			(Moreno-Danvila, 1983)
anionic	LAS	11	invertebrate	*Daphnia magna*	acute	24	LC50	30.00			"
anionic	LAS	12	invertebrate	*Daphnia magna*	acute	24	LC50	8.00			"
anionic	LAS	13	invertebrate	*Daphnia magna*	acute	24	LC50	2.00			"
anionic	LAS	14	invertebrate	*Daphnia magna*	acute	24	LC50	0.10			"
anionic	LAS		invertebrate	*Mya arenaria*	acute	96	LC50	70	6-8˚C		(Swedmark *et al.*, 1971)
anionic	LAS		invertebrate	*Mytilus edulis*	acute	96	LC50	>100	6-8˚C		"
anionic	LAS		invertebrate	*Cardium edule*	acute	96	LC50	15	6-8˚C		"
anionic	LAS		invertebrate	*Pecten maximus*	acute	96	LC50	<5	6-8˚C		"
anionic	LAS		invertebrate	*Leander adspersus*	acute	96	LC50	50	6-8˚C		"
anionic	LAS		invertebrate	*Leander squilla*	acute	96	LC50	>100	6-8˚C		"
anionic	LAS		invertebrate	*Eupagurus bernhardus*	acute	96	LC50	>100	6-8˚C		"
anionic	LAS		invertebrate	*Hyas araneus* (adult)	acute	96	LC50	>100	6-8˚C		"
anionic	LAS		invertebrate	*Hyas araneus* (larvae)	acute	96	LC50	9	6-8˚C		"
anionic	LAS		invertebrate	*Carcinus maenas*	acute	96	LC50	>100	6-8˚C		"
anionic	LAS		invertebrate	*Balanus balanoides* (adult)	acute	96	LC50	50	6-8˚C		"
anionic	LAS		invertebrate	*Balanus balanoides* (larvae)	acute	96	LC50	3	6-8˚C		"
anionic	LAS		invertebrate	*Mya arenaria*	acute	96	LC50	<25	15-17˚C		"
anionic	LAS		invertebrate	*Mytilus edulis*	acute	96	LC50	50	15-17˚C		"
anionic	LAS		invertebrate	*Cardium edule*	acute	96	LC50	<5	15-17˚C		"
anionic	LAS		invertebrate	*Pecten opercularis*	acute	96	LC50	<5	15-17˚C		"
anionic	LAS		invertebrate	*Leander adspersus* (intermoult)	acute	96	LC50	50	15-17˚C		"
anionic	LAS		invertebrate	*Leander adspersus* (postmoult)	acute	96	LC50	25	15-17˚C		"
anionic	LAS	10 to 13	invertebrate	*Biomphalaria glabrata*	acute	24	LC100	3.00			(van Emden *et al.*, 1974)
anionic	LAS	10 to 15	invertebrate	*Biomphalaria glabrata*	acute	24	LC100	1.00			"
anionic	LAS	10 to 13	invertebrate	*Aëdes aegypti*	acute	24	LC50	6.00			"
anionic	LAS	10 to 15	invertebrate	*Aëdes aegypti*	acute	24	LC50	2.00			"
anionic	LAS	12 to 13	invertebrate	*Ceriodaphnia cf dubia*	acute	48	LC50	3.24		Fig. 1	(Warne and Shifko, 1999)
anionic	LAS	12 to 13	invertebrate	*Ceriodaphnia cf dubia*	acute	48	LC50	5.96		Fig. 1	"

anionic	LAS	12 to 13		invertebrate	*Ceriodaphnia cf dubia*	acute	48	LC50	5.65		Fig. 1	"
anionic	LAS	12 to 13		invertebrate	*Ceriodaphnia cf dubia*	acute	48	LC50	7.81		Fig. 1	"
anionic	ABS	12		invertebrate	*Daphnia magna*	acute	20	LC100	30.00			(Degens *et al.*, 1950)
anionic	ABS			invertebrate	*Asellus sp.*	acute		LC100	10.00			(Mann, 1955)
anionic	ABS			invertebrate	*Carinogammarus sp.*	acute		LC100	10.00			"
anionic	ABS			invertebrate	*Chironomus sp.*	acute		LC100	100.00			"
anionic	ABS			invertebrate	*Tubifex sp.*	acute		LC100	2.50			"
anionic	ABS			invertebrate	*Physa heterostropha*	acute	96	LC50	34.20			(Patrick *et al.*, 1968)
anionic	ABS			invertebrate	*Carcinus maenas*	acute	96	LC50	>100	6-8˚C		(Swedmark *et al.*, 1971)
anionic	ABS			invertebrate	*Cardium edule*	acute	96	LC50	20	6-8˚C		"
anionic	ABS			invertebrate	*Eupagurus bernhardus*	acute	96	LC50	>100	6-8˚C		"
anionic	ABS			invertebrate	*Hyas araneus* (adult)	acute	96	LC50	>100	6-8˚C		"
anionic	ABS			invertebrate	*Leander adspersus*	acute	96	LC50	>100	6-8˚C		"
anionic	ABS			invertebrate	*Leander squilla*	acute	96	LC50	>100	6-8˚C		"
anionic	ABS			invertebrate	*Mytilus edulis*	acute	96	LC50	>100	6-8˚C		"
anionic	ABS /LAS	12		invertebrate	*Asellus aquaticus*	acute	96	LC50	74.00			(Strezlecka and Hubner, 1978)
anionic	ABS /LAS	12		invertebrate	*Daphnia magna*	acute	96	LC50	45.00			"
anionic	ABS /LAS	12		invertebrate	*Oligochaeta*	acute	96	LC50	24.00			"
anionic	AES	14 to 15	2.25	invertebrate	*Daphnia magna*	acute	96	LC50	1.89			(A D Little Co., 1977)
anionic	AES	15 to 15	2.25	invertebrate	*Gammarus sp*	acute	96	LC50	4.54			"
anionic	AES	12	3	invertebrate	*Daphnia magna*	acute	24	LC50	37.00			(Lundahl *et al.*, 1972)
anionic	AES	12 to 14	2.2	invertebrate	*Daphnia magna*	acute	24	LC50	21.00			"
anionic	AES	12 to 14	2	invertebrate	*Daphnia magna*	acute	24	LC50	16.30			(Maki and Bishop, 1979)
anionic	AES	10 to 11	2	invertebrate	*Daphnia magna*	acute	24	LC50	19.60			"
anionic	AES			invertebrate	*Daphnia pulex*	acute	48	LC50	24.61		Fig. 1	(Moore *et al.*, 1987)
anionic	AES			invertebrate	*Daphnia pulex*	acute	48	LC50	15.81		Fig. 1	"
anionic	AES	12	3	invertebrate	*Balanus balanoides (larvae)*	acute	96	LC50	5	6-8˚C		(Swedmark *et al.*, 1971)
anionic	AES	12	3	invertebrate	*Carcinus maenas*	acute	96	LC50	>100	6-8˚C		"
anionic	AES	12	3	invertebrate	*Cardium edule*	acute	96	LC50	50	6-8˚C		"
anionic	AES	12	3	invertebrate	*Eupagurus bernhardus*	acute	96	LC50	>100	6-8˚C		"
anionic	AES	12	3	invertebrate	*Hyas araneus* (adult)	acute	96	LC50	>100	6-8˚C		"

anionic	AES	12	3	invertebrate	*Hyas araneus* (larvae)	acute	96	LC50	>1000	6-8˚C		"
anionic	AES	12	3	invertebrate	*Leander adspersus*	acute	96	LC50	>100	6-8˚C		"
anionic	AES	12	3	invertebrate	*Leander squilla*	acute	96	LC50	>100	6-8˚C		"
anionic	AES	12	3	invertebrate	*Mya arenaria*	acute	96	LC50	50	6-8˚C		"
anionic	AES	12	3	invertebrate	*Mytilus edulis*	acute	96	LC50	>100	6-8˚C		"
anionic	AES		3	invertebrate	*Aëdes aegypti*	acute	24	LC50	11.00			(van Emden *et al.*, 1974)
anionic	AES		3	invertebrate	*Biomphalaria glabrata*	acute	24	LC100	12.00			"
anionic	AES	12 to 14	2	invertebrate	*Ceriodaphnia* cf *dubia*	acute	48	LC50	3.12		Fig. 1	(Warne and Shifko, 1999)
anionic	AES	12 to 16	2.7	invertebrate	*Ceriodaphnia* cf *dubia*	acute	48	LC50	3.43		Fig. 1	"
anionic	AOS	14 to 16		invertebrate	*Ceriodaphnia* cf *dubia*	acute	48	LC50	4.53		Fig. 1	"
anionic	AS	12		invertebrate	*Daphnia magna*	acute	46	LC50	1.80		Fig. 1	(Bishop and Perry, 1981)
anionic	AS			invertebrate	*Daphnia magna*	acute	20	LC100	2.50			(Degens *et al.*, 1950)
anionic	AS	12		invertebrate	*Daphnia pulex*	acute	48	LC50	27.04		Fig. 1	(Moore *et al.*, 1987)
anionic	AS	12		invertebrate	*Daphnia pulex*	acute	48	LC50	7.07		Fig. 1	"
anionic	AS	12		invertebrate	*Daphnia pulex*	acute	48	LC50	49.38		Fig. 1	"
anionic	AS	12		invertebrate	*Acartia tonsa*	acute	96	LC50	0.55			(Roberts *et al.*, 1982)
anionic	AS	12		invertebrate	*Eurytemora affinis*	acute	96	LC50	2.60			"
anionic	AS	12		invertebrate	*Mysidopsis bahia*	acute	96	LC50	6.10			"
anionic	AS	12		invertebrate	*Mysidopsis bahia*	acute	96	LC50	7.10			"
anionic	AS	12		invertebrate	*Neomysis americana*	acute	96	LC50	5.70			"
anionic	AS	12		invertebrate	*Neomysis americana*	acute	96	LC50	8.80			"
anionic	DDBSA			invertebrate	*Daphnia pulex*	acute	48	LC50	19.87		Fig. 1	(Moore *et al.*, 1987)
anionic	TAES		3	invertebrate	*Daphnia magna*	acute	96	LC50	0.7 to 2.47			(A D Little Co., 1977)
anionic	TAES		3	invertebrate	*Gammarus sp*	acute	96	LC50	29.40			"
nonionic	AE	12 to 18	7.4	invertebrate	*Daphnia magna*	acute	24	LC50	3.30			"
nonionic	AE	12 to 18	14	invertebrate	*Daphnia magna*	acute	24	LC50	1.10			"
nonionic	AE	12 to 18	11	invertebrate	*Daphnia magna*	acute	24	LC50	5.10			"
nonionic	AE	14	8	invertebrate	*Daphnia magna*	acute	24	LC50	2.00			"
nonionic	AE	14	6	invertebrate	*Daphnia magna*	acute	96	LC50	0.21			"
nonionic	AE	14	6	invertebrate	*Gammarus sp*	acute	96	LC50	2.07			"
nonionic	AE linear			invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	2.14		Fig. 1	(Ankley *et al.*, 1990)
nonionic	AE linear			invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	1.59		Fig. 1	"
nonionic	AE linear			invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	1.23		Fig. 1	"
nonionic	AE linear			invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	1.38		Fig. 1	"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	acute	48	LC50	0.70		Fig. 1	(Bishop and Perry, 1981)

