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BIOLOGY

Acute toxicity of nitrate in *Litopenaeus vannamei* juveniles at low salinity levels

Inácio Alves Neto[1*] Hellyjúnyor Brandão[1] Plínio Schmidt Furtado[1] Wilson Wasielesky Jr.[1]

[1]Programa de Pós-graduação em Aquicultura, Instituto de Oceanografia, Universidade Federal do Rio Grande (FURG), Cassino, 96210-030, RS, Brasil. E-mail: engpesca.inacio@gmail.com. *Corresponding author.

ABSTRACT: *Different technologies have been developed to improve the performance of Litopenaeus vannamei in low salinity, mainly in super-intensive systems like recirculation and BFT (Biofloc Technology System) systems. However, there is an accumulation of toxic nitrogenous compounds to the shrimps such as nitrate, that at high concentrations and depending on the salinity of the culture water can be lethal. Acute toxicity tests allow to analyze the relationship between the compound and other abiotic or biotic variables. The aim of this research was to determine the acute toxicity and safety level of nitrate ($N\text{-}NO_3^-$) for juveniles of L. vannamei at salinities of 5 and 10g.L^{-1}. For salinity of 5g.L^{-1}, a control and 5 treatments were tested, with nitrate concentrations of 100, 500, 1500, 2500 and 3500mg.L^{-1}.For salinity of 10mg.L^{-1}, a 4500mg.L^{-1}nitrate concentration was added. Juveniles were exposed to concentrations during 24, 48, 72, 96 hours in static system. The Mean Lethal Concentration (LC_{50}) was calculated and the recommended safety level for L. vannamei cultivation is 60.05 and 127.61mg.L^{-1} of nitrate for salinities 5 and 10g.L^{-1}, respectively.*
Key words: *median lethal concentration, Pacific white shrimp, brackish water.*

Toxicidade aguda do nitrato em juvenis de *Litopenaeus vannamei* em baixa salinidade

RESUMO: *Diferentes tecnologias foram desenvolvidas para melhorar o desempenho do Litopenaeus vannamei em baixa salinidade, principalmente em sistemas super intensivos como sistema de recirculação e BFT (Biofloc Technology System). No entanto, há um acúmulo de compostos nitrogenados tóxicos aos camarões, como o nitrato, que em altas concentrações e dependendo da salinidade da água pode ser letal. Os testes de toxicidade aguda permitem analisar a relação entre o composto e outras variáveis abióticas ou bióticas. O objetivo deste trabalho foi determinar a toxicidade aguda e o nível de segurança do nitrato ($N\text{-}NO_3^-$) em juvenis de L. vannamei nas salinidades de 5 e 10g.L^{-1}. Para a salinidade de 5g.L^{-1}, um controle e cinco tratamentos foram testados, com concentrações de nitrato 100, 500, 1500, 2500 e 3500mg.L^{-1}. Para salinidade de 10mg.L^{-1}, foi adicionada uma concentração de nitrato de 4500mg.L^{-1}. Os juvenis foram expostos às concentrações durante 24, 48, 72, 96 horas em sistema estático. A Concentração Letal Média (CL_{50}) foi calculada e o nível de segurança recomendado para o cultivo de L. vannamei é de 60,05 e 127,61mg.L^{-1} de nitrato para salinidadesde 5 e 10g.L^{-1}, respectivamente.*
Palavras-chave: *concentração letal mediana, camarão branco do Pacífico, água salobra.*

INTRODUCTION

The Pacific white shrimp *Litopenaeus vannamei* is an euryhaline species that tolerates a wide range of salinity (0.5 to 40g.L^{-1}), which allows it to be reared in low salinity and is already established in several countries, including the USA, Thailand, Ecuador and Israel (BOYD 2001, SAMOCHA et al., 2001). For this reason, studies on the minimum ionic concentrations required for its good zootechnical performance have been developed (CHONG-ROBLES et al., 2014; ROY et al., 2010) and together with super-intensive cultivation systems such as recirculation and Biofloc Technology System (BFT) provide ability to expand the activity. However, super-intensive systems of aquaculture production require high feed input and stocking densities of animals, increasing the deposition of deleterious compounds of water quality and toxic to the shrimp (MUHLERT et al., 2013). Among these compounds, generation and accumulation of nitrogenous products, which are inserted into the system through the degradation of the organic matter, unconsumed food and from the excretion resulting from the metabolism of the animals, are highlighted (BIANCHINI et al., 1996; EBELING et al., 2006).

