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Median lethal concentration for nitrogen compounds in scallops (*Nodipecten nodosus* Linnaeus, 1758) (Mollusca, Bivalvia)

Concentração letal mediana para compostos nitrogenados em vieiras (Nodipecten nodosus linnaeus, 1758) (Mollusca, Bivalvia)

SILVA, Renan Ribeiro e. Mestre em Ciência Animal
Universidade Federal Rural do Rio de Janeiro (UFRRJ), Campus Seropédica. BR-465, Km 07, Seropédica – Rio de Janeiro – Brasil. CEP: 23890-000 / Telefone: (21) 2681-4600 / E-mail: renanribeiro@iedbig.org.br / Orcid: https://orcid.org/0000-0003-3405-0746

SILVA, Rafael Carvalho da. Doutorando em Ciência Animal
Universidade Federal do Vale do São Francisco (UNIVASF), Campus Ciências Agrárias. Rodovia BR-407, Km 12, Lote 543, Projeto de Irrigação Senador Nilo Coelho – N-4, Petrolina – Pernambuco – Brasil. CEP: 56.300-990 / Telefone: (87) 2101-4845 / E-mail: rafaelcarvalho51@yahoo.com.br / Orcid: https://orcid.org/0000-0002-2543-3561

MELO, Pâmela Talita da Silva. Mestre em Ciência Animal
Universidade Federal Rural do Rio de Janeiro (UFRRJ), Campus Seropédica. BR-465, Km 07, Seropédica – Rio de Janeiro – Brasil. CEP: 23890-000 / Telefone: (21) 2681-4600 / E-mail: talita.pamella.ts@gmail.com / Orcid: https://orcid.org/0000-0002-9585-5516

PEDROSA, Virgínia Fonseca. Doutora em Ciências Veterinárias
Universidade Federal do Rio Grande (FURG), Instituto de Oceanografia. Rio Grande – Rio Grande do Sul – Brasil CEP: 96201-900 / Telefone: (53) 3233-6600/ E-mail: vikavetp@gmail.com / Orcid: https://orcid.org/0000-0003-3353-1994

RAMOS, Leonardo Rocha Vidal. Doutor em Aquicultura
Universidade Federal Rural do Rio de Janeiro (UFRRJ), Campus Seropédica. BR-465, Km 07, Seropédica – Rio de Janeiro – Brasil. CEP: 23890-000 / Telefone: (21) 2681-4600 / E-mail: rochavr.leo@gmail.com / Orcid: https://orcid.org/0000-0003-1348-6006

ABSTRACT

The objective of this study was to identify the median lethal concentration (LC_{50}) of the nitrogenous compounds ammonia, nitrite and nitrate in *Nodipecten nodosus* seeds, as well as to evaluate the incidence of behavioral and histological changes in scallop gills. Toxicity of nitrogenous compounds was investigated using a completely randomized experimental design, using seven concentrations of total ammonia, 3, 6, 12, 15, 18, 25, and 30 mg L^{-1} (equivalent to 0; 0.10, 0.21, 0.43, 0.54, 0.65, 0.90 and 1.0 mg L^{-1} of non-ionic ammonia) and a control (0 mg L^{-1}); 25, 50, 100, 500, 600, 750, 900 e 1000 mg L^{-1} for nitrite and 100, 500, 1000, 6000, 7500, 9000 and 10000 mg L^{-1} for nitrate. The 96 h LC_{50} of total ammonia was estimated at 8.614 mg L^{-1} (0.2972 mg L^{-1} NH_3) through the TSK test (Trimmed Spearman-Karber), and it was not possible to find the median lethal concentration for nitrite and nitrate from the concentrations used. During exposure to nitrogenous compounds, only scallops exposed to higher concentrations of total ammonia showed behavioral changes such as reduced ability to respond to external stimuli and loss of ability to attach to the container wall (polyethylene). Furthermore, gill histological analyzes showed the presence of necrosis, hemocyte infiltrate and hyperplasia at concentrations of 18, 25 and 30 mg L^{-1} of Ammonia. Through the nitrite and nitrate levels used, it was not possible to estimate the LC_{50}. Based on these results, the species can be used in intensive production systems.