nonionic	AE branched (4 methyls)	13	7	invertebrate	*Daphnia magna*	acute	48	EC50	14.00	immobilisation	Fig. 1	(Dorn *et al.*, 1993)
nonionic	AE branched (2 methyls)	13	7	invertebrate	*Daphnia magna*	acute	48	EC50	9.80	immobilisation	Fig. 1	"
nonionic	AE linear	12 to 15	9	invertebrate	*Daphnia magna*	acute	48	EC50	1.30	immobilisation	Fig. 1	"
nonionic	AE branched (4 methyls)	13	7	invertebrate	*Daphnia magna*	chronic	7 d	EC50	>6.0	immobilisation		"
nonionic	AE branched (2 methyls)	13	7	invertebrate	*Daphnia magna*	chronic	7 d	EC50	3.00	immobilisation		"
nonionic	AE branched (4 methyls)	13	7	invertebrate	*Daphnia magna*	chronic	7 d	LOEC	>4.0	growth		"
nonionic	AE branched (2 methyls)	13	7	invertebrate	*Daphnia magna*	chronic	7 d	LOEC	>2.0	growth		"
nonionic	AE branched (4 methyls)	13	7	invertebrate	*Daphnia magna*	chronic	7 d	NOEC	4.00	growth		"
nonionic	AE branched (2 methyls)	13	7	invertebrate	*Daphnia magna*	chronic	7 d	NOEC	2.00	growth		"
nonionic	AE linear	12 to 15	9	invertebrate	*Daphnia magna*	chronic	7 d	EC50	1.40	immobilisation		(Kravetz *et al.*, 1991; Dorn *et al.*, 1993)
nonionic	AE linear	12 to 15	9	invertebrate	*Daphnia magna*	chronic	7 d	LOEC	>1.0	growth		"
nonionic	AE linear	12 to 15	9	invertebrate	*Daphnia magna*	chronic	7 d	NOEC	1.00	growth		"
nonionic	AE	12 to 13	6.5	invertebrate	Cladocera	chronic	30 d	NOEC	1.99	pop. density-mesocosm		(Dorn *et al.*, 1997)
nonionic	AE	12 to 13	6.5	invertebrate	Cladocera	chronic	30 d	LOEC	5.15	pop. density-mesocosm		"
nonionic	AE	12 to 13	6.5	invertebrate	Copepoda	chronic	30 d	NOEC	1.99	pop. density-mesocosm		"
nonionic	AE	12 to 13	6.5	invertebrate	Copepoda	chronic	30 d	LOEC	5.15	pop. density-mesocosm		"

nonionic	AE	12 to 13	6.5	invertebrate	inertebrates-general	chronic	30 d	NOEC	<0.32	pop. density-mesocosm	"
nonionic	AE	12 to 13	6.5	invertebrate	inertebrates-general	chronic	30 d	LOEC	0.32	pop. density-mesocosm	"
nonionic	AE	12 to 13	6.5	invertebrate	Simuliidae	chronic	30 d	NOEC	<0.32	pop. density-mesocosm	"
nonionic	AE	12 to 13	6.5	invertebrate	Simuliidae	chronic	30 d	LOEC	0.32	pop. density-mesocosm	"
nonionic	AE	12 to 13	6.5	invertebrate	Copepoda, Cladocera	chronic	30 d	LOEC	5.15	pop. density-mesocosm	(Gillespie *et al.*, 1996)
nonionic	AE	14 to 15	7	invertebrate	inertebrates-general	chronic	28-30 d	NOEC	0.33	pop. density-mesocosm	"
nonionic	AE	14 to 15	7	invertebrate	Simuliidae	chronic	28-30 d	NOEC	0.08	pop. density-mesocosm	"
nonionic	AE	14 to 15	7	invertebrate	Simuliidae	chronic	28-30 d	LOEC	0.16	pop. density-mesocosm	"
nonionic	AE	12 to 13	6.5	invertebrate	Simuliidae	chronic	30 d	LOEC	0.32	pop. density-mesocosm	"
nonionic	AE	9 to 11	6	invertebrate	inertebrates-general	chronic	30 d	LOEC	4.35	pop. density-mesocosm	(Gillespie *et al.*, 1997)
nonionic	AE	9 to 11	6	invertebrate	*Daphnia magna*	chronic	21 d	IC50	4.00	reproduction	(Gillespie *et al.*, 1999)
nonionic	AE	12 to 13	6.5	invertebrate	*Daphnia magna*	chronic	21 d	IC50	1.30	reproduction	"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	chronic	21 d	IC50	1.40	reproduction	"
nonionic	AE	9 to 11	6	invertebrate	*Daphnia magna*	chronic	21 d	LC50	5.90	survival	"
nonionic	AE	12 to 13	6.5	invertebrate	*Daphnia magna*	chronic	21 d	LC50	2.20	survival	"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	chronic	21 d	LC50	1.20	survival	"
nonionic	AE	9 to 11	6	invertebrate	*Daphnia magna*	chronic	21 d	LOEC	5.57	survival	"
nonionic	AE	12 to 13	6.5	invertebrate	*Daphnia magna*	chronic	21 d	LOEC	2.69	survival	"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	chronic	21 d	LOEC	1.02	survival	"
nonionic	AE	9 to 11	6	invertebrate	*Daphnia magna*	chronic	21 d	LOEC	2.77	reproduction	"
nonionic	AE	12 to 13	6.5	invertebrate	*Daphnia magna*	chronic	21 d	LOEC	1.75	reproduction	"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	chronic	21 d	LOEC	0.79	reproduction	"
nonionic	AE	9 to 11	6	invertebrate	*Daphnia magna*	chronic	21 d	NOEC	2.77	survival	"
nonionic	AE	12 to 13	6.5	invertebrate	*Daphnia magna*	chronic	21 d	NOEC	1.75	survival	"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	chronic	21 d	NOEC	0.79	survival	"
nonionic	AE	9 to 11	6	invertebrate	*Daphnia magna*	chronic	21 d	NOEC	2.77	reproduction	"

nonionic	AE	12 to 13	6.5	invertebrate	*Daphnia magna*	chronic	21 d	NOEC	0.77	reproduction		"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	chronic	21 d	NOEC	0.79	reproduction		"
nonionic	AE	10	4	invertebrate	*Mysidopsis bahia*	acute	48	LC50	5.57		Fig. 1	(Hall *et al.*, 1989)
nonionic	AE	13	9.75	invertebrate	*Mysidopsis bahia*	acute	48	LC50	2.24		Fig. 1	"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	acute	48	LC50	0.34	hardness 35	Fig. 1	(Lewis and Perry, 1981)
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	acute	48	LC50	0.29	hardness 181	Fig. 1	"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	acute	48	LC50	0.40	hardness 340	Fig. 1	"
nonionic	AE	16 to 15	7	invertebrate	*Asellus sp.*	acute	48	LC50	6.20		Fig. 1	(Lewis and Suprenant, 1983)
nonionic	AE	18 to 15	7	invertebrate	*Dero sp.*	acute	48	LC50	2.60			"
nonionic	AE	17 to 15	7	invertebrate	*Dugesia sp.*	acute	48	LC50	1.00			"
nonionic	AE	15 to 15	7	invertebrate	*Gammarus sp.*	acute	48	LC50	1.40		Fig. 1	"
nonionic	AE	14 to 15	7	invertebrate	*Paratanytarsus parthenogenica*	acute	48	LC50	5.00			"
nonionic	AE	19 to 15	7	invertebrate	*Rhabditis sp*	acute	48	LC50	6.80			"
nonionic	AE	14	1	invertebrate	*Daphnia magna*	acute	48	LC50	0.83		Fig. 1	(Maki and Bishop, 1979)
nonionic	AE	14	2	invertebrate	*Daphnia magna*	acute	48	LC50	1.53		Fig. 1	"
nonionic	AE	14	3	invertebrate	*Daphnia magna*	acute	48	LC50	0.73		Fig. 1	"
nonionic	AE	14	4	invertebrate	*Daphnia magna*	acute	48	LC50	1.76		Fig. 1	"
nonionic	AE	14	6	invertebrate	*Daphnia magna*	acute	48	LC50	4.19		Fig. 1	"
nonionic	AE	14	9	invertebrate	*Daphnia magna*	acute	48	LC50	10.07		Fig. 1	"
nonionic	AE	14	1	invertebrate	*Daphnia magna*	acute	48	LC50	0.14		Fig. 1	"
nonionic	AE	14	4	invertebrate	*Daphnia magna*	acute	48	LC50	0.24		Fig. 1	"
nonionic	AE	14	1	invertebrate	*Daphnia pulex*	acute	48	LC50	0.10		Fig. 1	"
nonionic	AE	14	4	invertebrate	*Daphnia pulex*	acute	48	LC50	0.21		Fig. 1	"
nonionic	AE	9 to 11	6	invertebrate	*Daphnia pulex*	acute	48	LC50	5.36		Fig. 1	(Moore *et al.*, 1987)
nonionic	AE	12 to 15	7	invertebrate	*Daphnia pulex*	acute	48	LC50	0.76		Fig. 1	(Salanitro *et al.*, 1988)
nonionic	AE	12 to 15	9	invertebrate	*Carcinus maenas*	acute	6	LC50	>100			(Shell Chemical Co., 1983; Talmage, 1994)
nonionic	AE	12 to 15	9	invertebrate	*Carcinus maenas*	acute	48	LC50	>100			"
nonionic	AE	12 to 15	9	invertebrate	*Carcinus maenas*	acute	48	LC50	>100			"
nonionic	AE	12 to 15	9	invertebrate	*Crangon crangon*	acute	48	LC50	>3300			"
nonionic	AE	14 to 15	1	invertebrate	*Crangon crangon*	acute	48	LC50	500.00			"
nonionic	AE	14 to 15	3	invertebrate	*Crangon crangon*	acute	48	LC50	200.00			"
nonionic	AE	14 to 15	11	invertebrate	*Crangon crangon*	acute	48	LC50	3300.00			"

