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

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"Capsule": *Are frogs at risk from the toxic effects of agricultural surfactants?*

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 nonspecific 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). Replicate acute toxicity tests with *B. marinus* exposed to NPE at 30°C over 96 h 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) 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.

Keywords: Amphibian–tadpole; Nonionic surfactant; Acute toxicity; Nonylphenol ethoxylate; Narcosis

1. 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; Mann and Bidwell, 1999a; 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, 1999a). 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, 1999b). 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

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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*.

2. 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[El], Standard practice for conducting acute tests with fishes, macroinvertebrates and amphibians (ASTM, 1993).

2.1. 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.

2.2. 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. *X. 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). *B. 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). *C. insignifera* is a small (14–29 mm snout-vent length [s-v]) ground dwelling frog that inhabits areas temporarily inundated by water. *H. eyrei* is a medium sized (45–66 mm s-v) burrowing frog inhabiting sandy soils in areas prone to temporary inundation. *L. dorsalis* is a relatively large (60–73 mm s-v) ground frog which inhabits vegetation close to temporary and permanent water. *L. moorei* is also a relatively large (53–74 mm s-v) frog found in permanent waters where it inhabits emergent vegetation.

X. 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). *B. marinus* were induced to breed by intralymphatic administration of 10 μg luteinizing hormone–releasing hormone (LH–RH, Sigma, L45 13) 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. *C. 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. *L. dorsalis* and *L. moorei* were collected as egg masses from a single location in the Mandurah district south of Perth. *H. 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 these sites had large healthy populations of frogs.

2.3. 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, 1993) 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 7 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 produced by mating adults within the laboratory at 20°C, or transferred directly from field sites to the laboratory. Holding/acclimation periods ranged from 1 to 3 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 at 30°C within a climate control room for approximately 1 week before use. For the high temperature–low DO study, *B. marinus* tadpoles were obtained as described earlier 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 10–20% saturation DO. The observed fluctuation in DO was accounted for by the increased oxygen demand that 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 10 days.

2.4. 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 40–48 mg/l $CaCO_3$ and a conductivity of approximately 210 μS/cm (ASTM, 1993). Test concentrations were made up immediately prior to the beginning of the tests using soft water.

2.5. 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 1 s. If in response to prodding, a tadpole failed to swim strongly for at least 1 s, 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.

2.6. 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. *X. laevis* were at equivalent development stages 48 to 50 (Nieuwkoop and Faber, 1975). The average mass of at least 10 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, 1993). Either 400 ml or 600 ml acid-washed glass beakers with 200–500 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.

2.7. 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 12 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.

2.8. 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, Rochester, NY, 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 earlier. Temperature, pH, conductivity and DO were recorded at 2, 6, and 12 h in at least one replicate test chamber of each concentration.

2.9. Environmental conditions

All tests with native species were performed in a Conviron ClO 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 h. 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 9OFL multimeter.

2.10. 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 (Mann and Boddy, 2000). Briefly, NPE was extracted from the water samples using solid-phase extraction with 2.8 ml, 500 mg C_{18} cartridges (Alltech, Deerfield, IL, 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 8×10 Radial-Pak column was used for normal phase

separation of NPE oligomers. UV detection was carried out using a Waters™ 486 tuneable absorbance detector ($\lambda = 225$ 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. 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.

2.11. 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.

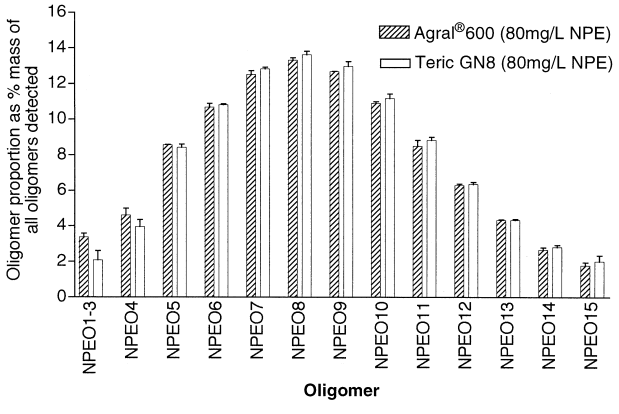

Fig. 1. Oligomer distribution for Agral®600 and Teric GN8. Error bars represent standard deviations for six measurements of Teric GN8 and 2 measurements of Agral®600.