The main product generated by the protein catabolism of most aquatic organisms and the

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decomposition of organic matter from over-feeding is ammonia (MAILLARD et al., 2005; REGNAULT, 1987). In water it is found in the ionized (NH_4^+) and non-ionized (NH_3) forms which together form the total ammoniacal nitrogen. In systems that reuse water, this compound goes through a nitrification process, resulting in a final form of nitrate-N through nitrite oxidation (SCHULER et al., 2010).

Nitrate is considered the less toxic form among nitrogen compounds, but at high concentrations and depending on the salinity of the water, it can be lethal. The conversion of pigments responsible for the transport of oxygen, such as hemocyanin, in forms incapable of carrying out transportation (metahemocyanin) is the main toxic action of nitrate in aquatic animals, reducing the availability of oxygen for metabolism and may induce hypoxia and mortality (CHENG & CHEN, 2002; JENSEN, 1996; SCOTT &CRUNKILTON, 2000; TAHON et al., 1988).

Acute toxicity tests represent a standard method for quantifying and comparing the relative toxicities of pollutants and are defined by the concentration that kills 50% of the test population, known as the LC_{50} value (APHA, 1998). The LC_{50} values can be presented from 12 to 24 hours exposure to pollutant concentrations at intervals of up to 96 hours. In addition to providing comparable data for other species or pollutants, these values allow us to analyze the relationships between the compound and other abiotic or biotic variables.

Several studies have been conducted to determine the acute toxicity of nitrogenous compounds in shrimp (CAMPOS et al., 2012; GROSS et al., 2004; SCHULER et al., 2010; TSAI & CHEN, 2002; WICKINS, 1976). However, until the present study, data for low salinities is still scarce. The aim of this research was to determine the acute toxicity and safety level for nitrate ($N\text{-}NO_3^-$) in juveniles of *Litopenaeus vannamei* at salinities 5 and 10g.L^{-1}.

MATERIALS AND METHODS

Animals and location

The study was conducted at the Shrimp Production Laboratory of Marine Aquaculture Station (Estação Marinha de Aquacultura - EMA) belonging to the Oceanography Institute of the Federal University of Rio Grande - FURG, located in the city of Rio Grande, Cassino - RS, Brazil (32º 19 'S, 52º 15 'W). Prior to the experiments, the animals were acclimated to the salinities of 5.0 and 10.0g.L^{-1}. *L. vannamei* juveniles (1.8±0.5g) were stocked in two 700L tanks in a stocking density of 700 shrimps/m^3. The initial salinity of 30g.L^{-1} was gradually reduced, five parts per day until reaching the desired final values. Water quality parameters were maintained at suitable values for species, with the temperature controlled by thermostatic water heaters (Aquarium Heater H-606) and dissolved oxygen kept near saturation point. Water renewals were performed (50%) whenever total ammonia nitrogen reached concentrations close to 0.8mg.L^{-1}. During acclimation, animals were fed 2x per day considering a feed rate of 10% of the biomass.

In the same period, toxicity pre-tests were carried out, exposing shrimp at the concentrations of 500, 1000, 2000 and 3000mg.L^{-1} of $N\text{-}NO_3^-$, in order to determine the minimum and maximum concentrations of nitrate and its effects on survival. From these data it was possible to delineate the experimental treatments.