nonionic	AE	11	5	invertebrate	*Daphnia magna*	acute	48	LC50	3.30			"
nonionic	AE	12 to 13	6.5	invertebrate	*Daphnia magna*	acute	96	LC50	0.57, 0.09			"
nonionic	AE	12 to 15	9	invertebrate	*Daphnia magna*	acute	96	LC50	1.23			"
nonionic	AE	12 to 14	6.3	invertebrate	*Daphnia magna*	acute	96	LC50	1.50			"
nonionic	AE tallow	16, 18	10	invertebrate	*Balanus balanoides* (larvae)	acute	96	LC50	1.2	6-8˚C		(Swedmark *et al.*, 1971)
nonionic	AE tallow	16, 18	10	invertebrate	*Carcinus maenas*	acute	96	LC50	>100	6-8˚C		"
nonionic	AE tallow	16, 18	10	invertebrate	*Cardium edule*	acute	96	LC50	<5	6-8˚C		"
nonionic	AE tallow	16, 18	10	invertebrate	*Eupagurus bernhardus*	acute	96	LC50	>100	6-8˚C		"
nonionic	AE tallow	16, 18	10	invertebrate	*Hyas araneus* (adult)	acute	96	LC50	>100	6-8˚C		"
nonionic	AE tallow	16, 18	10	invertebrate	*Hyas araneus* (larvae)	acute	96	LC50	800.00	6-8˚C		"
nonionic	AE tallow	16, 18	10	invertebrate	*Leander adspersus*	acute	96	LC50	>100	6-8˚C		"
nonionic	AE tallow	16, 18	10	invertebrate	*Leander squilla*	acute	96	LC50	>100	6-8˚C		"
nonionic	AE tallow	16, 18	10	invertebrate	*Mya arenaria*	acute	96	LC50	100.00	6-8˚C		"
nonionic	AE tallow	16, 18	10	invertebrate	*Mytilus edulis*	acute	96	LC50	50.00	6-8˚C		"
nonionic	AE	18	n	invertebrate	*Daphnia magna*	acute	48	LC50	0.19			(Talmage, 1994)
nonionic	AE	16 to 18	18	invertebrate	*Daphnia magna*	acute	48	LC50	20.00			"
nonionic	AE	16 to 18	30	invertebrate	*Daphnia magna*	acute	48	LC50	18.00			"
nonionic	AE	12 to 13	6.5	invertebrate	*Daphnia magna*	acute	96	LC50	1.14			"
nonionic	AE	14 to 15	7	invertebrate	*Daphnia magna*	acute	96	LC50	0.43			"
nonionic	AE	14-15	7	invertebrate	*Penaeus duoarum*	acute	96	LC50	0.80			"
nonionic	AE	14-15	7	invertebrate	*Penaeus duoarum*	acute	96	LC50	1.20			"
nonionic	AE	14-15	7	invertebrate	*Penaeus duoarum*	acute	96	LC50	1.10			"
nonionic	AE	12 to 15	9	invertebrate	*Aëdes aegypti*	acute	24	LC100	200.00			(van Emden *et al.*, 1974)
nonionic	AE	12 to 15	9	invertebrate	*Biomphalaria glabrata*	acute	24	LC100	12.00			"
anionic	AE	9 to 11	3	invertebrate	*Ceriodaphnia* cf *dubia*	acute	48	LC50	5.36	immobilisation	Fig. 1	(Warne and Shifko, 1999)
nonionic	AE	12 to 13	5	invertebrate	*Daphnia magna*	acute	48	EC50	0.46	immobilisation	Fig. 1	(Wong *et al.*, 1997)

nonionic	AE	12 to 13	4.5 to 6	invertebrate	*Daphnia magna*	acute	48	EC50	0.59	immobilisation	Fig. 1	"
nonionic	AE	12 to 13	6.5	invertebrate	*Daphnia magna*	acute	48	EC50	0.74	immobilisation	Fig. 1	"
nonionic	AE	9 to 11	6	invertebrate	*Daphnia magna*	acute	48	EC50	5.30	immobilisation	Fig. 1	"
nonionic	AE	9 to 11	8	invertebrate	*Daphnia magna*	acute	48	EC50	12.00	immobilisation	Fig. 1	"
nonionic	AE	11	7	invertebrate	*Daphnia magna*	acute	48	EC50	2.10	immobilisation	Fig. 1	"
nonionic	AE	11	9	invertebrate	*Daphnia magna*	acute	48	EC50	6.70	immobilisation	Fig. 1	"
nonionic	AE	12 to 15	12	invertebrate	*Daphnia magna*	acute	48	EC50	1.40	immobilisation	Fig. 1	"
nonionic	AE	14 to 15	13	invertebrate	*Daphnia magna*	acute	48	EC50	1.20	immobilisation	Fig. 1	"
nonionic	NPE			invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	1.20		Fig. 1	(Ankley *et al.*, 1990)
nonionic	NPE			invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	1.03		Fig. 1	"
nonionic	NPE			invertebrate	*Ceriodaphnia dubia*	acute	48	LC50	0.90		Fig. 1	"
nonionic	NPE branched		9	invertebrate	*Daphnia magna*	acute	48	EC50	14.00	immobilisation	Fig. 1	(Kravetz *et al.*, 1991; Dorn *et al.*, 1993)
nonionic	NPE branched		9	invertebrate	*Daphnia magna*	chronic	7 d	EC50	9.00	immobilisation		"
nonionic	NPE branched		9	invertebrate	*Daphnia magna*	chronic	7 d	LOEC	>10.0	growth		"
nonionic	NPE branched		9	invertebrate	*Daphnia magna*	chronic	7 d	NOEC	10.00	growth		"
nonionic	APE linear	9	1.5	invertebrate	*Mysidopsis bahia*	acute	48	LC50	2.00		Fig. 1	(Hall *et al.*, 1989)
nonionic	APE linear	9	1.5	invertebrate	*Mysidopsis bahia*	acute	48	LC50	3.34		Fig. 1	"
nonionic	APE linear	9	9	invertebrate	*Mysidopsis bahia*	acute	48	LC50	1.40		Fig. 1	"
nonionic	APE linear	9	9	invertebrate	*Mysidopsis bahia*	acute	48	LC50	1.89		Fig. 1	"
nonionic	APE linear	9	50	invertebrate	*Mysidopsis bahia*	acute	48	LC50	4148.00			"
nonionic	NPE branched		1.5	invertebrate	*Mysidopsis bahia*	acute	48	LC50	2.28		Fig. 1	"
nonionic	NPE branched		1.5	invertebrate	*Mysidopsis bahia*	acute	48	LC50	1.41		Fig. 1	"
nonionic	NPE branched		15	invertebrate	*Mysidopsis bahia*	acute	48	LC50	2.57		Fig. 1	"
nonionic	OPE		1.5	invertebrate	*Mysidopsis bahia*	acute	48	LC50	7.07		Fig. 1	"

