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Sodium chloride as a reference substance for the three growth endpoints used in the *Lemna minor* L. (1753) test

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ABSTRACT

Lemna sp. growth inhibition test standardized protocols suggest the use of compounds such as 3,5-dichlorophenol as reference substances for checking the test organism's sensitivity routinely. However, this and other recommended chemicals present risks to human health and to the environment. Sodium chloride (NaCl) appears as a less toxic alternative reference substance which has been successfully used in routine ecotoxicological tests. However, the evaluation of this compound in multiple growth endpoints used in the *L. minor* test, which is required for recommending it as a reference substance for this test organism, has not yet been reported. In the present study, NaCl was tested with *L. minor* for the growth endpoints frond number, total frond area and fresh weight. Results showed acceptable sensitivity and reproducibility (coefficient of variance < 15.0%) for all three of the measured endpoints. Statistically significant differences were observed between the EC_{50} values calculated based on the three endpoints ($p < 0.05$). Total frond area was the most sensitive one, with average EC_{50} value of 2742.80 ± 245.7 mg L^{-1}. It was anticipated that NaCl can be a suitable alternative reference substance and that total frond area should be the endpoint of choice for sensitivity toxicity tests using NaCl.

Keywords: Duckweed, phytotoxicity, quality assurance.

Cloreto de sódio como uma substância de referência para três parâmetros de crescimento usados no teste com *Lemna minor* L. (1753)

RESUMO

Protocolos padronizados do teste de inibição de crescimento com *Lemna* sp. sugerem a utilização de compostos, tais como 3,5-diclorofenol, como substâncias de referência para verificar a sensibilidade dos organismos rotineiramente. No entanto, este e outros produtos químicos apresentam riscos para a saúde humana e para o meio ambiente. O cloreto de sódio

(NaCl) surge como uma substância de referência alternativa menos tóxica que tem sido utilizada com sucesso na rotina em testes ecotoxicológicos. No entanto, a avaliação deste composto em múltiplos parâmetros de crescimento utilizados no teste com *L. minor*, que é necessária para recomendá-lo como substância de referência para este organismo-teste, não foi ainda relatada. No presente estudo, o NaCl foi testado com *L. minor* para os parâmetros de crescimento número de frondes, área total das frondes e peso fresco. Os resultados mostraram sensibilidade e reprodutibilidade aceitáveis (coeficiente de variação < 15,0%) para todos os três parâmetros avaliados. Foram observadas diferenças estatisticamente significativas entre os valores de CE_{50} calculados com base nos três parâmetros ($p < 0,05$). A área total das frondes foi o parâmetro mais sensível, com valor médio de CE_{50} de 2742,80 ± 245,7 mg L^{-1}. Estima-se que o NaCl pode ser uma substância de referência alternativa adequada e que a área total das frondes deve ser o parâmetro de escolha para testes de sensibilidade utilizando NaCl.

Palavras-chave: Fitotoxicidade, garantia de qualidade, lentilha d'água.

1. INTRODUCTION

Reference substances are standard chemicals used to assess organisms' sensitivity in ecotoxicological tests and to establish essential test conditions for each species (Zagatto, 2008; Amorim et al., 2012). They are also used to assess the precision and reliability of data obtained by a laboratory for the selected reference toxicant (Canada, 2007). Therefore, using reference substances in laboratories of ecotoxicology must be a routine procedure in analytical quality assurance schemes (Zagatto, 2008).

The criteria desired in a reference substance and used in recommending the appropriate reference toxicants in standardized protocols include chemical readily available in pure form, stable shelf life of chemical, highly soluble in water, stability of the chemical in aqueous solution, analytical methods available to verify concentrations, good dose-response curve for the test organism, ease to perform, measurable effects on test endpoints and minimal hazard posed to user (Canada, 2007; Amorim et al., 2012). However, some of the reference substances indicated in standardized protocols may represent an important risk for the researcher, with some of them even presenting carcinogenic potential, e.g. potassium dichromate (IARC, 1990).

