Relative uptake of urea and ammonium by dinoflagellates or
cyanobacteria in shrimp mesocosms

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Abstract

The relative role of the organic nitrogen source, urea, versus ammonium as a nitrogen source for two species of dinoflagellates was compared with one species of cyanobacteria. Experiments were conducted opportunistically in nutrient-rich marine water during blooms of either cyanobacteria or dinoflagellates in outdoor mesocosms. These replicate mesocosms, which were stocked with shrimp fed high-protein formulated feeds, contained high biomasses of phytoplankton (mean chlorophyll *a* concentrations, 439.2 – 811.2 $\mu\text{g}\cdot\text{l}^{-1}$). ^{15}N -urea and ammonium uptake rates for dinoflagellate-dominated blooms (*Gymnodinium pulchellum*-complex (Larsen), *Karlodinium micrum* (Larsen) (Dinophyceae)) were compared with blooms of the cyanobacterium, *Romeria* sp. (Cyanophyceae) in mesocosms with mean urea and ammonium concentrations ranging from 2.32 to 3.24 μM , and 7.39 to 64.85 μM respectively. Urea uptake rates were significantly ($P < 0.005$) lower than ammonium uptake rates irrespective of which algal species dominated the bloom. Additionally urea uptake rates were not significantly higher in *G. pulchellum*-complex or *K. micrum*-dominated blooms than in *Romeria* sp. blooms. These results suggest that *G. pulchellum*-complex and *K. micrum* may not be gaining a competitive advantage in waters high in dissolved organic matter simply by preferentially utilizing urea. The periodic dominance of these species in highly organic environments, such as shrimp ponds, is likely to have a more complex explanation.

Introduction

Nitrogen is a key nutrient controlling phytoplankton production in marine waters (Howarth, 1988; Smith, 1999). In physiological terms, ammonium is taken up more efficiently by phytoplankton, requires less energy to assimilate and has a lower saturation constant than nitrate or urea (Raven, 1984; Dortch, 1990). However, more recently the development of dinoflagellate blooms has been linked to the availability of dissolved organic nitrogen (DON), including urea (Carlsson et al., 1998; Glibert & Terlizzi, 1999, Kudela & Cochlan, 2000; Dyhrman & Anderson, 2003). Many dinoflagellate species are capable of mixotrophic growth under conditions of high organic loads and/or low inorganic nutrient availability (Antia 1991).

Aquaculture has become an important industry in the coastal zone in many countries. However, there are concerns about the environmental sustainability of aquaculture, including the effects of waste nutrients on stimulating harmful or nuisance algal blooms (Naylor et al., 1998; Alonso-Rodríguez & Páez-Osuna, 2003). Nitrogen is a nutrient of key concern as only 22 to 31% of nitrogen fed to aquaculture species is retained by the animals (Funge Smith & Briggs, 1998; Gross et al., 2000; Jackson et al., 2003). The remaining nitrogen enters the water and sediment potentially stimulating phytoplankton blooms. Dissolved organic nitrogen is a major (30-40%) component of the waste and is derived principally from the protein-rich feed given to cultured species (Burford & Williams, 2001).

One component of the DON pool utilized by phytoplankton in aquaculture systems is urea. Rates of urea uptake comparable with those of ammonium have been measured

in shrimp ponds (Burford & Glibert, 1999). However, it is not clear whether particular phytoplankton species are gaining a competitive advantage over other species by utilizing urea. This study examined this issue by comparing urea and ammonium concentrations and uptake rates in organic rich mesocosms which simulated shrimp ponds.

Materials and methods

Preconditioning

The study was conducted during the Australian summer (Jan-Feb 2001) in 10 x 2500 L round outdoor mesocosms stocked with the shrimp, *Penaeus monodon* Fabricius (75 per mesocosm, individual weight 3.11 ± 0.43 g). All mesocosms were set up and maintained in an identical fashion, and were designed to mimic commercial shrimp ponds as closely as possible. Shrimp feed was added to all mesocosms to satisfy the nutritional requirements of the shrimp and provided the main nitrogen and phosphorus input to the mesocosms (Burford et al., 2004). Each mesocosm contained a 50 mm deep layer of sediment taken from the bottom of an established shrimp pond and was filled with water from a 1 ha commercial shrimp production pond (20%), and sand-filtered oceanic water (80%). The maximum water temperature at this time was 29°C and the salinity was 33. Upon filling, the mixed algal community in all mesocosms was dominated by the green algae, *Nannochloropsis*, with low numbers of gymnodinoid dinoflagellates. Airlift pumps with regulated flow rates were used to aerate the mesocosms at rates equivalent to those in shrimp ponds.

