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The salinity tolerance of riverine microinvertebrates from the southern Murray-Darling Basin.

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Abstract

Concern about the effect of rising salinity on freshwater biodiversity has led to studies investigating the salt tolerance of macroinvertebrates and fish, with less attention given to microinvertebrates. We investigated the acute lethal effects of salinity on 12 microinvertebrate species from rivers in the southern Murray-Darling Basin in central Victoria, Australia. For a sub-set of these species, sub-lethal salinity effects and the effect of water temperature on salinity tolerance were also investigated. The most sensitive microinvertebrates had broadly similar 72-h LC₅₀ values to the most sensitive macroinvertebrates, reported in other studies. However, the most tolerant microinvertebrates tested were much more sensitive than the most tolerant macroinvertebrates and the microinvertebrates studied were more sensitive than most freshwater fish. Temperature affected the acute lethal toxicity of salinity but only to a small degree. In three of four species (the exception being *Hydra viridissima*), the effects of

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salinity on growth, development and/or reproduction at levels below their 72-h LC_{50} values were observed. However, different endpoints responded differently to salinity. The demonstrated effect of salinity on microinvertebrates has the potential to indirectly affect fish and salt tolerant macroinvertebrates via changes to their prey species or ecological functions performed by

5 microinvertebrates.

Additional keywords: microcrustaceans, zooplankton, *Newnhamia fenestra*, *Simocephalus*, salinization

Running heading: The salinity tolerance of riverine microinvertebrates

Introduction

The impact of increasing salinity on inland rivers and wetlands from rising saline groundwaters is recognised as a major issue for the conservation of freshwater biodiversity in Australia and elsewhere (MDBMC 1999; Williams 1999; Halse *et al.* 2003). Measuring the responses of freshwater organisms to changes in salinity is vital to understand, forecast and thus manage the effects of rising salinity on freshwater biodiversity. Laboratory studies can provide a causal link between a particular salinity and the measured responses. Recent laboratory experiments have demonstrated that high salinity adversely affects riverine macroinvertebrates (e.g. Williams *et al.* 1999; Kefford *et al.* 2003, 2005a, 2006a) and freshwater fish at various life stages (reviewed by James *et al.* 2003). There have been fewer experimental studies on the salinity tolerance of freshwater microinvertebrates. The experimental studies conducted have tended to relate to wetlands, rather than rivers, and measure the effect of salinity on the emergence of microinvertebrates from sediment egg banks (e.g. Skinner *et al.* 2001; Nielsen *et al.* 2003a) and not the effect of salinity on non-dormant life-stages. Other studies have considered the effect of tide-induced salinity changes on microinvertebrates (e.g. Schallenberg and Burns 2003; Schallenberg *et al.* 2003). The processes of inland salinity and estuarine salinity and the salinity tolerances of organisms, which inhabit these environments, are likely to differ, therefore the relevance of this work to inland salinity is unknown.

Our main objective was to use laboratory experiments to measure the relative salinity tolerance of a range of riverine microinvertebrates (Table 1) using a method allowing direct comparisons with existing data on the relative salinity tolerance of macroinvertebrates and fish. These groups were compared to assess the potential for indirect effect, which are defined here as effects of salinity on a species via ecological interactions with more sensitive species. While the theoretical possibility of indirect effects of salinity is

widely acknowledged (e.g. Hart *et al.* 1990; James *et al.* 2003; Kefford *et al.* 2006a), their prevalence and ecological importance is unknown. Microinvertebrates are the prey of predatory fish and macroinvertebrates. Thus if the salinity sensitivity of microinvertebrates differed substantially from that of fish and macroinvertebrates, there is potential for indirect effects of salinity on the latter groups.

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The null hypothesis tested is that the species sensitivity distributions (SSDs) of riverine microinvertebrates exposed to salinity do not differ from the SSDs for macroinvertebrates and fish. For most species we considered acute (≤ 96 h) lethal effects of salinity only, because for fish and macroinvertebrates these effects have the most extensive datasets. Furthermore, lethal levels of salinity for freshwater macroinvertebrates and fish (Kefford *et al.* 2004a, 2006a) and estuarine microinvertebrates (Schallenberg *et al.* 2003) have been related to the distribution of species in the field with respect to salinity. However, salinity can also cause sub-lethal effects and its effect can be influenced by other water quality factors. Therefore for a subset of species the secondary objective was to measure sub-lethal effects of salinity and the effect of water temperature on salinity tolerance (Table 1).

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Materials and methods

Microinvertebrates were collected from five river sites (Table 1) in the southern Murray-Darling Basin (MDB) in central Victoria, Australia. This area spans the rainfall range of 600 to 1 200 mm year⁻¹ falling mostly in winter and spring. Two species (the rotifer, *Epiphanes macrourus*, and green hydra, *Hydra viridissima*) were also obtained from a commercial supplier (Southern Biological, Nunawading, Victoria) originally sourced from a local site (Southern Biological, pers. comm.).

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The salt source used in all experiments, the aquarium salt Ocean Nature (Aquasonic, Wauchope, NSW), has ionic proportions similar to sea water, the most common ionic proportions in saline waters of south-eastern Australia (but see Radke *et al.* 2002; Zalizniak *et al.* 2006). Where possible, experiments were

preferentially conducted in carbon-filtered and aged Melbourne tap water, hereafter referred to as wet lab water (WLW) with salinity of 0.1-0.15 mS cm⁻¹ and a typical chloride concentration of approximately 9 mg L⁻¹ (Kefford *et al.* 2004b). This medium was preferentially used for consistency with experiments on macroinvertebrates (Kefford *et al.* 2003, 2005a, 2006a; Dunlop *et al.* in press). Further information on typical ionic proportions in WLW and WLW with added Ocean Nature can be found in Kefford *et al.* (2004b) and Zalizniak *et al.* (2006). However, some microinvertebrate species had poor survivorship (regardless of salinity) in WLW and for these species (Table 2) we used water from their collection site, referred to as river water (RW).

Throughout this paper salinity is expressed as electrical conductivity (EC). The relationships between EC and other measures of salinity for Ocean Nature salt are: $TDS = 0.754 \cdot EC$, and $osmolarity = 0.0184 \cdot EC$, where EC is in mS cm⁻¹ @ 25°C, TDS is total dissolved solids in g L⁻¹ and osmolarity is in Osmol kg⁻¹ H₂O as calculated over the salinity range of 0.1-67 mS cm⁻¹ by Kefford *et al.* (2003). All EC values reported are presented as specific EC i.e. @ 25°C.