nonionic	OPE	5	invertebrate	*Mysidopsis bahia*	acute	48	LC50	1.83		Fig. 1	"
nonionic	NPE	4	invertebrate	*Daphnia sp.*	acute		Tox. thresh.	5.00			(Janicke *et al.*, 1969)
nonionic	NPE	6	invertebrate	*Daphnia sp.*	acute		Tox. thresh.	5.00			"
nonionic	NPE	7	invertebrate	*Daphnia sp.*	acute		Tox. thresh.	10.00			"
nonionic	NPE	10	invertebrate	*Daphnia sp.*	acute		Tox. thresh.	10.00			"
nonionic	NPE	20	invertebrate	*Daphnia sp.*	acute		Tox. thresh.	1000.00			"
nonionic	NPE	30	invertebrate	*Daphnia sp.*	acute		Tox. thresh.	10000.00			"
nonionic	NPE	7	invertebrate	*Daphnia magna*	acute	96	LC50	4.10			(Markarian *et al.*, 1989)
nonionic	NPE	10	invertebrate	*Daphnia pulex*	acute	48	LC50	15.43		Fig. 1	(Moore *et al.*, 1987)
nonionic	NPE	10	invertebrate	*Daphnia pulex*	acute	48	LC50	10.95		Fig. 1	"
nonionic	NPE	10	invertebrate	*Daphnia pulex*	acute	48	LC50	11.18		Fig. 1	"
nonionic	NPE branched	7	invertebrate	*Daphnia pulex*	acute	48	LC50	2.87		Fig. 1	(Salanitro *et al.*, 1988)
nonionic	NPE	10	invertebrate	*Balanus balanoides* (adult)	acute	96	LC50	<25	6-8˚C		(Swedmark *et al.*, 1971)
nonionic	NPE	10	invertebrate	*Balanus balanoides* (adult)	acute	96	LC50	<25	15-17˚C		"
nonionic	NPE	10	invertebrate	*Balanus balanoides* (larvae)	acute	96	LC50	2	6-8˚C		"
nonionic	NPE	10	invertebrate	*Carcinus maenas*	acute	96	LC50	>100	6-8˚C		"
nonionic	NPE	10	invertebrate	*Cardium edule*	acute	96	LC50	5	6-8˚C		"
nonionic	NPE	10	invertebrate	*Cardium edule*	acute	96	LC50	<<10	15-17˚C		"
nonionic	NPE	10	invertebrate	*eupagurus bernhardus*	acute	96	LC50	>100	6-8˚C		"
nonionic	NPE	10	invertebrate	*Hyas araneus* (adult)	acute	96	LC50	>100	6-8˚C		"
nonionic	NPE	10	invertebrate	*Hyas araneus* (larvae)	acute	96	LC50	10	6-8˚C		"
nonionic	NPE	10	invertebrate	*Leander adspersus*	acute	96	LC50	>100	6-8˚C		"
nonionic	NPE	10	invertebrate	*Leander adspersus* (intermoult)	acute	96	LC50	50	15-17˚C		"
nonionic	NPE	10	invertebrate	*Leander adspersus* (postmoult)	acute	96	LC50	10	15-17˚C		"
nonionic	NPE	10	invertebrate	*Leander squilla*	acute	96	LC50	>100	6-8˚C		"
nonionic	NPE	10	invertebrate	*Mya arenaria*	acute	96	LC50	18	6-8˚C		"

nonionic	NPE	10	invertebrate	*Mya arenaria*	acute	96	LC50	<10	15-17˚C	"
nonionic	NPE	10	invertebrate	*Mytilus edulis*	acute	96	LC50	12	6-8˚C	"
nonionic	NPE	10	invertebrate	*Mytilus edulis*	acute	96	LC50	<10	15-17˚C	"
nonionic	NPE	10	invertebrate	*Pecten maximus*	acute	96	LC50	<<5	15-17˚C	"
nonionic	NPE	10	invertebrate	*Pecten opercularis*	acute	96	LC50	<<10	15-17˚C	"
nonionic	NPE	10	invertebrate	*Daphnia magna*	acute	24	LC50	44.20		(Talmage, 1994)
nonionic	NPE	11	invertebrate	*Aëdes aegypti*	acute	24	LC50	500.00		(van Emden *et al.*, 1974)
nonionic	NPE	11	invertebrate	*Biomphalaria glabrata*	acute	24	LC100	23.00		"

Appendix 2

Toxicological issues for amphibians in Australia

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in

Declines and Disappearances of Australian Frogs:

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Declines and Disappearances of AUSTRALIAN frogs

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Toxicological issues for amphibians in Australia

Reinier Mann[1] and Joseph Bidwell[2]

ABSTRACT

As a consequence of agricultural, urban and industrial development, the chemical profiles of Australian soils and waterways are being altered. One could expect that this kind of habitat alteration may have deleterious effects on native fauna such as frogs. At present however, it is unclear whether environmental contaminants pose a threat to Australian amphibian populations. To date there have been very few toxicological studies examining the effects of environmental chemicals such as pesticides, fertilisers and metals, or the effects of changes in soil chemistry on Australian fauna.

For some of these contaminants, there is a reasonable volume of literature for northern hemisphere species, although these studies are lacking in uniformity, preventing useful comparisons. Furthermore, these studies may not reflect the risks posed to Australian species which are phylogenetically distinct. Interspecific variation in chemical sensitivity has been demonstrated amongst northern hemisphere phylogenetic groupings.

The discipline of ecotoxicology has much to contribute to a better understanding of these risks, with several methodological approaches available, although standardised protocols still need development.

INTRODUCTION

In recent years amphibians have been proffered as good indicators of environmental contamination because of their unique physiology. Amphibians are the highest vertebrate group to retain an essentially "naked" egg, and the only vertebrate group which has an aquatic larval stage and a terrestrial adult phase. Furthermore, the skin of an adult amphibian is a permeable organ used for respiration and water-balance whereas the tadpole stage relies predominantly on gills for respiration. This dual life cycle implies that amphibians may have more opportunities for exposure and

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more modes of exposure to environmental contaminants than other vertebrates, although the notion that amphibians exhibit greater sensitivity to environmental contaminants than other phylogenetic groups has yet to be verified (see below).

Amphibians do however, provide an extremely visible indicator of pollution. The recent discovery by a school group of several deformed frogs in Minnesota (Schmidt 1997) brought public focus to a water contamination issue. Indeed, the incidence of frog limb abnormalities has been proposed as a useful indicator of environmental contamination (Tyler 1994; Ouellet *et al.* 1997; Read 1997) and has been used to evaluate the hazards to wildlife posed by mining operations at Olympic Dam in South Australia (Read and Tyler 1990; 1994; Read 1997) and Jabiru in the Northern Territory during the 1970s (Tyler 1994).

Amphibians as a group have only recently been included in routine toxicological assessment of environmental chemicals. More than 45% of the peer reviewed literature which focuses on amphibians in toxicology oriented studies has been published in the last ten years. Presumably this reflects the increased scientific and community interest in amphibians following much publicity regarding their apparent global decline, as well as a greater appreciation of the importance of these animals as an integral part of the food chain in many of the world's ecosystems. Amphibians may actually constitute the largest fraction of vertebrate biomass in some ecosystems, making them an important source of food for higher vertebrates such as fish, birds, reptiles and mammals, as well as important herbivores (tadpoles) and carnivores in these ecosystems (Blaustein *et al.* 1994).

Australia has one of the most diverse amphibian assemblages in the world with more than 210 species of frogs representing 29 genera and four families (Cogger 1992; Barker *et al.* 1995; Tyler, 1997). While it is surprising that more attention has not been paid to the effects of environmental pollutants upon these animals, environmental toxicology is itself a relatively new field in Australia, with few toxicological studies carried out using native fauna of any kind. Consequently, regulatory authorities such as the National Registration Authority for Veterinary and Agricultural Chemicals (NRA) and the Environmental Protection Agency (EPA) rely on studies conducted with North American, South African or European species. More than 80% of the amphibian toxicological literature describes studies using representatives of only three genera — *Rana* spp, *Bufo* spp or *Xenopus laevis*. In Australia, only one species belongs to the genus Rana (a relatively recent colonist from New Guinea, restricted to Cape York Peninsula) and the only *Bufo* species is represented by the introduced pest species *Bufo marinus* (cane toad).

This review is intended to highlight some of the potential risks to Australian frogs from exposure to chemical contaminants and environmental imbalances. Moreover, we hope to place the current body of literature dedicated to toxicology in Australian species of frogs within the context of the general amphibian toxicological literature. In doing so, we also intend to provide an introduction to the amphibian toxicological literature. It is not however, an exhaustive treatment of the available literature. Other valuable sources of information include the list of amphibian and reptile toxicology references accumulated by the Canadian Wildlife Service (http://www.cciw.ca/green-lane/herptox/reference-list.html), Power et al. (1989), Ferraro and Burgin (1993) and Tyler (1994). This review will cover three main questions:

1. Which chemicals pose a threat to Australian frogs?
2. What tools are available to study toxic effects in frogs?
3. What are the problems associated with amphibian toxicology?

WHICH CHEMICALS POSE A THREAT TO AUSTRALIAN FROGS ?

Pesticides

The largest single group of potential chemical pollutants that Australian frogs might encounter are the various pesticides employed in agriculture and pest management in Australia. Much of the recent work examining the effects of pesticides on amphibians has concentrated on the newer generations of pesticides such as pyrethroids, carbamates, and organophosphates (see below), although there has been a resurgence of interest in the older organochlorine insecticides (i.e. DDT) because of their environmental persistence and possible links to amphibian decline. For example, studies in the Sierra Nevada and Cascade Mountains of the USA indicate that wind-born topsoil bearing pesticide residues from the central Californian agricultural valley, may be responsible for declines of several species of amphibians (Cory *et al.* 1970; Bradford *et al.* 1994; Fellers 1997). At this stage a direct causal relationship has not been well established. Dramatic declines of the high altitude species *Rana muscosa* in Kings Canyon National Park in the Sierra Nevada mountains in California were preceded by an outbreak of redleg disease caused by the pathogen *Aeromonas hydrophila* (Bradford 1991). The reason for the outbreak is unknown, however, given that immunosuppression has been reported in other vertebrate species following exposure to DDT and other pesticides (Barnett and Rodgers 1994), one can speculate about the immunosuppressive effects of pesticide exposure in Rana muscosa. One possible contributing factor in recently documented declines in high elevation species along the eastern slopes of the Great Dividing Range of eastern Australia (Richards *et al.* 1993) is that of a fungal pathogen (Berger *et al.* 1999) and this also may be related to the immunosupressive effects of environmental contaminants.