Water exchanges were conducted to prevent algal blooms from becoming too unstable and causing anoxic conditions. 10% of the volume of the mesocosms was

simultaneously removed from each mesocosm on one day each week, and filtered oceanic water was used to simultaneously refill the mesocosms. When the mesocosm salinities increased above 36, freshwater was used to replace water lost through evaporation.

Uptake experiments

Phytoplankton uptake rates of ammonium and urea were determined using ^{15}N -enriched ammonium and urea incubations of water from the 10 replicated mesocosms. All mesocosms had been established for 1 mo under identical conditions prior to commencing incubations that were conducted within a 2 week period. Experiments were conducted opportunistically when 10 individual mesocosms became dominated by only one phytoplankton genus, either dinoflagellates or cyanobacteria,. This reflected a natural divergence from the dominance of *Nannochloropsis* sp. at the time of filling.

Water from each replicate mesocosm was used to fill two 1 L polycarbonate bottles. ^{15}N -ammonium chloride and ^{15}N -urea were added to one of each of the bottles at a concentration calculated to be 10% of ambient ammonium and urea concentrations. Bottles were incubated for 1 h during the middle of the day by floating them on the surface of the mesocosms. At the end of the 1 h incubation period, known volumes of water were filtered onto precombusted GF/F glass fibre filters for mass spectrometry to determine $^{15}\text{N}/^{14}\text{N}$ nitrogen ratios, and CHN analysis to determine the N content. Ammonium and urea uptake rates were calculated using the equations of Glibert et al., (1991). Relative preference indices (RPI) were calculated using the equations of McCarthy et al., (1977).

Water temperature, salinity, pH, dissolved oxygen (DO) and turbidity were measured with a datalogger (Yeokal model 611) in all mesocosms in the preconditioning period of the first month and during the two weeks that incubations were performed. When mesocosms were established, water samples were taken weekly for ammonium, nitrate, DON and fluorescence measurements, fortnightly for phosphate. At the start of the incubations, water samples were taken for algal counts, chlorophyll *a*, urea, ammonium, nitrate, dissolved organic nitrogen (DON) and phosphate analyses. Water samples were processed as follows: for algal counts, samples were fixed in 1% glutaraldehyde and stored at 4°C; for chlorophyll analysis, samples were filtered onto GF/F glass fibre filters and frozen; and for nutrient analyses, samples were filtered through disposable filters (cellulose acetate, 0.45 µm pore size) and frozen.

Algae in fixed samples were counted using a Sedgewick Rafter counter under phase-contrast microscopy. For identification of dinoflagellates, samples were fixed in osmium tetroxide, critical point dried and observed with scanning electron microscopy. Ammonium and phosphate concentrations in water samples were analyzed using standard methods (American Public Health Association, 1995). Nitrate and nitrite were analyzed using a spongy cadmium method (Jones, 1983). Dissolved nitrogen (DN) was measured using the simultaneous persulfate oxidation method (Hosomi & Sudo, 1986). Urea was analyzed by the diacetyl monoxime method (Rahmatullah & Boyde, 1980). Frozen filters, for chlorophyll *a* analysis, were extracted into acetone using a sonicator and measured spectrophotometrically (Jeffrey & Welshmeyer, 1997).

Nutrient and algal data was log transformed, as required, to ensure normality.

Treatments were compared using analysis of variance (SAS software).

Results

In the five weeks prior to commencing the incubations, fluorescence units, ammonium and DON concentrations generally increased (Fig. 1). There was a high level of variability between the mesocosms used but no obvious differences in nitrogen concentrations or fluorescence leading up to the monospecific blooms. Phosphate and nitrate concentrations were undetectable or near the limits of detection during this time.