Acute lethal effects of salinity at 20°C

To allow direct comparisons with existing data on the salinity tolerance of macroinvertebrates, the methods of Kefford *et al.* (2003, 2005a, 2006a) were followed, except that smaller containers (Table 1) were used to house the microinvertebrates, and thus the methods are only briefly outlined. We used a rapid test approach (Kefford *et al.* 2005b), which aims to determine the approximate tolerance of a number of species from a range of taxonomic and other groups, including rare species (Table 1), rather than the standard ecotoxicological approach of obtaining accurate estimates for a few species. Rapid testing implies that it is better to have an approximate estimate of sensitivity, calculated for a few individuals of a taxon, than no estimate at all. By attempting to test species from particular taxonomic

groups in approximate proportion to their occurrence at the collection sites, the estimated SSD should better reflect the SSD for microinvertebrates of the southern MDB (see Kefford *et al.* 2005b).

Most acute salinity tolerance data for Australian macroinvertebrates are from 72-h tests. Most of our tests on microinvertebrates were for this period. A subset of experiments were, however, conducted for 96 h.

Some taxa were collected from multiple sites or from the same site on multiple occasions (Table 1) allowing for spatial and temporal variation to be incorporated into the estimated salinity tolerance. Where species level identification is not reported (Table 1) we treat each taxon as a species, even though it is possible that it may comprise individuals from multiple species. Results suggest that, if multiple species were tested, all had similar acute lethal salinity tolerance. Where fewer than 50 individuals of a taxon were obtained in every collection, they were arbitrarily defined as rare. For common taxa, multiple individuals were exposed concurrently to control (WLW or RW) and treatments of various salinities as in standard ecotoxicological tests. Salinity treatments were chosen depending on assumed salinity tolerance of each taxon (initially from Hart *et al.* 1991; Skinner *et al.* 2001; Nielsen *et al.* 2003a,b; but later from the results of previous tests). The up-down test (see Bruce 1985, 1987; Sunderam *et al.* 2004) was modified for rare taxa. On each occasion when a rare taxon was collected it was exposed to one or more treatments. If this taxon was collected on a subsequent occasion, previous survivorship results were used to select salinity treatments (as per Kefford *et al.* 2003, 2005a).

The volume of water was determined by the size of the species (Table 1). Lids on vessels minimised evaporation. Animals were not fed in acute experiments nor were the test waters changed. Mortality was defined by immobility and failure to respond to prodding.

For common taxa, logistic regression equations were fitted to daily mortality data and LC₅₀ values were calculated from these equations. For rare taxa, approximate LC₅₀ values were determined as a point estimate, greater than a specified value (censored data) or as a range (as per Kefford *et al.* 2003, 2005a). When given as a range, the LC₅₀ values were assumed to be the midpoint of this range. Due to the inclusion of censored data, SSD were constructed using the Kaplan-Meier method, a form of survival analysis.

Effect of temperature on salinity tolerance

For three species, *H. viridissima*, *Simocephalus* sp. and *E. macrourus*, acute lethal experiments were conducted at different water temperatures to investigate whether temperature influences salinity tolerance (Table 1). Unless otherwise specified, these experiments were conducted in an identical manner to those described above for common species. The experiments were conducted in constant temperature rooms (± 1 °C). There were minor differences in the methods used for each species depending on their specific requirements (Table 1). While this is suboptimal for comparisons between species, it will still give an indication of the potential for changes in water temperature to alter salinity tolerance.

This study was designed to investigate the effect of water temperature on salinity tolerance and not the effect of temperature shock. *Epiphanes macrourus* were therefore added to their test solutions at 20 °C and then transferred to 10 and 30 °C constant temperature rooms, and their test water adjusted to the test temperatures. At 10 °C *E. macrourus* were slow moving and it was difficult to ascertain whether non-moving individuals were actually dead. Thus after the completion of the experiment (96 h), test solutions at 10 and 30 °C were moved to 20 °C and assessment on survivorship confirmed after a further hour.

Hydra viridissima were cultured at 20 °C and then acclimated to their respective temperatures for 72 hours before commencing the salinity tolerance experiment. The *H. viridissima* experiment comprised 3 replicates per salinity and temperature treatments, each containing four non-budding *H. viridissima*.

5 *Simocephalus* sp. cultures were established at 20 and 12 °C in RW and neonates (<72 h old) were collected from both temperatures and then allocated to salinity treatments to conduct a 48h LC₅₀ experiment. The unicellular green algae *Pseudokirchneriella subcapitata* was periodically added to the *Simocephalus* sp. cultures as a food source and other food sources were likely to be present in the RW.

Egg hatching and sub-lethal effects of salinity

10 To facilitate comparisons of the methods used for each species they are summarised in Table 3 and described in more detail below. *Simocephalus* sp. neonates (<72h old) from the 20°C culture were studied for their sub-lethal salinity tolerance. *Simocephalus* sp. were housed individually for 16 days and number of offspring produced, the number of moults and the final size determined. *Pseudokirchneriella subcapitata* was provided daily as a food source.

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Hydra viridissima were fed (ad lib brine shrimps, *Artemia salina*) for 5 days, to ensure they were budding, and then used in a sub-lethal experiment. Changes in population size after 5 days of 10 *H. viridissima* and 10 *E. macrourus* were determined at a range of salinities. For both species, the per capita rate of population increase (r) was calculated for each treatment using the formula:

20 $r = [\ln(N_t + 1) - \ln(N_{t-1} + 1)] / \Delta t$ where N_t is the number of animals at time t , N_{t-1} is the number of animals at time of the previous observation and Δt is the time between two observations (days). If a populations is increasing $r > 0$, in a constant sized population $r = 0$ and in a declining population $r < 0$. In a separate experiment with *H. viridissima*, non-budding individuals were used and the proportion with abnormal

tentacle contraction was determined daily over 5 days in different salinity treatments (as per Pollino and Holdway 1999).

