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POTASSIUM CHLORIDE AND SODIUM CHLORIDE AS REFERENCE TOXICANTS TO ASSESS QUALITY OF TOXICITY TESTS CARRIED OUT WITH THE MICROCRUSTACEAN CLADOCERA *Ceriodaphnia dubia*

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ABSTRACT

Two reference toxicants (potassium chloride-KCl and sodium chloride-NaCl) were used in the present study in order to assess the sensitivity of the microcrustacean cladocera *Ceriodaphnia dubia*. Chronic toxicity tests were carried out with those two salts and the sensitivity expressed as IC50;7D - concentration that causes inhibition of 50% of reproduction ability, in seven days exposure time. The medium values estimated for IC50;7D were 200.70 and 499.60 to KCl and NaCl, respectively. Those data have demonstrated that KCl can be more restrictive and more suitable than NaCl, when used as standard reference toxicant for plotting control charts of sensitivity.

Key words: sensitivity; reference toxicant; KCl; NaCl; microcrustacean.

CLORETO DE POTÁSSIO E CLORETO DE SÓDIO COMO SUBSTÂNCIAS DE REFERÊNCIA PARA AVALIAR A QUALIDADE DO TESTE DE TOXICIDADE COM O MICROCRUSTÁCEO CLADOCERA *Ceriodaphnia dubia*

RESUMO

No presente estudo foram utilizadas duas substâncias de referência (cloreto de potássio-KCl e cloreto de sódio-NaCl), para avaliar a sensibilidade do microcrustáceos cladocera *Ceriodaphnia dubia*. Testes de toxicidade crônica foram realizados com esses dois sais e a sensibilidade expressa como IC50;7D - concentração que causa inibição de 50% da capacidade de reprodução, em sete dias de tempo de exposição. Os valores médios estimados para IC50;7D foram 200,70 e 499,60 para KCl e NaCl, respectivamente. Esses resultados demonstraram que o KCl pode ser mais restritivo e mais adequado do que o NaCl, quando utilizado como substância de referência padrão para traçar gráficos de controle de sensibilidade.

Palavras-chave: sensibilidade; substância de referência; KCl; NaCl; microcrustácio.

INTRODUCTION

The micro-crustacean *Ceriodaphnia dubia* is a typical freshwater cladocera largely employed in short-term tests for estimating chronic adverse effects of toxicant substances, which endpoint is essentially to determine the decrease of reproduction, generally expressed by IC50;7D – concentration that causes inhibition of 50% of reproduction ability (COOPER *et al*., 2009). Such test follows some criteria to assess test quality and to validate test results (USEPA, 2002; MOREIRA *et al*., 2014).

In the majority of aquatic bioassays the health of test organisms is primarily assessed by their performance (survival, growth, and/or reproduction) in the control group from individual tests (PAKRASHI *et al*., 2013). However, to provide good condition of method testing and validation, the sensitivity of test-organisms needs to be verified in order to control the precision of results from toxicity tests.

A reference toxicant is a chemical that is used in toxicity testing to provide general measure of the reproducibility of a toxicity test method within a single laboratory over time (SADIQ *et al*., 2011). Generally it is made by plotting, in a control chart, some data from at least the last five monthly short-term chronic toxicity tests, using the same reference toxicant and control conditions (USEPA, 2002; ZAGATTO and BERTOLETTI, 2006; MOREIRA *et al*., 2014; SANTOS *et al*., 2014). In other words, the control chart is used to make comparisons among individual and historical test performances to identify whether they fall within an acceptable range of variability, i.e. the numerical results obtained in each test performed with the same reference toxicant, should be within the range limited by the cumulative mean, and upper and lower control limits of two times the standard deviation (± 2S). Those data are re-calculated with each successive test result. Therefore, the control chart is useful in order to demonstrate the acceptance of the results obtained for all toxicity tests performed with the same strain of test-organisms, under the same test conditions. Such criteria have also been used in inter-laboratory testing to judge comparability of results among different laboratories.