There were two dominant algal classes represented in the study: dinoflagellates and cyanobacteria, at the time of the incubations. The dinoflagellate blooms were dominated by a combination of *G. pulchellum* complex and *K. micrum* (n=5) (mean cell densities of $8.59 \times 10^4 \text{ ml}^{-1}$, mean chlorophyll *a* concentration of $811.2 \mu\text{g l}^{-1}$ (Fig 2, Table 1)) whereas only one species within the cyanobacterial class, *Romeria* sp. (n = 5) dominated in the other treatment (mean cell density of $26.6 \times 10^4 \text{ ml}^{-1}$, mean chlorophyll *a* concentration of $439.2 \mu\text{g l}^{-1}$). Chlorophyll *a* concentrations were significantly higher ($P < 0.005$) in the dinoflagellate treatments than the cyanobacterial treatments despite the higher cell counts in the cyanobacterial treatment. Other algal groups were present at densities < 10% of the dominant algal class with the exception of the cyanobacteria treatments (*Romeria* sp.) which also had 25% *Nannochloropsis* sp.

The mean water temperatures, salinities and pH during the incubations were statistically the same in all mesocosms (Table 1). Only pH was statistically different between treatments ($P < 0.05$). Mean ammonium and urea concentrations were 7.4 and 3.2 $\mu\text{M N}$, and 64.8 and 2.3 $\mu\text{M N}$ for dinoflagellate and cyanobacterial-dominated mesocosms respectively. Phosphate concentrations were statistically higher ($P < 0.005$) in the dinoflagellate mesocosms. DON was the dominant form of dissolved N in the mesocosms with mean concentrations of 220.5 and 144.4 $\mu\text{M N}$ for dinoflagellate and cyanobacteria mesocosms respectively (Table 1). Urea represented up to 5.5% of the DON pool.

Mean particulate N (PN) concentrations in the mesocosms were 597.5 and 452.3 $\mu\text{M N}$ respectively for dinoflagellate and cyanobacteria mesocosms (Table 1). Across all mesocosms, PN concentrations were significantly correlated with chlorophyll *a* concentrations ($R^2 = 0.78$, $p < 0.05$).

Mean ammonium uptake rates (Rho , $\mu\text{M N h}^{-1}$) were highly variable (10.3 ± 4.0 and $24.1 \pm 28.6 \mu\text{M N h}^{-1}$ for dinoflagellate and cyanobacteria mesocosms respectively) with no statistical difference between algal classes (Table 1). The same was true of urea uptake rates (1.0 ± 0.7 and $0.9 \pm 0.2 \mu\text{M}\cdot\text{N h}^{-1}$ for dinoflagellate and cyanobacteria mesocosms respectively).

Both algal classes had a statistically higher preference for ammonium than urea ($P < 0.005$) (Table 1). Additionally across all mesocosms, there was no evidence of increasing urea uptake with increasing urea concentrations.

Discussion

There was no evidence in our study that the dinoflagellate species, *G. pulchellum* complex and *K. micrum* utilized urea more rapidly than the cyanobacteria, *Romeria* sp. in water used to culture shrimp. This contrasts with the finding that some other dinoflagellate species can grow on urea as the preferred nitrogen source (Kudela & Cochlan, 2000; Rosser & Thompson, 2001; Dyhrman & Anderson, 2003). However the ability to utilize urea is not exclusive to dinoflagellates as a range of other algal species have also been shown to utilize urea effectively (Berg et al., 1997; Berman & Chava 1999).

However, the presence of dinoflagellate blooms in shrimp mesocosms is evidence of optimal conditions for their growth. A number of studies have shown shrimp ponds to be generally dominated by dinoflagellates, diatoms and cyanobacteria (Burford, 1997; Tookwinas & Songsangjinda, 1999; Alonso-Rodríguez & Páez-Osuna, 2003). A number of dinoflagellate genera, including *Gymnodinium* and *Prorocentrum*, have been associated with anoxia and/or toxin production causing a decrease in shrimp growth or mortality (Alonso-Rodríguez & Páez-Osuna, 2003). Other factors such as the high concentrations of higher molecular-weight dissolved organic matter may be contributing to the dominance of dinoflagellates. Other studies have found that a range of sources of dissolved organic matter can promote dinoflagellate blooms (Palenik & Morel, 1990; Lewitus et al., 1999; Purina et al., 2004).