Keywords: Behavioral, Pectinides, Toxicity, Water quality.

RESUMO

O objetivo deste estudo foi identificar a concentração letal mediana (CL_{50}) dos compostos nitrogenados amônia, nitrito e nitrato em sementes de *Nodipecten nodosus*, bem como avaliar a incidência de alterações comportamentais e histológicas em brânquias de vieiras. A toxicidade dos compostos nitrogenados foi investigada utilizando um delineamento experimental inteiramente casualizado, utilizando sete concentrações de amônia total, 3, 6, 12, 15, 18, 25 e 30 mg L^{-1} (equivalente a 0; 0,10, 0,21, 0,43, 0,54, 0,65, 0,90 and 1,0 mg L^{-1} de amônia não-iônica), e um controle (0 mg L^{-1}); 25, 50, 100, 500, 600, 750, 900 e 1000 mg L^{-1} para nitrito e 100, 500, 1000, 6000, 7500, 9000 e 10000 mg L^{-1} para nitrato. A CL_{50} de 96 h da amônia total foi estimada em 8,614 mg L^{-1} (0,2972 mg L^{-1} NH_3) através do teste TSK (Trimmed Spearman-Karber), e não foi possível encontrar a concentração letal mediana para nitrito e nitrato a partir das concentrações utilizadas. Durante a exposição aos compostos nitrogenados, apenas as vieiras expostas a concentrações mais elevadas de amônia total apresentaram alterações comportamentais, como redução da capacidade de resposta a estímulos externos e perda da capacidade de fixação à parede do recipiente (polietileno). Além disso, análises histológicas branquiais mostraram presença de necrose, infiltrado de hemócitos e hiperplasia nas concentrações de 18, 25 e 30 mg L^{-1} de Amônia. Através dos teores de nitrito e nitrato utilizados não foi possível estimar a CL_{50}. Com base nesses resultados, a espécie pode ser utilizada em sistemas de produção intensivos.

Palavras-chave: Comportamento, Pectinídeos, Toxicidade, Qualidade de água.

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Introduction

According to the FAO (2024), aquaculture has increased significantly over the years, with production of 94 million tons in 2022, representing 51% of the total fish produced by both aquaculture and fisheries, surpassing the first time the total of fisheries capture. In this scenario, the world production of mollusks plays an important role, reaching the mark of 18.9 million tons produced in 2022; of which, bivalves represent more than 90% of farmed mollusks.

On the Brazilian coast, which extends for approximately 8500 km, pectinids are represented by 15 species (RIOS, 2009), with emphasis on *Euvola ziczaz* (Linnaeus, 1758) and *Nodipecten nodosus* (Linnaeus, 1758) due to their zootechnical potential for cultivation in a natural environment (MANZONI; RUUP, 1993), high commercial value, and acceptance in the consumer market (RUPP, 2007; MARQUES et al., 2018).

However, to promote the activity and population of marine farms with *Nodipecten nodosus,* it is necessary to produce seeds (young forms) in the laboratory, considering that their low natural density and dispersed populations do not allow a high rate of collection through collectors installed in a natural environment (Uriarte et al., 2001). Thus, for the development of pectin farming in other states of Brazil and the consequent increase in this activity, seeds must be transported over long distances in transport boxes. However, under these conditions and during laboratory production, the parameters that determine water quality, such as temperature, dissolved oxygen, hydrogen potential, salinity, and nitrogen compounds, and play decisive roles can have different effects on the metabolism and physiological processes of organisms, which are easily altered (RUPP; PARSONS, 2016).