Standard reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate (K_2Cr_2O7) are suitable for carrying out tests focusing on the construction of control charts (ENVIRONMENT CANADA, 1990; USEPA, 2002; ABNT, 2005; ZAGATTO and BERTOLETTI, 2006; FREITAS and ROCHA, 2011). However, the choice of using a particular substance should take into account some aspects, including the facility of handle and the risk of environment contamination.

Pollution prevention and waste management should be the main criteria for selecting reference toxicants. In this positive aspect, potassium chloride (KCl) and sodium chloride (NaCl) are being evaluated by several laboratories (commercial or research approach) for their suitability as reference toxicants. Those salts may be superior to other toxicants because water quality has lower or no effect on its toxicity and therefore easier to handle in the laboratory than many other reference toxicants as they are rapidly biodegradable and meet regulatory agencies requirements.

There are many studies focusing on the use of NaCl as reference toxicant for freshwater toxicity tests. Also, the coefficients of variation reported for most inter-and intralaboratory studies are frequently better than for any other toxicant. But, this can indicates two contrasting possibilities: that sodium chloride is an excellent material, or that sodium chloride tests are not sufficiently sensitive to indicate abnormal conditions (ENVIRONMENT CANADA, 1990; AGUILAR-ALBEROLA and MESQUITA-JOANES, 2012). On the other hand, despite of having the same positive aspects previously mentioned for both salts, KCl has not been well studied so far (ENVIRONMENT CANADA, 1990; USEPA, 2002; UTZ and BOHRER, 2001; MOREIRA *et al*., 2014; REZENDE *et al*., 2018).

The objective of the present study was evaluate the *Ceriodaphnia dubia* 7-day chronic reproduction toxicity tests using standard reference toxicant substances (potassium chloride and sodium chloride) in order to determine the pattern of sensitivity for those two salts, which data may be useful for establishing best methods for controlling test precision inter-and intra-laboratories.

MATERIAL AND METHODS

The microcrustacean cladocera *Ceriodaphnia dubia* was used in the present study as test organism by the main characteristic of having a parthenogenetic potential, that produces a large number of neonates (essentially females), in a great natural frequency, when maintained under suitable environment conditions. The techniques for culturing organisms and carrying out tests were based on the standard operating procedures reported by the international reference (USEPA, 2002), as well as the adapted techniques reported by the Brazilian reference (ABNT, 2005).

Experiments were carried out at the Laboratory of Ecotoxicology of the Instituto de Pesca in Sao Paulo/SP - Brazil. Both culturing and dilution water were collected from the same source, i.e. a natural pond located at Itatiba-SP-Brazil, which quality parameters were previously assessed and approved for such use. After arriving at the laboratory and/or right before performing culture/test the water was adjust for pH range of 7.2 to 7.6, and total hardness of 40-48 mg L^{-1} as $CaCO_3$. Test organisms (females of *C. dubia*) were cultured in dismissable polystyrene cups (type beaker) containing one organism per 30 mL beaker, filled with 20 mL culturing water. Those vessels were kept inside incubator chambers for controlling photoperiod of 16-hour light and 8-hour darkness and temperature at 25 (±1)°C. Feeding consisted of 0.04 mL of suspension solution of the green microalgae *Pseudokirchneriella subcapitata* ($2.0x10^5$ cells mL^{-1}), plus 0.02 mL of a nutritional composition made of digested ration of fish (TetraMin™). Water was changed and the young organisms (neonates) removed on every Monday, Wednesday, and Friday, when feed was supplied. Each culture group was kept at the maximum of 14 days, before being replaced by new ones. Prior to start tests, all young neonates, with the maximum of 30-hour old were collected from culture beakers and reserved (ARAGÃO and PEREIRA, 2003).

The methodology selected for carrying out this experiment was the chronic 7-days test with *C. dubia*, based on the standard references of USEPA (2002) and ABNT (2005). Test conditions and criteria are summarized in Table 1.

