Bull Environ Contam Toxicol (2007) 78:63–66
DOI 10.1007/s00128-007-9007-z

Effects of Ammonia on Cellular Biomarker Responses in Oysters (*Crassostrea virginica*)

C. J. Keppler

Published online: 28 February 2007

Ammonia occurs naturally in seawater and exists in two forms: as un-ionized ammonia (NH_3) and as ammonium ion (NH_4^+). The concentrations of each in seawater is dependant on pH and temperature, with the proportion of un-ionized ammonia increasing at a higher pH and temperature (Emerson et al., 1975). Findings have shown that un-ionized ammonia is the more toxic of the two forms. It is acutely toxic to a number of marine organisms including fish, bivalves, and crustaceans. Oftentimes, fish are more sensitive to un-ionized ammonia than invertebrates (Arthur et al., 1987). Recent studies also have shown the sublethal, chronic effects of ammonia exposure on marine organisms, with end points such as growth and feeding rates greatly affected at low concentrations (Boardman et al., 2004; Huber et al., 1997).

In this study, the sublethal effects of ammonia exposure were tested using two well-established cellular biomarkers in oysters (*Crassostrea virginica*). The two biomarkers included an indicator of cellular damage (lysosomal destabilization) and an indicator of cellular detoxification (glutathione concentration). Previous findings have shown lysosomal destabilization to be a sensitive biomarker of physiologic stress in *Crassostrea virginica*, and to be affected by both natural and anthropogenic stressors (Keppler et al., 2006; Ringwood et al., 1998). Glutathione serves numerous essential functions within the cell, including detoxification of metals and oxy-radicals, and there is evidence that glutathione depletion is associated with exacerbation of adverse effects in marine bivalves (Conners and Ringwood, 2000; Meister and Anderson, 1983). This study serves to highlight the sublethal cellular toxicity of ammonia for the oyster, *Crassostrea virginica*.

C. J. Keppler (✉)
South Carolina Department of Natural Resources, Marine Resources Research Institute, 217 Fort Johnson Road, Charleston, SC 29412, USA
e-mail: KepplerC@dnr.sc.gov

Materials and Methods

Oysters (4.8 ± 0.8 cm) and seawater were collected from a control site on Lighthouse Creek, South Carolina, an area unpolluted by metals, polycyclic aromatic hydrocarbons, or ammonia, according to extensive sediment analysis (Hyland et al., 1998). An initial subset of oysters was dissected at the beginning of each exposure to determine the lysosomal destabilization levels and glutathione concentrations at day zero. Treatment oysters were scrubbed and randomly placed into 3.0 L of control seawater (filtered and adjusted to 25 parts per thousand [ppt]).

Each treatment consisted of two replicates comprising five oysters each (10 oysters per treatment). Each replicate was continuously aerated. The pH, salinity, temperature, and total ammonia were measured daily for each replicate in the 48-h exposure, and on days 2, 5, and 7 in the 7-day exposure. Total ammonia was measured using a Hach DR/700 (Hach Company, Loveland, Colorado) colorimeter and Hach method 8155. Un-ionized ammonia (NH_3) was calculated using the total measured ammonia values together with the tables and equations found in Emerson et al. (1975). Each replicate was fed approximately 4.5×10^7 cells of *Isochrysis galbana* paste every 24 h.

Table 1 Measured (total) and calculated (un-ionized) ammonia concentrations for each exposure assay[a]

48-h exposure			7-Day exposure		
mg/L	Total	Un-ionized	mg/L	Total	Un-ionized
0	0.54 ± 0.37	0.01 ± 0.01	0	0.60 ± 0.37	0.01 ± 0.01
10	8.92 ± 0.50	0.30 ± 0.08	10	8.93 ± 0.97	0.25 ± 0.06
20	15.93 ± 0.74	0.58 ± 0.15	20	15.93 ± 0.74	0.52 ± 0.09
40	34.58 ± 0.74	1.31 ± 0.19	40	31.94 ± 1.21	1.02 ± 0.08

[a] Values in mg/L; total expressed as mean ± standard deviation

A 48-h range-finding study was conducted with three nominal concentrations of ammonia (0, 10, 20, and 40 mg/L) prepared from a stock solution of American Chemical Society (ACS) grade ammonium chloride. A 7-day exposure also was performed, using the same nominal ammonia concentrations and assay conditions, to determine effects of longer term exposure. Test solutions (50% per replicate) were removed and replaced with fresh solutions on day 5. At the end of the exposure period, oyster digestive gland tissues were dissected out for immediate lysosomal destabilization analysis, and the remaining digestive gland tissue was frozen at –80°C for glutathione concentration analysis.

The lysosomal destabilization assay was conducted following the methods described in Ringwood et al. (1998). Briefly, digestive gland tissue was disaggregated in Ca^{++}, Mg^{++} free saline and trypsin solution, then washed. The cell suspensions then were incubated in neutral red (NR) for 1 h. Cells were examined with a light microscope to evaluate NR retention in the lysosomes, and data were expressed as the percentage of cells with destabilized lysosomes per oyster.

