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Acute sensitivity to *Nitokra* sp benthic copepod to potassium dichromate and ammonia chloride

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Abstract

The present study assesses acute toxicity of non-ionized ammonia and potassium dichromate using the estuarine benthic copepod *Nitokra* sp from laboratory cultures. Bioassays with non-ionized ammonia sensitivity were carried out, and the mean. Bioassays with non-ionized ammonia sensitivity were carried out, and the mean of $LC(I)_{50\text{-}96h}$ was 1.7 mg.L^{-1}. – $LC(I)_{50\text{-}96h}$ was 1.7 mg.L^{-1}. Potassium dichromate was used as reference substance in two distinct periods of exposure and the mean $LC(I)_{50\text{-}48h}$ and $LC(I)_{50\text{-}96h}$ values were 55.61 mg.L^{-1}and 21.70 mg.L^{-1}, respectively. The organism showed sensitivity to the toxic agents used and good reproducibility of results, indispensable factors for use in ecotoxicological assays.

Keywords: bioassay, acute sensitivity test, potassium dichromate, ammonium chlorinate, estuarine copepod

Resumo

Sensibilidade aguda do copépodo bentônico *Nitokra* sp ao dicromato de potássio e cloreto de amônia

O presente estudo avalia a toxicidade aguda da amônia não ionizada e do dicromato de potássio ao copépodo bentônico estuarino *Nitokra* sp cultivado em laboratório. O valor médio da $CL(I)_{50\text{-}96h}$ foi 1,7 mg.L^{-1} de amônia não ionizada, e para a substância de referência os valores médios foram $CL(I)_{50\text{-}48h}$ = 55,61 mg.L^{-1} e $CL(I)_{50\text{-}96h}$ = 21,70 mg.L^{-1} de dicromato de potássio. O organismo apresentou sensibilidade aos agentes tóxicos utilizados e boa reprodutibilidade de resultados, fatores esses indispensáveis para uso em ensaios ecotoxicológicos**.**

Palavras-chave: bioensaio, testes de sensibilidade, dicromato de potássio, cloreto de amônia, copépodo estuarino

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INTRODUCTION

An important step for advancing the field of marine ecotoxicology is finding appropriate organisms for the development of toxicity tests (Kwok *et al*., 2008). Some benthic harparcticoid copepods have many characteristics that make them suitable for organism-tests in evaluating toxicity in sediment and water samples.

Benthic copepods are broadly applied in chronic and acute toxicity evaluation of environmental samples and specific contaminants. As examples of the use of benthic copepods in toxicity testing, *Nitocra lacustris* and *Schizopera knabeni* were used in analyses of sublethal toxicity with phenanthrene (Lotufo & Fleeger, 1997); *Amphiascus tenuiremis* species are used in tests with sediment spiked with metals; organophosphorous pesticides azimphosmethyl and a crude oil soluble fraction (Hagopian-Schlekat *et al*., 2001; Klosterhaus *et al.,* 2003; Bejarano *et al*., 2004); *Nitocra spinipes* was used in the evaluation of mixtures with organic polymers (Breitholtz *et al*., 2008); *Tisbe battagliai*, *Tigriopus fulvus* and *Robertsonia propinqua* were used in estuarine sediment toxicity assays (Hack *et al.,* 2008; Pane *et al.,* 2008; Araújo *et al.,* 2010).

The availability of organisms in sufficient number from laboratory cultures, ease of culture maintenance, tolerance to a broad range of abiotic factors such as granulometry, salinity and temperature, and their permanence during the entire life cycle in sediment and reduced size, are desirable test species characteristics (Lamberson *et al*., 1992; Rand *et al*., 2000; Domingues & Bertoletti, 2006). The harpacticoid copepod species listed above possess these characteristics.

Coull (1990), states that harparcticoids are the second most abundant meiofauna taxon and are an important link in the trophic chain, serving as food for a variety of fish and invertebrates in the juvenile phase.

Ecological studies in the structure of this community consider these organisms as sensible indicators of pollution in the benthic environment. Chemical-effect study of contaminants in benthic organisms indicate that copepods are frequently more vulnerable to the effect of some classes of contaminants than other benthic invertebrates (Coull & Chandler, 1992).

The benthic harparcticoid copepod *Nitokra* sp was isolated from several species collected in the sediment of Cananéia (SP) estuarine region and fully adapted to culture conditions in the Laboratory of Marine Ecotoxicology and Microphytobenthic (LEcotox) of the Oceanographic Institute of the University of São Paulo in Brazil.