Among the parameters mentioned above, some nitrogen compounds are toxic to aquatic organisms. Non-ionic ammonia (NH3) is highly toxic, followed by nitrite (NO2) and nitrate, which are toxic only at high concentrations. Albeit in the natural environment there is little probability of the occurrence of LC_{50} of nitrogen compounds for scallops and other species of bivalves mollusks, its well know that in rearing conditions the concentrations could reach harmful levels. To date, the tolerance limits for *Nodipecten nodosus* to nitrogen compounds have not been identified, and their determination is of fundamental importance for the success of long-distance transport and the development of new technologies for the intensive

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production of *Nodipecten nodosus*, such as those in closed systems. Due to the lack of information on the impact of nitrogen on seeds, this study aimed to determine the median lethal concentrations for ammonia, nitrite, and nitrate after 96 h of exposure, to evaluate behavioral changes during the exposure period, and to evaluate the presence of gill histological alterations in *Nodipecten nodosus* seeds at 90 days of life.

Material and methods

Experiment Location

The experiment was carried out at the Shellfish Larviculture Laboratory of the Instituto de Ecodesenvolvimento da Baía da Ilha Grande (IED-BIG), municipality of Angra dos Reis, RJ, at 23°00`24.55``S – 44°14`06.10`` W.

Scallop Seeds

Nodipecten nodosus seeds (1500) with a size and an average weight of 16.15 ± 1.98 mm and 0.84 ± 0.25 g, respectively, were produced and supplied by the Shellfish Larviculture Laboratory of IED-BIG. Generally, scallops of this size are already transported and sold to growth out farms. Seeds were transferred from the marine farm located at Angra dos Reis, RJ, to the laboratory.

For acclimatization to laboratory conditions, the seeds were initially placed in 500-liter boxes in a semi-static system, with a density of 500 individuals per box (~1 animal L^{-1}), with constant aeration by air diffusion (blower), maintained at an average temperature of 24°C controlled by an air conditioner, a salinity of 35 mg L^{-1}, pH 8, and photoperiod of 8 Light:16 Dark. Feed was supplied once a day after the partial exchange of 30% of the water with 3 liters of microalgae (*Chaetoceros* sp.) at a concentration of 50,000 cell ml^{-1} box^{-1}.

After 48 h of acclimatization, the seeds were transferred to the bioassay, where they were submitted to experiments.

Acute toxicity of nitrogen compounds in *Nodipecten nodosus* Seeds

Ammonia, nitrite, and nitrate toxicity for *Nodipecten nodosus* was determined with 96 h exposures to each nitrogen compounds. Scallop seeds were transferred to

1 L tanks (n = 10 each concentration) and exposed to the following concentrations: 0, 3, 6, 12, 15, 18, 25 and 30 mg L^{-1} of total ammonia, which are equivalent to 0; 0.10, 0.21, 0.43, 0.54, 0.65, 0.90 and 1.0 mg L^{-1} of non-ionic ammonia (NH3) at experimental conditions (pH 8, temperature 22ºC, and salinity 30) (Bower; Bidwell, 1978); 25, 50, 100, 500, 600, 750, 900, and 1000 mg L^{-1} of nitrite and 100, 500, 1000, 6000, 7500, 9000 and 10000 mg L^{-1} of nitrate. All concentrations tested were evaluated in quadruplicate, and during the tests, the animals were not fed.

To obtain these concentrations, stock solutions previously prepared from ammonium chloride (NH4Cl, P.A.), sodium nitrate (NaNO2, PA), and sodium nitrate (NaNO3, PA) were used. When necessary, the pH and salinity of the seawater after the inclusion of nitrogen compounds were adjusted using sodium bicarbonate and distilled water, respectively. The nitrogen concentrations used in the tests were based on values found in the literature for other bivalves species (Epifanio; Srna, 1975; Reddy; Menon, 1979; Widman et al., 2007) and were regulated according to the resistance demonstrated by *Nodipecten nodosus*. The death of the scallops was assessed every 24 h, by opening the valves and by the lack of response to tactile stimuli (touch with a glass rod), as described in Widman et al. (2007), after confirmation of death, the animals were identified and removed.