Glutathione concentrations of individual oysters were determined using the enzymatic recycling assay (Anderson, 1985; Ringwood et al., 1999). Briefly, digestive gland tissues were homogenized in 5% sulfosalicyclic acid and centrifuged. The supernatant was mixed with reduced β-nicotinamide adenine dinucleotide phosphate buffer containing 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB). Glutathione reductase was added, and the rate of DTNB formation was monitored at 405 nm over a 100-s interval. Glutathione concentrations were calculated from a standard curve.

The data were analyzed using Sigma Stat. (Systat Software, Inc., Point Richmond, California) Differences between treatments were examined using analysis of variance, with the Student–Newman–Keuls method used for multiple comparisons. Pearson product–moment correlations and linear regressions were used to determine relationships between cellular biomarker responses and ammonia concentrations.

Results and Discussion

Water chemistry was similar for both exposure experiments. The temperature, salinity, and pH averaged, respectively, 20.8 ± 0.7°C, 24.0 ± 0.9 ppt, and 7.93 ± 0.11 for the 48-h study, and 21.3 ± 0.6°C, 25.0 ± 0.3 ppt, and 7.86 ± 0.10 for the 7-day study. The total and un-ionized ammonia concentrations for each exposure are listed in Table 1. The total ammonia concentrations were relatively stable throughout the course of the exposures. The slightly elevated ammonia levels measured in the 0-mg/L treatment may indicate normal ammonia excretion by the oysters (Srna and Baggaley, 1976).

In both experiments, exposure to elevated ammonia concentrations caused a significant increase in lysosomal destabilization (Fig. 1). Oysters exposed to 20 and 40 mg/L of ammonia had significantly elevated lysosomal destabilization rates, ranging from 38% to 53%. Lysosomal destabilization levels exceeding 35% have been associated with significant impairment in digestive gland tissues. Such levels also have been linked to reduced gamete viability and poor rates of embryonic development (Ringwood et al., 2004).

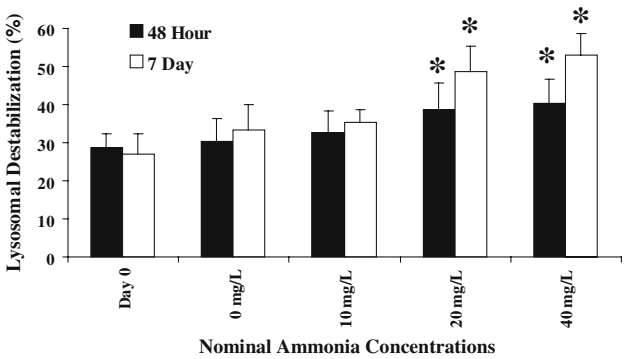

Fig. 1 Effect of ammonia exposures on lysosomal destabilization in *Crassostrea virginica*. An asterisk (*) indicates a significant difference from the control oysters (0 mg/L) during the same exposure period. Values are mean + standard deviation

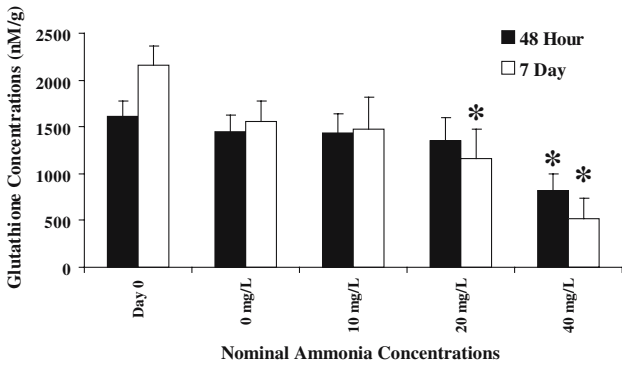

Fig. 2 Effect of ammonia exposures on glutathione concentrations in *Crassostrea virginica*. An asterisk (*) indicates a significant difference from the control oysters (0 mg/L) during the same exposure period. Values are mean + standard deviation

There was a positive trend between lysosomal destabilization and total ammonia concentration after the exposures (48 h: $R^2 = 0.88$, $p = 0.06$; 7 days: $R^2 = 0.88$, $p = 0.06$). The similarity in lysosomal destabilization rates between the day 0 and 0-mg/L oysters indicate that the oysters were not stressed before the exposures.

In both experiments, ammonia exposures caused a significant decrease in glutathione concentrations (Fig. 2). After 48 h, glutathione concentrations in the 40-mg/L treatment were decreased by approximately 43%, as compared with the control. However, these glutathione concentrations (~825 nM/g) may still be within the normal range of healthy oysters. After 7 days, however, glutathione concentrations in both the 20- and 40-mg/L treatments were significantly less than in the control, decreasing by 26% and 67%, respectively. The oysters exposed to total ammonia of 40 mg/L were significantly stressed, with glutathione concentrations decreasing to 513 nM/g. Findings have shown that oysters with low glutathione concentrations have an increased susceptibility to contaminant exposure, both in the adults and in the embryos from glutathione-depleted adults (Conners and Ringwood, 2000; Ringwood et al., 2004). There was a positive trend between glutathione concentrations and ammonia concentrations after 48 h ($R^2 = 0.87$; $p = 0.07$), and a significant relationship at 7 days ($R^2 = 0.97$; $p = 0.02$). The 0-mg/L and day 0 oysters had similar glutathione concentrations, again indicating that the oysters were not stressed before the exposures.