Insecticides — organochlorines

Organochlorines were the first synthetic chemical pest control agents, with DDT being the archetypal organochlorine insecticide. DDT was used extensively in developed nations until it was banned from use in the USA in 1972 and in Australia in the mid 1980s. DDT is still widely used as an insecticide in third world countries (Lambert 1993; 1997). While there are a few early studies on the effects of DDT on amphibians (Ellis *et al.* 1944; Herald 1949; Langford 1949; Logier 1949; Speirs 1949; Tarzwell 1950; Vinson *et al.* 1963; Isaacson 1968) research only commenced in earnest in the 1970s. One of the main proponents of these studies was A.S. Cooke of the Monks Wood Experimental Station in Huntington England, who published much of the work on DDT toxicity in amphibians (Cooke 1970; 1971; 1972; 1973a; 1973b; 1974; 1979; Osborn *et al.* 1981).

DDT and most other organochlorines are characterised by high environmental persistence. Their persistence and subsequent biomagnification through the food chain (Meeks 1968; Licht 1976; Niethammer *et al.* 1984; Russell *et al.* 1995) are among the factors which have led to their reduction in use. However, more than 20 years after it was banned from use in North America, DDT is still being detected in amphibians from those areas in which the pesticide was applied (Russell *et al.* 1995). In Australia, DDT and other organochlorines were also widely used, as indicated by a survey carried out in Western Australia which detected, amongst other organochlorines, DDT and dieldrin in 39.6% and 39.0% of 11 248 soil samples respectively (EPA WA 1989). Despite this widespread contamination, we are aware of only one study that has measured organochlorine residues in a native Australian amphibian (Birks and Olsen 1987).

Organochlorines are still being used to some extent in Australia. Reports of fish kills in September 1997 in the Ord River agricultural zone of the Kimberly region of north-western Australia, were attributed to the application of endosulfan to control cotton pests. We found only six studies examining the effects of endosulfan on amphibians (Cockbill 1979; Gopal *et al.* 1981; Hashimoto and Nishiuchi 1981; Vardia *et al.* 1984; Abbasi and Soni 1991; Berrill *et al.* 1998). One of these studies (Gopal *et al.* 1981) found endosulfan to be an order of magnitude more acutely toxic to *Rana tigrina* tadpoles than to the catfish *Clarias batrachus* and damselfly nymphs (*Enallagma* spp.). Mulla *et al.* (1963), also noted that endosulfan/toxaphene application was effective in producing an "almost complete kill" of "public nuisance" bullfrogs. Furthermore, in their review of the amphibian toxicological literature, Power *et al.* (1989) ranked endosulfan as the second most acutely toxic chemical thus far tested on amphibians, being surpassed only by the heavy metal mercury. To date there has been no research into the possible consequences of endosulfan exposure to frog species of Australia's Kimberly region or other agricultural areas where this chemical is still being applied.

Organochlorines are known to produce developmental abnormalities in amphibians (Cooke 1970; 1972; 1973b; Brooks 1981; Marchal-Ségault and Remande 1981; Osborn *et al.* 1981; Gavilan *et al.* 1988; van der Bercken *et al.* 1989). One of these references (Brooks 1981) examined the teratogenic effects of dieldrin on the Australian frog *Limnodynastes tasmaniensis*, however this is the only study of this kind for an Australian species.

Insecticides — organophosphates

Organophosphates replaced organochlorines by virtue of their lower environmental persistence. The first notable studies to examine the effects of organophosphates on amphibians were published in the early sixties (Edery and Schatzberg-Porath 1960; Mulla 1962; Mulla *et al.* 1963). More recently, several studies indicated that standard field application rates of organophosphate insecticides may have a deleterious effect on amphibian populations (Anguiano *et al.* 1994; Berrill *et al.* 1994; 1995; Schuytema *et al.* 1995; Sparling *et al.* 1997).

The established mechanism of organophosphate toxicity is through non-reversible acetylcholinesterase inhibition (Dekins *et al.* 1978; Llamas *et al.* 1985; Balasundaram and Selvarajan 1990; Swann *et al.* 1996). Interestingly, adult amphibians appear to be tolerant of severe acetylcholinesterase inhibition (Balasundaram and Selvarajan 1990; Wang and Murphy 1982) and the developmental toxicity of organophosphates (Elliot-Feeley and Armstrong 1982; Snawder and Chambers 1990; 1993; Alvarez *et al.* 1995) may be more ecologically important. Also, there are a few studies which have indicated that these chemicals can bioaccumulate (Hall and Kolbe 1980; Fleming *et al.* 1982; Powell *et al.* 1982; Hall 1990).

In Australia, organophosphate insecticides are used extensively in agriculture and within urban areas (e.g. golf courses, turf clubs etc.). There are no studies which have examined their potential risks to Australian frogs.

Insecticides — carbamates

Like organophosphates, the low persistence of carbamate insecticides has led to their widespread acceptance as a replacement for the more traditionally used organochlorines. Again however, there has been very little research to evaluate the toxicity of these chemicals to amphibians, although there are some indications that field concentrations of carbamates following application of these insecticides may be detrimental to amphibians (Tucker and Crabtree 1969; Flickinger *et al.* 1980; Marian *et al.* 1983; Bridges 1997). Furthermore, carbaryl has been demonstrated to penetrate amphibian skin more rapidly than organochlorines (dieldrin and DDT), organophosphates (parathion) or pyrethroids (permethrin) (Shah *et al.* 1983). Carbamate based insecticides have also been found to produce developmental malformations in skeletal tissue (Alvarez *et al.* 1995) and musculature (Rzehak *et al.* 1977; Cooke 1981). The effects on Australian species have yet to be investigated.

Insecticides — pyrethroids

Pyrethroids have gained a reputation as "safe" insecticides and are widely used in agricultural, aquatic and household products (Elliot *et al.* 1978; Smith and Stratton 1986). There is some indication however, that field application of these chemicals may be deleterious to amphibians (Jolly *et al.* 1978; Thybaud 1990; Berrill *et al.* 1993; Materna *et al.* 1995). Pyrethroids appear to affect voltage-dependent neuromuscular sodium channels producing tremors, hyperexcitation and convulsions (van den Bercken 1977; Vijverberg *et al.* 1982; Ruigt and van den Bercken 1986).

Although pyrethroids are used extensively in Australia, there are no published studies on the effects of these chemicals on Australian frogs. As far as we are aware, a B.Sc. honours thesis by Millen (1995) presents the only information available for an Australian species that we are aware of. It indicated temporary increases in acetylcholinesterase, growth inhibition and behavioural effects following exposure to cypermethrin.

Herbicides and Fungicides

The first publication to investigate the potential hazards of a herbicide to amphibians emerged in 1970 (Hazelwood 1970). Since then approximately 70 articles dealing with the toxicology of herbicides and fungicides have been published. The scope of chemical species which these references cover amounts to more than 40 different compounds. There are however, more than 100 chemical compounds registered for

use as herbicides in Australia and more than 60 registered for use as fungicides (Department of Agriculture 1994). It is reasonable to suggest that no particular herbicide or fungicide has been adequately studied.

Two of the more wide-ranging studies by Johnson (1976) and Sanders (1970), have examined the acute toxicity of numerous herbicides to tadpoles or adult frogs and provide a useful overview of herbicide toxicity. The only compounds that have received a notable level of attention are the:

- phenoxyacid herbicides (i.e. 2,4-D and MCPA) (Sanders 1970; Cooke 1972; Zaffaroni *et al*. 1986a; 1986b; Zavanella *et al.* 1988; Arias et al. 1989; Leone *et al.* 1994; Vismara *et al.* 1995; Bernardini et al. 1996; Vismara *et al.* 1996; Vismara and Garavaglia 1997);
- dithiocarbamate fungicides (Prahlad et al. 1974; Zaffaroni *et al.* 1978; Arias and Zavanella 1979; Zavanella *et al.* 1979, 1984; Seugé *et al.* 1983; Birch and Prahlad 1986a, 1986b); and
- paraquat/diquat herbicides (Sanders 1970; Anderson and Prahlad 1976; Johnson 1976; Cooke 1977; Paulov 1977b; Bimber and Mitchell 1978; Hashimoto and Nishiuchi 1981; Dial and Bauer 1984; Dial and Bauer-Dial 1987; Lindquist *et al.* 1988; Linder *et al.* 1990; Dial and Dial 1995; Lajmanovich *et al.* 1998).

Many of these studies have indicated that these three classes of chemicals have teratogenic effects.

Three published studies have incorporated Australian species. Johnson (1976) used four species of Australian frogs: Adelotus brevis, *Limnodynastes peronii*, *Limnodynastes tasmaniensis* and *Litoria ewingii*. This particular study represents the only comprehensive toxicological study for Australian species with regard to any environmental contaminant. More recently, Bidwell and Gorrie (1995) and Mann and Bidwell (1998), presented data on the acute toxicity of glyphosate formulations to tadpoles or adult frogs of the south-western Australian species, *Litoria moorei, Litoria adelaidensis, Crinia insignifera, Heleioporus eyrei*, and *Limnodynastes dorsalis*.

Pesticide mixtures

Pesticides are rarely applied in isolation. Usually a combination of pesticides is mixed together and applied as a single application cocktail. Furthermore, commercial preparations are often a combination of two or more pesticides, or they incorporate various solvents, carriers or surfactants. These various combinations may have additive, synergistic or antagonistic toxicological effects (Landis and Yu 1995). A few studies have examined the toxicology of pesticide mixtures on amphibians (Anderson and Prahlad 1976; Berrill *et al.* 1993, 1994; Howe *et al.* 1998) while others have noted differences in toxicity between technical grade pesticides and formulated products (Bidwell and Gorrie 1995; Schuytema *et al.* 1995; Swann *et al.* 1996; Mann and Bidwell 1998).

