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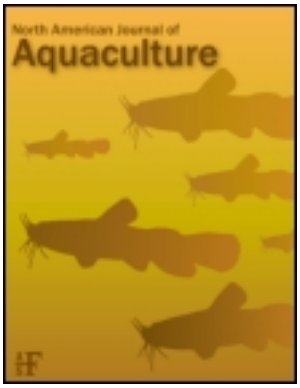
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North American Journal of Aquaculture
Publication details, including instructions for authors and subscription information:
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Evaluation of a Sodium Hydroxymethanesulfonate Product for Reducing Total Ammonia Nitrogen in a Small-Scale Rotifer Batch Culture System

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Published online: 09 Jan 2011.

To cite this article: Marty Riche , Timothy J. Pfeiffer & Javier Garcia (2006) Evaluation of a Sodium Hydroxymethanesulfonate Product for Reducing Total Ammonia Nitrogen in a Small-Scale Rotifer Batch Culture System, North American Journal of Aquaculture, 68:3, 199-205

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North American Journal of Aquaculture 68:199–205, 2006
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DOI: 10.1577/A05-063.1

[Communication]

Evaluation of a Sodium Hydroxymethanesulfonate Product for Reducing Total Ammonia Nitrogen in a Small-Scale Rotifer Batch Culture System

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Abstract.—The total ammonia nitrogen (TAN) produced as metabolic waste in rotifer batch culture systems can reach toxic concentrations as un-ionized ammonia (UIA). Sodium hydroxymethanesulfonate has been used to neutralize TAN in hauling and shipping containers for fish, crustaceans, and mollusks. The objective of this study was to determine the efficacy of a sodium hydroxymethanesulfonate product to neutralize ammonia in a rotifer batch culture system. Rotifers *Brachionus rotundiformis* were stocked at an initial concentration of 80 individuals/mL and were batch-cultured without water exchange for 7 d. We evaluated response variables that included TAN concentration, rotifer production, egg count, and egg : female ratio. Mean maximum TAN was 3.3 mg/L (0.08 mg UIA per liter of water) and 9.5 mg/L (0.84 mg/L UIA) for treated and control groups, respectively. Significant differences were detected in TAN, rotifer density, egg count, and egg : female ratio beginning on day 3 ($P < 0.05$). Beginning on day 2, mean rotifer density declined exponentially in the control group ($y = 293.4 \cdot e^{-0.249x}$; $R^2 = 0.98$) but remained constant in the treated group. Correlation coefficients indicated that TAN explained 72% of the population decrease. We conclude that the use of a sodium hydroxymethanesulfonate product as an ammonia neutralizer is effective in decreasing TAN and UIA in a small-scale rotifer batch culture system.

It is estimated that 80×10^9 fry were required to support world aquaculture production in 1998 (Lee 2003). Given the current limitations in larval feed technology, it is accepted that the production of larval marine fish requires the use of rotifers as an initial food source. Demand in some large-scale hatcheries in Japan requires a daily production in excess of 20×10^9 rotifers (Fu et al. 1997).

The culture systems and techniques used for culturing rotifers are classified as batch, semicontinuous, or continuous. Batch culture systems are used for low-density production (10–300 rotifers/mL), and all rotifers are harvested at the end of the culture cycle. Semicontinuous culture is employed for low- to high-density production (100–1,000 rotifers/mL), and the population is routinely reduced by removing 10–30% of the volume, which is replaced with seawater. Continuous culture requires recirculating systems and is used for production of high- and ultra-high densities reaching 1.6×10^5 rotifers/mL (Yoshimura et al. 1997).

Rotifer metabolic waste products increase total ammonia nitrogen (TAN) and decrease pH (Schlüter and Groeneweg 1985; Nagata 1989; Suantika et al. 2003). Approximately 50% of the dissolved nitrogen excreted by rotifers is ammonium (Hirata and Nagata 1982). Although rotifers can tolerate a wide range in pH (Epp and Winston 1978), TAN can reach toxic concentrations (Perry et al. 2003; Rombaut et al. 2003; Yoshimura et al. 2003). Yu and Hirayama (1986) demonstrated an inversely proportional relationship between rotifer density and un-ionized ammonia (UIA) concentration and suggested that UIA should be maintained below 2.1 mg per liter of water. Moreover, removal of accumulated ammonia from the culture system was shown to increase productivity of the rotifers *Brachionus rotundiformis* (Yoshimura et al. 2003) and *B. plicatilis* (Rombaut et al. 2003; Suantika et al. 2003). Recirculating systems with biofiltration can support sustained production of 5,500–6,000 rotifers/mL (Fu et al. 1997; Rombaut et al. 2003).