In this study, ammonium was the preferred nitrogen source for both dinoflagellates and cyanobacteria. This contrasts with a previous study in shrimp ponds that showed similar preferences for urea and ammonium (Burford & Glibert, 1999). The urea

concentration also did not appear to be a factor in determining urea uptake since uptake rates did not increase with increasing urea concentrations. This contrasts with Glibert & Terlizzi (1999) who found a correlation between dinoflagellate dominance and urea concentrations higher than 1.5 μM . Our study highlights that the role of phytoplankton in nutrient cycling can be difficult to infer based on measurements of nutrient concentrations alone. A previous study found that growth and succession of dinoflagellates and diatoms in shrimp ponds was the same irrespective of whether urea or nitrate fertilizer was used (Burford & Pearson, 1998).

Bacteria may also have contributed to urea and ammonium uptake since bacteria are likely to be at least partially trapped on glass fibre filters. Previous studies have shown that bacteria can play an important role in urea uptake (Jørgensen et al., 1999). However the high correlation between chlorophyll *a* and PN suggests that phytoplankton are the dominant component of the PN on the filters.

The phosphate concentrations in this study were low, resulting in a high dissolved nitrogen:phosphate ratio relative to the requirements for phytoplankton growth (Redfield, 1958). However, phosphate is typically released from bacterial remineralization in shrimp pond sediments at rates up to 0.6 $\mu\text{M P d}^{-1}$ (Burford & Longmore, 2001), and mineralization of phosphorus-rich organic matter by bacterioplankton may also provide a source. Additionally, there was little evidence of phosphorus limitation in the cells (PN:particulate phosphorus ratio of 18:1). Therefore the high nitrogen:phosphorus ratios are more likely to reflect very high nitrogen concentrations than phosphorus limitation. Interestingly, the phosphate concentrations were statistically higher in the dinoflagellate-dominated mesocosms

than the cyanobacteria-dominated mesocosms. This may be the result of the higher cyanobacterial cell densities utilizing phosphate more rapidly than dinoflagellates, or differences in the bacterial abundance between treatments.

In summary, the high organic loads in shrimp ponds may contribute to the high densities of dinoflagellates often observed in these ponds. However there is no evidence from our study that the presence of the bioavailable organic nitrogen source, urea, was used preferentially over the dominant inorganic source, ammonium by dinoflagellates, *G. pulchellum* complex and *K. micrum*, or the cyanobacteria, *Romeria* sp.

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References

- Alonso-Rodríguez, R. & F. Páez-Osuna, 2003. Nutrients, phytoplankton and harmful algal blooms in shrimp ponds: a review with special reference to the situation in the Gulf of California. *Aquaculture* 219: 317-336.
- American Public Health Association, 1995. Standard Methods for the Examination of Water and Wastewater. 19th ed. Clesceri, L. S., Greenberg, A. E., Trussell, R. R. (eds) APHA, Washington D.C., USA.
- Antia, N. J., P. J. Harrison & L. Oliveira, 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. *Phycologia* 30: 1-89.
- Berg, G. M., P. M. Glibert, M. W. Lomas, & M. A. Burford, 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. *Marine Biology* 129: 377-387.
- Berman, T. & S. Chava, 1999. Algal growth on organic compounds as nitrogen sources. *Journal of Plankton Research* 21: 1423-1437.
- Burford, M. A. 1997. Phytoplankton dynamics in shrimp ponds. *Aquaculture Research* 28: 351-360.
- Burford, M. A. & D. C. Pearson, 1998. Effect of different nitrogen sources on phytoplankton composition in aquaculture ponds. *Aquatic Microbial Ecology* 15: 277-284.
- Burford, M. A. & P. M. Glibert, 1999. Short-term N cycling in early and late growth-phase shrimp ponds. *Aquaculture Research* 30: 215-227.

