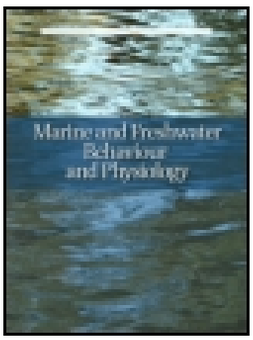

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SHORT COMMUNICATION

Acute toxicity of carbon dioxide to juvenile marine shrimp *Litopenaeus vannamei* (Boone 1931)

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ABSTRACT

Elevated concentrations of dissolved carbon dioxide (CO_2) and reduced pH levels are observed during the culture and transportation of aquatic organisms. Studies on the toxicity effects of CO_2 in penaeid shrimp are scarce when compared to the amount of research in fish. The objective of the present study was to determine the lethal concentration and safety levels of CO_2 for juvenile white shrimp *Litopenaeus vannamei*. Juveniles (1.76 ± 0.36 g) were exposed for 96 h to one of six concentrations of dissolved CO_2 (14.5, 23.8, 59.0, 88.0, 115.0, and 175.0 mg/L) or a control condition (without the addition of CO_2), and their survival was monitored for 96 h. The LC_{50} values with 95% confidence limits at 24, 48, 72, and 96 h were 130.05 (104.2–162.1), 77.2 (73.8–80.02), 69.65 (65.47–74.32), and 59.12 (53.08–66.07) mg/L of CO_2, respectively. The calculated safety level was 5.9 mg/L of CO_2, and the highest concentration that did not induce significantly higher mortality than that observed in controls (NOEC) was 23.8 mg/L of CO_2. We recommend that CO_2 levels should be kept below the safety level obtained in this study.

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Toxicity; carbon dioxide; safety level; pH; *Litopenaeus vannamei*

Introduction

Researchers have recently observed an increase in the concentration of dissolved carbon dioxide (CO_2) in the oceans and a reduction of 0.1 pH unit in the water surface since the pre-industrial era (Caldeira and Wickett 2003). The pH in the oceans is predicted to be reduced by 0.2–0.3 units by the year 2100 (Feely et al. 2009).

Several studies were conducted to analyze the effects of ocean acidification on larval, juvenile, and adult invertebrates (Kurihara 2008; Bierbower and Cooper 2010; Carter et al. 2013). The results obtained by Kurihara (2008) showed that, with chronic exposure of the shrimp *Palaemon pacificus* to the predicted concentrations of CO_2 over the next centuries, the survival, growth and possibly the reproduction of these organisms may be reduced.

The consumption of oxygen and the release of CO_2 occur during the transportation of aquatic organisms in closed environments such as plastic bags and generate a stressful condition of hypoxia and hypercapnia (Jensen et al. 2014). In fish and shrimp cultures, CO_2

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concentrations are increased mainly due to their biomass and decaying organic matter in the culture environment (Timmons and Ebeling 2010). The Pacific white shrimp, *Litopenaeus vannamei*, is the most widely farmed species of shrimp in superintensive bioflocs technology (BFT) systems. Stocking densities in these systems are between 150 and 600 shrimp/m^2, and the biomass is between 2 and 8 kg/m^2 (Silva et al. 2013).

In a BFT system, if the number of heterotrophic organisms exceeds the number of photoautotrophic microorganisms, then CO_2 production also exceeds the production of dissolved oxygen. This may accumulate CO_2 and reduce the pH levels of the culture water (Vinatea et al. 2010; Furtado et al. 2011). Under fault conditions in the aeration system or power failure, dissolved oxygen concentrations decrease rapidly, CO_2 concentrations increase and the water is acidified (Furtado et al. 2014). According to Van Wyk and Scarpa (1999), CO_2 concentrations below 5 mg/L in the water are ideal and 20 mg/L is considered acceptable for penaeid shrimp. Concentrations between 20 and 60 mg/L are not lethal but can interfere in the exchange of CO_2 in the gills, and concentrations above 60 mg/L can be lethal. Dissanayake and Ishimatsu (2011) found a synergistic effect of elevated CO_2 concentrations on temperature, which can impair the breathing and swimming capacities of penaeids.