In marine and estuarine environments, ammonia is a natural element, discharged as a product of excretion by invertebrates and other animals from these environments (Mackin & Aller, 1984). In this form, its toxic effect is practically insignificant compared with the amount released from industrial effluent and sewage.

Even occurring naturally as excretion product or nutrient, ammonia can interfere with the survival and reproduction of marine organisms, some of which are sensitive planktonic species and others, are less sensitive benthic invertebrates, favorable to higher levels of this nutrient (Lapota *et al*., 2000). Knowledge of sensitivity of marine species to ammonia used in toxicity tests is very important because this nutrient can be present in higher concentrations in environmental samples.

In all aquatic environments, ammonia can occur in two modes; the ionized (NH_4^+) mode, which is assimilated as nutrient by primary producers and has low permeability across the cellular membrane, and the non-ionized mode (NH_3), which is usually toxic because of high lipid solubility, favoring diffusion across cell membranes (Bower & Bidwell, 1978; Thurston *et al*., 1979; Rebelo, 1996; Frias-Espericueta *et al*., 2000; Boardman *et al*., 2004).

Toxicity tests with reference substances are frequently used to evaluate the sensitivity and physiological state of a test-organism lot. Moreover, they serve to verify the reproducibility, and are employed in interlaboratory analyses (Rand *et al*., 2000).

Potassium dichromate is frequently used as a reference substance. Therefore, according to Rand, *et al*. (2000), the contaminant must be stable and cause, in low concentrations, a fast lethal effect.

The aim of this work is to present results about the acute sensitivity of *Nitokra* sp to ammonia chloride and potassium dichromate, providing valuable information in widening the use of this species in ecotoxicological studies.

MATERIAL AND METHODS

Test-organism

Nitokra sp is typically benthic and estuarine in areas with salinity varying between 5 and 30 (Lotufo & Abessa, 2002). It feeds on microorganisms, organic matter associated with the sand grains and seaweed and grazing on single food particles (Ruppert *et al*., 1996). According to Giere (2009), harpacticoids have often been considered mainly "detritus feeders", ingesting primarily bacteria, protozoires and diatom cells.

The culture of *Nitokra* sp is monospecific and was established in 1998 from organisms collected from the sediment surface from the intertidal zone of *Spartina* sp banks in the Cananéia Estuary in the southern coast of the state of São Paulo (Lotufo & Abessa, 2002).

The organisms were maintained in a 1 L glass with 750 mL of reconstituted seawater and salinity of 23 ± 3. The temperature varied between 20º and 25ºC. The cultures were fed three times a week with a microalgae mixture containing *Chaetoceros, Chlorophucea, Dunaniela, Hillea, Isochrysis, Nannochloropsis, Odontela, Pavlova, Pyrocystis, Thalassiosira, Prorocentrum, Synecococcus* and supplemental food made of 0.5 mg fish ration diluted in 100mL of reconstituted seawater prepared at salinity 22 ± 2.

Acute toxicity test

Adult males and non-ovigerous females were used as test-organisms. Organisms were selected under stereomicroscope (25x) using Petri plates with reconstituted seawater, after sieving part of the culture content using a 125 µm mesh.

The culture water was reconstituted seawater, prepared by adding artificial sea salts (RED SEA Fish farm) to de-ionized water, aged for at least 1 day and maintained under constant aeration until the moment of its use.

Five tests of acute effect with ammonia chloride reagent (MERCK®), in salt form, were performed. The stock solution was prepared by adding 500 mg of ammonium chloride per 1L of reconstituted seawater. Test solutions were prepared by adding appropriate amounts of stock solution in concentrations 5.0; 10.0; 50.0; 100.0 and 500.0 mg.L^{-1} of ammonium chloride. The tests were conducted during a 96-hour exposure period with 5 replicates per concentration, in Petri plates with 20 mL capacity. Ten adult males and non-ovigerous were exposed in each replicate. The entire test system was kept at 25°C in a acclimatized room with a natural photoperiod.

After the exposure period, live and dead organisms were observed and counted under a stereomicroscope. Physical-chemical parameters, i.e., salinity, pH and temperature, were measured at the beginning and at the end of each test. These parameters and total ammonia (nominal concentration) were used to determine non-ionized ammonia concentration according to Whitfield (1978). The Trimmed Spearman-Karber program was used to calculate the LC_{50} values (Hamilton *et al*., 1977).