Two reference toxicants were tested according to the following concentrations: potassium chloride (KCl) = 0, 250, 500, 1000, and 2000 mg L^{-1} and sodium chloride (NaCl) = 0, 50, 100, 200, and 400 mg L^{-1}. Those concentrations were determined by previous exploratory tests. Once the cups were filled with test solution, test organisms were randomly assigned to each container. Test organisms were daily transferred into new fresh solution and the number of live/dead female counted and recorded, as well as the number of neonates produced by each female. Prior all organisms had been transferred, food was added to each new test container. All tests were performed during distinct weeks. The total amount of 21 tests was performed with the two reference toxicants: twelve with KCl and nine with NaCl.

Since the reproduction was the endpoint of the present study, the IC50;7D (concentration that causes 50% of inhibition in the reproduction of test organisms in relation to control group) was the index used for determining the 7-day chronic toxicity effect of the two reference toxicants. Those results were estimated by the Computer Program Icpin (NORBERG-KING, 1993).

Control charts of sensitivity were calculated according to the recommendation of USEPA (2002), based on the distribution of numerical results of IC50;7D obtained in each test performed with the same reference toxicant, considering the range limited by the cumulative mean, and upper and lower control limits of two times the standard deviation (± 2S), and plotted in graph. In order to avoid large deviation, the observations that were numerically distant from the rest of the data (outliers: minimum and maximum) were not considered for calculating and plotting control charts. Data obtained in the present study were used for stating the construction of the control charts, which re-calculation is required as basic methodology for actualizing data.

RESULTS

The overall results obtained along all tests are registered in Tables 2 and 3. A comparison between the two reference toxicants is summarized in Table 4. Data showed that *C. dubia* was more

Table 1. Summary of test conditions and criteria used for carrying out 7-day chronic toxicity tests with *Ceriodaphnia dubia*.

Test parameter	condition / criteria
Test Type	Static-renewal
Renewal of Test Solution	Daily
Test Duration (exposure time)	7 days
Temperature	25 (±1°)C (controlled by incubator chamber)
Photoperiod	16 hrs light: 8 hrs dark (controlled by incubator chamber)
Type of test vessel	30 mL dismissable polystyrene cups (type beaker)
Test solution volume	20 mL
Age of test organisms	< 30 hrs old
Number of organisms/chamber	1
Number of replicates	10 vessels by each concentration
Feeding routine	Daily - 0.04 mL of suspension of *P. subcaptata* ($2.0x10^5$ cells/mL), plus 0.02 mL of digested commercial fish ration
Test solution aeration	None
Dilution water	Natural pond water (same source of culture water)
Number of test concentrations	4 plus control group
Dilution Series	0.5
Endpoint	Reproduction (number of neonates produced by parthenogenesis)
Test acceptability	80% or greater control survival and average of 15 neonates produced from control females

Table 2. Results from chronic tests carried out with potassium chloride (KCl) as reference toxicant substance to *Ceriodaphnia dubia*.

Test number	IC50; 7D(mg L^{-1})*	Confidence Limit (95%) Lower	Upper
1	298.19	277.63	300.00
2	271.33	241.56	281.16
3	277.47	247.27	291.60
4	284.83[a]	246.21	300.00
5	184.94	142.59	248.65
6	190.22	116.91	268.05
7	70.69[b]	33.33	75.00
8	159.65	152.16	164.11
9	87.77	70.67	208.04
10	118.82	88.17	136.04
11	230.38	141.03	267.42
12	201.56	49.24	252.69

*IC50;7D = concentration that causes inhibition of 50% of reproduction ability, in seven days exposure time; [a]outlier (lower observation); [b]outlier (upper observation).

sensitive to KCl than NaCl, which averages of IC50;7D were 200.70 mg L^{-1} and 499.60 mg L^{-1}, respectively.