Experimental design

Experimental units with 20 liters of useful volume were used with aeration supplied by radial blower and distributed by two air stones diffusers inside each experimental unit. Sea water was diluted with chlorinated and dechlorinated fresh water for each desired salinity. For salinity 5g.L^{-1}, a control (without addition of sodium nitrate) and 5 treatments were tested, with concentrations of $N\text{-}NO_3^-$: 100, 500, 1500, 2500 and 3500mg.L^{-1}. For salinity of 10g.L^{-1}, a control and 6 treatments in differentconcentrations of $N\text{-}NO_3^-$ were determined: 100, 500, 1500, 2500, 3500 and 4500mg. L^{-1}. For both salinities, nitrate concentration was obtained through the dilution of 8.5, 42.52, 127.57, 212.62, 297.66 and 382.70g (for 4500mg.L^{-1} treatment in salinity 10) of Sodium nitrate ($NaNO_3^-$– P.A. Synth) in the water, respectively. To maintain the Na: K ratio (28: 1),0.156, 0.78, 2.34, 3.9 and 5.46g of Potassium Chloride (KCl^-), respectively, was added in both experiments and 7.02g in the treatment with the highest concentration of nitrate in the salinity 10g.L^{-1}. Treatments were completely randomized distributed.

Forty-five juveniles were stocked per treatment and monitored daily to verify mortality - death criteria adopted was the absence of any type of movement or reaction to mechanical stimuli when touched by a glass rod. Dead animals were immediately removed from experimental units. The exposure times at concentrations were 24, 48, 72, 96 hours in static system. During exposure times, shrimps were not fed.

Water quality

The parameters of water quality were monitored daily. Temperature and dissolved oxygen were measured with the aid of multiparameter water quality meter (YSI - ProODO). The salinity was measured using digital refractometer (Atago, PAL-UREA). A table top pHmeter (Mettler Toledo) was used to measure pH and alkalinity was measured according to the methodology proposed by APHA (1998). Concentrations of ammonia (NAT) were monitored daily and nitrate ($N\text{-}NO_3^-$) was measured to verify the concentrations of the compound after addition of $NaNO_3$, according to methodologies proposed by UNESCO (1983) and AMINOT & CHAUSSEPIED (1983), respectively.

Statistical analysis

Water quality data were submitted to Levene's homoscedasticity and Shapiro-Wilk normality tests and after confirmation of these premises a One-Way Analysis of Variance (ANOVA) was performed to verify the significant differences in water quality parameters. When differences were reported ($p<0.05$), Tukey's test for comparison was applied (ZAR, 1996).

Median Lethal Concentration (LC_{50}) and confidence interval (95% CI) were calculated using the Trimmed Spearman-Karber Method software (HAMILTON et al., 1977). To calculate the safety levels (concentration of the compound that does not have an adverse effect on the organisms) for each salinity, the value of LC_{50} 96h was multiplied by an application factor proposed by SPRAGUE (1971) (LC_{50} 96h * 0.1).

RESULTS

Water quality parameters remained within the ranges considered appropriate for the species studied (LIN AND CHEN, 2003, 2001; WYK et al., 1999) and presented differences only in dissolved oxygen levels. The data are presented in tables 1 and 2.

Actual concentrations of nitrate measured were similar to the nominal concentrations, with small variations between nominal concentrations and replicates. The mean values of NO_3^- ranged from -0.39% to 2.38% of the nominal concentrations (99.61±0.83, 503.15±2.77, 1507.92±7.95, 2504.57±26.41, 3547.13±55.43 and 4606.94±104.75mg.L^{-1} of nitrate). To obtain compound concentrations in the experimental units, high purity reagents were used which was representative by comparing actual and nominal concentrations. The differences reported in this study for the variations between nominal and real concentrations were smaller than those described in other studies such as TSAI & CHEN (2002) and SOUCEK & DICKINSON (2012).

Figures 1 and 2 illustrate that there was mortality in the control group (without addition of nitrate) in both experiments starting from 60h. In 24 hours, the treatment with 3500mg.L^{-1} of nitrate reached 100% of mortality when in salinity of 5g.L^{-1}. For the same period and at the highest concentration, 4,500mg.L^{-1}, 93.33% mortality was observed in salinity 10.

After 96h, 100% of mortality was reported in the highest concentration, 3,500mg.L^{-1} followed by 97.77%, 80%, 44.44%, 13.33%, 8.88% for 2,500, 1,500, 500 and 100mg.L^{-1} NO_3^- concentrations. As in the previous one, 100% lethality was obtained for the highest nitrate concentration, 4,500mg.L^{-1}, followed

Table 1 - Water quality parameters during the LC_{50} test for salinity 5g.L^{-1} under different concentrations of nitrate ($N\text{-}NO_3^-$).