Batch and continuous culture methods are practiced by most hatcheries but require substantial investments in space and labor, which increase operational costs (Lee 2003). Although recirculating technologies can decrease costs of rotifer production (Suantika et al. 2000, 2003), interest remains in small-scale batch culture, particularly for small-scale hatcheries, short-term seasonal use, or situations where capital, resources, and training are limited. In addition, batch culture

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Received June 30, 2005; accepted November 17, 2005
Published online May 30, 2006

is the simplest method, and the use of multiple small-scale systems can reduce the impact on hatchery production if the rotifer culture crashes (Lubzens et al. 1997).

Where biofiltration is unavailable or impracticable, such as in hauling tanks or shipping bags, water conditioners can be used to remove or neutralize ammonia. One mechanism for neutralization of ammonia is the use of sodium hydroxymethanesulfonate,

$$NaCH_2(OH)SO_3 + NH_3 \rightarrow Na^+ + H_2O + CH_2(NH_2)SO_3^-,$$

where the reaction product, aminomethanesulfonate, is further oxidized to nitrate by nitrifying bacteria. Such products have been used successfully on fish, crustaceans, and mollusks.

The objective of this study was to determine the efficacy of a sodium hydroxymethanesulfonate product for neutralizing ammonia in a rotifer batch culture system.

Methods

Experimental design.—Brachionus rotundiformis were obtained from Oceans, Reefs, and Aquariums, Inc., Fort Pierce, Florida. Prior to the experiment, stock cultures were maintained at 170 rotifers/mL, fed with algae *Nannochloropsis oculata*, and provided daily enrichment with Selco (Inve Aquaculture Nutrition, Ogden, Utah).

During the experimental period, rotifers were batch-cultured without water exchange for 7 d. Experimental units were 20-L vessels containing 6 L of saltwater (34 g/L) that had been filtered through a sand filter, a charcoal filter, and a cartridge filter (1 μm) and then sterilized with ultraviolet light. Each unit was stocked with 80 rotifers/mL and supplied with oxygen at a rate of approximately 1 L/min. Low aeration was also provided to maintain a homogenous distribution of rotifers and to suspend food in the water column. Rotifers were fed once per day (75,000 cells/rotifer) in the morning with Instant Algae *Nannochloropsis* 3600 (Reed Mariculture, Campbell, California).

Sodium hydroxymethanesulfonate (ClorAm-X, AquaScience Research Group, Inc., North Kansas City, Missouri) was added to the batch culture and served as the treatment. The treatment and control were run with six replicates each. We evaluated response variables that included TAN concentration, rotifer production, egg count, and egg : female ratio. Twice-daily TAN measurements were used to determine ClorAm-X application rates. Applications were carried out per the manufacturer's recommendations by dissolving 32 mg of ClorAm-X in 50 mL of saltwater for each ppm of TAN per liter of tank volume, representing a fourfold excess of the stoichiometric amount to react on a one-to-one molar basis of ammonia.

Temperature, pH, and dissolved oxygen (DO) were measured three times daily (0830, 1200, and 1630 hours). Temperature and pH were measured with a pH/temperature probe pHep (Tri-Ess Sciences, Inc., Burbank, California), and DO was measured with a YSI 55 DO meter (Yellow Springs Instruments, Inc., Yellow Springs, Ohio). Total ammonia nitrogen was measured twice daily (morning and afternoon) with a HACH test kit and DR/890 colorimeter (HACH Co., Loveland, Colorado). Un-ionized ammonia was calculated according to methods described by Emerson et al. (1975).

Rotifer density and egg production were monitored daily prior to feeding. A 200-mL sample of the culture suspension was collected, from which a 1-mL sample was drawn by pipette, and five aliquot sub-samples were used to determine density. A drop of Lugol's solution was used to immobilize rotifers for enumeration of eggs and individuals.

Statistical analysis.—A completely randomized design with repeated measures was used for analysis. Temperature, salinity, pH, and DO were analyzed with repeated measures for day and time of day (Cody and Smith 1997). Analysis was conducted utilizing the general linear models procedure and the REPEATED statement in the Statistical Analysis System version 8 (Cary, North Carolina). Wilk's lambda (multivariate statistic) and a significance level of 0.05 were used for declaring significant differences.