5 *Newnhamia fenestra* in RW (0.6 mS cm^{-1}) were observed to lay eggs. As the younger life-stages, such as eggs, are often considered more salt sensitive than older life-stages (James *et al.* 2003; Kefford *et al.* 2004b) eggs were hatched and grown at a range of salinities. Eggs of *N. fenestra* were laid in clusters. Since egg clusters were limited and may exhibit genetic differences, some clusters were divided (as done with *Chironomus tepperi* egg masses by Kefford *et al.* 2004b) into individual embryos and the embryos from several clusters mixed before allocating them to treatments. As a procedural control other clusters
10 were exposed whole.

Newnhamia fenestra eggs took > 72 hours to hatch. To directly compare their tolerance to the adults' 72h-LC₅₀ value (above), some eggs (both clusters and single embryos) were exposed to high salinity treatments for 72 hours and then returned to RW. Other eggs, and their subsequent hatchlings, were
15 continuously exposed for 21 days. For all groups of eggs, hatching success, survival of hatchlings and size of hatchlings at day 21 of exposure was determined. On the first sighting of hatchlings, a suspension of *P. subcapitata* was added for feeding and was renewed weekly after water changes.

Results

Acute lethal effects of salinity at 20°C

20 Where the same taxon was collected from different sites and on different collections from the same site, the salinity responses were very similar and the following analysis was conducted with individuals from all sources pooled.

The most salt sensitive species tested was *H. viridissima*, while the most tolerant was *N. fenestra* (Table 2). For Chydoridae and *Simocephalus* sp. LC₅₀ values were not calculable after 72 hours of exposure due to poor survival in all salinity treatments but were calculable after 24 and 48 hours of exposure, respectively (Table 2). Mortality of these taxa was probably due to starvation, and for the purpose of constructing the SSD their 72-h LC₅₀ values were assumed to equal those at 24-h or 48-h. Despite the lack of food, there was some reproduction of *Paramecium* over the course of the experiment and since it was impossible to tell which individuals were the parent and daughter *Paramecium*, their LC₅₀ values actually increased slightly through time (Table 2).

The mean and median (95% CI) 72-h LC₅₀ values of all taxa tested were 12.9 (9.0-16.8) and 13.5 (5.7-21.3) mS cm⁻¹, respectively, with 91% of taxa having a 72-h LC₅₀ value greater than 4.2 mS cm⁻¹ and 82% > 4.9 mS cm⁻¹ (Fig. 1a). Overall the crustaceans were more tolerant (a mean 72-h LC₅₀ value of 15.3 (11.2-19.6) mS cm⁻¹) than non-crustaceans (6.4 (4.1-8.6) mS cm⁻¹) (Fig. 1b). Copepods and ostracods were the richest crustacean groups (both n=3). The mean 72-h LC₅₀ value for copepods (11.1 (6.1-16.2) mS cm⁻¹) was significantly lower than that for ostracods (20.3 (19.7-29.8) mS cm⁻¹) while the mean value for ostracods was significantly higher than that for other crustaceans (12.4 (5.7-19.1) mS cm⁻¹). The cladocerans appeared more salt sensitive than other crustaceans (Table 2), however, with data for only two species, it is not possible to generalise. Although the mean 72-h LC₅₀ value for common taxa, 10.5 (4.0-17) mS cm⁻¹, was less than that for rare taxa, 16.5 (12-21) mS cm⁻¹, their 95% CIs overlapped.

The Weibull distribution, with scale and shape parameters of 14.1 and 1.9, respectively, (see Bain and Engelhart 1987) described the non-censored 72-h LC₅₀ values reasonably well (Fig. 1c).

Effect of water temperature on salinity tolerance

Salinity had a similar effect on the survival of the rotifer, *E. macrourus*, at two of the three temperatures investigated (Fig. 2a). At 20 and 30 °C the salinity concentration-response curves were very similar and

the 96-h LC₅₀ values were much the same 6.1 (5.0-7.6) and 5.6 (2.1-16.4) mS cm⁻¹, with overlapping 95% CIs. At 10 °C the 96-h LC₅₀ value was lower at 3.0 mS cm⁻¹ (95% CI not calculable). However, at this temperature, while all individuals survived for 96 hours at 2 mS cm⁻¹, at lower salinities the survivorship was less than 70% (Fig. 2a).

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Simocephalus sp. had lower salinity tolerance at 20 °C than at 12 °C (Fig. 2b). The 48-h LC₅₀ values for the two temperatures do not have overlapping 95% CIs, 5.7 (5.2-6.2) mS cm⁻¹ and 7.3 (6.8-7.8) mS cm⁻¹, (calculated with a single logistic regression with temperature as a fixed factor).

- 10 All *H. viridissima* survived for 96 hours in 4 mS cm⁻¹ at 12 °C while at the same salinity at 20 and 26 °C 100% died within 24 hours. The 96-h LC₅₀ value for 12 °C exposure was 4.9 (4.1-6.0) mS cm⁻¹, while for 20 and 26 °C it was 3.5 (3.1-3.9) mS cm⁻¹. However, it should be noted that none of the *H. viridissima* exposed to 4 mS cm⁻¹ at 12 °C had normal tentacles (Fig. 2c). *Hydra viridissima* also appeared to be slightly more tolerant of high salinity in terms of tentacle contractions at cool water temperatures (Fig.
- 15 2c) but differences were not statistically significant. The 50% effect concentration (EC₅₀ value) for tentacle contractions at 12 °C was 2.5 (2.0-3.0) mS cm⁻¹, for 20 °C 1.9 (CI not calculable) mS cm⁻¹ and for 26 °C 1.8 (1.3-2.1) mS cm⁻¹.

Egg hatching and sub-lethal effects of salinity

- Increases in *E. macrourus* population were evident in all treatments except 8 mS cm⁻¹ on each day of the
- 20 experiment. However, the trend in population growth was clearest when calculated over the entire experiment (Fig. 3), where there was a statistically significant difference between salinity treatments (F=34, df=5&54, P<0.001). Population growth was not maximal in the lowest salinity (0.5 mS cm⁻¹) but at an intermediate salinity (2 mS cm⁻¹) before decreasing at 4 mS cm⁻¹; no population growth occurred at 8 mS cm⁻¹ (Fig. 3). It is noteworthy that survivorship of *E. macrourus* after 96 hours at 20 and 30 °C in 4

mS cm⁻¹ was > 80% (Fig. 2a), while at this salinity its population growth was about half of that at 2 mS cm⁻¹.