The OECD guideline for the testing of chemicals protocol 221 (OECD, 2006) recommends the use of reference substances such as 3,5-dichlorophenol as a chemical for checking the *Lemna* sp. growth inhibition test procedure. However, this compound may be harmful to human health causing severe irritations or burns of the eyes, skin and respiratory tract. 3,5-dichlorophenol chronic toxicity includes headache, neurological disturbances and liver and kidney damage (NLM, 2015). It also represents hazard to the environment, because it emits toxic fumes of carbon monoxide, carbon dioxide and hydrogen chloride gas when it is heated to decomposition (NOAA, 2015). Therefore, the use of alternative substances is necessary in order to avoid unnecessary risks to human health.

In this sense, sodium chloride (NaCl) has been successfully used as an alternative reference substance for assessing the sensitivity of some species such as the midge *Chironomus xanthus*, the water flea *Daphnia magna*, the cnidarian *Hydra attenuata* and the freshwater green algae *Raphidocelis subcapitata* (Santos et al., 2007). The acceptable coefficients of variation (C.V.) obtained in those tests (≤ 20.08%) indicated adequate reproducibility of results when NaCl was used as a reference toxicant for those species. Moreover, it must be highlighted that advantages in using this salt in sensitivity tests include its relative low cost, relatively low toxicity for human and the ease of disposal of wastes.

Despite this, sensitivity and consistency of response to the NaCl effects have not been extensively assessed in tests using the macrophyte *Lemna minor*. An appropriate reference substance should be applicable to evaluate the sensitivity of multiple endpoints. However, the few data available in the literature concerning the NaCl toxicity effects for this test organism address just one endpoint, typically frond number (Chastinet and Silva, 2000; Keppeler, 2009; Souza et al., 2011). Although frond number is the primary measurement endpoint, the OECD guideline for the testing of chemicals protocol 221 (OECD, 2006) recommends measuring at least one other endpoint, such as total frond area and dry or fresh weight, since they may be affected in different intensities depending on the chemical tested.

It is also worthy to mention that, as stated by Pattard et al. (2009), the number of sensitivity tests performed for test systems other than algae, *Daphnia* sp. and luminescent bacteria is very limited. Therefore, additional reference data for test systems such as *Lemna* sp. can be a valuable source of information. *L. minor* is an important test organism commonly used for regulatory toxicity testing of chemicals. Therefore, growth inhibition tests performed with this macrophyte are very useful as an additional source of information about phytotoxicity for higher plants (Cleuvers, 2003).

The aim of the present study was to evaluate the sensitivity and the consistency of *L. minor* responses to NaCl as a reference substance on three growth endpoints indicated in the OECD 221 guideline (OECD, 2006) in order to provide NaCl toxicity data for multiple growth endpoints, including the ones generally neglected in routine tests, in order to contribute to the evaluation of the suitability of this compound as an alternative reference substance. In addition, the EC_{50} values obtained in the tests for frond number, total frond area and fresh weight were statistically compared in order to determine possible differences in sensitivity between the growth endpoints to the NaCl effects.

2. MATERIAL AND METHODS

2.1. Test Chemical

NaCl with 99.0% of purity was purchased from Vetec®. The stock solutions used in the sensitivity tests were prepared by dissolving NaCl in Steinberg medium. The concentration of the stock solutions was 100 g L^{-1}. Stock solutions were prepared immediately before each test. The nominal concentrations of NaCl used in the tests were 1,500; 2,500; 3,500; 4,500; 5,500; 6,500 and 7,500 mg L^{-1}. Three replicates per treatment were used in all the tests.