Fertilisers

The potential hazards associated with agricultural fertilisers have only recently been proposed as a potential threat to amphibians (Berger 1989). Berger (1989) noted a correlation between amphibian declines and environmental increases in nitrates and ammonia. Subsequent laboratory studies have concentrated on the effects of nitrate on amphibian survival and growth (Baker and Waights 1993, 1994; Hecnar 1995; Watt and Oldham 1995; Hecnar and McLoskey 1996; de Wijer *et al.* 1997; Oldham *et al.* 1997; Xu and Oldham 1997) but the results are somewhat equivocal. Hecnar (1995) reported 96 hour LC_{50}s of 13.6-39.3 mg/L for *Bufo americanus* when exposed to ammonium nitrate. Similarly, in two studies, one of which is the only study to have examined an Australian species, Baker and Waights (1993, 1994) reported reduced feeding and weight loss in *Bufo bufo* and *Litoria caerula* tadpoles at 40 and 100 mg/L sodium nitrate respectively. In contrast, Xu and Oldham (1997) reported a 96 hour LC_{50} of over 1000 mg/L $N03^{2-}$ (as ammonium nitrate) for *Bufo bufo* tadpoles and increased growth at 50 mg/L $N03^{2-}$, while de Wijer *et al.* (1997) reported a disparity in the toxicity of ammonium nitrate and calcium nitrate. Until these discrepancies are clarified the role of agricultural fertilisers in amphibian decline remains contentious.

It is worth noting that the Australian frog fauna has evolved in an environment that is comparatively depauperate in nitrate and phosphate (Lamont 1994) which may accord Australian frogs a greater sensitivity to these chemicals.

Impurities in fertilisers include cadmium, lead and mercury (State of the Environment Advisory Council 1996) and will be addressed in the following section on metals.

Metals

Metal contamination of agricultural land/waterways occurs as a consequence of fertiliser impurities such as cadmium, lead and mercury (State of the Environment Advisory Council 1996). Similarly, coastal wetlands are contaminated by various metals through urban and industrial runoff. The effect that these metals may have on Australian frogs has yet to be investigated. Wetland bioremediation systems are slowly being incorporated as integral parts of urban and industrial waste management. Such wetlands could provide a valuable laboratory for the examination of metal exposure effects on Australian frogs.

The literature which deals with the effects of metal contaminants is relatively expansive and has been treated comprehensively by Power *et al.* (1989). Recent studies of note include several which have examined the teratogenic effects of various metals including heavy metals, particularly cadmium and lead (Pérez Coll and Herkovits 1990; Nebeker *et al.* 1994; Plowman *et al.* 1994; Sunderman *et al.* 1995; Herkovits *et al.* 1997; Rowe *et al.* 1998) and divalent metals such as zinc, copper, cobalt and nickel (Hopfer *et al.* 1991; Plowman *et al.* 1991; Luo *et al.* 1993, 1994; Sunderman *et al.* 1995).

Endocrine disrupting chemicals (EDCs)

Many environmental contaminants are now known to behave as hormone mimics, and there is much concern that wildlife is being affected (Raloff 1994). One class of chemicals of concern is the alkylphenolic surfactants. World-wide, approximately 500 000 tonnes of alkylphenol based surfactants are produced annually for use in detergents, paints, pesticides, textile and petroleum recovery chemicals, metal working lubricants and personal care products (Renner 1997). While not directly concerned with amphibians, one of the most notable studies involving alkylphenols is that by Jobling *et al.* (1996), which reported induction of vitellogenesis (a process normally dependent on endogenous oestrogens) and concomitant inhibition of testicular growth in male rainbow trout following exposure to alkylphenolic compounds at the 30 ppb level.

Very little work has been published on the potential effects of EDCs in amphibians and only one has appeared in the refereed literature (Palmer and Palmer 1995). A few conference abstracts however, indicate that amphibian development may be affected by oestrogen mimics such as DDT (Palmer and Palmer 1995; Hayes and Noriega 1997), polychlorinated hydrocarbons (Glennemeier 1997) and alkylphenolic compounds (Palmer *et al.* 1996; Ramsdell *et al.* 1996).

Salinity

Dry land salinity and irrigation-induced salinity are possibly the most important environmental problems facing Australia. By 1994 approximately nine percent of land cleared for agriculture in south-west Western Australia (1.6 million hectares) was affected by dryland salinity, with an average increase of 0.07% per year. In 1992, 200 000 hectares of the Murray-Darling Basin were similarly affected. In South Australia and Victoria, 1993 estimates stood at 400 000 and 150 000 hectares respectively, with smaller estimates beginning to come from other states. Shallow water-tables with accompanying salinity problems were estimated to affect 360 000 hectares of the Murray-Darling Basin and 199 000 hectares of the Wakool, Deniliquin and Murrumbidgee irrigation areas (State of the Environment Advisory Council 1996).

The severity of salinity induced land degradation in Australia is perhaps analogous to that of acid rain in the northern hemisphere. Certainly the widespread nature of the two phenomena is comparable and the consequential changes in water chemistry can be expected to exert comparable physiological stresses on amphibians. A considerable volume of literature (more than 140 journal articles) is dedicated to the examination of acid tolerance/sensitivity amongst amphibians (for reviews see Pierce 1985; Freda 1986; Ferraro and Burgin 1993). In contrast to this impressive body of literature there are fewer than 30 studies which have examined salt tolerance/sensitivity in amphibians.

TABLE 1: Studies which have investigated the toxic effects of environmental contaminants in Australian species. OC organochlorine, P pyrethroid.

Toxin	Species	Study type	Reference
Organochlorines	*Limnodynastes tasmaniensis*	Fat residues	Birks and Olsen 1987
Dieldrin (OC)	*Limnodynastes tasmaniensis*	Sublethal – development	Brooks 1981
Cypermethrin (P)	*Litoria ewingi*	Sublethal – growth, behaviour and acetylcholinesterase levels	Millen 1995
Herbicides	*Adelotus brevis; Limnodynastes peronii; L. tasmaniensis; Litoria ewingi*	Acute toxicity and Sublethal – thermal tolerance	Johnson 1976
Glyphosate-based herbicide	*Crinia insignifera; Litoria adelaidensis; L. moorei*	Acute toxicity	Bidwell and Gorrie 1995
Glyphosate-based herbicides	*Crinia insignifera; Heleioporus eyrei; Limnodynastes dorsalis; Litoria moorei*	Acute toxicity	Mann and Bidwell 1998
Sodium fluoroacetate (1080)	*Limnodynastes tasmaniensis*	Intraperitoneal administration	McIlroy et al. 1985
Sodium nitrate	*Litoria caerulea*	Sublethal- growth and behaviour	Baker and Waights 1994
Sodium chloride	*Crinia pseudinsignifera; Heleioporus albopunctatus; Pseudophryne guentheri*	Acute toxicity and Sublethal – avoidance Field correlations	Baumgarten 1991
Sodium chloride	*Limnodynastes peronii; Uperoleia laevigata*	Actute toxicity and Sublethal – growth	Ferraro 1992
Sodium chloride	*Limnodynastes tasmaniensis*	Sublethal – growth	Quincy 1991
Sodium chloride	Multiple species	Sublethal – righting reflex	Tyler 1972
Radiation	*Limnodynastes tasmaniensis*	Sublethal – development	Panter 1986
Radiation	*Limnodynastes tasmaniensis*	Sublethal – oxygen consumption	Panter et al. 1987
Metals	*Neobatrachus centralis*	Limb abnormalities – monitoring	Read 1997
Metals	*Neobatrachus centralis*	Limb abnormalities – monitoring	Read and Tyler 1990
Metals	*Neobatrachus centralis*	Limb abnormalities – monitoring	Read and Tyler 1994
Copper	*Limnodynastes dorsalis; Litoria raniformis*	Liver residues	Beck 1956

A wide variation in salt tolerance in amphibians has been reported (see Liggins and Grigg 1985; Ferraro and Burgin 1993). In general however, amphibians must maintain hyperosmoticity to their environment and salinities greater than 25% sea-water present an osmotic challenge. This challenge is occasionally met by increasing plasma concentrations of chloride and urea, thereby increasing plasma osmotic pressure (Liggins and Grigg 1985). Such a response indicates a degree of adaptability in those species examined, including *Bufo marinus* (Liggins and Grigg 1985), *Bufo viridis* (Katz 1973), *Bufo bufo* (Ferreira and Jesus 1973), *Rana temporaria* (Ackrill *et al.* 1969), *Rana cancrivora* (Gordon *et al.* 1961) and *Xenopus laevis* (Romspert 1976). It is not known whether Australian species show a similar level of plasticity. In an examination on the likelihood of frogs crossing the Torres Strait, Tyler (1972) examined salt tolerance in several Australian and New Guinean species by exposing them to seawater and observing the time required for loss of righting-reflex, but the results were somewhat equivocal. Apart from Tyler (1972) we are aware of a further three unpublished studies that have examined salt tolerance in Australian species (Baumgarten 1991; Quincy 1991; Ferraro 1992 — see Table 1).

SUMMARY OF TOXICOLOGICAL STUDIES INVOLVING AUSTRALIAN SPECIES

A summary of Australian studies is presented in Table 1. There are eighteen studies listed, four of which are unpublished theses. It is likely that there are other unpublished theses residing within Australian universities. This table has been included to highlight the paucity of information available for Australian species.

WHAT TOOLS ARE AVAILABLE TO STUDY TOXICOLOGY IN FROGS?