While there was a considerable range in the hatching success of *N. fenestra* eggs exposed to low salinity (<2.5 mS cm⁻¹), a general reduction, well described by linear regression ($r=-0.84$, $P<0.001$, $n=46$) was observed in hatching with increasing salinity (Fig. 4a). This reduction in hatching was similar and not significantly different (Fig. 4a) between egg clusters and single embryos ($r=-0.82$, $P<0.001$, $n=35$; $r=-0.89$, $P<0.001$, $n=11$, respectively). The concentration at which 50 % of eggs hatched (HC₅₀) was about 10 mS cm⁻¹.

Exposing *N. fenestra* eggs to salinity for 72 hours only and then returning them to RW (0.6 mS cm⁻¹) had a similar effect on hatching success as continuous salinity exposure (Fig. 4a,b). The only effect of exposure appeared to be that with continuous exposure at ≥ 20 mS cm⁻¹ no hatching occurred (Fig. 4a), while with 72-h exposure a minority (20-40%) of embryos hatched at 20 and 22.5 mS cm⁻¹ (Fig. 4b). No hatching occurred at 25 mS cm⁻¹ regardless of exposure type.

There were no differences between egg types (egg clusters and single eggs), therefore the following results are presented ignoring egg type. Eggs of *N. fenestra* hatched within 4 to 17 days from the commencement of exposure (Fig. 4c). There were significant positive linear correlations between the number of days to complete hatching and EC for both exposure types: continuous ($r=0.67$, $P<0.001$, $n=32$) and 72-h ($r=0.52$, $P=0.032$, $n=16$). There was no significant difference between these correlations ($F=0.001$, $df=1\&45$, $P=0.972$).

Exposing eggs to salinity for 72 hours appeared to have no effect on the consequent survival of *N. fenestra* hatchlings (Fig. 5a) with 100% of hatchlings from 5-22.5 mS cm⁻¹ surviving for 21 days. In

contrast, no *N. fenestra* survived for 21 days continuous exposure at 17.5 mS cm⁻¹ and about 50% survived at 15 mS cm⁻¹ (Fig. 5a). The length of individual *N. fenestra* that survived 21 days appeared to follow an inverted 'U' shaped response curve (Fig. 5b). Limited hatching or survival in the lowest and highest salinities precludes statistical analysis.

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The size of *Simocephalus* sp. after 16 days differed between WLW (control and WLW with salt added to be the same salinity as RW) and RW (control and RW treatments with added salt) (Fig. 6a) and in the following analysis data from WLW were eliminated. After 16 days of salinity exposure in treatments with salt added to RW, *Simocephalus* sp. size decreased approximately linearly with EC ($r=-0.49$, $n=52$, $P<0.001$). There was no relationship between number of moults of *Simocephalus* sp. after 16 days exposure and salinity.

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There appeared to be no difference in *Simocephalus* sp. reproduction (number of neonates produced) in WLW and RW (Fig. 6b) and for this response all data were combined. Reproduction of *Simocephalus* sp. that survived the 16 days of the experiment poorly fitted a linear response ($r=0.18$, adjusted $r^2=0.033$, $df=1\&82$, $F=2.8$, $P=0.099$) but was better described by quadratic ($r=0.58$, adjusted $r^2=0.32$, $F=20$, $df=2\&81$, $P<0.001$) and cubic ($r=0.62$, adjusted $r^2=0.37$, $F=17$, $df=3\&80$, $P<0.001$) regressions (Fig. 6b). The issue here is not whether the response is cubic or quadratic because these responses rarely have direct biological meaning. Instead the main implication is that a non-linear response was observed with maximum reproduction in *Simocephalus* sp. occurring at intermediate salinities.

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After five days there was no statistically significant difference in *H. viridissima*'s population growth rate (r) in the various salinity treatments (1.5-4 mS cm⁻¹) ($F=1.3$, $df=5\&54$, $P=0.29$). In a separate experiment, within one hour of exposure *H. viridissima* had responded to salinity ≥ 4 mS cm⁻¹ by contracting their tentacles (Fig. 7). All individuals ≥ 8 mS cm⁻¹ subsequently died within 24 hours of exposure. There was

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a recovery by 24 hours in the tentacle contraction in a minority of *H. viridissima* exposed to 4 and 6 mS cm⁻¹. Even then, all *H. viridissima* exposed to 6 mS cm⁻¹ died within 96 hours. Of those exposed to 4 mS cm⁻¹ between 30 and 90 % survived 96 hours of exposure. In two of the three replicates none of these survivors had normal tentacles, while 90% of survivors from the third replicate had normal tentacles. At lower salinity no mortalities occurred and most had normal tentacles (Fig. 7).

Discussion

Acute lethal effects of salinity

The riverine microinvertebrates tested had 72-h LC₅₀ values ranging from 3.8-20.8 mS cm⁻¹, which is within the range of salinity recorded for Victorian rivers (Kefford *et al.* 2006a). Considering these LC₅₀ values do not incorporate sub-lethal and chronic effects and reduced salinity tolerance of young life-stages, it is likely that adverse effects of salinity on freshwater microinvertebrates currently occur in Victorian rivers.

The most sensitive microinvertebrates tested had similar 72-h LC₅₀ values to the most salt sensitive macroinvertebrates (Kefford *et al.* 2003, 2005a, 2006a; Dunlop *et al.* in press). The upper end of this range of 72-h LC₅₀ values is, however, considerably lower than that of most salt tolerant macroinvertebrates (76 mS cm⁻¹ Kefford *et al.* 2003). Likewise, the mean 72-h LC₅₀ value of microinvertebrates (12.9 mS cm⁻¹), is less than half the mean values (31 and 32 mS cm⁻¹) recorded in comparable studies with macroinvertebrates (Kefford *et al.* 2003, 2005a). There is currently considerably more experimental and observational salinity sensitivity information available on Australian freshwater macroinvertebrates than microinvertebrates. While not ideal, for interim management decisions it may be acceptable to use data from sensitivity macroinvertebrate data as surrogates for microinvertebrates.

Several reviews (e.g. Hart *et al.* 1991; James *et al.* 2003; Nielsen *et al.* 2003b) state that salinity tolerance in freshwater invertebrates is negatively related to the length of time since the species evolved from marine ancestors. This conclusion was based on studies with groups of related species, (e.g. Sutcliffe 1974). Our study across a wider range of taxa, however, provides little support for this idea across all invertebrates. Freshwater crustaceans have a more recent marine origin than insects (which comprise the majority of the macroinvertebrates), yet the microcrustaceans investigated here were more sensitive than many insects (Kefford *et al.* 2003, 2005a, 2006a; Dunlop *et al.* in press).