Daily morning TAN and the response variables of rotifer density, egg production, and egg : female ratio were analyzed with repeated measures for day. The morning TAN measurements were used for statistical analysis, as this period corresponded with egg counts and density determinations. Means were separated with Tukey's test, and significance is reported at P-values less than 0.05 unless otherwise indicated.

Stepwise multiple regression was performed on rotifer density and egg production (dependent variables). Independent variables for analysis of rotifer density were egg production, temperature, pH, TAN, and NH_3. The multiple regressors for egg production included temperature, pH, TAN, NH_3, and rotifer density. Data used for the environmental parameters were collected at 1630 hours. Only those independent variables that had P-values less than 0.15 were selected to remain in the model, and a P-value of 0.05 was used for rejecting the null hypothesis.

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TABLE 1.—Range and mean ($n = 126$) dissolved oxygen (DO), temperature, and salinity measured at 0830, 1200, and 1630 hours in a rotifer batch culture system for 7 d with (treatment) or without (control) the use of a sodium hydroxymethanesulfonate ammonia neutralizer.

	DO (mg/L)		Temperature (°C)		Salinity (g/L)	
Group	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)
Control	3.3–20.0	10.7 (4.1)	22.6–31.0	26.3 (2.2)	34.0–35.0	34.1(0.3)
Treatment	4.2–20.0	10.0 (4.2)	22.5–30.6	26.2 (2.2)	34.0–35.0	34.1(0.3)

Results

The range and mean values for DO, temperature, and salinity are shown in Table 1. No significant treatment effects or interactions were detected for these variables. Significant differences in pH were detected for the effects of treatment, time, and day as well as for the treatment × time interaction. Mean pH values at 0830, 1200, and 1630 hours indicated that pH decreased during the day regardless of treatment (Table 2). The pH decreased over the course of the trial. It stabilized in the control group at day 3 but continued to fall in the treated group; differences between the groups were initially observed on day 3.

Total ammonia nitrogen increased during the trial in both treatments (Figure 1). Mean maximum TAN was 3.3 and 9.5 mg/L for the treated and control groups, respectively. Significant differences were detected between the two groups beginning on day 3.

Mean rotifer density significantly increased during the first day of the trial, reaching 226 rotifers/mL in each group (Figure 2). Beginning on day 2, mean density declined exponentially in the control group ($y = 293.4 \cdot e^{-0.249x}$; $R^2 = 0.98$) but remained constant in the treated group; the slope of the regression for the treated group was not significantly different from zero ($P = 0.1259$). Differences in density developed at day 3 and continued until the end of the trial.

The observed number of eggs significantly increased on day 1 and returned to the initial level by day 2 (Figure 3). Significant differences were observed in number of eggs between the control and treated groups by day 3, when the lowest level of egg production was observed for both treatments. The highest number of eggs observed in both treatments was on day 1; however, the egg : female ratio on day 1 decreased due to increased female density. The egg : female ratio was significantly higher in the treated group beginning on day 3. A summary table for the multiple-regression analyses for egg production and rotifer density is given in Table 3.

Discussion

Dissolved oxygen and salinity in our experiment were always within acceptable limits for *Brachionus* spp. (Esparcia et al. 1989; Fielder et al. 2000). Despite diurnal fluctuations, temperature remained optimal for reproduction and growth (Lubzens 1987). Although pH steadily decreased in the treated group from day 0 to 7 at all sampling periods, both treatments had values within the range reported to have no effect on

TABLE 2.—Daily pH (±SD; $n = 6$ replicates/treatment) measured at 0830, 1200, and 1630 hours from stocking (day 0) to harvest (day 7) in a small-scale rotifer batch culture system with (treatment) or without (control) the addition of a sodium hydroxymethanesulfonate ammonia neutralizer. Different letters across a row represent significant differences ($P < 0.05$). Values in bold italics were significantly lower than control values at the same sampling time.