Acute toxicity tests

One of the most widespread protocols for assessing toxicity of a chemical to an aquatic species is the acute toxicity test. Standard procedures for conducting these tests can be found in a range of sources (U.S.EPA 1991; ASTM 1993a; OECD 1993). ASTM (1993a) includes a specific protocol for testing tadpoles, fish and macroinvertebrates (ASTM 1993c). Acute Toxicity tests will usually run for multiples of 24 h, with 48 and 96 h tests being most common. The results of such tests are usually expressed as point estimates such as the LC50- a statistically or graphically estimated concentration that is expected to be lethal to 50% of a group of organisms under specified conditions. Other endpoints include the LD50 which is a lethality endpoint in which the toxicant is administered orally. The use of a 50% effect on the test population is for statistical reasons only, not an indication that only 50% of the population should be protected.

Acute toxicity tests provide an inexpensive, rapid and simple method for accumulating base-line data on the toxicity of a chemical to a specific organism. Such data can be used to compare the relative toxicity of different chemicals or the relative sensitivities of different species. These tests demand the availability of large numbers of test animals. This requirement precludes the use of rare or endangered species. Indeed, it may be preferable if this approach were restricted to a suite of representative species which could be reared in adequate numbers in captivity. Interspecific variation in acute responses is unlikely to be great enough to invalidate data collected in common or cultured species, provided there is strict adherence to established protocols.

About 30% of the amphibian toxicology studies reviewed present acute toxicity data in one form or another. Since the majority of this work has remained in the realm of "herpetological studies" rather than "toxicological studies" there has been virtually no adherence to standard methodology. Sanders (1970) presented a reasonably detailed account of a methodology used to generate LC_{50} data for several different pesticides on two species of anurans. This protocol was subsequently adopted by Johnson (1976) in his studies with Australian animals and his data are somewhat comparable to later more rigorous studies (e.g. Wohlgemuth 1977; Jolly *et al.* 1978; Hall and Swineford 1980; Gopal *et al.* 1981; Thurston *et al.* 1985; Holcombe *et al.* 1987; Materna *et al.* 1995; Schuytema *et al.* 1995; Sparling *et al.* 1997; Xu and Oldham 1997; Howe *et al.* 1998; Mann and Bidwell 1998).

Sub-chronic, chronic and non-lethal exposures

The amphibian toxicological literature includes a number of sub-chronic and chronic exposure studies in which either long term survival or sub-lethal effects are documented. A selection of representative studies is presented in Table 1. Unfortunately the absence of methodological uniformity precludes much useful comparison between studies, although the tabulation system employed by Power *et al.* (1989) provides a useful basis for comparing much of the earlier work. Various life stages have been used in these studies with most focusing on various aspects of embryo and tadpole growth, development or survival. Studies with adults have been uncommon and will be discussed further in a later section.

Frog Embryo Teratogenesis Assay-Xenopus (FETAX)

The African clawed toad (*Xenopus laevis*) is one of the most widely used laboratory animals in the world. It is both extremely easy to maintain and breed in captivity, and its embryological development has been described in detail (Nieuwkoop and Faber 1975). FETAX is a recently developed technique (Dumont *et al.* 1983) which uses *Xenopus laevis* embryos to ascertain the teratogenic potential of environmental chemicals and has been adopted as a standard bioassay for teratogenicity (ASTM 1993b). It has not however, been developed with frog conservation as the primary motivation. Of approximately 70 studies published by the end of 1997, a number focus on validation of the test as a bioassay for teratogenicity, and only a few have examined chemicals which may pose a threat to amphibians in the field (Dawson *et al.* 1985; Birch and Prahlad 1986b, 1988; Snawder and Chambers 1989, 1990; Hopfer *et al.* 1991; Sunderman *et al.* 1991; Hauptman *et al.* 1993; Luo *et al.* 1993; Presutti *et al.* 1994; Bernardini *et al.* 1996; Morgan *et al.* 1996; Vismara *et al.* 1996; Winchester *et al.* 1996; Dumont and Bantle 1997; Schrock *et al.* 1997). Furthermore, *Xenopus laevis* is a somewhat atypical amphibian belonging to the family Pipidae, and may not provide information relevant to other species. The technique does however, provide a template for a stringent and highly reproducible methodology for evaluating embryotoxic potentials, and could be adapted to other species.

TABLE 2: A representative sample of sub-chronic and chronic studies or studies in which sub-lethal endpoints have been observed. OC organochlorine, OP organophosphate, C carbamate, H herbicide, F fungicide, DOC dissolved organic carbon.

Endpoint	Toxicant	Exposure stage and duration	Reference
Hatching Success	pH	Embryos – hatching	Freda and Dunson 1985
	Petroleum oil	Embryos – hatching	Mahaney 1994
	pH/aluminium	Embryos – hatching	Tyler Jones et al. 1989
	pH/temperature	Embryos- hatching	Griffiths and De Wijer 1994
Growth retardation and survival	pH/aluminium	Newly hatched – metamorphosis	Cummins 1986
	Triphenyltin/pH	Newly hatched – metamorphosis	Fioramonti et al. 1997
	pH/metals/DOC	Newly hatched – metamorphosis	Horne and Dunson 1995b
	pH/aluminium	Newly hatched – 96h	Jung and Jagoe 1995
	2,3,7,8-tetrachlorodibenzo -*p*-dioxin (TCDD)	Embryos-metamorphosis	Jung and Walker 1997
	Petroleum oil	Larvae/Embryos – metamorphosis	Lefcort et al. 1997; Mahaney 1994
	Lindane (OC)	Embryos – metamorphosis	Marchal-Ségault and Remande 1981
	Organophospates	Embryos – metamorphosis	Mohanty-Hejmadi and Dutta 1981
	N-methyl-N'-methyl urea (H)	2 days post hatch – 80 days post hatch	Paulov 1977a
	Pyrasophos (F)	2 days post hatch – 80 days post hatch	Paulov 1981
	pH	Larvae – metamorphosis	Rowe *et al.* 1992
	pH/aluminium	Embryos – metamorphosis	Tyler Jones *et al.* 1989
	Ammonium nitrate	Larvae – metamorphosis	Watt and Oldham 1995
Morphological effects and deformities (see also section on FETAX)	Herbicides; fungicides	Embryos for up to 5 days	Anderson and Prahlad 1976
	Dithiocarbamate fungicides	Embryos for up to 10 days	Bancroft and Prahlad 1973
	Dieldrin (OC)	Embryos for up to 35 days	Brooks 1981
	DDT; Dieldrin (OC); 2,4D (H)	Various stages for various time spans	Cooke 1970; 1972; 1973b
	Methyl mercury	Embryos for up to 5 days	Dial 1975
	Paraquat (H)	Various stages for various time spans	Dial and Bauer 1984; Dial and Bauer-Dial 1987; Lajmanovich *et al.* 1998
	Organophosphates	Embryos	Fulton and Chambers 1985
	DDT (OC)	Embryos	Gavilan *et al.* 1988
	Nickel	Embryos	Hauptman *et al.* 1993
	Corticosterone	Larvae (Gosner 39-40) for 9 days	Hayes *et al.* 1997
	Cadmium	Various stages for 72 hours	Herkovits *et al.* 1997
	Primacarb (C)	Embryos for up to 9 weeks	Honrubia *et al.* 1993
	Lindane (OC)	Embryos-metamorphosis	Marchal-Ségault and Remande 1981
	Ethanol	Embryos	Nakatsuji 1983
	DDT (OC)	Larvae for 2 days	Osborn *et al.* 1981
	Malathion; fenitrothion (OP); benzene hexachloride (OC); carbofuran (C)	Embryos for up to 96 hours	Pawar *et al.* 1983; Pawar and Katdare 1984
	Lead	Various stage embryos for 20 hours	Pérez Coll and Herkovits 1990
	Cadmium	Embryos	Pérez Coll *et al.* 1986
	Dithiocarbamate fungicide	Embryos for 7 days	Prahlad *et al.* 1974
	Thiosemicarbazide	Various stages for various time spans	Riley and Weil 1987
	Coal-ash polluted water	Embryos – 80 days post hatch	Rowe *et al.* 1998
	Carbaryl (C)	Early stage larvae for various time spans	Rzehak *et al.* 1977
	Malathion (OP)	Embryos for up to 96 hours	Snawder and Chambers 1993
Postmetamorphic persistence of deformities	Parathion-methyl (OP); Pirimicarb (C)	Embryos – postmetamorphic juveniles (14 wks)	Alvarez et al. 1995
	Nickel; cadmium; cobalt	Embryos – postmetamorphic juveniles (14 wks)	Plowman *et al.* 1994
Enzyme activity	Parathion (OP)	Embryos or 22 day old larvae for 120 hrs	Anguiano *et al.* 1994
	Organophosphates	Tadpoles for 96 hours	Hall and Kolbe 1980
	DDT (OC)	Embryos for 31 days	Juarez and Guzman 1986
	Pyrasophos (F)	2 days post hatch-22 days post hatch	Paulov 1981
	Temephos (OP)	Early stage larvae for 96 hours	Sparling *et al.* 1997
	Malathion (OP)	Early stage larvae for up to 144 hours	Venturino *et al.* 1992
Behavioural effects	Carbaryl (C)	Larvae (Gosner 25) for up to 48 hours	Bridges 1997
	DDT; Dieldrin (OC); 2,4D (H)	Various stages for various time spans	Cooke 1970; 1972; 1973a
	Napthalene	Three week old larvae for 96 hours	Edmisten and Bantle 1982
	pH	Late stage larvae (hind paddles) for 5-8 days	Griffiths 1993
	Distillery effluent	Larvae	Haniffa and Augustin 1989
	pH/aluminium	Newly hatched larvae for 96 hours	Jung and Jagoe 1995
	pH	Early stage larvae for less than 24 hours	Kutka 1994
	Triphenyltin	20 day old larvae for 48 hours	Semlitsch *et al.* 1995
	Lead	Larvae for 6 days	Steele *et al.* 1989

Mesocosms, Microcosm and Artificial Ponds

The inadequacies of single species, laboratory based assays for predicting the ecosystem consequences of anthropogenic pollutants has been voiced by leading ecotoxicologists and amphibian ecologists (Kimball and Levin 1985; Rowe and Dunson 1994; Cairns *et al.* 1996). Microcosms and mesocosms are more complex systems which provide more realistic exposure while allowing some level of experimental control. The categorical terminology denotes the scale of the system. Microcosms are generally small systems which can be set up on a laboratory bench, while mesocosms or artificial ponds are large tanks or permanent outdoor systems. Some may even take the form of in situ enclosures in streams or ponds. The relative benefits of the various systems are still being assessed, with the inherent problem being that the greater complexity often makes it more difficult to develop causal relationships between the presence of a contaminant and response.