The acute lethal salinity effects range in microinvertebrates recorded in the current study is similar to that of early life-stages of Australian freshwater fish 2.1-20 g L⁻¹ or 2.7-26 mS cm⁻¹ (James *et al.* 2003). The acute tolerance testing of the microinvertebrates was conducted using individuals of undetermined age and younger individuals, which are potentially more salt sensitive (see Kefford *et al.* 2004b), were not preferentially selected. Therefore it is probably appropriate to compare our data to that from adult freshwater fish, which are clearly more tolerant than the microinvertebrates in the current study. Acute (mostly 96-h) LC₅₀ value range of adult freshwater fish is 8-59 g L⁻¹ or about 10.6-78 mS cm⁻¹ (James *et al.* 2003). Small rises in salinity will thus likely have greater direct effects on freshwater microinvertebrates than fish.

Environment Canada (2001) reported the 96-h LC₅₀ values for sodium chloride from three North American and European cladocerans (*Ceriodaphnia dubia*, *Daphnia pulex* and *D. magna*) to be generally lower (range 2.3-6.7 g L⁻¹ NaCl or about 3.1-8.9 mS cm⁻¹) than those for nine North American freshwater fish (range 5.0-22 g L⁻¹ NaCl or about 9.2-40 mS cm⁻¹). Although sea water is predominantly sodium chloride, sea water is less toxic (Kefford *et al.* 2004c). We therefore caution against direct comparisons of this data with ours. However, as the toxicity of sodium chloride and sea water is correlated (Kefford *et al.* 2004c), it is possible to compare the relative sensitivity LC₅₀ values from these two salt sources. The

most sensitive fish species reported by Environment Canada (2001) had a 96h LC₅₀ value for sodium chloride similar to that of the cladocerans, yet the more tolerant fish were clearly more tolerant than the cladocerans, which is consistent with the current study.

5 As microinvertebrates also inhabit saline lakes (e.g. Pinder *et al.* 2002, 2005; Blinn *et al.* 2004), there clearly exist some species more salt tolerant than those tested here. Furthermore, in regions like south-western Australia, where there is a longer history of primary (natural) and secondary (anthropogenic) salinization (see Pinder *et al.* 2005 and references therein), organisms may have evolved greater salinity tolerance or more salt sensitive species may have been eliminated. Indeed in the current study

10 *Mytilocypris* sp. had a 96-h LC₅₀ value of 15 mS cm⁻¹, but in south-western Australia this genus has been recorded in salinity up to about 160 mS cm⁻¹ (Pinder *et al.* 2005). Our results, however, suggest that most microinvertebrates inhabiting freshwater rivers of the southern Murray-Darling Basin are generally more salt sensitive than most freshwater macroinvertebrates and fish. Small rises in salinity in this region may thus have greater direct effects on riverine microinvertebrates than on macroinvertebrates or fish. It is

15 unknown whether, and if so over what time scale, the loss of salt sensitive riverine microinvertebrates would be replaced by more tolerant organisms.

Hart *et al.* (1991) cites examples where reproductive status, stages within the moult cycle, sex, body size and nutrition influence salinity tolerance of invertebrates, resulting in variation in salinity tolerance of

20 apparently identical individuals within a population. Acclimation to elevated, but non-lethal, salinity levels can also increase salinity tolerance (Hart *et al.* 1991; James *et al.* 2003). There is also the possibility of genetic differences contributing to salinity tolerance within a species. Yet when the same macroinvertebrate (Kefford *et al.* 2003, 2005a, 2006a) or microinvertebrate (this study) taxa were collected from different sites and from the same site on different dates, there were no detectable

25 differences in acute salinity tolerances (despite a range of collection salinities). This finding held true

even when macroinvertebrates at sites separated by 1 000's km (Dunlop *et al.* in press). Therefore, while such factors may be important in determining the salinity tolerance of individuals, the available evidence suggest that for most taxa either they have a small effect or the variation is enough to obscure any spatial and temporal difference.

5

Although the use of rapid ecotoxicity testing (Kefford *et al.* 2005b) allowed the testing of more species than usual, microinvertebrates proved more difficult to work with than macroinvertebrates and we obtained acute salinity tolerance data from relatively few microinvertebrates (12 taxa), compared to macroinvertebrates (Kefford *et al.* 2003, 2005a, 2006a: 57, 49 and 110 taxa respectively). We thus note

10 only preliminary differences in the relative salinity tolerance between microinvertebrate taxa. *H.*

viridissima was the most salt sensitive species investigated, which along with Fields (2002), supports the finding of Hart *et al.* (1991) that *Hydra* is one of the most salt sensitive invertebrate genera. In general, the microcrustaceans studied were more salt tolerant than other microinvertebrate taxa, the only exception being two cladocerans (Table 2). Within the microcrustaceans, ostracods were more tolerant

15 than copepods and other crustaceans. In south-western Australia, Pinder *et al.* (2005) found that most rotifers and other non-arthropod microinvertebrates tended to be more restricted to freshwater wetlands than microcrustaceans. Kefford *et al.* (2003, 2005a) observed that macrocrustaceans tended to be more salt tolerant than insects which in turn were generally more salt tolerant than non-arthropods. However, the microcrustaceans studied here were considerably more sensitive (72-h LC_{50} range 4.9-20.8) than the
20 macrocrustaceans (72-h LC_{50} range 38-76 $mS\ cm^{-1}$) studied by Kefford *et al.* (2003, 2005a).

Unlike macroinvertebrates (Kefford *et al.* 2003, 2005a), rare microinvertebrates were not significantly more salt tolerant than common microinvertebrates. However the trend for the greater tolerances or rare species was observed in both invertebrate groups and, with their small sample size, the possibility of a
25 type-2 error should be considered.

Our study shows that salinity could rise to a concentration lethal to many inland riverine microinvertebrates yet not lethal to most macroinvertebrates and adult fish from the same or comparable environments. Microinvertebrates are prey for many predatory macroinvertebrates and fish, they perform different ecological functions to macroinvertebrates and fish and their populations' can more quickly expand in size when conditions are favourable. Salinity could, therefore, indirectly affect macroinvertebrates and fish by directly affecting microinvertebrates.

