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Effects of potassium on nitrate mediated alterations of osmoregulation in marine crabs

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Abstract

Determining the acute nitrate tolerance of marine animals is important for conservation as high levels of nitrate may be discharged into aquatic ecosystems via various anthropogenic sources. Typically, sodium nitrate ($NaNO_3$) is used to determine the acute nitrate toxicity of marine animals. The standard procedure involves dissolving $NaNO_3$ salt in distilled water to create a stock solution, which is then diluted in seawater to obtain the desired nitrate concentration for the toxicity test. However, due to the relatively low toxicity of NO_3^-, large volumes of the stock solution are required to create high $NaNO_3$-N concentrations in the test solutions for LC_{50} (median lethal concentration) calculations. As the stock solution contains no other elements, other than Na^+ and NO_3^-, this can lead to drastically altered Na^+/K^+ ratios (compared to natural seawater) of the test solutions, which could significantly affect the osmo-ionoregulation of the animals, and subsequently bias survival data.

Consequently, experiments were performed to determine if incorporating potassium chloride (KCl), at a K^+ level equaling natural seawater at 30‰, to the $NaNO_3$-N stock solution influences the haemolymph osmolality, ion composition and LC_{50} values of two commercially important crab species, the mud crab *Scylla serrata* and the blue swimmer crab *Portunus pelagicus*. In each experiment with *S. serrata* and *P. pelagicus*, a total of 20 replicate crabs were exposed to $NaNO_3$-N concentrations of 2000, 3000, 4000, 5000 and 6000 mg l^{-1} with and without incorporated KCl. Mortality observations were made at 12-h interval for 96-h. After 96-h, the haemolymph osmolality, Na^+, K^+ and Ca^{2+} of the surviving crabs were measured. The 96-h LC_{50} values for early juveniles of *S. serrata* and *P. pelagicus* were 3601 (3314–3902) mg l^{-1} versus 4339 (4056–4518) mg l^{-1} and 3355 (3085–3620) mg l^{-1} versus 4132 (3864–4409) mg l^{-1}, respectively for the treatments without and with incorporated KCl. Statistical analysis showed that the sole utilisation of $NaNO_3$ led to a significantly ($p < 0.01$) lower LC_{50} value for both crabs, likely a consequence of their significantly lower ($p < 0.05$) haemolymph K^+ levels. In contrast, no significant differences ($p > 0.05$) in haemolymph K^+ was detected between crabs from the control and the treatment with incorporated KCl. It is therefore likely that previously reported acute nitrate toxicity tests have substantially underestimated the nitrate tolerances of marine animals. To avoid this problem, we propose incorporating KCl to the $NaNO_3$-N stock solution as a standard protocol for future acute nitrate toxicity experiments on marine animals.

Keywords: *Portunus pelagicus*; *Scylla serrata*; Early juvenile crabs; Nitrate toxicity; Hypokalemia; Na^+/K^+ ratios; Protocol revision

1. Introduction

Rises of various nutrients in ground and surface waters, primarily via increased anthropogenic activities including agricultural fertilizers, animal waste and urban run-off, are a worldwide concern (Camargo et al., 2005; Ju et al., 2006). Among the major nutrients, *i.e.* ammonia, nitrite, nitrate, phosphorous and potassium, nitrate is typically less toxic (Meade and Watts, 1995; Romano and Zeng, 2007a) although it is often discharged at higher levels (Camargo et al., 2005). Furthermore, as the end product of the nitrification process, rapid accumulation of nitrate can occur on closed recirculating aquaculture systems to levels of 500 mg l^{-1} and higher (Otte and Rosenthal, 1979).

It has been previously demonstrated that elevated nitrate levels could have negative physiological consequences to aquatic animals (Jensen, 1996; Cheng and Chen, 2002; Chen et al., 2002), and in severe cases, can be lethal (Epifano and Srna, 1975; Camargo and Ward, 1992; Hamlin, 2006; McGurk et al., 2006; Romano and Zeng, 2007a). Therefore, determining the nitrate tolerance of marine organisms, and assessing their physiological response to elevated nitrate levels, can provide valuable information for both marine ecosystem conservation and management (McGurk et al., 2006) and, for commercially important

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species, fisheries and aquaculture management (Tsai and Chen, 2002; Hamlin, 2006).

The current practice of acute nitrate toxicity experiments normally involves preparing a stock solution by dissolving sodium nitrate ($NaNO_3$) salt in distilled water to equal a nitrogen concentration, in the form of NO_3^-, at 10,000 mg l^{-1} $NaNO_3$-N. Typically, $NaNO_3$ is used as the nitrate source (Epifano and Srna, 1975; Brownell, 1980; Meade and Watts, 1995; Camargo and Ward, 1992; Tsai and Chen, 2002; Hamlin, 2006; McGurk et al., 2006) due to the relatively non-toxic ionic species of Na^+ compared to the associated elements in other potential nitrate sources, such as ammonium nitrate (NH_4NO_3) (Schuytema and Nebeker, 1999; Johansson et al., 2001) or potassium nitrate (KNO_3) (Dowden and Bennet, 1965; Romano and Zeng, 2007a). Furthermore, distilled water is used to make up the stock solution as the addition of $NaNO_3$, to equal a concentration of 10,000 mg l^{-1} $NaNO_3$-N, will result in a salinity of approximately 36‰. This stock solution is then added to seawater to equal the desired nitrate concentration in each test solution. However, as nitrate is comparatively less toxic than other nutrients, such as ammonia or nitrite, high nitrate concentrations in the test solutions are often required to obtain adequate mortality data for LC_{50} (median lethal concentration) calculations (Epifano and Srna, 1975; Brownell, 1980; Meade and Watts, 1995; Tsai and Chen, 2002; McGurk et al., 2006; Romano and Zeng, 2007a). Subsequently, the high volume additions of the $NaNO_3$-N stock solution, which contain only Na^+ and NO_3^-, results in substantially altered ionic composition/ratios of the test solutions (Romano and Zeng, 2007a) likely leading to severe osmo-ionoregulatory stress, and biased survival data, during acute nitrate toxicity tests on marine animals.