- Burford, M. A. & A. R. Longmore, 2001. High ammonium production from sediments in hypereutrophic aquaculture ponds. *Marine Ecology Progress Series* 224: 187-195.
- Burford, M. A. & K. C. Williams, 2001. The fate of nitrogenous waste from shrimp feeding. *Aquaculture* 198: 79-93.
- Burford, M. A., D. M. Smith, S. J. Tabrett, F. E. Coman, P. J. Thompson, M. C. Barclay & P. J. Toscas, 2004. The effect of dietary protein on the growth and survival of the shrimp, *Penaeus monodon* in outdoor tanks. *Aquaculture Nutrition* 10: 15-23.
- Carlsson, P., H. Edling & C. Béchemin, 1998. Interactions between a marine dinophyceae (*Alexandrium catenella*) and a bacterial community utilizing riverine humic substances. *Aquatic Microbial Ecology* 16: 65-80.
- Dortch, Q. 1990. The interaction between ammonium and nitrate uptake in phytoplankton. *Marine Ecology Progress Series* 61: 183-201.
- Dyhrman, S. T. & D. M. Anderson, 2003. Urease activity in cultures and field populations of the toxic dinophyceae *Alexandrium*. *Limnology and Oceanography* 48: 647-655.
- Funge-Smith, S. J. & M. R. P. Briggs, 1998. Nutrient budgets in intensive shrimp ponds: implications for sustainability. *Aquaculture* 164: 117-133.
- Glibert, P. M., C. Garside, J. A. Fuhrman & M. R. Roman, 1991. Time-dependent coupling of inorganic and organic nitrogen uptake and regeneration in the plume of the Chesapeake Bay estuary and its regulation by large heterotrophs. *Limnology and Oceanography* 36: 895-909.

- Glibert, P. M. & D. E. Terlizzi, 1999. Cooccurrence of elevated urea levels and dinophyceae blooms in temperate estuarine aquaculture ponds. *Applied Environmental Microbiology* 65: 5594-5596.
- Gross, A., C. E. Boyd & C. W. Wood, 2000. Nitrogen transformations and balance in channel catfish ponds. *Aquacultural Engineering* 24: 1-14.
- Hosomi, M. & R. Sudo, 1986. Simultaneous determination of total N and total phosphorus in freshwater samples using persulphate digestion. *International Journal of Environmental Studies* 27: 267-275.
- Howarth, R. W. 1988. Nutrient limitation of net primary production in marine ecosystems. *Annual Reviews of Ecology* 19: 89-110.
- Jackson, C., N. Preston, P. Thompson & M. Burford, 2003. Nitrogen budget and effluent nitrogen components at an intensive shrimp farm. *Aquaculture* 218: 397-411.
- Jeffrey, S. W. & N. A. Welshmeyer, 1997. Spectrophotometric and fluorometric equations in common use in oceanography. In: Jeffrey, S. W., Mantoura, R. F. C. & Wright, S. W. (eds) *Phytoplankton Pigments in Oceanography*. Monographs on Oceanographic Methodology No.10. UNESCO Publishing, Paris: pp. 597-615.
- Jones, M. N. 1983. Nitrate reduction by shaking with cadmium, alternative to cadmium columns. *Water Research* 8: 643-646.
- Jørgensen, N. O. G., N. Kroer, R. B. Coffin & M. P. Hoch, 1999. Relations between bacterial nitrogen metabolism and growth efficiency in an estuarine and an open-water ecosystem. *Aquatic Microbial Ecology* 18: 247-261.
- Kudela, R. M. & W. P. Cochlan, 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquatic Microbial Ecology* 21: 31-47.