3. Results

3.1. Chemical analysis

HPLC analysis of NPE indicated that oligomer distribution did not change over 24 h (Fig. 2) at either 20 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.

3.2. Water quality-comparative study

Water temperature, pH, DO, and conductivity were consistent across all tests. Recorded temperatures ranged between 18.9 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. DO occasionally dropped below 80% (but never below 70%) if the presence of dead tadpoles resulted in high oxygen demand.

3.3. Water quality-high temperature trials

Water temperature, pH, DO, and conductivity were also consistent across all tests. Recorded temperatures ranged from 29.0 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). DO occasionally

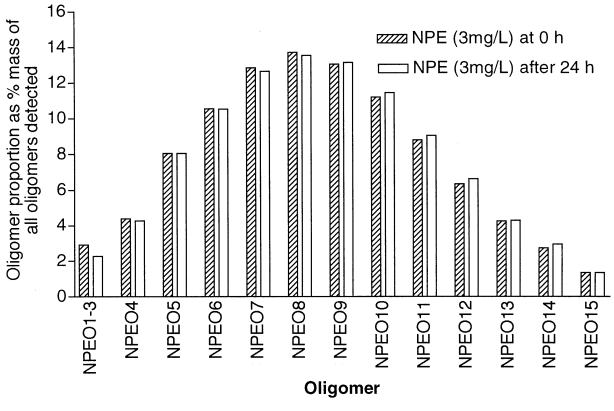

Fig. 2. Oligomer distribution for Teric GN8 at 0 and 24 h at 20°C.

dropped below 75% (but never below 65%) if the presence of dead tadpoles resulted in a high oxygen demand.

3.4. 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 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).

3.5. Acute toxicity

The comparative 48-h EC50 values obtained in this study are presented in Table 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*. *X. 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*, were 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 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 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 2). Tadpoles with hind limbs were no more or less sensitive than less developed tadpoles (Table 2). The mean wet weight of tadpoles in this test was 97.8 mg/l (Table 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). These values are similar to the 24, 48, 72 and 96-h

Table 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

Species	GN8			Agral®600			BS1000®		
	Average wet weight (mg; S.D.)	EC50 (95% CI; mg/l)[a]		Average wet weight (mg; S.D.)	EC50 (95% CI; mg/l)[a]		Average wet weight (mg; S.D.)	EC50 (95% CI; mg/l)[a]	
		Mild narcosis	Full narcosis		Mild narcosis	Full narcosis		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)[c]	6.0 (5.5–606)c
Limnodynastes dorsalis				8.8 (2.0)		4.1 (3.9–4.4)[c]	8.8 (2.0)	< 6.0[bc]	14.3 (13.8–14.8)[c]
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)			
Litora moorei				17.2 (3.0)		4.6 (4.2–5.0)[c]	17.2 (3.0)	< 11.0[bc]	
Helioporus eyrei				32.6 (7.4)	< 10.6[b]	12.1 (11.8–12.5)	41.6 (11.0)	< 10.0[bc]	25.4 (24.2–26.6)[c]

[a] EC50 values are expressed as mg/l nonylphenol ethoxylate (GN8 and Agral®600) or mg/l alcohol alkoxylate (BS1000®).
[b] Data for which there were insufficient lower concentrations to generate an EC50.
[c] Data for which nominal concentrations were used to calculate an EC50.

Table 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[a]

	Average wet weight (mg; S.D.)	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[b]	3.5 (3.4–3.6)	3.5[b]	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)	4.3 (4.2–4.4)
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 (S.D.)		0.36	0.25	0.27	0.15	0.38	0.38	0.41	0.34
Coefficient of variation		10%	6.57%	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)		

[a] All EC50 values are based on nominal concentrations.
[b] Insufficient data to calculate 95% confidence interval.

EC50 values generated in static-renewal tests at normal DO (Table 2). The 12-h EC50 (FN) at low DO and 30°C ranged from 1.4 to 2.2 mg/l (Table 3, Fig. 3).

At low DO, a maximal narcotic effect was produced after 2–6 h (Fig. 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. 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. 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. 3a).

4. 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, 1979; 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. Similar difficulties were encountered by Calamari and Marchetti (1973) in 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).