The CO_2 is an essential carbon source for photosynthesis but is also the final product of the respiration of aquatic organisms (Boyd 2008). Initially, the conditions for culture in a BFT system are predominantly photoautotrophic. CO_2 concentrations can fluctuate between 5 and 10 mg/L in the morning but may exceed 20 mg/L in ponds with high biomass (Boyd 2008). Over a 24-h period, dissolved oxygen (DO), pH and carbon dioxide levels can oscillate. During the light phase of the day, CO_2 is removed and pH and DO increase as a result of photosynthetic processes; however, at night, photosynthesis stops and respiration predominates, and the release of CO_2 by aerobic organisms results in reduced pH and DO in the culture water (Vinatea 2004). Over time, CO_2 is permanently produced, and there is minimal production of DO by phytoplankton within the dominant heterotrophic communities in the BFT system.

Excretion of CO_2 in the gill epithelium is hampered when CO_2 concentrations exceed 20 mg/L, resulting in a decline of the hemolymph pH. This process adversely affects the transport oxygen from the hemolymph, reduces tissue oxygenation and increases the ventilation rate (Taylor and Whiteley 1989; Van Wyk and Scarpa 1999). An intra- or extra-cellular maladjustment in the acid-base balance can damage the metabolism of shrimp (Wang et al. 2012). This is possible even if decapod crustaceans possess the ability to tolerate basic and slightly acidic conditions, due to the exchange of Na^+/H^+ and Cl^-/HCO_3^- occurring in their gills (Henry et al. 1981).

Studies on the effects of CO_2 in penaeid shrimp are scarce when compared to the amount of research in fish (Grottum and Sigholt 1996; Santos et al. 2013). The aim of this study was to determine the lethal concentrations and safety level of carbon dioxide for *L. vannamei* juveniles.

Materials and methods

Location and facilities

This study was conducted at the Marine Aquaculture Station Prof. Marcos Alberto Marchiori (EMA) of the Oceanographic Institute – Federal University of Rio Grande (FURG), located at Cassino Beach, Rio Grande (32°11′ S, 52°10′ W), Rio Grande do Sul, Brazil.

Water, shrimp, and experimental design

The water used in the experiment was pumped from the beach and filtered through a sand filter and a cartridge with a pore size of 5 µm. No water was exchanged throughout the study. Dechlorinated fresh water using 1 ppm ascorbic acid and intense aeration for 12 h and was added to restore the volume lost through evaporation.

The shrimp used in this study was obtained from the laboratory Aquatec Ltda (Canguaretama, Rio Grande do Norte, Brazil). After the *L. vannamei* nauplii arrived at EMA, they were maintained in the larviculture sector until they reached the post-larvae 15 (pL15) stage. Thereafter, they were cultivated in a nursery (1500 pL/m^2) in a BFT system, where they reached a mean weight of 1.76 ± 0.36 g. A total of 210 *L. vannamei* juveniles in the intermolt stage (2 days after molting) were acclimated to the experimental conditions for 48 h to reduce the stress of capture and handling. During this period, they were fed twice per day with commercial feed (Guabi® 38% crude protein and 8% ethereal extract) in the proportion of 8% of biomass/day. The experimental photoperiod was 12 h light: 12 h dark at 27 °C.

The acute test to determine the median lethal concentration (96 h-LC_{50}) was conducted in a semi-static system. Animals were not fed during the test. Each rectangular tank had a usable volume of 30 L and was stocked with 10 shrimp, and each treatment had three replicates (n = 30 shrimp). Six concentrations of carbon dioxide were tested (14.5, 23.8, 59.0, 88.0, 115.0, 175.0 mg/L) in addition to a control treatment (without the addition of CO_2).