	Day							
Group or time	0	1	2	3	4	5	6	7
Control								
0830 hours		8.01 z	7.84 yz	7.78 x	7.75 x	7.65 x	7.81 x	7.83 x
		(0.17)	(0.06)	(0.11)	(0.12)	(0.24)	(0.18)	(0.15)
1200 hours	7.98 z	7.90 yz	7.77 x	7.66 x	7.65 x	7.65 x	7.80 xy	
	(0.11)	(0.10)	(0.06)	(0.12)	(0.12)	(0.17)	(0.11)	
1630 hours	8.03 z	7.63 y	7.54 y	7.58 y	7.60 y	7.56 y	7.68 y	
	(0.12)	(0.21)	(0.12)	(0.12)	(0.14)	(0.19)	(0.19)	
Treatment								
0830 hours		8.10 z	7.77 y	7.66 y	***7.49 x***	***7.27 w***	***7.21 w***	***7.08 w***
		(0.04)	(0.20)	(0.10)	***(0.12)***	***(0.15)***	***(0.07)***	***(0.13)***
1200 hours	7.95 z	8.01 z	7.76 y	7.53 x	***7.29 w***	***7.13 v***	***7.15 u***	
	(0.07)	(0.04)	(0.09)	(0.11)	***(0.13)***	***(0.11)***	***(0.07)***	
1630 hours	8.02 z	7.57 y	7.43 xy	***7.39 x***	***7.18 w***	***7.06 w***	***6.93 v***	
	(0.10)	(0.05)	(0.20)	***(0.10)***	***(0.15)***	***(0.11)***	***(0.10)***	

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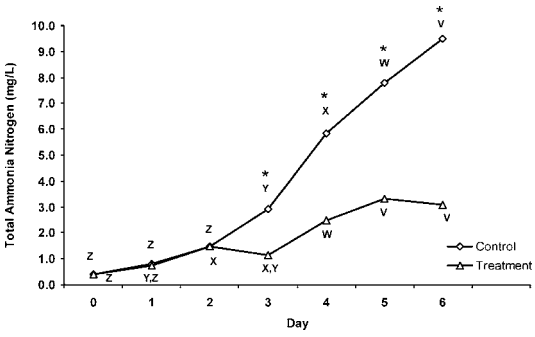


FIGURE 1.—Daily mean ($n = 6$ replicates/treatment) total ammonia nitrogen concentration in a 7-d rotifer *Brachionus rotundiformis* batch culture with (treatment) or without (control) the addition of a sodium hydroxymethanesulfonate ammonia neutralizer. Data points with different letters represent significant differences within a treatment, and asterisks represent differences between treatments ($P < 0.05$).

metabolism and performance of *B. plicatilis* (Epp and Winston 1978) or *B. rotundiformis* (Yoshimura et al. 1995). The observed decrease in pH is common with the use of ClorAm-X and can be reversed with the addition of bicarbonate.

Rotifers are usually harvested on 4-d cycles in batch cultures when TAN approaches 10 mg/L and when rotifer growth is declining (Suantika et al. 2000). In the control group, TAN was approximately 6 mg/L on day 4 and did not reach 10 mg/L until day 6 (Figure 1). Significant differences in rotifer density appeared on day 3 and coincided with significant differences in TAN (Figure 1).

The calculated UIA concentration was 0.011–0.078 mg/L in the treated group and 0.013–0.840 mg/L in the control group. The UIA concentration that was lethal for 50% of *B. plicatilis* over 24 h was reported as 20.4 mg/L, but a reduction in swimming activity, an indicator of stress, was reported to occur at UIA levels as low as 2.3 mg/L (Snell et al. 1987). Moreover, 2.4 mg/L was the UIA concentration above which an effect on reproduction was reported (Yu and Hirayama 1986). Hamada et al. (1993) fed *N. oculata* algal paste to *B. rotundiformis* in a batch culture and observed UIA concentrations of 0.51–1.14 mg/L, which were coincident with a decrease in population after 6 d. The researchers suggested that the decline was attributable to UIA and that *B. rotundiformis* was more sensitive to UIA than was *B. plicatilis*.

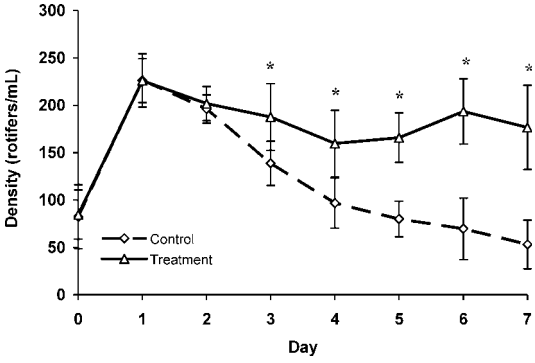


FIGURE 2.—Daily mean (±SE; $n = 6$ replicates/treatment) density of rotifers *Brachionus rotundiformis* in a 7-d batch culture with (treatment) or without (control) the addition of a sodium hydroxymethanesulfonate ammonia neutralizer. Asterisks represent significant differences between treatments ($P < 0.05$).