2.2. Cultivation of the Test Species

L. minor was originally collected from cultivation tanks kept at Federal University of São Carlos (São Carlos-SP, Brazil). For long-term cultivation, the macrophytes were periodically sterilized in sodium hypochlorite solution followed by sterile water (OECD, 2006). Prior to experiment for sensitivity testing, the plants were allowed to adapt to the culture medium Steinberg (OECD, 2006) for a 3-week period. The macrophytes were cultured in glass crystallizer filled with 2 L Steinberg medium (pH 5.5), kept in a climate chamber at 24 ± 2 °C with a photoperiod of 12 h light/12 h dark instead of continuous lighting in order to provide conditions closer to the environment (Godoy et al., 2015). Light intensity used was 6550 lux (104.8 µmol photons/m^2 s^{-1}). Once a week a number of young, light-green plants were removed to fresh medium aseptically. The culture vessel was covered with plastic film having perforations of approximately 1 mm diameter to minimize evaporation and to avoid contamination, while allowing adequate air exchange. Only young, rapidly growing plants without visible lesions or chlorosis were selected for the tests.

2.3. Test Performance

Sensitivity tests were carried out according to OECD guideline 221 for the testing of chemicals - *Lemna* sp. growth inhibition test (OECD, 2006). The temperature and photoperiod conditions used in the sensitivity tests were the same ones used in the cultivation. The tests were carried out in 250 mL beakers filled with 100 mL of test solution. Inoculum for each beaker was 12 fronds using only macrophytes with three fronds, all of them with similar total area. Three control and treatment replicates were performed for each test concentration. The test period was 7 days (168 h). Eight independent assays were performed. After exposure, the specific growth average rates were calculated based on the endpoints frond number, total frond area and fresh weight. In addition, visual changes in plant development, e.g., chlorosis, necrosis and colony break-up, were observed and registered.

Frond number was counted daily using a stereomicroscopic (Zeiss®, Stemi 2000-C model), at 80-fold increase. The fresh weight was calculated at the end of the tests using an analytical balance after removing excess moisture by placing the macrophytes between layers of paper towels. The difference between the weight of the plant material at the beginning and at the end of the experiments was calculated according to OECD (2006) in order to determine the fresh weight. The pH was measured at the beginning and at the end of the tests.

For the calculation of the total frond area, the test organisms were photographed at days 0, 3, 5 and 7 with a digital camera alongside a 1- x 1-cm red paper square (standard comparison scale). All the images were handled through the GIMP (GNU Image Manipulation Program, 2.8.14) free software (Godoy et al., 2015). Any visual signs of phytotoxicity were observed and registered.

2.4. Statistical Analysis

ECx-values (effect concentration at x% level) and respective 95% confidence intervals were calculated for all the three endpoints assessed in the sensitivity tests based on the specific growth rates compared to the respective controls, according to OECD (2006). These values were determined with the free software ToxCalcMix (Barata et al., 2006), using non-linear curve fitting based on a sigmoid model (three-parameter logistic function). Statistically significant differences between the endpoints frond number, total frond area and fresh weight were tested using a one-way ANOVA followed by the Scott-Knott's multiple comparison test ($P < 0.05$). Prior to perform ANOVA, the data were tested for normality using Shapiro-Wilk test with the free software SISVAR 5.3.

3. RESULTS AND DISCUSSION

The pH of the control medium did not increase by more than 1.5 units during the test (5.5 - 6.8), as recommended by the 221 OECD guideline (OECD, 2006). The EC_{50} values and their respective 95% confidence intervals for the growth endpoints frond number, total frond area and fresh weight are summarized in the Table 1.

The frond number is the most commonly used endpoint to express duckweed test results because its evaluation is simple, rapid and noninvasive (Wang et al., 1990). Regarding the average EC_{50} result obtained in this study for this endpoint (Table 1), the value is similar to the average $EC_{50\text{-}7\ days}$ value of 4,308 mg L^{-1} obtained by Souza et al. (2011) under similar test conditions as used in this study, except for the culture medium applied (Hoagland medium instead of Steinberg medium). EC_{50} values for the other two growth endpoints were quite lower and results from the one-way ANOVA test indicated that there was a statistically significant difference between the sensitivities of the evaluated endpoints to the NaCl effects ($p < 0.05$). However, the growth rate of *L. minor* was significantly decreased at the LOEC of 1,500 mg L^{-1} for all three of the evaluated endpoints (Figure 1) (A, B, C).