In our previous investigation, it had been demonstrated that for early juvenile blue swimmer crabs, *Portunus pelagicus*, $NaNO_3$-N levels of 2000 mg l^{-1} (or 142.76 mmol l^{-1}) and above caused significantly increased haemolymph Na^+ levels while significantly decreasing haemolymph K^+ levels, leading to hypokalemia (Romano and Zeng, 2007a). However, it remained unclear at that time if such haemolymph ionic imbalances were a direct result of NO_3^- toxicity or due to the increased Na^+ ratios to other ions, specifically the Na^+/K^+ ratios, of the test solutions used. As it has been previously shown that increased Na^+/K^+ ratios can negatively influence the osmo-ionoregulatory responses, and consequently the survival of various penaeid shrimp species (Pan et al., 2006; Prangnell and Fotedar, 2006; Tantulo and Fotedar, 2007), the current common practice of solely utilising $NaNO_3$ in acute nitrate toxicity experiments with marine animals may similarly disrupt normal osmo-ionoregulation and therefore compound the LC_{50} values.

Consequently, the current experiments were performed to determine if the sole utilisation of $NaNO_3$ compared to when K^+ was incorporated, at a level equal to a natural seawater salinity of 30‰, in a $NaNO_3$-N stock solution, would lead to differences in the haemolymph osmolality, Na^+, K^+ and Ca^{2+} levels, as well as the LC_{50} values. The experimental animals chosen for the present experiments were early juveniles of two commercially important marine crab species, the mud crab *Scylla serrata* and the blue swimmer crab *P. pelagicus*, which were selected based on the strong osmoregulatory ability of the former (Chen and Chia, 1997a,b) and the weak osmoregulatory ability of the latter (Romano and Zeng, 2006).

2. Materials and methods

2.1. Source of experimental animals

Both *S. serrata* and *P. pelagicus* early juveniles were cultured from newly hatched larvae in the laboratory according to procedures described in detail by Holme et al. (2006) and Romano and Zeng (2006), respectively. The larval culture of the two crab species were carried out separately at the Marine and Aquaculture Facility Unit (MARFU), James Cook University, Townsville, Queensland, Australia. Briefly, the broodstock were caught in estuary areas in Townsville, north Queensland, Australia and held in outdoor recirculating tanks until spawning. When a newly spawned female was observed, it was transferred to an indoor 300-l tank until hatching. On the day of hatching, the Zoea I larvae were stocked at a density of approximately 500 larvae l^{-1} in five indoor 300-l tanks at a salinity of 25 ± 1‰ and at a temperature of 28 ± 1 °C. Initially the larvae were fed rotifers (*Branchionus* sp.) at 20–40 individuals ml^{-1}, which was maintained by daily additions of microalgae *Nannochloropsis* sp. From the Zoea II stage and onwards, newly hatched and enriched brine shrimp *Artemia* sp. were added at increasing densities of 1–5 individuals ml^{-1} until larvae settled as first stage crabs, which took approximately 23–25 days for *S. serrata* and 15–16 days for *P. pelagicus*.

Both newly settled first stage crabs of *S. serrata* and *P. pelagicus* were then nursery cultured according to the methods described by Romano and Zeng (2007b,c). Briefly, all newly settled crabs were transferred from the indoor larval culture tanks to five outdoor 1000-l recirculating tanks at a salinity of 30 ± 2‰ and at a temperature of 28 ± 3 °C. Initially, the crabs were fed frozen *Artemia* sp. and each tank contained various shelters to reduce cannibalism and aggressive behavior. After the third day, the juvenile crabs were fed formulated pelleted feeds (Ridley) designed for the black tiger shrimp, *Penaeus monodon*, to satiation. By the crab 3–4 stage, the crabs were transferred to individual containers with numerous holes to facilitate adequate water exchanges. When the desired juvenile stage was reached, which took approximately 2 weeks, healthy intermolt crabs of similar size were randomly selected, weighed (to 0.001 g) and moved indoors for the commencement of the experiments. Using identical procedures, two separate experiments were performed in succession with early juvenile *S. serrata* (0.232 ± 0.014 g) and early juvenile *P. pelagicus* (0.229 ± 0.011 g), respectively.

2.2. Preparation of stock solutions and test solutions

Two identical stock solutions of 10,000 mg l^{-1} nitrogen, were made daily by dissolving 607.07 g $NaNO_3$ (Ajax Finechem, analytical reagent) into 10-l of distilled water. However, for one of the stock solutions, 6.33 g KCl (Ajax Finechem, analytical reagent) was directly dissolved into the 10-l $NaNO_3$-N stock solution to equal a KCl-K concentration of 332 mg l^{-1} (or