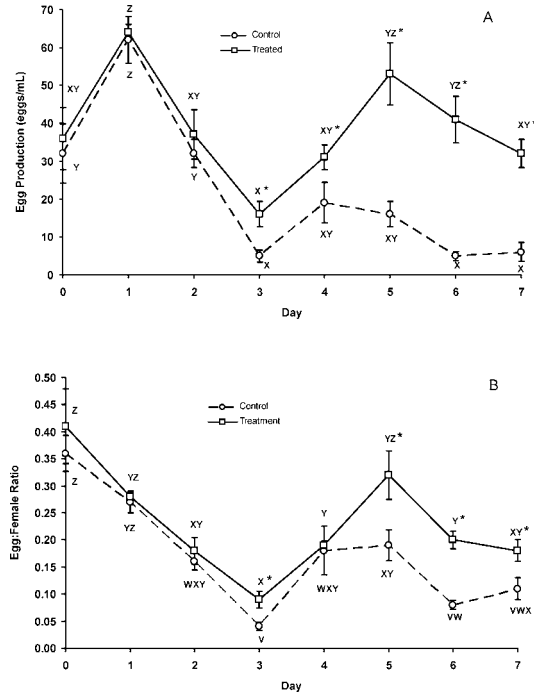


FIGURE 3.—Mean (±SE; $n = 6$ replicates/treatment) **(A)** rotifer *Brachionus rotundiformis* egg production and **(B)** egg : female ratio in a 7-d batch culture with (treatment) or without (control) the addition of a sodium hydroxymethanesulfonate ammonia neutralizer. Data points with different letters represent significant differences within a treatment, and asterisks represent differences between treatments ($P < 0.05$).

Rotifer density had a significant effect on egg production for both treated ($P = 0.0011$) and control groups ($P < 0.0001$). Inhibition of reproduction occurs at low densities (James et al. 1983), and *B. plicatilis* populations with egg ratios below 0.13 are unable to sustain themselves (Snell et al. 1987). The egg : female ratio was significantly higher in the treated group (Figure 3) when the ratios in the control group were

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TABLE 3.—Summary of stepwise multiple-regression analyses of egg production and densities of the rotifer *Brachionus rotundiformis* reared in batch culture for 7 d with (treatment) or without (control) the addition of a sodium hydroxymethanesulfonate ammonia neutralizer (TAN = total ammonia nitrogen).

Dependent variable	Independent[a] variable	Partial R^2	Model R^2	F-value	P-value
Egg production					
Control	Density	0.647	0.647	62.41	<0.0001
	TAN	0.028	0.675	2.79	0.1042
	pH	0.023	0.698	2.43	0.1285
Treatment	Density	0.274	0.274	12.81	0.0011
	TAN	0.098	0.372	5.15	0.0299
	Temperature	0.050	0.422	2.79	0.1062
Rotifer density					
Control	TAN	0.724	0.724	89.10	<0.0001
	Egg production	0.137	0.861	32.27	<0.0001
	pH	0.022	0.883	5.86	0.0213
Treatment	Egg production	0.274	0.274	12.81	0.0011
	TAN	0.171	0.444	10.16	0.0031

[a]Independent variables included temperature, pH, TAN, un-ionized ammonia, rotifer density (for egg production), and egg production (for rotifer density). Variables that did not meet a significance level of 0.15 were excluded from the model.

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0.19, 0.08, and 0.11 for days 5, 6, and 7, respectively. Multiple regression suggested that decreased density explained 65% of reduced egg production in the control group and 27% in the treated group (Table 3).

Although statistical significance ($P = 0.0299$) was detected for the effect of TAN on egg production in the treated group, the low partial correlation coefficient and lack of statistical significance ($P = 0.1042$) in the control group would suggest that TAN was not the causative agent. The maximum UIA concentration detected was 0.85 mg/L, which is below the UIA concentration reported as reducing fertility in *B. plicatilis* (Snell et al. 1987). However, reproduction in *B. rubens* was inhibited at a UIA concentration of 3 mg/L (Schlüter and Groeneweg 1985), and a concentration of 2.4 mg/L reduced fecundity in *B. plicatilis* by approximately 50% (de Araujo et al. 2000). These data suggest that decreased egg production may have been attributable to a factor not measured in this experiment.