CO_2 was injected into the experimental units through a pressurized gas cylinder weighing 30 kg and equipped with a manometer, control valve and carbon dioxide flow meters (scale of 0–15 L/min). Each treatment consisted of a recirculation system with three rectangular tanks of 30 L and a 200 L circular reservoir, which had a submerged pump (capacity of 1000 L/h) with a Venturi tube that injected CO_2 from the cylinder (Figure 1). The pump transferred water from the reservoir to the rectangular tanks, solubilizing CO_2 into the water during the process. The water exchange rate in each experimental unit was 150 L/h and water returned to the reservoir by gravity. Six air diffusers provided aeration in each 200 L reservoir, and a thermostat was used to maintain the temperature at 27 °C.

Temperature, pH and oxygen were measured every 2 h using a pH meter model 100 YSI® (Yellow Springs Instruments, USA) and an oximeter 55 YSI® (Yellow Springs Instruments, USA). Salinity was verified at the beginning and end of the experiment with an optical refractometer (Atago®, Japan). Total ammonia (TAN) concentrations (NH_3 + NH_4^+) and alkalinity were measured every 24 h according to the methodologies recommended by UNESCO (1983) and APHA (1998), respectively. The dissolved carbon dioxide concentrations were measured according to APHA (1998) and calculated with the software CO_2 Analysis Salt® (Timmons and Ebeling 2010). CO_2 concentrations and shrimp survival were monitored every 2 h (12 times per day) over a 96 h period. Shrimp were considered dead when they were still and did not respond to mechanical stimulation with a glass rod (Lin and Chen 2003). The dead shrimp were removed from the tank as soon as they were detected.

Statistical analyses

The cumulative data of mortality for the periods of 24, 48, 72, and 96 h were used to estimate the median lethal concentration (LC_{50}) and its confidence limits (95%) using the Trimmed Spearman Karber Method software (Hamilton et al. 1977). The safety level was calculated

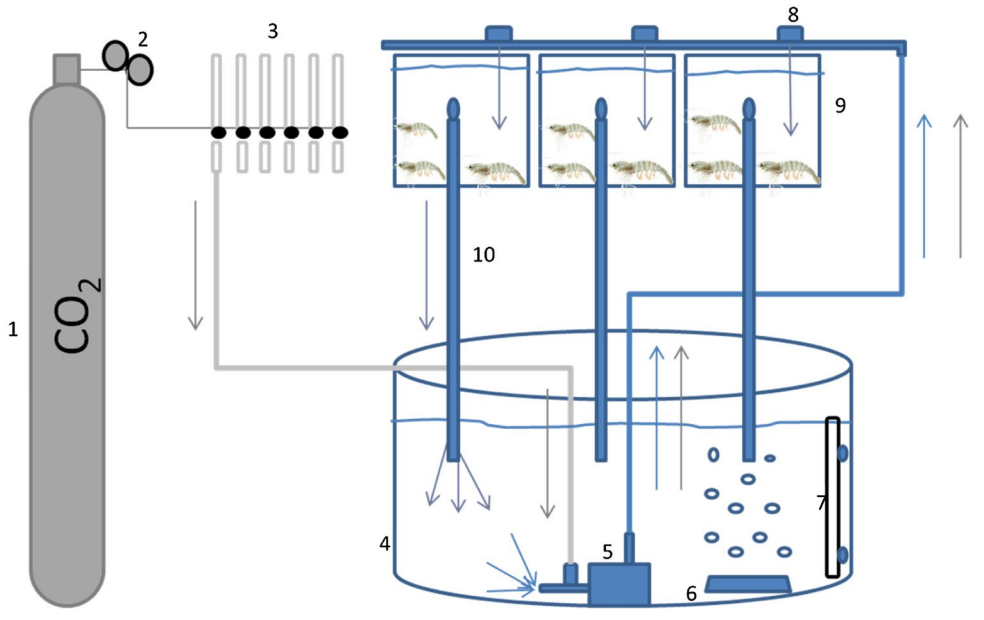

Figure 1. Diagram illustrating the experimental design where: (1) a carbon dioxide cylinder (CO_2); (2) a pressure gage and regulating valve; (3) flow meters; (4) a circular tank with 200 L of usable volume; (5) a submerged pump with a Venturi tube for CO_2 entry; (6) an air diffuser for water oxygenation; (7) a heater with a thermostat; (8) a water register for flow adjustment; (9) 30 L experimental tanks for the exposure of the shrimp to CO_2; and (10) a water pipe to return water from the experimental units to the circular tank.

using a 0.1 factor, according to Sprague (1971). The NOEC (i.e. the highest concentration that did not induce significantly higher mortality than that observed in controls) (EPA 2002) was calculated by comparing the number of survivors in each treatment at the end of the test.