To confirm the nitrogen concentrations, Hanna Checker HI733 and HI708 digital colorimeters were used for ammonia and nitrite, respectively, and Hanna Probe HI72911 was used for nitrate. At higher concentrations, serial dilutions were performed to obtain a reading of concentrations within the working range of each instrument.

During the experiment, water quality parameters were monitored. Salinity was maintained at 30.33 ± 0.42 mg L^{-1}, measured with a handheld refractometer. The pH, temperature, and dissolved oxygen were measured using a HANNA HI98194 multiparameter probe. Nitrogen was measured daily to confirm the concentrations initially proposed. All parameters were measured twice a day at 9:00 am and 4:00 pm.

Behavioral changes

The scallops were observed during prolonged exposure to nitrogenous compounds (ammonia, nitrite and nitrate) to detect the incidence of abnormal

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behaviors such as retraction of the mantle borders and prolonged opening of the shell. The behavioral assessment was carried out according to the definitions of Rupp and Parsons (2004).

Histological Analysis

The seeds that died during the tests were immediately fixed in buffered formalin diluted in seawater (20%) and identified by treatment for histological analysis. The collected material was sent to the Laboratory of Immunology and Pathology of Aquatic Organisms (LIPOA) of the Federal University of Rio Grande (FURG/RS), where it was subjected to classical histological processing. The presence of damage to the gills, cellular hypertrophy, and the presence of hemocyte infiltrates were analyzed.

Statistical Analysis

For the analysis of toxicity and determination of the (LC_{50}) of total ammonia at 96 hours of exposure, the TSK Test (Trimmed Spearman–Karber) was used with the elimination of the lower limit (control—0 mortality) from the base and calculation (HAMILTON et al., 1977).

Results and discussion

In this study, water quality parameters remained stable between treatments, recording a temperature of 22.4 ± 0.68°C, pH 8.02 ± 0.08, salinity 30.33 ± 0.42 mg L^{-1} and dissolved oxygen around 5.88 ± 0.27 mg L^{-1}. According to Alves Neto et al. (2019) water parameters constitute a determining factor for the maintenance of cultivation systems, and knowledge about the limits tolerated by different species, especially to nitrogen compounds, is extremely important.

Despite the importance of evaluating ammonia concentration in crops, information on the toxicity of this compound to marine organisms is often neglected. This is because, although toxicity varies proportionally with the elevation of temperature and pH, but the effect of salinity is inverse. The higher the salinity, the lower the toxicity of ammonia, nitrite, and nitrate.

These trends occur in three ways; for ammonia, the acid hydrolysis is higher with elevated salinities, favoring the prevalence of $NH4^{+}$ to NH3 (Bower; Bidwell,

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1978); for nitrite and nitrate, the presence of chloride ions (Cl^-) in seawater competes with it in the hemolymph receptors, impairing the nitrogen $NO2^-$ and $NO3^-$ ions influx and further oxidation of hemocyanin pigment in invertebrates (Camargo et al., 2006); at last, most of freshwater bivalves lacks these respiratory pigments, inhibiting the toxicity effects of nitrite and nitrate. The same mechanism should be investigated in seawater shellfish species furthermore (Soucek; Dickinson, 2012).

At the end of exposure for 96 h, the mortality rate observed for the control group (0 mg L^{-1}) and concentrations of 0.10, 0.21, 0.43, 0.54, 0.65, 0.90 and 1.0 mg L^{-1} of non-ionic ammonia (NH3) were 7.5, 0.0, 0.0, 72.5, 77.5, 92.5, 100, and 100%, respectively (Table 1). At 0.43 mg L^{-1} of non-ionic ammonia, mortality was observed only after 72 h of the experiment, while at concentrations of 0.54, 0.65, 0.90 and 1.0 mg L^{-1} of non-ionic ammonia, mortality was observed in the first 24 h of exposure.