A possible explanation for the lack of population increase was that decreased egg production was a function of feed rate. A variety of life history strategies are employed by rotifers in response to insufficient quantity or quality of food (Kirk 1997; Kirk et al. 1999). *Brachionus plicatilis* regulate their fecundity schedule and life span by suppressing reproduction (Yoshinaga et al. 2000; 2003). When food becomes limiting, fecundity falls quickly (Yúfera and Pascual 1989; Yoshinaga et al. 2003), and increasing the feeding rate increases the reproductive rate in *B. plicatilis*. Moreover, the egg : female ratio is used as an indicator of the nutritional state of rotifers (Øie and Olsen 1997); ratios below 0.1 are associated with populations under a nutritional limitation (Yúfera et al. 1997). In the current study, rotifers were fed 75,000 algal cells/individual per day ($4–17 \times 10^6$ cells/mL daily), which was determined to be sufficient for obtaining maximum growth and maximum intrinsic growth rates in *B. plicatilis* fed a variety of live microalgal species (Scott and Baynes 1978). However, feed quality as well as quantity can result in poor reproductive performance. Hamada et al. (1993) demonstrated that *B. plicatilis* ingest *N. oculata* algal paste similar to that used in this study at the same rate as fresh *N. oculata*. However, *B. plicatilis* fed dried or frozen *N. oculata* exhibited lower reproductive rates than those fed fresh algae (Lubzens et al. 1995). Therefore, we might have observed greater egg production if we had used a live *N. oculata* culture.

Decreasing populations are a function of increased mortality and decreased reproduction. Regression analysis indicated that decreasing rotifer density and reduced egg numbers were correlated ($R^2 = 0.47$, $P < 0.0001$); this relationship was stronger in the control group ($R^2 = 0.56$, $P < 0.0001$). Conversely, the egg : female ratio was independent of density ($R^2 = 0.01$, $P < 0.3005$), which suggests that the reduction in egg numbers observed in the control group beyond day 2 was a function of lower rotifer density. Therefore, lower production in the control group was probably a function of increased mortality, which subsequently resulted in decreased reproduction.

The multiple-regression analysis with rotifer density as the dependent variable suggests that TAN and reduced egg production significantly contributed to decreased rotifer density ($P < 0.0001$). Partial correlation coefficients indicated that TAN explained 72% of the decrease in population, while 14% was explained by the reduction in egg production, further supporting the effects of TAN on adult mortality rather than inhibition of reproduction. Surprisingly, the NH_3 concentration did not meet the criteria for inclusion in

the model. This is probably due to the confounding effects of temperature and pH on the NH_3/NH_4^+ equilibrium. Diel fluctuations in temperature and pH may have masked the constituent effect of UIA in favor of the more easily measured TAN.

The experimental units receiving sodium hydroxymethanesulfonate product had a lower pH. Since the reaction product, aminomethanesulfonate, is further oxidized to nitrate by nitrifying bacteria, the full reaction would produce hydrogen ions (Wheaton et al. 1994). However, cultures were initiated with sterilized seawater, suggesting that the drop in pH was due to higher CO_2 production. It is unclear whether the observed difference in density can be attributed to TAN reduction or a pH shift in the NH_3/NH_4^+ equilibrium, and further investigation is warranted. Although rotifer cultures without the sodium hydroxymethanesulfonate product exhibited significantly reduced density, the efficacy of this product for enhancing rotifer batch cultures could not be ascertained because rotifer density did not increase with its use.

We conclude that the use of a sodium hydroxymethanesulfonate product as an ammonia neutralizer is effective in decreasing TAN and UIA in a small-scale rotifer batch culture system. Although elevated TAN resulted in lower rotifer densities, it could not be determined whether the use of sodium hydroxymethanesulfonate could be used to increase rotifer density under the experimental conditions used.

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Acknowledgments

The authors would like to thank the staff at Harbor Branch Oceanographic Institution and express appreciation to the Link Foundation for their support of this investigation. Special thanks are extended to Jeffrey Wilcox, Jerry Ludwig, and Ken Riley for their expertise and critical review of the manuscript during preparation. This is Harbor Branch Oceanographic Institution contribution number 1611. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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