The tests of acute effect with potassium dichromate were carried out similarly to the tests with ammonia chloride. Five tests were performed during a period of 48 hours of exposure with 5 replicates per concentration between April and May of 2004. From 2004 to 2009, twelve more tests were performed, for a period of 96 hours/test, with 4 replicates per concentration. The nominal concentration was 6.25; 12.5; 25.0; 50.0 and 100.0 mg.L^{-1} of potassium dichromate ($K_2Cr_2O_7$), for both periods of exposure.

After each test, the live and dead organisms were observed and counted under stereomicroscope. The Trimmed Spearman-Karber program was used to calculate the $LC_{50\text{-}48h}$ and $LC(I)_{50\text{-}96h}$ values (Hamilton *et al*., 1977). The sensitivity limits for both periods of exposure were calculated (average ± 2DP), according to Zagatto & Bertoletti (2006). Subsequently, a sensitivity control chart was developed for *Nitokra* sp to $K_2Cr_2O_7$, for the exposure period of 96 hours.

RESULTS AND DISCUSSION

Evaluation of acute toxic effects must be carried out using accurate and reproducible methods. Mortality during laboratory exposure is a widely used endpoint for comparing the effects of organisms exposed to an environmental sample or specific chemicals with exposure to control or reference material.

The LC_{50} - lethal concentration that causes 50% organism mortality during a determined period of time - is frequently used to express the concentration effect of a chemical agent to test-organisms and also to compare toxicity between test-species and toxic agents.

According to Buratini & Bertoletti (2006), the LC_{50} percentage calculation of effect are based on transformed data and assume a normal distribution, making it possible to trace a straight line through the points, allowing the attainment of a median concentration, which corresponds to the concentration that causes 50% mortality of the exposed organism. In the event that similar curves were established, the optimum level of correlation would be presented in the 50% level of effect. Therefore, the choice of this percentage is related to accuracy and reproducibility results.

According to Whitfield (1974), Frias-Espericueta *et al*. (2000) and Boardman *et al*. (2004), the concentration of ionized ammonia in the marine environment is highly influenced by pH and also varies according to temperature, salinity and pressure. The rise of temperature, pH and salinity cause the dissociation of NH_4^+ ionized to NH_3 non-ionized.

In regard to *Nitokra* sp sensitivity to non-ionized ammonia, the $LC(I)_{50\text{-}96h}$ rate varied between 0.56 and 2.89 mg.L^{-1} of NH_3, and the average value was 1.7 mg.L^{-1} of NH_3.

The analyzed environmental parameters such as salinity, pH and temperature at the beginning and at the end of the tests influenced the equilibrium between NH_4^+ and NH_3 levels and in $LC_{50\text{-}96h}$ results. The higher the salinity, the lower the rates of LC_{50} (Table 1), and, therefore, the ammonia chloride was more toxic. The rise in temperature corresponded to higher LC_{50}, and consequently, to lower toxicity.

The average NH_3 $LC_{50\text{-}96h}$ values for *Nitokra* sp derived in this study, 1.7 mg.L^{-1} , was similar to $LC_{50\text{-}72h}$ values of 1.83 mg.L^{-1} reported by Abessa (2002) for the dweller amphipod *Tiburonella viscana,* and similar to the $LC_{50\text{-}48h}$ value of 1.03 mg.L^{-1} reported by Boardman *et al*. (2004) for mysid shrimp (*Mysidopsis bahia*) and a $LC_{50\text{-}96h}$ value of 1.66 mg.L^{-1} for ghost shrimp *(Palaemonetes pugio)*. Both *Nitokra* sp and *Tiburonella viscana* are benthic invertebrates which are relatively more tolerant to ammonia and non-ionized ammonia (Lapota *et al*., 2000).

For *Leptocheirus plumulosus*, a benthic amphipod frequently used as a test-organism in sediment exposures, levels above 60.0 mg.L^{-1} of NH_4^+ and 0.8 mg.L^{-1} of NH_3 with pH 7.7 lead to effects in survival and reproduction USEPA (2001). *Kalliapseudes schubartii*, a presented higher tolerance to non-ionized ammonia, as evidenced by the reported $LC_{50\text{-}7days}$ value for NH_3 of 3.16 mg.L^{-1} (Maraschin *et al*., 2008).