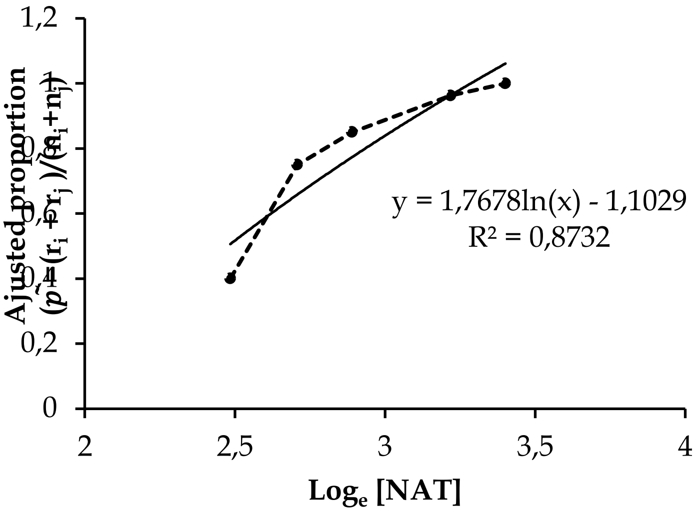
Figure 1 highlights the mortality curve from the logarithmic conversion of concentrations and the application of the TSK test. According to the results, the 96 h LC_{50} of total ammonia for scallop seeds was estimated at 8.614 mg L^{-1}.

From the data of toxic ammonia, the values were estimated since there was no way to perform the direct determination of this compound in water. Bower and Bidwell (1978) provided several tables estimating the concentration of toxic ammonia in water from different temperatures, pH, and salinity ranges. Considering the toxicity of LC_{50} 96 h for total ammonia in scallops found in the present work, 8.614 mg L^{-1}, converting for toxic ammonia at environmental conditions of the study (22.4°C and pH 8.01), the value estimated was 0.2972 mg L^{-1} $N\text{-}NH_3$.

Table 1. Total accumulated mortality during 96 days of *Nodipecten nodosus* seeds exposed to different concentrations of non-ionic ammonia (NH3).

NH3 (mg L^{-1})	24h	%	48h	%	72h	%	96h	%
0	0	0	0	0	1	2.5	3	7.5
0.10	0	0	0	0	0	0	0	0
0.21	0	0	0	0	0	0	0	0
0.43	0	0	0	0	9	22.5	29	72.5
0.54	2	5	7	17.5	16	40	31	77.5
0.65	2	5	29	72.5	34	85	37	92.5
0.90	12	30	27	67.5	40	100	40	100
1.00	9	22,5	25	62.5	39	97.5	40	100

Figure 1. Graph and equation generated from the logarithmic conversion of total ammonia concentrations as a function of the adjusted proportion of animal mortality according to the TSK test.



The toxic ammonia LC_{50} for *Nodipecten nodosus* assessed in the present study was superior to the tolerance limits observed by Epifanio and Srna (1975) in juveniles and adults of *Mercenaria mercenaria* and *Crassostrea virginica* at 96 h of exposure, in the range of 3.3 to 6 mg L^{-1} of NAT, respectively. These values were lower than those with juveniles of *Perna viridis*, verified by Reddy and Menon (1979), who determined the LC_{50} at 96 h of exposure to 13 mg L^{-1} NAT. These values also presented a lower LC_{50} of N-NH3 to that observed by Widman et al. (2007), who found an LC_{50} of 0.43 mg L^{-1} of N-NH3 after 72 h of exposure for the scallop *Argopecten irradians irradians*. Despite presenting lower tolerance to ammonia when compared to certain species of bivalves, these values can be considered high for *N. nodosus*.

Recent studies with ammonia toxicity in shellfish are not common, with works focused on some freshwater species and a few marine species (Soucek; Dickinson, 2012; Kleinhenz et al., 2019; Miao et al., 2010; Zhang et al., 2020).