	Temperature	DO	pH	TAN	Alkalinity
Control	27.95±1.62	7.20±0.15^{ab}	8.13±0.11	0.40±0.26	97.5±2.54
100	27.84±1.80	7.32±0.15^{ab}	8.12±0.11	0.40±0.26	97.5±2.55
500	28.14±0.11	7.41±0.09^{a}	8.12±0.11	0.42±0.27	97.5±2.56
1500	27.99±2.26	6.86±0.06^{b}	8.17±0.11	0.41±0.26	97.5±2.59
2500	28.03±1.69	6.98±0.03^{b}	8.15±0.11	0.42±0.26	97.5±2.55
3500	27.89±1.79	7.22±0.18^{ab}	8.13±0.10	0.41±0.26	97.5±2.55

The data correspond to mean values of three replicates±standard deviation. Different letters on the same column indicate that means differ significantly ($p<0.05$). Temperature (°C); DO - Dissolved oxygen (mg.L^{-1}); TAN - Total ammonia nitrogen (mg.L^{-1}); Alkalinity (mg of $CaCO_3$.L^{-1}).

Table 2 - Water quality parameters during the LC_{50} test for salinity 10g.L^{-1} under different concentrations of nitrate (N-NO_3^-).

	Temperature	DO	pH	TAN	Alkalinity
Control	27.01±0.50	6.74±0.1[a]	8.20±0.09	0.35±0.25	97.5±2.54
100	26.9±0.49	6.75±0.11[a]	8.20±0.10	0.34±0.23	97.5±2.58
500	27.06±0.51	6.70±0.09[a]	8.18±0.10	0.35±0.26	97.5±2.57
1500	26.7±0.46	6.8±0.13[a]	8.18±0.07	0.37±0.24	97.5±2.61
2500	27.02±0.52	6.72±0.10[a]	8.20±0.10	0.33±0.26	97.5±2.57
3500	26.9±0.49	6.75±0.12[a]	8.19±0.09	0.35±0.24	97.5±2.54
4500	28.27±0.22	7.46±0.00[b]	8.17±0.12	0.36±0.26	97.5±2.73

The data correspond to mean values of three replicates±standard deviation. Different letters on the same column indicate that means differ significantly ($p < 0.05$). Temperature (°C); DO - Dissolved oxygen (mg.L^{-1}); TAN – Total ammonia nitrogen (mg.L^{-1}); Alkalinity (mg of $CaCO_3$.L^{-1}).

by 95.55%, 88.88%, 60%, 13.33%, 26.66 % and 13.33 respectively for the treatments with 3500, 2500, 1500, 500 and 100mg.L^{-1} NO_3^- in salinity of 10.

Figures 3 and 4 a linear negative relationship between the LC_{50} lethal concentration and exposure time was shown in two salinities, 5 and 10g.L^{-1}. Results of Mean Lethal Concentration (LC_{50}) as well as the confidence intervals for each salinity (95% CI) are presented in table 3. The acute toxicity of nitrate was tested for several species of crustaceans, especially for penaeids, as shown in table 4.

DISCUSSION

The material presented in this research adds to the current knowledge more data about the sensitivity of the penaeid shrimp *Litopenaeus vannamei* to the nitrate. Although, there are studies on acute toxicity to some aquatic organisms (CAMARGO et al., 2005; PANDEY et al., 2011; SOUCEK & DICKINSON, 2012) there is still a demand for more information of the effects on this species, especially at low salinity. In aquaculture it is important to know the limits of tolerance of the species that is aimed to be cultivated since the parameters of water quality are determinant factors for the maintenance of cultivation systems.

CAMPOS et al. (2012) tested LC_{50} values for juveniles of *Farfantepenaeus brasiliensis* and their respective confidence intervals at salinity 28g.L^{-1} and found 912.07mg.L^{-1} (LC_{50}-96h). Based on the application factor proposed by SPRAGUE (1971), the calculated safety level for juveniles of *F. brasiliensis* is 91.20mg.L^{-1}.TSAI & CHEN (2002) conducted an acute nitrate toxicity test in *Penaeus monodon*, using the same empirical application factor presented to estimate safe chronic levels and reported a value of 145mg.L^{-1} of nitrate in 15g.L^{-1} of salinity.