Effect of water temperature on salinity tolerance

In all three species where the effect of water temperature on acute salinity sensitivity was investigated, some relative minor temperature effects were observed. The salinity tolerance of *E. macrourus* at 20 and 30°C was similar but appeared more sensitive at 10°C. However, at 10°C *E. macrourus* had poor survivorship at salinity $< 2 \text{ mS cm}^{-1}$ (including in the control). *Epiphanes macrourus* could be more sensitive to salinity at 10°C, than at 20 and 30°C, producing an inverted 'U' shaped concentration response (see Kefford and Nugegoda 2005) at 10°C only. Alternatively survivorship of *E. macrourus* is generally reduced at 10°C, irrespective of salinity, with the high survivorship at 2 mS cm^{-1} being an outlier. If the latter is true, the lower estimated salinity tolerance is due to poor survival at 10°C, rather than a lower salinity tolerance at this temperature. *Simocephalus* sp. 48-h LC_{50} value at 20°C was 20% lower than at 12°C. While this difference is statistically significant, its practical significance is uncertain.

In contrast to *E. macrourus* and *Simocephalus* sp., which appeared more salt sensitive in low temperature treatments, *H. viridissima* was more salt tolerant at low temperatures. These seemingly contradictory results may reflect differences in the temperature tolerances of the species investigated. Dorgelo (1974) found little effect of temperature on lethal salinity tolerance in three gammarid amphipods. Aladin and Potts (1995) stated that for some Cladocera species within their normal temperature range, changes in

temperature has little, or no, effect on osmoregulation. However, in other cladoceran species osmoregulation ability increases with temperature due to complex and uncertain physiological processes.

When a species is under temperature stress (either too hot or too cold), it may have less tolerance to an added salinity stress. The specific temperatures that induce higher and lower salinity tolerance most likely vary between species, and possibly also within species, depending on factors such as seasonal acclimation (e.g. Dorgelo 1974).

Common ionic compositions (Radke *et al.* 2002) appear to affect freshwater macro- and micro-invertebrate chronic sub-lethal salinity tolerance but not their acute lethal salinity tolerance (Zalizniak *et al.* 2006; Zalizniak, Kefford and Nugegoda unpublished data). A greater effect of water temperature on sub-lethal salinity tolerance, than acute tolerance, should not be ruled out. We note however that pH had a similar and minor effect on both lethal and sub-lethal salinity tolerance (Zalizniak, Kefford and Nugegoda unpublished data).

Egg hatching and sub-lethal effects of salinity

As would be expected, investigation of sub-lethal effects of salinity suggested that *E. macrourus*, *Simocephalus* sp. and *N. fenestra* had lower tolerances than indicated by acute lethal effects. Likewise Cowgill and Milazzo (1990) found two North American cladocerans, *Daphnia magna* and *Ceriodaphnia dubia*, were more sensitive to NaCl when several reproductive parameters were studied than indicated from acute lethal effects. The current study, however, found no evidence of effects of salinity on *H. viridissima* below those that induced mortality. Low salinity treatments ($<1.5 \text{ mS cm}^{-1}$) were not used with *H. viridissima*, so whether this species is affected by low salinities is unknown.

As with studies on fish (James *et al.* 2003) and some macroinvertebrates (Kefford *et al.* 2004b), the eggs of *E. macrourus* were more salt sensitive than their older life-stages. However, the difference in tolerance between these life-stages in *E. macrourus* was reduced when the eggs were exposed for the same period (72-hours) as their older life-stages. The survival of *N. fenestra* hatchlings from eggs exposed for 72 hours only was high even at salinity where continuous exposure resulted in complete mortality. However, this 72-hour exposure increase the time that eggs took to hatch and reduced the size of the resulting hatchlings after 21 days. Thus non-lethal acute exposure can go on to have chronic consequences.

Population growth of the rotifer *E. macrourus* was greatest in intermediate salinity and reduced in both low and high salinity i.e. they had an inverted 'U'-shaped concentration-response curve (Kefford and Nugegoda 2005). Likewise Nielsen *et al.* (2003a) found that other rotifers had maximum emergence from an egg bank at intermediate salinities. These responses are similar to those observed in a range of taxa including gastropods (Duncan 1966; Jacobsen and Forbes 1997; Kefford and Nugegoda 2005), fish (see review Boeuf and Payan 2001), tadpoles (Chinathamby *et al.* 2006) and an odonatan (Kefford *et al.* 2006b). With an inverted 'U'-shaped response, there is no minimum, or threshold, salinity below which changes in salinity will have no effect (Kefford and Nugegoda 2005) and thus its presence makes the interpretation of sub-lethal effects difficult (see Chapman 1998).

Simulations of the effects of stressors and toxicants on sub-lethal responses (e.g. Calow *et al.* 1997; Grant 1998, Forbes *et al.* 2001a) have assumed that they affect only one life-history trait (e.g. growth or reproduction) or, if multiple traits are considered, that they are all adversely affected. However, different sub-lethal responses with different optima were observed for some species in the current study and elsewhere (Linke-Gamenick *et al.* 2000; Forbes and Calow 2002; Hassell *et al.* 2006). *Simocephalus* sp. followed an inverted 'U' response for reproduction (maximum around 2-4 mS cm⁻¹); however, the size of individual *Simocephalus* sp. after 16 days of exposure had an inverse linear relationship with salinity. It

is still possible that size of *Simocephalus* sp. is reduced below 0.9 mS cm^{-1} (the lowest RW treatment salinity tested) but growth and reproduction seem to have different optimal salinities. Likewise, while the size of *N. fenestra* appears to follow an inverted 'U' response, the time it took for their eggs to hatch increased linearly with increasing salinity. The proportion of larval *Chironomus* sp. (Diptera: Chironomidae) that emerged as flying adults followed an inverted 'U' response with increasing salinity, yet the duration of the larval life-stage followed a threshold response and their growth rate had a non-linear decreasing response with salinity (Hassell *et al.* 2006). Therefore for species where the effects of salinity on only one, or a few, responses are measured, it is unwise to conclude that other endpoints will respond similarly (see Forbes and Calow 2002a).