The distributions of different results of sensitivity of *C. dubia* in response to two reference toxicants were used to start plotting the two different models of control chart of sensitivity (Figures 1 and 2). From those pictures it was possible to observe that the acceptable limits of IC50;7D stated by the interval of two times the standard deviation (± 2 S) is shorter for KCl than it is for NaCl. It means that KCl can be more restrictive than NaCl, when used as reference toxicant for plotting control charts of sensitivity. This aspect is even emphasized by the coefficient of variation, which was higher for NaCl (56.04%), and also by the large amplitude of variation (215.26 to 1039.22 mg L^{-1}) observed for the same reference toxicant (Table 4). Therefore, KCl may be a rigorous and more restrictive choice for the use as reference toxicant. In addition, large amplitude of variation determines higher standard deviation, which can lead to unacceptable positions of the lower limit ($CL50_{,7d}$ – 2 S), when it is plotted in the negative

Table 3. Results from chronic tests carried out with sodium chloride (NaCl) as reference toxicant substance to *Ceriodaphnia dubia*.

Test number	IC50; 7D (mg L^{-1})*	Confidence Limit (95%) Lower	Confidence Limit (95%) Upper
1	215.26	181.09	1103.98
2	1233.01[b]	867.79	1433.45
3	588.71	466.22	1028.35
4	169.76[a]	136.29	251.52
5	500.00	190.91	1214.29
6	317.83	195.75	386.63
7	269.53	217.53	439.52
8	566.67	400.00	798.08
9	1039.22	830.46	1258.84

*IC50;7D = concentration that causes inhibition of 50% of reproduction ability, in seven days exposure time; [a]outlier (lower observation); [b]outlier (upper observation).

Table 4. Summary of results from chronic tests carried out with potassium chloride (KCl) and sodium chloride (NaCl) as reference toxicant substances to *Ceriodaphnia dubia*.

Toxicant	IC50;7D (mg L^{-1})*						
					Confidence Limit		C.V.
Substance	Minimum	Maximum	Average	S	Lower	Upper	(%)
KCl	87.77	284.83	**200.70**	66.95	149.58	241.78	33.36
NaCl	215.26	1039.22	**499.60**	280.02	354.57	889.96	56.04

*IC50;7D = concentration that causes inhibition of 50% of reproduction ability, in seven days exposure time; C.V. = coefficient of variation; S = standard deviation. OBS: outliers observed in Tables 2 and 3 were not considered in the present table.

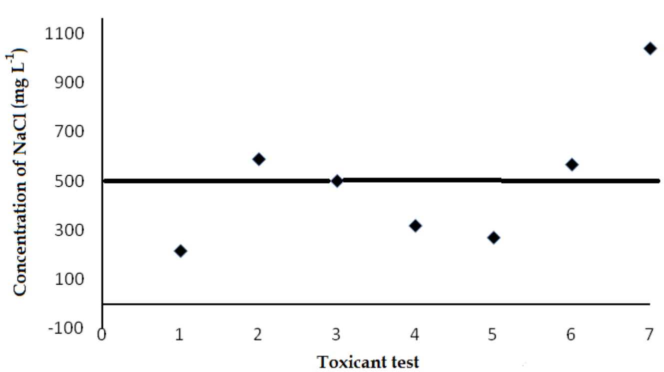

Figure 1. Model of control chart plotted with original data from chronic tests carried out with NaCl as reference toxicant to *Ceriodaphnia dubia*. (—) = average; (---) = average ± 2x standard deviation.

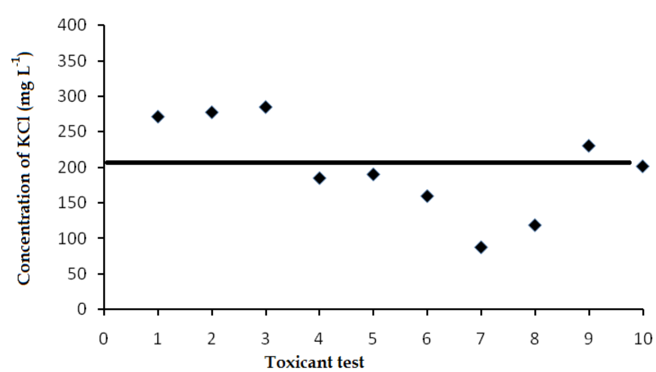

Figure 2. Model of control chart plotted with original data from chronic tests carried out with KCl as reference toxicant to *Ceriodaphnia dubia*. (—) = average; (---) = average ± 2x standard deviation.

area of the vertical axis "Y" in the graph. This negative aspect in the control chart was observed to NaCl (Figure 2).