The use of mesocosms for amphibian studies was pioneered by Morin (1981) by employing 1000 litre cattle watering tanks. Such tanks have been used extensively since then for the study of amphibian community dynamics (for review see Rowe and Dunson 1994). More recently, similar tanks have been used to examine the effects of acidity (Clark and Hall 1985; Warner *et al.* 1991, 1993; Rowe *et al.* 1992; Sadinski and Dunson 1992; Horne and Dunson 1995a; 1995b), pyrethroid contamination (Materna *et al.* 1995), hydrocarbon contamination (Mahaney 1994; Lefcort *et al.* 1997) and fertilisers (de Wijer *et al.* 1997) on multiple species systems.

WHAT ARE THE PROBLEMS ASSOCIATED WITH AMPHIBIAN TOXICOLOGY?

Adult/Larvae (Terrestrial /Aquatic) dichotomy

One of the reasons that amphibians are considered good bioindicators of environmental contaminants is that the permeable skin of the adult terrestrial phase of their life cycle confers greater sensitivity than other vertebrates. One would expect therefore, that equal weight would be given in the literature to studies that examine toxicity in adults. In actuality, it has been rare for researchers to test the toxicity of pollutants on adults. There are two reasons for this. Firstly, various studies have indicated that the larval or tadpole stages are more sensitive to pollutants than eggs (Cooke 1972; Pritchard-Landé and Guttman 1973; Dial 1975; Greenhouse 1976; Bimber and Mitchell 1978; Saber and Dunson 1978; Birge *et al.* 1979; Hall and Swineford 1980; Davis *et al.* 1981; Mohanty-Hejmadi and Dutta 1981; Dial and Bauer 1984; Herkovits and Jatimliansky 1986; Dial and Bauer-Dial 1987; Anguiano *et al.* 1994; Berrill *et al.* 1994) while others have shown that post-metamorphic adults are less susceptible than larval stages (Hall and Swineford 1980; Schultz *et al.* 1983; Bidwell and Gorrie 1995; Mann and Bidwell 1998). Consequently, most work has concentrated on the larval tadpole stages.

Another factor which has led to a reluctance on the part of researchers to examine toxicity in adult amphibians is deciding on the most likely mode of exposure. Although the permeability of amphibian skin provides the obvious point of entry, oral intoxication by consumption of contaminated food must also be considered. Only a few studies have examined toxicity through oral administration (Rosato and Ferguson 1968; Tucker and Crabtree 1969; Hall and Swineford 1979; Dial and Dial 1995) with most concentrating on skin exposure.

The latter studies can be split into those which have used isolated skin preparations (Yorio and Bentley 1973; Celentano *et al.* 1979; Webb *et al.* 1979; Fromm 1981; Ferreira and Hill 1982; Salibian 1983; Ardizzone *et al.* 1990; Lippe *et al.* 1992; Natochin and Jones 1992) and whole animal studies. Among the whole animal studies, acute toxicity protocols are rare (i.e. Kaplan and Yoh 1961; Kaplan and Glaczenski 1965; Kaplan *et al.* 1967; Zaffaroni *et al.* 1986a; Mudgall and Patil 1987; Mann and Bidwell 1998) and most studies have examined an extremely wide range of sub-lethal effects such as behavioural effects (i.e. Cooke 1974; Hall and Swineford 1980; Roudebush 1988; Haniffa and Augustin 1989; Antony and Ramalingam 1990;), or physiological parameters including thermal tolerance (Johnson and Prine 1976), enzyme activity (Guzman and Guardia 1978; Deshmukh and Keshavan 1987; Joseph and Rao 1990; 1991; Mendiola and De Costa 1991), tissue metallothionein levels (Suzuki *et al.* 1986; Vogiatzis and Lombourdis 1998), limb regeneration (Manson and O'Flaherty 1978; Zavanella *et al.* 1984; Pfeiffer *et al.* 1985; Arias *et al.* 1989; Nebeker *et al.* 1994), and metabolism (Mudgall and Patil 1987).

Finally, approximately 50 studies were located which have utilised administration by hypodermic injection to examine a similarly wide variety of sub-lethal effects (e.g. Nagel and Urich 1981; Woodall and Maclean 1992; Scadding 1996). One study examined the acute responses to intraperitoneal injection of sodium fluoroacetate (1080) in the Australian species, *Limnodynastes tasmaniensis* (McIlroy *et al.* 1985), although the ecological significance of such a study is questionable. Indeed the existence of numerous nephrostome like structures associated with frog kidneys allowing rapid egress of water from the body cavity (Tyler pers. comm.) suggests that protocols that utilise intraperitoneal injection of a toxic agent may severely underestimate the toxicological threat posed by that toxin.

One notable similarity in all of those studies which have utilised adult subjects is the absence of any uniform methodology. Clearly a range of physiological and behavioural biomarkers have to be developed as standard test parameters for adult animals.

Species variability

Toxicological testing has for a long time relied on a small suite of aquatic test species. Prominent amongst these are salmon, trout, sunfish, daphnids and amphipods. Several studies have attempted to evaluate the relative sensitivity of amphibian larvae when compared to such species (Jolly *et al.* 1978; Thurston *et al.* 1985; Holcombe *et al.* 1987; Thybaud 1990; Herkovits *et al.* 1995; Deyoung *et al.* 1996; McCrary and Heagler 1997) but the issue remains contentious, since the results of such studies are often dependent upon the toxin to which test species are exposed (Thurston *et al.* 1985; Holcombe *et al.* 1987). Also, only a limited range of amphibian phylogenetic groups has been assessed in this manner.

Species variation amongst amphibians has received even less attention, although a few studies that have examined several species in parallel tests have reported species differences. Berrill *et al.* (1993, 1994, 1995) reported differences in the sensitivity of *Bufo* sp, *Rana* sp, and *Ambystoma maculatum* to the organophosphate pesticide fenitrothion and the pyrethroid pesticide permethrin. Hall and Swineford (1981) reported differences in sensitivity of up to one order of magnitude amongst *Bufo* sp, *Rana* sp, *Ambystoma* sp and *Acris crepitans* following exposure to the halogenated pesticides endrin and toxaphene. Hoppe and Mottl (1997) noted species-specific differences in the occurrence, type and severity of malformations amongst field collected animals in Minnesota. Wyman (1988) correlated distribution differences for several species of amphibians with soil pH. Interspecific variation in acid tolerance has also been reviewed in Freda (1986) and Pierce (1985).

Apart from a recent study examining variation in sensitivity between representatives of four temperate Australian frog genera (Mann and Bidwell 1998), the degree of species variation amongst Australian frogs, or between Australian species and standard test species like *Xenopus laevis* or *Rana* sp. is unknown. A comparative study by Mann and Bidwell (unpubl. data) indicated little variation in sensitivity to agricultural surfactants between the tadpoles of four Australian species and the two exotic species *Bufo marinus* and *Xenopus laevis* under standard test conditions. Such studies however, fail to consider the influence of high temperature, low oxygen environments inhabited by many Australian species. The extreme conditions experienced by Australian species need to be considered in the development of appropriate toxicity tests using Australian species.

It is worth emphasising here that the sensitivity of an organism is stage dependent. Not only do embryos and adults differ from tadpoles in their relative sensitivities (see previous section), but tadpoles at different stages of development will also display differences in sensitivities (Sanders 1970; Cooke 1972; Johnson 1976; Jordan *et al.* 1977; Wohlgemuth 1977; Mohanty-Hejmadi and Dutta 1981; Rao and Madhyastha 1987; Howe *et al.* 1998).

Animal supply

Many commonly used aquatic test species are either cultured in the laboratory, or, as in the northern hemisphere, are supplied by commercial companies. The advantage of such an approach is a constant supply of test organisms with a uniform age structure and known history with respect to nutrition and disease. In Australia there are no commercial supply houses able to provide native or exotic frogs in the large numbers required for toxicology (although the Amphibian Research Centre in Victoria may have that potential in the future) and, consequently, research on amphibian toxicology is largely dependent on field collected animals. This presents a number of problems. Firstly, animal collection in some states and territories is illegal or regulated by legislative authorities. Second, field collection is time consuming and can be relatively expensive. Third, there is always uncertainty about the health of field collected animals and their field exposure histories. Finally, it is becoming increasingly difficult to justify the removal of animals from natural populations. While field collection has not been singled out as an important factor in amphibian decline, it must be considered as a potential factor if animals are to be removed from an already declining population base. Commercial breeding of native species needs to be encouraged if amphibian susceptibility to environmental contaminants is to be pursued as a line of research in Australia.

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