Ammonia is typically naturally present in environmental samples, especially sediments. Therefore, knowledge of sensitivity of test-organisms is desirable and valuable. Evaluation of species sensitivity to chemicals used as reference substance, such as potassium dichromate, is likewise desirable and warranted. Despite this, it is necessary to have a parallel, in this case. The mean $LC(I)_{50\text{-}48h}$ value for *Nitokra* sp was 55.61 mg.L^{-1} for $K_2Cr_2O_7$ and the sensitivity limits were 10.19 and 101.03 mg.L^{-1}. The $LC(I)_{50\text{-}48h}$ rates obtained in the 5 replicate tests are presented in Table 2.

Table 1 - Physical chemical parameters, ammonium chloride $LC(I)_{50\text{-}96h}$ and non ionized ammonia $LC_{50\text{-}96h}$.

Number of Test	Salinity		pH		Temperature (°C)		$LC(I)_{50\text{-}96h}$ NH_4^+ (mg.L⁻¹)	$LC_{50\text{-}96h}$ NH_3 (mg.L⁻¹)
	Initiate	End	Initiate	End	Initiate	End		
1	25.00	27.00	8.71	7.51	23.00	17.00	>100	>0.836
2	27.00	26.00	8.54	7.46	23.00	17.00	217.61 (191.05-247.87)	1.47 (1.32-1.64)
3	23.17	23.83	8.54	7.79	29.00	28.00	93.45 (69.00–126.55)	2.89 (2.27-3.66)
4	26.50	27.50	8.54	7.61	22.00	21.00	38.37 (21.96-67.04)	0.56 (0.35-0.88)
5	23.17	24.17	8.30	7.86	23.00	23.00	60.27 (39.18–92.70)	1.87 (1.29-2.71)

Table 2 - Potassium dichromate $LC(I)_{50\text{-}48h}$ and its respective confidence limits, mean value, standard deviation and variation coefficients of each test.

Number of Test	$LC(I)_{50\text{-}48h}$ $K_2Cr_2O_7$ (mg.L⁻¹)	Confidence Limits	
		Lower	Higher
1	46.29	29.96	71.54
2	20.69	16.00	26.74
3	62.09	46.95	82.12
4	75.15	69.48	81.33
5	73.84	67.81	80.41
Mean Value	55.61	46.04	68.43
Standard Deviation	22.71	23.37	23.69
Coefficient of Variation	40.83	50.76	34.61

For the 96-hour exposure period, the average $LC(I)_{50}$ was 21.70 mg.L⁻¹ of $K_2Cr_2O_7$ and the sensitivity limits were 10.26 and 33.13 mg.L⁻¹ of $K_2Cr_2O_7$. These results have been used to assemble a control chart for the sensitivity of this test organism to $K_2Cr_2O_7$ (Table 3 and Figure 1).

The $LC(I)_{50}$ mean value and the higher and lower limits of sensitivity were lower for 96-h exposures than for 48-h exposures. Moreover, the standard deviation and coefficient of variation (standard deviation expressed as a percentage of the mean) values were also lower for 96-h exposures, likely because of the higher number of tests performed to comprise the mean value.

In addition, according to USEPA (1992) many factors such as culture and physiological conditions could contribute to the variability of these toxicity results from short-term exposures when using different pools of organisms.

According to Environment Canada (1990), results are considered adequate when the coefficient of variation is between 20% and 30%. The experiment with *Nitokra* sp and potassium dichromate presented a coefficient of variation value of 23.56%, which indicates success in reproducibility.

Nitokra sp showed more sensitivity to potassium dichromate compared to freshwater fish such as *Piracactus mesopotamicus* (pacu); *Hyphessobrycon eques* (weed-thick) and *Phalloceros. caudimaculttus* (guarú) (Cruz *et al*., 2008), and less sensitivity than embryos of echinoids such as *Mellita quinquesperforata*, *Arbacia lixula* and *Lytechinus variegatus*, frequently used in aquatic tests in studies developed by Resgalla & Laitano (2002); Laitano *et al*. (2008) and Maximo *et al*. (2008), respectively (Table 4).

In regard to other crustaceans, *Nitokra* sp showed more tolerance to potassium dichromate than *Tiburonella viscana* (Abessa & Sousa, 2003) and the harpacticoid copepod *Tisbe biminiensis* (Araújo-Castro *et al*., 2009). The measure of sensitivity between each species to potassium dichromate through the LC_{50} rates is expressed in Table 4.

Although *Nitokra* sp showed less sensitivity to potassium dichromate, in regard to other species of crustaceans used in toxicity tests, the use of this benthic copepod in ecotoxicological assays is very promising due to its versatility. According to Lotufo & Abessa (2002), this organism survives and reproduces in a wide range of salinities, enabling it to be applied in evaluations of the toxicity of isolated substances,

Table 3 - Potassium dichromate $LC(I)_{50\text{-}96h}$ and its respective confidence limits, mean value, standard deviation and variation coefficients.