DISCUSSION

The use of cladocerans in toxicity tests has many advantages, including their ease of handling, the possibility of obtaining clones by parthenogenesis, their short life cycle and high sensitivity to toxicants (MOREIRA *et al*., 2014). These characteristics make them suitable test organisms for bioassays (ADEMA, 1978; MOREIRA *et al*., 2014). Higher sensitivity of *C. dubia* to KCl was also detected by UTZ and BOHRER (2001) in chronic tests carried out in order to compare the toxic effect between potassium chloride and potassium acetate.

Some laboratories have performed a lot of chronic tests with a sort of reference toxicant (FREITAS and ROCHA, 2011). However it is rarely reported in the literature, since the use of those data is restricted to the intern control of test precision. Nevertheless, NORBERG-KING (1988) reported data of toxicant tests carried out with the aim of assessing sensitivity of *Ceriodaphnia dubia* to NaCl, and their study reveled averages of IC50;7D from 1200 to 1400 mg L^{-1}, which is slightly similar to maximum concentration observed in the present study for the same toxicant (1233.01 mg L^{-1}). Those authors also observed high coefficients of variation in their results (up to 39,9%). In the same way, DEGRAEVE *et al*. (1992) reported $CI50_{,7d}$ of NaCl to *C. dubia* from 730 to 1550 mg L^{-1}, also with high coefficients of variation (up to 30.8%).

In another study developed by ARAGÃO and PEREIRA (2003) the $IC50_{,7d}$ of NaCl to *C. dubia* varied from 570 to 1130 mg L^{-1} among twelve chronic toxicity tests, which denotes a large similarity to the results obtained in the present study, including the large amplitude of variation observed for the same reference toxicant.

STRUEWING *et al*. (2015) established a 48 h acute and a 14-day short-term chronic testing procedure for *C. triangulifer* and compared its sensitivity to two model invertebrates, *Ceriodaphnia dubia* and *Daphnia magna*. Toxicity tests were conducted to determine mortality and growth effects using standard reference toxicants: NaCl, KCl and $CuSO_4$. In 48-h acute tests, the average LC50 for the mayfly was 659 mg L^{-1} NaCl, 1957 mg L^{-1} KCl, and 11 µg L^{-1} $CuSO_4$. IC25 values, using dry weight as the endpoint, were 228 mg L^{-1} NaCl, 356 mg L^{-1} KCl and 5 µg L^{-1} $CuSO_4$ In our study in seven days exposure time demonstrated that the effect of lethality of NaCl and to *C. dubia* can occur at concentrations higher than 200.70 and 499.60 mg L^{-1}. COWGIL and MILAZZO (1990) and FREITAS and ROCHA, 2013 have demonstrated that the effect of lethality of NaCl to *C. dubia* can occur at concentrations higher than 1200 mg L^{-1}. HARMON *et al*. (2003) and OLIVEIRA-FILHO *et al*. (2008) reported data of LC50 and EC50-48h of (730 to 1330 mg L^{-1}) and 1590 mg L^{-1}, respectively. While HARMON *et al*. (2003) have also registered $IC50_{,7d}$ of 1350 mg NaCl L^{-1} to the same test organism. From those finds in the literature it was possible to notice that acute and chronic concentrations of NaCl to *C. dubia* are quite similar, and it may be other negative aspect to consider at the time of choosing such salt as standard reference toxicant.

CONCLUSION

Potassium chloride (KCl) showed to be more suitable than sodium chloride (NaCl) for the use as standard reference toxicant in chronic tests carried out with *Ceriodaphnia dubia*, in order to estimate limits for assessment of test precision inter-and intra-laboratory.

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