We used the software STATISTICA 7.0® (StatSoft Inc. 2004, Tulsa, Oklahoma, USA) for the statistical analysis. To check for significant differences between the data, an analysis of variance (ANOVA, one-way) was performed after the assumptions of homocedasticity of variances (Levene) and data distribution normality (Kolmogorov-Smirnov) were confirmed. Tukey's test was used to compare the means when significant differences were detected between treatments ($p < 0.05$).

Results

The mean values ± the standard deviation of the water quality parameters monitored throughout the test are shown in Table 1. There were no significant differences in the salinity, temperature, and alkalinity ($p > 0.05$) between treatments. However, significant differences were found ($p < 0.05$) for dissolved oxygen, pH, and TAN. The mean values of dissolved oxygen were above 6 mg/L at CO_2 concentrations below 59.0 mg/L and differed significantly from other treatments with higher CO_2 concentrations. The treatment with 175.0 mg/L showed the lowest dissolved oxygen level (5.6 mg/L). There were significant differences in pH among all of the treatments (Figure 2), and pH showed an inverse relationship between the values of CO_2. Thus, pH values decreased as the concentrations of

Table 1. Water quality parameters (mean ± SD) measured during a 96 h test with different concentrations of carbon dioxide (CO_2) with juvenile *L. vannamei*.

CO_2 treatment (mg/L)	Salinity (‰)	Temperature (°C)	DO (mg/L)	pH	TAN (mg/L)	Alkalinity ($mgCaCO_3$/L)
0.6 ± 0.3[a]	30.2 ± 0.3	27.2 ± 0.6	6.24 ± 0.18[a]	8.06 ± 0.05[a]	0.20 ± 0.13[a]	105.2 ± 1.4
14.5 ± 0.9[b]	30.3 ± 0.3	27.0 ± 0.54	6.21 ± 0.15[a]	6.79 ± 0.08[b]	0.14 ± 0.09[a]	105.1 ± 1.7
23.8 ± 1.5[c]	30.5 ± 0.2	26.9 ± 0.87	6.22 ± 0.14[a]	6.57 ± 0.13[c]	0.18 ± 0.11[a]	104.7 ± 2.6
59.0 ± 3.6[d]	30.1 ± 0.6	26.96 ± 0.7	6.0 ± 0.12[ab]	6.17 ± 0.1[d]	0.12 ± 0.06[a]	104.7 ± 2.5
88.0 ± 6.5[e]	30.4 ± 0.3	27.1 ± 0.70	5.75 ± 0.25[b]	6.03 ± 0.04[e]	0.08 ± 0.04[ab]	104.6 ± 2.5
115.0 ± 10.5[f]	30.6 ± 0.3	26.95 ± 0.9	5.65 ± 0.27[b]	5.88 ± 0.1[f]	0.06 ± 0.03[b]	104.2 ± 2.0
175.0 ± 15.6 [g]	30.8 ± 0.5	26.81 ± 0.7	5.61 ± 0.3[b]	5.7 ± 0.05 [g]	0.05 ± 0.01[b]	103.9 ± 1.5

Notes: Different superscript letters in each column indicate significant differences ($p < 0.05$) among the treatments. DO: dissolved oxygen. TAN: total ammonia nitrogen.

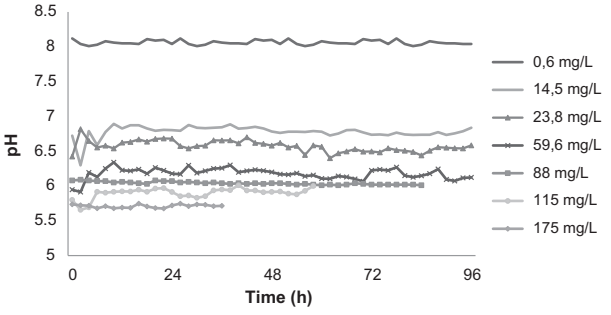

Figure 2. pH in the water during 96 h of study in the different concentrations of CO_2 (mg/L).