- Lewitus, A. J., B. M. Willis, K. C. Hayes, J. M. Burkholder, H. B. Glasgow, P. M. Glibert, & M. K. Burke, 1999. Mixotrophy and nitrogen uptake by *Pfiesteria piscicida* (Dinophyceae). *Journal of Phycology* 35: 1430-1437.
- McCarthy, J. J., W. R. Taylor & J. L. Taft, 1977. Nitrogenous nutrition of the plankton in the Chesapeake Bay. I. Nutrient availability and phytoplankton preferences. *Limnology and Oceanography* 22: 996-1011.
- Naylor, R. L., R. J. Goldburg, H. Mooney, M. Beveridge, J. Clay, C. Folke, N. Kautsky, J. Lubchenco, J. Primavera & M. Williams, 1998. Nature's subsidies to shrimp and salmon farming. *Science* 282: 883-884.
- Palenik, B. & F. M. M. Morel, 1990. Comparison of cell-surface L-amino acid oxidases from several marine phytoplankton. *Marine Ecology Progress Series* 9: 195-201.
- Purina, I., M. Balode, C. Bechemin, T. Poder, C. Verite & S. Maestrini, 2004. The influence of dissolved organic matter from terrestrial origin on the changes of dinoflagellate species composition in the Gulf of Riga, Baltic Sea. *Hydrobiologia* 514: 127-137.
- Rahmatullah, M. & T. R. C. Boyde, 1980. Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clinica Chimica Acta* 107: 3-9.
- Raven, J. A. 1984. A cost-benefit analysis of photon absorption by photosynthetic unicells. *New Phytologist* 98: 593-625.
- Redfield, A. C. 1958. The biological control of chemical factors in the environment. *American Science* 46: 205-222.
- Rosser, S. M. J. H. & P. A. Thompson, 2001. Phytoplankton of the Swan-Canning Estuary: a comparison of nitrogen uptake by different bloom assemblages.

Hydrological Processes 15: 2579-2594.

Smith, V. H., G. D. Tilman & J. C. Nekola, 1999. Eutrophication: impacts of excess nutrient inputs on freshwater, marine and terrestrial ecosystems. *Environmental Pollution* 100: 179-196.

Tookwinas, S. & P. Songsangjinda, 1999. Water quality and phytoplankton communities in intensive shrimp culture ponds in Kung Krabaen Bay, Eastern Thailand. *Journal of the World Aquaculture Society* 30: 36-45.

Figure Legends

Figure 1: Mean ($\pm$ SD) ammonium and DON concentrations (μM), and fluorescence units for the dinoflagellate and cyanobacteria treatment tanks for the five weeks before the commencement of the study. $n = 10$.

Figure 2: Mean ($\pm$ SD) cell counts ($\times 10^4 \text{ mL}^{-1}$) in the mesocosms at the start of the ^{15}N -urea and ammonium incubations. $n = 5$.

Table 1: Mean ($\pm$ SD) values for physical data, nutrients, chlorophyll and particulate nitrogen concentrations, and nutrient uptake rates for mesocosms during incubations.

* $P < 0.05$, *** $P < 0.005$

	Dinoflagellates	Cyanobacteria
	<i>G. pulchellum</i> / <i>K. micrum</i>	<i>Romeria</i> sp.
Parameter	(n=5)	(n=5)
Temperature ° C	28.9 (1.3) ^{ns}	29.2 (1.8)
Salinity	32.7 (1.3) ^{ns}	33.0 (0.8)
pH	8.3 (0.2)*	7.9 (0.2)
Ammonium (μ M N)	7.4 (9.7) ^{ns}	64.8 (67.1)
Urea (μ M N)	3.2 (3.8) ^{ns}	2.3 (0.8)
Nitrate (μ M N)	0.4 (0.8) ^{ns}	0.8 (0.4) ^{ns}
DON (μ M N)	220.5 (87.3) ^{ns}	144.4 (44.6)
Phosphate (μ M P)	2.3 (1.2)***	0.2 (0.2)
Chlorophyll <i>a</i> (μ g L ⁻¹)	811.2 (128.7)***	439.2 (139.2)
PN (μ M)	597.5 (153.7) ^{ns}	452.3 (120.0)
NH ₄ uptake, Rho (μ M N h ⁻¹)	10.3 (4.0) ^{ns}	24.1 (28.6)
Urea uptake, Rho (μ M N h ⁻¹)	1.0 (0.7) ^{ns}	0.9 (0.2)
Relative preference index		
Ammonium	2.0 (0.1) ^{ns}	1.7 (1.4)
Urea	0.2 (0.1) ^{ns}	0.6 (0.6)

Fig. 1

Fig. 2