There is little information available about the sublethal effects of ammonia exposure on bivalves. Epifanio and Srna (1975) found that total ammonia concentrations of approximately 4 and 8 mg/L affected the algal clearance rate in juvenile and adult *Crassostrea virginica*. In a different bivalve, *Mulinia lateralis*, Huber et al. (1997) found the growth EC50 for total ammonia to be 11 mg/L (0.3 mg/L of un-ionized ammonia). The results of this study show that *C. virginica* also is significantly affected by short-term sublethal concentrations of ammonia. The effects of chronic ammonia exposure could result eventually in an increased susceptibility to contaminant stress in both the adults and the embryos, or a decrease in reproductive capability.

Recent studies have found that ammonia concentrations can range from 0.01 to 0.22 mg/L in undeveloped, urbanized, and industrialized estuarine watersheds (Lewitus et al., 2004; Scharler and Baird 2003). Although these values are comparatively lower than the exposure concentrations used in this study, ammonia concentrations have been measured upward of 85 mg/L at untreated animal wastewater outfalls (Stone et al., 2004). Further studies are needed to determine what sublethal effects occur in oysters exposed to lower concentrations of ammonia over a longer period.

Acknowledgments The author thanks J. Hoguet (NOAA-CCEHBR) and two anonymous reviewers for providing valuable editorial commentary. This research was supported in part by the South Carolina Sea Grant Consortium and CDC grant V2C6. This is Marine Resources Research Institute publication #592.

References

Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 113:548–555

Arthur JW, West CW, Allen KN, Hedtke SF (1987) Seasonal toxicity of ammonia to five fish and nine invertebrate species. Bull Environ Contam Toxicol 38:324–331

Boardman GD, Starbuck SM, Hudgins DB, Li X, Kuhn DD (2004) Toxicity of ammonia to three marine fish and three marine invertebrates. Environ Toxicol 19:134–142

Conners DE, Ringwood AH (2000) Effects of glutathione depletion on copper cytotoxicity in oysters (*Crassostrea virginica*). Aquat Toxicol 50:341–349

Emerson K, Russo RC, Lund RE, Thurston RV (1975) Aqueous ammonia equilibrium calculations: Effect of pH and temperature. J Fish Res Board Canada 32:2379–2383

Epifanio CE, Srna RF (1975) Toxicity of ammonia, nitrite ion, and orthophosphate to *Mercenaria mercenaria* and *Crassostrea virginica*. Mar Biol 33:241–246

Huber M, Pelletier MC, Charles JB, Burgess RM (1997) Ammonia tolerance of the bivalve *Mulinia lateralis* sublethal sediment toxicity test. Bull Environ Contam Toxicol 59:292–297

Hyland JL, Balthis L, Hackney CT, McRae G, Ringwood AH, Snoots TR, Van Dolah RF, Wade TL (1998) Environmental quality of estuaries of the Carolinian Province: 1995. NOAA Technical Memorandum NOS ORCA 123. NOAA/NOS, Office of Ocean Resources Conservation and Assessment, Silver Springs, Maryland

Keppler CJ, Lewitus AJ, Ringwood AH, Hoguet J, Staton T (2006) Sublethal cellular effects of short-term raphidophyte and brevetoxin exposures on the eastern oyster *Crassostrea virginica*. Mar Ecol Prog Ser 312:141–147

Lewitus AJ, Kawaguchi T, DiTullio GR, Keesee JDM (2004) Iron limitation of phytoplankton in an urbanized vs forested southeastern U.S. salt marsh estuary. J Exp Mar Biol Ecol 298:233–254

Meister A, Anderson ME (1983) Glutathione. Ann Rev Biochem 52:711–760

Ringwood AH, Conners DE, Hoguet J (1998) The effects of natural and anthropogenic stressors on lysosomal destabilization in oysters *Crassostrea virginica.* Mar Ecol Prog Ser 166:163–171

Ringwood AH, Conners DE, Keppler CJ (1999) Cellular responses of oysters, *Crassostrea virginica*, to metal contaminated sediments. Mar Environ Res 48:427–437

Ringwood AH, Hoguet J, Keppler CJ, Gielazyn M (2004) Linkages between cellular biomarker responses and reproductive success in oysters—*Crassostrea virginica*. Mar Environ Res 58:151–155

Scharler UM, Baird D (2003) The influence of catchment management on salinity, nutrient stochiometry and phytoplankton biomass of Eastern Cape estuaries, South Africa. Estuarine, Coastal and Shelf Sci 56:735–748

Srna RF, Baggaley A (1976) Rate of excretion of ammonia by the hard clam *Mercenaria mercenaria* and the American oyster *Crassostrea virginica*. Mar Biol 36:251–258

Stone KC, Poach ME, Hunt PG, Reddy GB (2004) Marsh–pond–marsh constructed wetland design analysis for swine lagoon wastewater treatment. Ecol Eng 23:127–133