Tests	$LC(I)_{50\text{-}96h}$ $K_2Cr_2O_7$ (mg.L⁻¹)	Confidence Limits	
		Lower	Higher
may/04	23.21	18.65	28.89
jun/04	29.88	23.14	38.59
aug/04	16.08	12.94	19.99
oct/04	17.50	14.36	21.34
nov/04	16.28	12.28	21.57
may/05	22.93	19.65	26.74
aug/05	15.75	13.3	18.65
jun/06	26.94	23.22	31.26
jul/06	25.00	21.86	28.58
aug/06	15.12	13.8	15.57
apr/07	23.21	18.65	28.89
jul/07	15.16	13.21	17.4
aug/07	24.80	20.88	29.46
jun/09	31.90	23.44	43.42
Mean Value	**21.70**	**17.81**	**26.45**
Standard Deviation	5.72	4.33	8.02
Coefficient of Variation	26.84	24.77	30.90

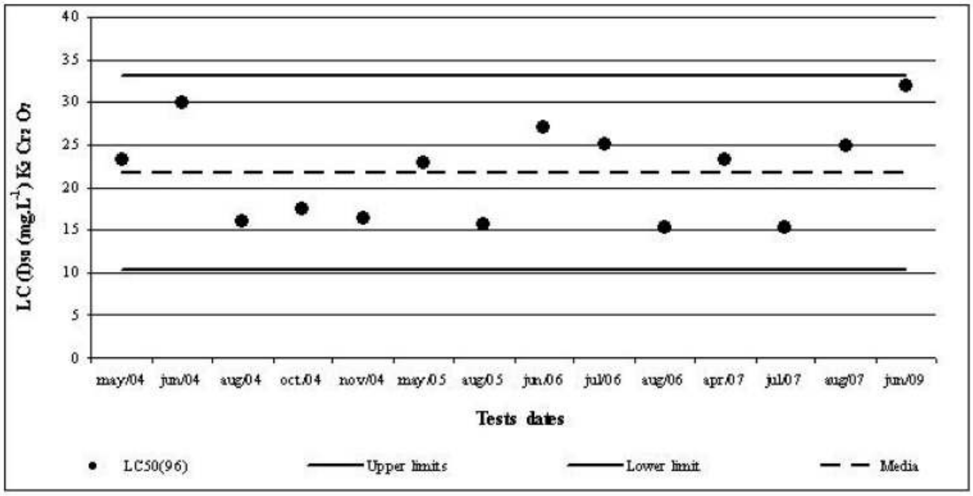

Figura 1 - Sensitivity control chart to *Nitokra* sp for potassium dichromate.

Table 4 - Sensitivity of some test-organisms to potassium dichromate.

Test-Organism		$K_2Cr_2O_7$ (mg.L^{-1})	Exposure Time and Effect	References
Echinoids	*Lytechinus variegatus*	1.41	24h - Chronic	Resgalla & Laitano, 2002
	Arbacia Lixula	2.08	24h - Chronic	Maximo *et al*., 2008
	Mellita quinquiesperforata	1.46	24h - Chronic	Laitano *et al*., 2008
Fishes	*P. mesopotamicus* (Pacu)	144.5	96h - Acute	Cruz *et al*., 2008
	H. eques (Matogrosso)	130.79	96h - Acute	
	P. caudimaculatus (Guarú)	154.39	96h - Acute	
Crustaceans	*Tiburonella viscana*	11.21	48h - Acute	Abessa & Sousa, 2003
	Tisbe biminiensis	22.2	48h - Acute	Araújo-Castro *et al*., 2009
		13.84	72h - Acute	
		9.45	96h - Acute	
	***Nitokra* sp**	**55.61**	**48h - Acute**	**Present study**
		21.70	**96h - Acute**	

as well as in ecotoxicological evaluations of environmental samples with variable salinity.

CONCLUSIONS

Nitokra sp is a native species of the Cananéia Estuary - São Paulo State, Brazil, and it is easily maintained in culture. Its short life cycle allows obtaining a great number of organisms in a short period of time, optimizing it use in routine toxicity testing.

Toxicity evaluation of ammonia toxicity and a reference compound was successful. *Nitokra* sp has proved to be a value species for use in environmental sample evaluation, especially in those obtained from estuarine regions.

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