In the present study we reported values for salinities 5 and 10g.L^{-1} of 600.51 and 1276.29mg.L^{-1}, respectively, with safety levels 60.05 and 127.61, thus showing that *L. vannamei* is sensitive to nitrate; however, according to the data generated in this

Table 3 - LC_{50} (mg.L^{-1}) and confidence intervals of N-NO_3^- at different times and salinities.

Time (h)	5g.L^{-1}	10g.L^{-1}
24	1385.38 (1163.29–1649.87)	2716.12 (2445.69–3016.46)
48	1052.58 (859.72–1288.72)	1860.64 (1611.66–2148.08)
72	768.09 (614.49–960.09)	2006.89 (1764.00–2282.70)
96	600.51 (467.23–771.81)	1276.09 (1090.07–1495.07)

Table 4 - LC_{50} - 96h values (mg $N\text{-}NO_3^-.L^{-1}$) of nitrate for juveniles of different species of crustaceans, including results generated by the present study.

Species	Life stage	Salinity	LC_{50}	References
Penaeus monodon	Juvenile	15 with K^+	1411	ROMANO & ZENG (2009)
		35 no K^+	2213	ROMANO & ZENG (2009)
		35 with K^+	2337	ROMANO & ZENG (2009)
Penaeus monodon	Juvenile (0.30g)	15	1449	TSAI & CHEN (2002)
		35	2316	TSAI & CHEN (2002)
Scylla serrata	-	30 no K^+	3601	ROMANO & ZENG (2007)
		30 with K+	4339	ROMANO & ZENG (2007)
Portunus pelagicus	Juvenile (0.24 g)	30 no K^+	3355	ROMANO & ZENG (2007)
		30 with K+	4132	ROMANO & ZENG (2007)
Farfantepenaeus brasiliensis	Juvenile (0.30g)	28	912.07	CAMPOS et al (2012)
Litopenaeus vannamei	Juvenile (1.8g)	5	600.51	This study
		10	1276.09	This study

LC_{50}=Median Lethal Concentration ($mg.L^{-1}$).

study, it is more resistant than Brazil pink shrimp and tiger shrimp.

Also, according to LC_{50} it was possible to show in this study that salinity is a determining factor for nitrate toxicity, since it is directly proportional to salinity. According to TSAI & CHEN (2002), nitrate toxicity decreases with increasing salinity. KUHN et al. (2010) showed an increase in mortality of *L. vannamei* when submitted to the reduction of salinity in higher nitrate concentrations.