Although we do not know the physiological reasons why salinity affects particular life-history traits differently, it may be related to salinity acting on different physiological mechanisms (see Calabrese 2005). If particular physiological systems affect individual life-history traits differently, then this could explain why for some species the response of life-history traits to salinity varies. Calow and Forbes (1998), for example, suggested that variation in feeding rate was the main cause of changes in reproduction in the snail *Potamopyrgus antipodarum* at different salinities, while for growth some other (unknown) physiological processes appeared to be involved. Other physiological responses to salinity change that could affect individual life-history traits differently include: deficiencies of essential elements (such as Na, Ca, Mg and Cl) at low salinities; energetics of osmoregulation; interactions between osmoregulation, pH balance and respiration; and changes in hormone levels at different salinities (see Boeuf and Payan 2001; Kefford and Nugegoda 2005).

Whatever the physiological explanations, the different responses of life-history traits create a problem. It is not immediately apparent which trait is most important for the persistence of natural populations. It is possible for an individual life-history trait that is sensitive to a toxicant to have no effect on population

growth while another trait that is less sensitive has a large effect (Kammernga *et al.* 1996). Measures of population growth rates (as determined for *E. macrourus* and *H. viridissima*) account for attributes of individual traits (e.g. growth, reproduction and time for eggs to hatch). Measuring the effect of salinity on population changes may therefore be preferential to measuring effects at the individual level (Forbes and Calow 1999). However, except for organisms with short life-cycles, changes in populations are time consuming to observe thus few species will be investigated. Some balance is thus needed between the incompatible goals of allocating limited resources to both investigating sub-lethal effects in many species and investigating each species in detail (see Kefford *et al.* 2005b).

10 Additionally, even when population growth rates are experimentally measured, the effects of salinity on populations in laboratory experiments may differ from effects on natural populations. The effect any individual trait has on population growth will depend on whether: (1) the population is growing, constant or decreasing; (2) its population is under density-dependent control; and (3) seasonal and stochastic variation in environmental conditions (Grant 1998; Grant and Benton 2000; Forbes *et al.* 2001a,b). These factors will likely differ between laboratory and natural populations. Therefore changes to laboratory populations may not realistically describe changes in natural populations.

15

In their review of the effects of toxicants on both population growth and individual life-history traits, Forbes and Calow (1999) found that in only 5% of cases did a toxicant affect population growth by a (statistically) detectable amount at a concentration lower than that which affected any individual life-history trait. They also found that in only 16% of cases was the percentage change in the population growth rate greater than the percentage change in the most sensitive individual life-history trait. Subsequently, Forbes *et al.* (2001a) showed by simulations that under the assumptions of approximately stable population size in the absence of some toxicant and density-independent control, the population effects of a toxicant were less than or equal to effects on individual life-history responses. Indeed, Forbes

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and Calow (2002) concluded that the hypothesis, that small effects on individual survival, growth or reproduction will generally cause large effects on populations, is not supported. However they also stated that this conclusion is only valid when a number of individual life-history responses are measured.

Therefore, preventing salinity, and other toxicants, from affecting several individual life-history

5 responses, should in most cases be overprotective. Further work is needed to determine how salinity-induced changes in individual life-history traits translate into population level effects which can then be used to predict extinction probabilities of natural populations at various salinity levels (see Calow *et al.* 1997).

Conclusion

10 The most salt sensitive riverine microinvertebrates, macroinvertebrates and early life-stages of freshwater fish are likely to be killed by exposure to similar elevated salinities. However, most salt tolerant riverine microinvertebrates are likely to be less salt tolerant than adult freshwater fish and salt tolerant macroinvertebrates. Small rises in salinity will have greater direct effects on microinvertebrates than macroinvertebrates or fish, thus creating the potential for indirect effects on macroinvertebrates and fish
15 via changes to microinvertebrate prey and other ecosystem functions. There are, however, many microinvertebrate species that thrive in saline waters. The potential for these species to move into salinized rivers, and perform the ecological roles of microinvertebrates that currently inhabit freshwater rivers, is unknown.

20 Effects of water temperature on acute salinity sensitivity were noted in all three species studied. However, they appeared to be of minor importance.

In three of the four species where sub-lethal effects were investigated, effects of salinity on growth, reproduction or development were observed at salinities below those acutely lethal. However, where

multiple endpoints were investigated, different endpoints indicated different salinity-response relationships and different optimal salinities. The ecological importance of these different optimal salinities will depend on the life-history characteristics of particular species and factors that control their population size.

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Table 1. Information on taxa investigated. Collection sites are: Broken River @ Bridge Cr Rd (37° 13'S 146° 17'E), Campaspe River @ Kyneton-Heathcote Rd (37° 23'S 144° 31'E), Goulbourn River @ Gardiners Rd (37° 20'S 145° 18'E), King Parrot Creek @ Flowerdale (Goulbourn Catchment) (37° 23'S 145° 16'E), Loddon River @ Pyrenees Hwy (37° 07'S 144° 05'E) and Sundays Creek @ Deckerys Rd (Goulbourn Catchment) (37° 10'S 145° 06'E). Culture = commercial laboratory culture.

Taxa	Collected from	EC (mS cm ⁻¹) at collection	Container held (acute mortality)	Abundance	Effect of temperatures °C investigated	Effects studied other than acute mortality.
<i>Acanthocyclops</i> sp. (Copepoda: Cyclopoida)	Broken	0.22	80 mL	rare	No	No
Chydoridae (Cladocera)	Broken	0.51	80 mL	rare	No	No
Cyclopoidae (Copepoda)	Campaspe, Goulbourn	0.062-0.86	80 mL	common	No	No
<i>Cypretta</i> sp. cf. (Ostracoda: Cyprididae)	Broken	0.03-0.22	80 mL	rare	No	No
<i>Daphnia</i> sp. (Cladocera: Daphniidae)	Campaspe	1.4	individually in 5 mL	rare	No	No
<i>Epiphanes macrourus</i> (Rotifera: Epiphanidae)	Culture		individually in 2 mL	common	Yes 10, 20 & 30 - transferred from 20	Yes
<i>Eucyclops</i> sp. (Copepoda: Cyclopoida)	Broken	0.16-0.51	individually in 5 mL	rare	No	Yes

<i>Hydra viridissima</i> (Hydrozoa), green hydra	Campaspe, Sundays, culture	1.4	6-10 in 30 mL	common	Yes 12, 20 & 26 (Campaspe only) - 72h acclimation	Yes
<i>Mytilocypris</i> sp. (Ostracoda: Cyrididae)	Broken	0.16-0.51	individually in 5 mL	rare	No	No
<i>Newnhamia fenestra</i> cf. (Ostracoda: Notodromadidae)	Broken, Sundays, Loddon, King Parrot	0.03-1.4	80 mL	common	No	Yes (Loddon)
<i>Paramecium</i> (Ciliata)	Campaspe	0.5	individually in 2 mL	common	No	No
<i>Simocephalus</i> sp. (Cladocera: Daphniidae)	Campaspe, Broken, Loddon	0.4-1.6	80 mL	common	Yes 12 & 20 (Campaspe only) - cultured at temperatures	Yes (Campaspe)

Table 2. Summary of lethal salinity (EC mS cm⁻¹) for acute tests conducted at 20°C. Where NC = not calculated (due to poor survivorship in control/low salinity treatments), WLW = wet lab water and RW = river water.