Lu et al. (2022) observed many toxic effects exposing *C. hongkongensis* to high total ammonia nitrogen concentrations (LC50 60 mg L^{-1} TAN), mainly in the immune system, such as phagocytic activity and mitochondrial mass, oxidative and metabolic pathways. Boardman et al. (2004) determined acute and chronic non-ionized ammonia LC50 for quahog clams *M. mercenaria*, with levels for 48h and 96h of exposure at 216 and 36.6 mg L^{-1} for acute test. For chronic exposition it was not

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possible to determine the toxicity effects. According to the authors, from all species tested (three marine fishes and three marine invertebrates), the clam was the most tolerant species, and one reason is that this bivalve closed its shell and consequently minimized the exposure to toxic ammonia. The same trend was observed in the present study with the scallops.

In the present study, the tests performed with nitrite and nitrate showed a high resistance of the scallop *N. nodosus* to these nitrogenous compounds, even when challenged at very high concentrations.

N. nodosus seeds showed no mortality or behavioral changes after 96 h in the tests performed with exposure to nitrite at a concentration of 1000 mg L^{-1} and nitrate at a concentration of 10000 mg L^{-1}. These results differ from those verified by Epifanio and Srna (1975), who, in 96 h tests for acute toxicity in the bivalves *M. mercenaria* and *C. virginica*, determined tolerance to nitrogen compounds in the range of 532–756 mg L^{-1} of NO_2^- for juveniles and adults of both species and 2600–3800 mg L^{-1} of NO_3^- for *C. virginica*. These results differ from those found by Widman et al. (2007), who determined the acute toxicity in the scallop *A. irradians irradians* after exposure to nitrite and nitrate for 72 h. For nitrite, concentrations of 800 mg L^{-1} caused 100% mortality, and the LC_{50} for nitrite was 345 mg L^{-1}. For nitrate, concentrations of 5000 mg L^{-1} caused 100% mortality, and the calculated 72 h LC_{50} was 4453 mg L^{-1}.

Few other studies to date have demonstrated the direct effects of nitrogen on bivalves. Because they are animals with low or no locomotor activity (except scallops) and because they present anaerobic metabolism for long periods through valve closure, which prevents the animal from accessing the outside environment as a form of protection against predators and variation of environmental parameters, such as salinity, dehydration, and pollution (Boyd, 2012), these characteristics make bivalves naturally resistant to the toxic effects of nitrogen.

Dybas (2014) considered the ability of bivalve mollusks to close valves and isolate themselves from the environment to be one of the factors for high tolerance to nitrogen compounds. In the present study, total valve closure was not observed during the period of exposure to nitrite and nitrate compounds, which suggests that for *N. nodosus*, high tolerance is related to physiological resistance.

Other behavioral changes were observed in scallop seeds exposed to 18, 25 and 30 mg L^{-1} of NAT, in these groups we observed a lower capacity to react to external stimuli, and loss of the ability to attach to the container wall (polyethylene). Thus, in animals exposed to such concentrations, the shell closing response was slow or even absent. These behavioral observations are unprecedented in the literature on the impact of nitrogen toxicity on bivalves, but Rupp and Parsons (2004) reported similar behavior in *N. nodosus* when challenged at low temperatures for up to 96 h, according to the authors in conditions of low water temperatures and for a long period of time, these animals may present a lower response to touch and a small open shell, in addition to the retracted edges of the mantle.

It is known that in aquatic organisms, nitrogen compounds act directly on the gills, the main organ of nitrogen excretion and absorption, especially ammonia, with both actions depending on the concentration gradient of this compound in the water in addition to abiotic conditions, especially the pH of the water (TomassO, 1994).

Thus, in an environment with a high level of ammonia, the excretion of this product by the body of the aquatic organism is reduced, causing accumulation in the hemolymph (invertebrates) and tissues, which in turn causes an increase in pH (metabolic acidosis), a reduction in cellular enzymatic activity, and alteration of membrane permeability (Boyd, 2012). These actions, as observed in the present study, are intensified in tissues and organs that are in direct contact with water, such as the gills, resulting in histological damage. In the short term, animals show behavioral responses, such as a reduction in activity and opening and closing of valves, and in the long term, mortality occurs, mainly caused by impacts on respiration.