CO_2 increased. The measured TAN concentrations were higher in the treatments from 0.6 to 88.0 mg/L, which differed from the treatments of 115 and 175 mg/L.

The accumulated mortality data along the 96-h test presented in the Figure 3. All *L. vannamei* juveniles exposed to the concentrations of 175, 115, and 88 mg/L of CO_2 died within 36, 48, and 84 h of exposure, respectively. There was mortality of shrimp exposed to 59.0 mg/L of CO_2 for 64 h, and it reached 33% after 96 h. No mortalities were observed in the treatments with 0.6, 14.5 and 23.8 mg/L of CO_2.

The calculated 96 h LC_{50} was 59.12 mg/L of CO_2 with a 95% confidence interval from 53.08 to 66.07 mg/L of CO_2. LC_{50} values of 24, 48, 72, and 96 h are shown in Table 2. The safety level was 5.9 mg/L of CO_2, approximately 25% of the highest concentration tested which did not cause mortality (NOEC) (23.8 mg/L of CO_2).

Discussion

The chemical and physical parameters of water quality measured in this study remained within the range considered adequate for the survival of juvenile *L. vannamei*, except for the treatments with CO_2 concentrations greater than 23.8 mg/L (Ponce-Palafox et al. 1997; Van Wyk and Scarpa 1999; Lin and Chen 2001).

TAN concentrations increased over time but did not reach levels that could affect the survival of shrimp (Lin and Chen 2001). There was a difference in TAN levels due to the premature mortality of shrimp in the treatments with concentrations greater than 88.0 mg/L of CO_2; therefore, the treatments with lower CO_2 concentrations showed higher TAN levels because the shrimp survived and excreted for a longer time.

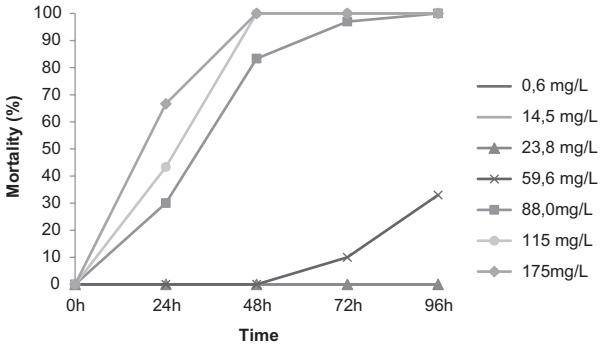

Figure 3. Cumulative mortality (%) of shrimp during 96 h of exposure to different concentrations of CO_2.

Table 2. Median lethal concentration (LC_{50}) data for marine shrimp *L. vannamei* juveniles exposed to CO_2. Confidence limits are 95%.

Time (h)	LC_{50} CO_2 (mg/L)	Confidence limits (mg/L)
24	130.05[a]	(104.2–162.1)
48	77.2[b]	(73.8–80.02)
72	69.65[bc]	(65.47–74.32)
96	59.12[c]	(53.08–66.07)

Note: Different superscript letters in the same column indicate significant differences ($p < 0.05$).

According to Van Wyk and Scarpa (1999), dissolved oxygen concentrations above 5.0 mg/L are considered ideal for the growth of shrimp. In the present study, DO concentrations remained above 5.0 mg/L in all of the treatments, even those with higher CO_2 concentrations. DO concentrations decreased with the increase in CO_2, which was also verified by Hall and Van Ham (1998). Under hypoxic conditions, the toxicity of CO_2 was increased, as it interfered in the oxygen absorption capacity (Boyd and Tucker 1998). To avoid lethal concentrations of CO_2 during the transportation of shrimp, which typically has hypoxic conditions, the biomass must be reduced and the pH must be adjusted with alkalizing compounds (Jensen et al. 2014).