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 2). This phenomenon was also quite distinct over the 2-h intervals in the low DO trials (Fig. 3a). None of the observed recoveries were statistically significant since

Table 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[a]

Species/test no.	Average wet weight (mg; S.D.)	12-h EC50 (95% CI; mg/l)		DO (mg/L)[b]	
		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 no. 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 no. 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 no. 3)	43.0 (9.3)	–	1.4 (1.3–1.5)	0.7–1.7	0.8–1.3

[a] All EC50 values are based on initial measured concentrations. DO, dissolved oxygen.
[b] DO was measured at 2, 6 and 12 h, in at least one replicate chamber for each exposure concentration.

EC50 values varied less than the 95% confidence intervals of individual values. However, the observation remains noteworthy since 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 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 8 hours exposure is not surprising, since changes in membrane fluidity and phospholipid composition were observed in trout following 6 hours exposure to cold water (Williams and Hazel, 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 for these differences in sensitivity 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 reflect 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). *X. 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* has been found to be 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.

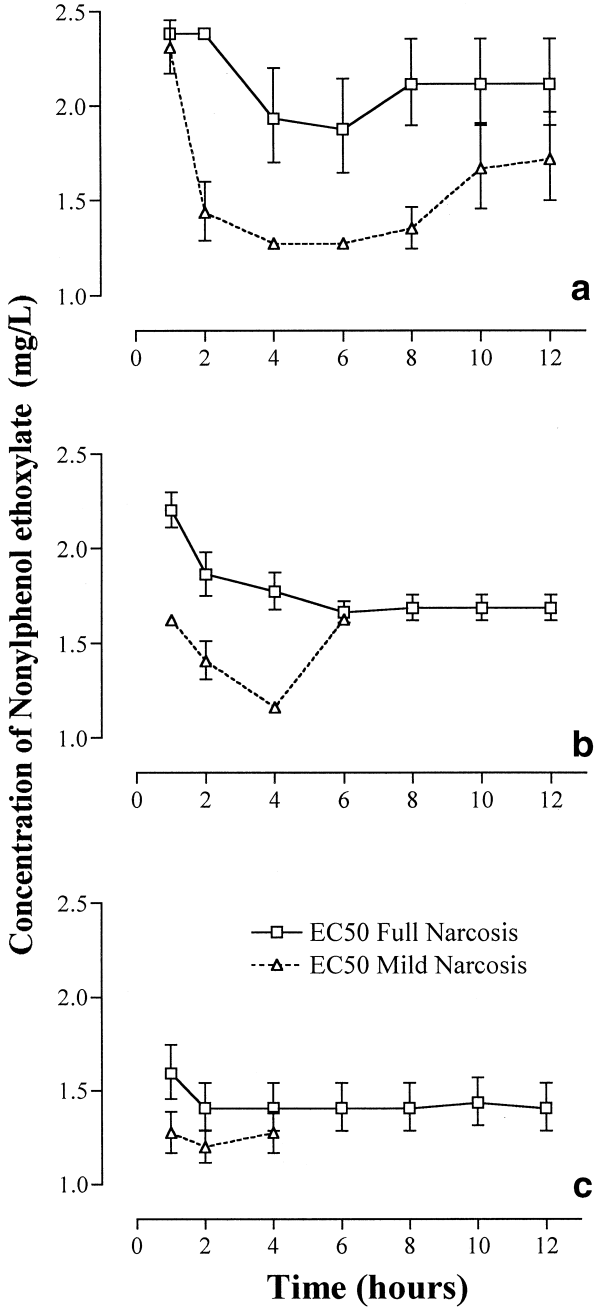

Fig. 3. EC50 values for *Bufo marinus* exposed to Teric GN8 (100% nonylphenol ethoxylate) in 12-h flow-through acute toxicity tests at 30°C and (a) 1.7 to 2.3 mg/l DO, (b) 1.2 to 1.7 mg/l DO, (c) 0.8 to 1.3 mg/l DO. Error bars represent 95% confidence intervals. Absence of error bars indicates insufficient data for the calculation of a 95% confidence interval.

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 that indicate that late stage, pre-metamorphic 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 10 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 (unpublished observation). Similar nonspecific 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 2) may therefore reflect the detrimental effects of cumulative pathology.

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. 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. 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 to *Lepomis macrochirus*.

High temperature–low DO conditions are characteristic of tropical Australian 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.

5. 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. *X. 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.

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