Also, in a serial study, Cong et al. (2017, 2018, 2019, and 2021) show a wide range of ammonia nitrogen gill effects in adult clam *Ruditapes philippinarum*. The concentrations of ammonia nitrogen utilized as a toxic indicator varied from 0.1, 0.5, and 1.0 mg $L^{-1,}$ and 3 days after the exposure, even at the minor level, were observed effects such as damage to membrane system, elevation in apoptosis rate, decreased in ATP content, disruption of gill structure and metabolism, affecting mainly the feed behavior, gas exchange and osmoregulation.

The analyses carried out on the slides containing the gill tissue of the scallops showed the existence of qualitative differences between the treatments and the

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control (Figures 2 and 3). In the control and the treatment with 12 mg L^{-1} of NAT, no lesions were found (Figure 2). On the hand, in the treatments with 18, 25 and 30 mg L^{-1} of total ammonia, hyperplasia, necrosis, and hemocyte infiltrates were observed in the gill tissue (Figure 3, B, C and D), with the hemocyte infiltrates being more intense in the treatment with 25 and 30 mg L^{-1} of total ammonia compared with the treatments with lower concentrations.

Despite no mortalities were observed in seeds exposed to nitrite and nitrate concentrations considered high for aquaculture standards, it should be important to suggest some hypothesis of such resistance, based on physiological effects of nitrite and nitrate on aquatic organisms. The main effect of these compounds on aquatic organisms is on respiratory activity since both bind to invertebrate hemocyanin, causing the oxidation of the copper molecule responsible for carrying oxygen through the hemolymph to the tissues and losing its functionality (Boyd, 2012).

Figure 2. Histological analysis of the gill tissue of *Nodpecten nodosus* scallop seeds from the control treatment, without alterations. HE staining. 40x Dark spots on the blade are fixture artifacts.