Studies by FURTADO et al. (2014) showed lethal effect on penaeid shrimp exposed

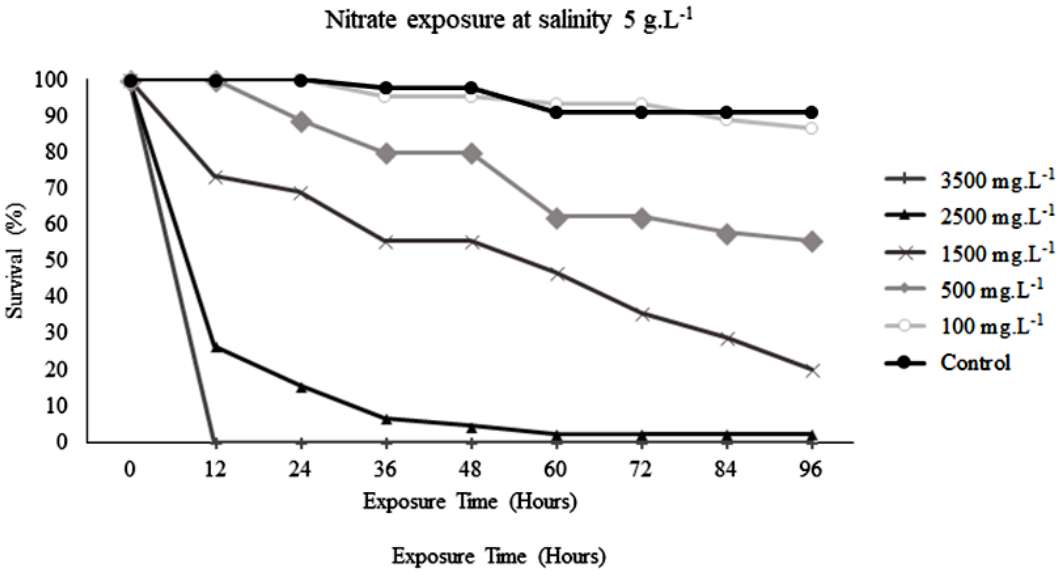

Figure 1 - Survival of *Litopenaeus vannamei* throughout 96h nitrate exposure at different concentration in salinity $5g.L^{-1}$.

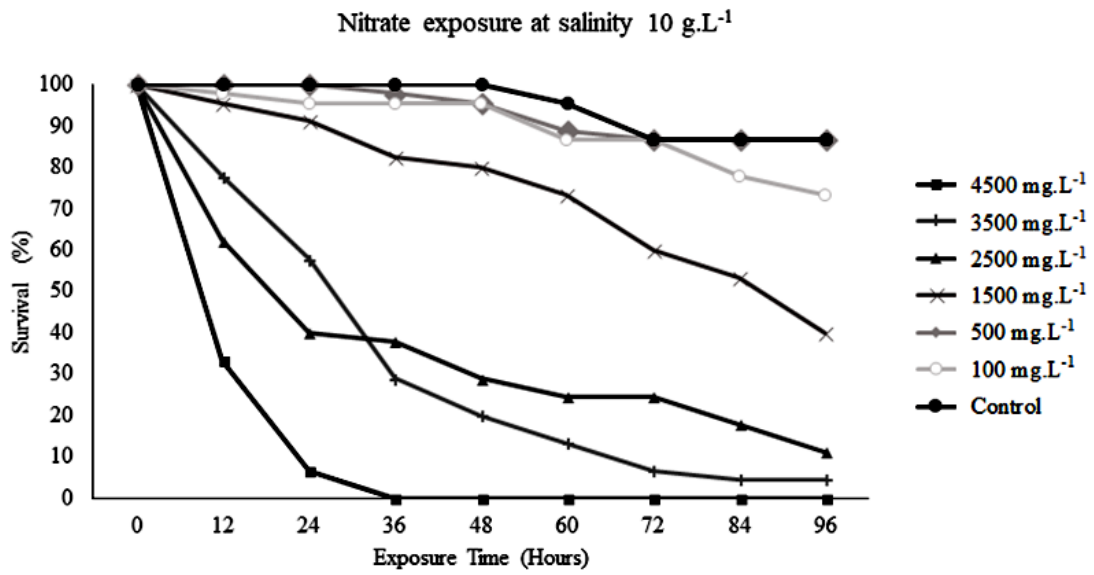

Figure 2 - Survival of *Litopenaeus vannamei* throughout 96h nitrate exposure at different concentration in salinity 10g.L^{-1}.

to concentrations above 300mg.L^{-1} in salinity of 24 and levels below 220mg.L^{-1} for salinity 11 was reported by KUHN et al. (2010). In addition, the accumulation of this nitrogen compound can cause sublethal effects on organisms, such as affecting antenna length, gill injury, hepatopancreas lesions, and other deleterious effects (FURTADO et al. 2014).

Closed aquaculture systems with little or no water exchange, such as recirculation and bioflocs systems, depend on nitrification for the conversion of ammonia nitrogen to nitrate, which