Suitable pH values for the culture of penaeid shrimp are in the range of 7–9 (Van Wyk and Scarpa 1999). However, penaeids have a resistance to acidic conditions generated by the addition of hydrochloric acid, the values of lethal pH_{50} at 24, 48, 72, and 96 h for juvenile *L. vannamei* had acidic pH values of 3.86, 3.92, 3.94, and 4.04 (Furtado et al. 2015). Hall and Van Ham (1998) stated that the incorporation of CO_2 reduces water pH from 8.5 to 5.9, generating behavioral changes and increasing glucose levels in the hemolymph of *Penaeus monodon* when compared to the same pH reduction by the addition of sulfuric acid. The lowest pH values measured in this study were higher than those generating mortality in the studies developed by Furtado et al. (2015). However, mortality rates in this study were 100% at a pH 6.03.

This difference is due to a higher diffusion capacity of CO_2 than that of hydrogen ions (H^+), as molecular CO_2 directly diffuses faster than protons through cell membranes (Gutknecht et al. 1977). A change in the hemolymph pH creates favorable conditions for the production of reactive oxygen species, resulting in oxidative stress and damage to cell structures such as lipid membranes, proteins, and DNA (Lushchak 2011; Wang et al. 2012).

Kikkawa et al. (2008) evaluated the tolerance of juvenile Kuruma prawn, *Marsupenaeus japonicus*, to acute exposure to CO_2 and calculated the 72 h LC_{50} as 14.3% of CO_2. A LC_{50} value of approximately 50% for the same exposure period was found in this study, which was lower than those obtained in the Kikkawa et al. study. Therefore, juvenile *L. vannamei* are more sensitive to CO_2. The results of this study contradict the literature recommendations (Van Wyk and Scarpa 1999; Boyd 2008) because shrimp exposed to concentrations below 60 mg/L of CO_2 had a mortality rate of 30%. During the present study, we observed a loss of equilibrium in the shrimp before death. Such behavior was also observed by Kikkawa et al. (2008).

An acute toxicity test provides information about the relative lethality of a toxin but cannot predict the concentration that has sublethal and chronic effects on organisms (Buikema et al. 1982). In the present study, we calculated the safety level to be 5.9 mg/L of CO_2 using the factor 0.1 as suggested by Sprague (1971); therefore, *L. vannamei* juveniles can be cultivated in the presence of that level of CO_2 without mortalities. However, we do not recommend levels as NOEC of 23.8 mg/L of CO_2 to be maintained in farming systems for longer periods than those tested in this study.

Wickins (1984) studied the chronic effects of hypercapnia and found that juvenile *P. occidentalis* exposed for 56 days to pH 7.3 showed reduced growth when compared to shrimp farmed in sea water without the addition of CO_2 (pH 7.6). That study also showed a decrease in the growth of *P. monodon* juveniles as the pH decreased with the injection of CO_2 and longer intervals between molts (8.2 and 6.6 days for pH 6.4 and 7.4, respectively).

Circadian monitoring of pH and DO during culture is important for decision-making to help prevent high concentrations of CO_2 and hypoxic conditions, which can cause shrimp mortality and hinder the success of the culture. Several alkalizing compounds may be applied to correct pH and CO_2 levels such as calcium hydroxide, sodium carbonate and bicarbonate (Furtado et al. 2011). Based on chemical reactions, approximately 0.84 mg/L of calcium hydroxide or 0.64 mg/L of calcium oxide are needed to remove 1 mg/L of CO_2. As calcium hydroxide does not readily dissolve to react completely with CO_2, the concentrations mentioned above must be doubled (Boyd 2008). Another strategy to remove CO_2 is the use of degassing columns (Moran 2010).

Conclusion

In this study of the acute toxicity of CO_2, the 96 h LC_{50} was found to be 59.12 mg/L of CO_2, and the safety level was 5.9 mg/L of CO_2. The highest non-lethal concentration was 23.8 mg/L of CO_2. We recommend that CO_2 levels are maintained below the safety limit calculated for the species to avoid hypercapnia conditions, which can cause immunosuppression in the shrimp, making them more susceptible to diseases.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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