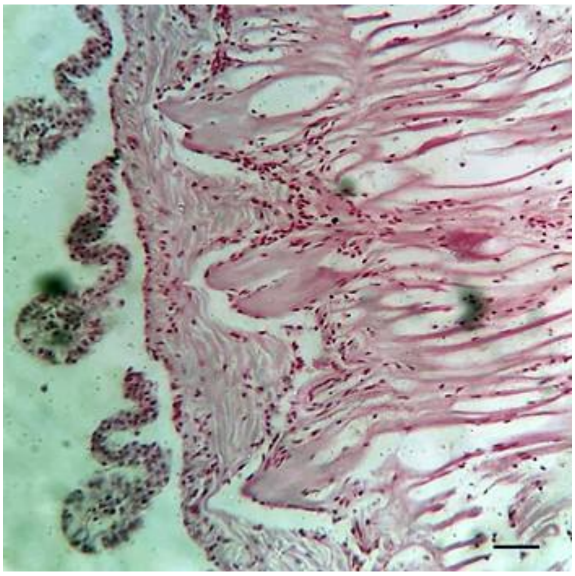

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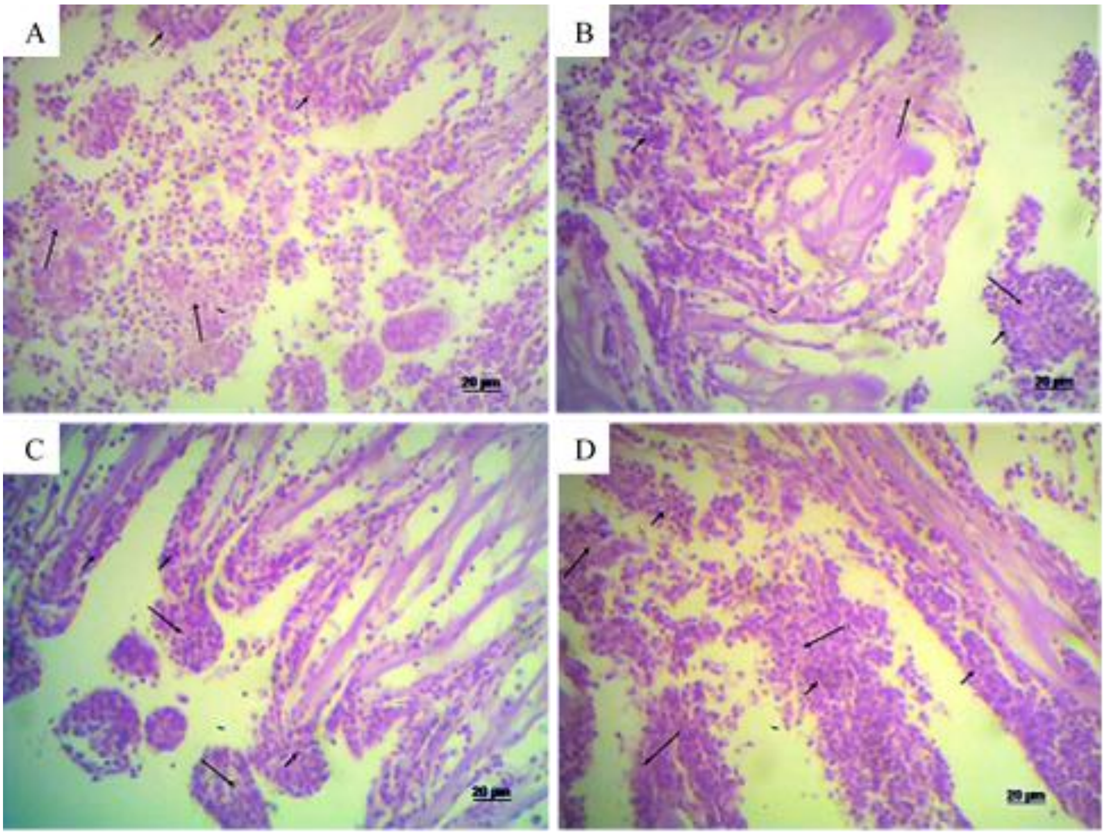
Figure 3. Gill histological analysis (HE, 40x) of *Nodpecten nodosus* scallop seeds exposed to increasing levels of ammonia. Clockwise, A: 15 mg L^{-1}, B: 18 mg L^{-1}, C: 25 mg L^{-1}, D: 30 mg L^{-1}. In all images, long arrows indicate necrosis and short arrows indicate hemocytic infiltrate. In C and D, the observed tissue enlargement characterizes hyperplasia.



The main way for nitrite ions to enter the body of aquatic organisms is via gill transporters, usually the same used for chloride ions, since both are monovalent anions and have approximately the same molecular weight (Tomasso, 1994). In addition, pH is directly related to toxicity: the more acidic the pH, the more toxic the effect of nitrite on aquatic organisms. In this way, marine and estuarine aquatic organisms naturally present greater resistance to nitrite toxicity, and, according to the author, there is species-specific resistance that allows animals to discriminate the absorption of nitrite from chloride. These same trends have been observed for the effect of nitrate on aquatic invertebrates (Camargo et al., 2006).

It has already been demonstrated that some fish species are capable of reversing methemoglobin through the activity of methemoglobin reductase (BOYD, 2012), and future studies are recommended to verify the presence of enzymes with similar activities in *Nodpecten nodosus*.

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Conclusions

This study showed that CL50 after 96 h exposure to the toxic non-ionic ammonia concentration for seeds of *Nodpecten nodosus* scallops, under the conditions, was estimated as 0.2972 mg L^{-1}.

Furthermore, the toxicity resistance to nitrite and nitrate was evident, given that there was no observation of mortality even after exposure to extremely high concentrations for aquaculture standards, 1000 and 10000 mg L^{-1}, respectively, within the reading range of the equipment that presents greater measurement sensitivity present in the domestic market.

Based on these results, the species can be recommended for use in intensive or super-intensive closed seed production systems during the laboratory production stages and for transport in boxes over long distances.

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