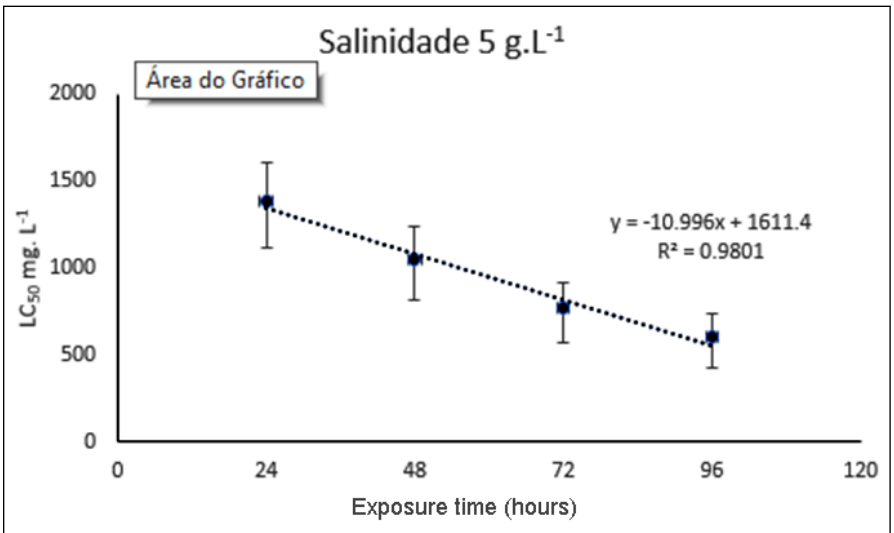

Figure 3 - Linear regression of LC_{50} of nitrate–N to juvenile of *L. vannamei* exposed to different concentrations of nitrate–N at salinity 5g.L^{-1}.

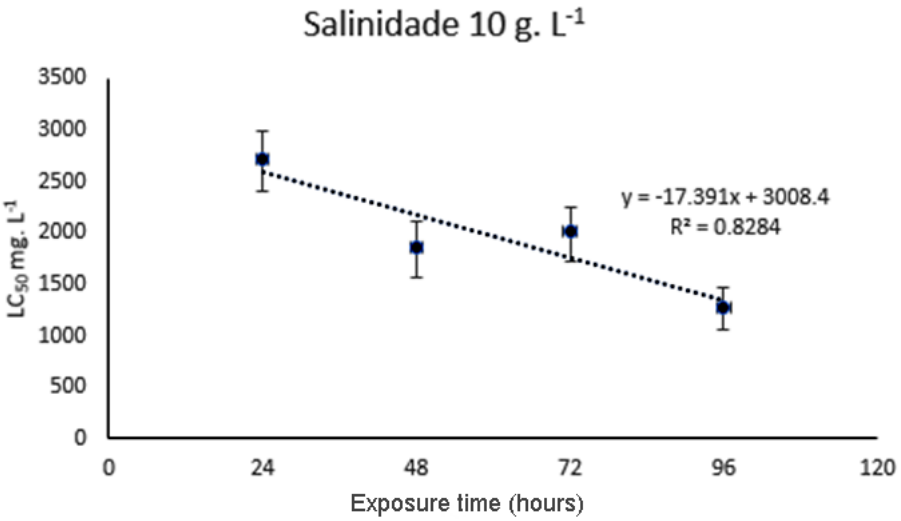

Figure 4 - Linear regression of LC_{50} of nitrate–N to juvenile of *L. vannamei* at salinity 10g.L^{-1} exposed to different concentrations of nitrate–N.

leads to the accumulation of the last one. Although less toxic, nitrate has lethal effect for organisms when in high concentrations. Some ways have been studied to remove and use this compound, such as the use of biofilters and integrated multi-trophic aquaculture systems, which t is the production of food such as fish, shrimp, mollusk, combined with an organism with market value, using plants for nitrate assimilation, reducing and/or recycling this compound (RAKOCY et al., 2006; VAN RIJN, 2013). Another technology under investigation for application in aquaculture is the electrochemical treatment (MOOK et al., 2012), where the nitrate is reduced to the nitrogen gas at the cathode.

CONCLUSION

Based on the values found for LC_{50} in 96h, the recommended safety level for *L. vannamei* cultivation is 60.05 and 127.61mg.L^{-1} of nitrate for the salinities of 5 and 10g.L^{-1}, respectively. To reuse water during several cycles of cultivation with low salinity in super-intensive systems, it is recommended the use of mechanisms and management to remove or immobilize this compound so that its toxicity does not compromise the development of the organisms cultivated, avoiding losses in the production.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

Alves Neto, Furtado and Wasielesky Jr conceived and designed experiments. Brandão performed statistical analyses of experimental data. All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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