Taxa	Dilutant water	24-h LC50	48-h LC50	72-h LC50	96-h LC50	# of individuals tested (72h)
<i>Acanthocyclops</i> sp. (Copepoda: Cyclopoida)	WLW	>15	>15	>15		12
Chydoridae (Cladocera)	WLW	6-12	NC	NC		4
Cyclopoidae (Copepoda)	RW	18.3	17.1	15.8		93
<i>Cypretta</i> sp. cf. (Ostracoda: Cyprididae)	WLW	15-25	15-25	15-25	NC	20
<i>Daphnia</i> sp. (Cladocera: Daphniidae)	WLW	>0.13	>0.13	>0.13		2
<i>Epiphanes macrourus</i> (Rotifera: Epiphanidae)	RW	8.0 (6.4-9.0)	8.0 (6.4-9.0)	8.0 (6.4-9.0)	6.1 (5.0-7.6)	103
<i>Eucyclops</i> sp. (Copepoda: Cyclopoida)	WLW	15	15	12-15		23
<i>Hydra viridissima</i> (Cnidaria: Hydrozoa)	RW	5.9 (5.6-6.2)	4.8 (4.5-5.1)	4.2 (3.7-4.9)	3.8 (3.1-4.9)	299
<i>Mytilocypris</i> sp. (Ostracoda: Cyrididae)	WLW	20-25	20	20	15	35
<i>Newnhamia fenestra</i> cf. (Ostracoda: Notodromadidae)	WLW	>30	26.4 (22.9-	20.8 (18.3-	15.4 (12.1-	402

			33.5)	24.2)	19.0)	
<i>Paramecium</i> (Ciliata)	RW	6.5 (5.6-7.4)	6.5 (5.8-7.3)	7.0 (6.3-7.9)	8.1	60
<i>Simocephalus</i> sp. (Cladocera: Daphniidae)	WLW & RW	8.4 (7.2-9.7)	4.9 (3.5-6.7)	NC		150

Table 3. Summary of methods used in experiments investigating sub-lethal effects of salinity. Where r = per capita rate of population increase.

Taxa	Life-stage at start	Duration (d) of experiment	Duration (d) of exposure	Fed	Held	Water changed	Responses measured
<i>E. macrourus</i>	Undetermined	5	5	Boiled lettuce daily	Individually in 2 mL	No	r
<i>H. viridissima</i>	Budding	5	5	Daily <i>A. salina</i>	Individually in 2 mL	Daily	r
<i>H. viridissima</i>	Non-budding	5	5	Not fed	30 mL	No	Abnormal tentacle contraction
<i>N. fenestra</i> cf.	Egg masses & single embryos	21	3 & 21	Weekly <i>P.</i> <i>subcapitata</i>	80 mL	Weekly	Hatching, chronic survival, size at end of experiment
<i>Simocephalus</i> sp.	neonates (<72h old) cultured	16	16	Daily <i>P. subcapitata</i>	Individually in 20 mL	Weekly	size at end of experiment, reproduction

(a)

(b)

(c)

Fig. 1. Species sensitivity distribution (SSD). (a) Kaplan-Meier function of 72h LC50 values for all microinvertebrates tested. (b) Kaplan-Meier functions of 72h LC50 values for crustacean and non-crustacean microinvertebrates tested. (c) Weibull probability plot of non-censored 72-h LC50 values. Censored marks indicate the presence of species with censored LC50 values.

(a)

(b)

(c)

Fig. 2. The effect of temperature on acute salinity toxicity, where O=10°C, △=12 °C, □ =20 °C, +=26 °C and ×=30 °C. (a) Survival of *Epiphanes macrourus* after 96 hours. (b) Survival of *Simocephalus* sp. after 48 hours. (c) Tentacle contraction of *Hydra viridissima* after 96 hours of exposure (where all individuals are dead values are not plotted).

Fig. 3. The effect of salinity on *Epiphanes macrourus* population growth (r) after 5 days, values plotted are mean and 95% CI. Treatments labelled with the same letter are not statistically different at the 0.05 level.

(a)

(b)

(c)

Fig. 4. Effect of salinity on *Newnhamia fenestra* eggs. (a) Percentage hatching in continuous exposure to various salinities, where ○=egg clusters and □=single embryos and solid line is regression equation for egg clusters and dashed line for single embryos. (b) Percentage hatching after 72 hours of exposure to various salinities and then returned to RW, symbols as for above. (c) Time (days) required for complete hatching, where ★=72-hour exposure and □=continuous exposure and dashed line is the regression for 72-hour exposure for and solid line for continuous exposure.

(a)

(b)

Fig. 5. Effect of salinity on *Newnhamia fenestra* hatchlings, where ★=72-hour exposure and □ = continuous exposure. (a) Of those that hatched, their survival after 21 days from commencement of exposure as eggs. (b) Mean length of individual after 21 days of exposure.

(a)

(b)

Fig. 6. Sub-lethal effect of salinity on *Simocephalus* sp. after 16 days, where ○ = river water (RW) and □ = wet lab water (WLW) based treatments. (a) Size of individual. Regression line is calculated excluding data from WLW prepared media. (b) Total number of neonates produced by each individual which survived 16 days. Regression lines are calculated using all data with the solid line being quadratic and the dashed line cubic.

Fig. 7. Effect of salinity on tentacle contraction of *Hydra viridissima* after various periods of exposure, where ○=1 h, ×=24 h, □=48 h, △=72 h and +=96 h. Note where all individuals are dead values are not plotted.