Table 1. EC_{50} values (mg L^{-1}) obtained for the endpoints frond number, total frond area and fresh weight in *L. minor* growth inhibition test with NaCl, calculated from average specific growth rates. In brackets are indicated the 95% confidence intervals estimated for EC_{50} point estimates.

	Endpoint		
	Frond number	Fresh weight	Total frond area
Test number	EC_{50}[a] values		
1	3810.86 (257.40)	2789.95 (301.29)	2514.59 (163.07)
2	3689.86 (245.68)	3132.79 (218.28)	2975.07 (166.10)
3	3916.76 (181.83)	2768.48 (355.16)	2569.38 (235.46)
4	3674.81 (158.17)	2411.81 (357.24)	2589.96 (197.77)
5	4245.57(140.75)	3728.82 (291.81)	2836.52 (195.82)
6	4280.01 (233.93)	3619.80 (201.98)	3200.46 (164.78)
7	4159.25 (263.41)	3441.31 (378.17)	2724.99 (243.20)
8	4074.54 (265.45)	3295.43 (249.38)	2531.44 (355.16)
Average (± S.D.[b])	3981.46 (242.46)	3148.55 (460.15)	2742.80 (245.73)
C.V.[c] (%)	**6.09**	**14.61**	**8.96**

[a] **EC_{50}** - Effect concentration at a 50% level;
[b] **S.D**. - Standard deviation; and
[c] **C.V**. - Coefficient of variation.

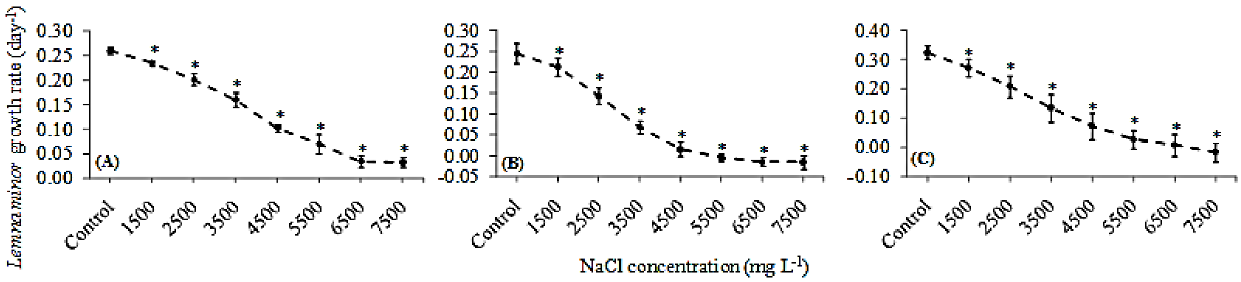

Figure 1. Growth rates of *L. minor* based on different endpoints upon exposure to increasing concentrations of the reference substance NaCl: (A) frond number; (B) total frond area; (C) fresh weight. Standard deviation is indicated by the vertical bars on each circle. Asterisks refer to a significant difference from the control ($p < 0.05$).

The C.V. values obtained for all three of the growth endpoints evaluated in this study were lower than 15.0%, indicating a relatively consistent response to NaCl, since in Environment Canada guidelines there are advisements that variation in a set of test results of a reference substance must be preferably ≤ 20% (Donnevert et al., 2009). Buckley et al. (1996) also found a relatively low C.V. value of 5.8% in four tests carried out using *L. minor* dry weight as an endpoint in order to assess the NaCl toxic effects. These results indicate that stable variance measures can be obtained for independent sensitivity tests with *L. minor*, based on growth endpoints, using the NaCl as a reference substance.

Comparing the EC_{50} values obtained in this study using NaCl with results reported for the reference substance 3,5-dichlorophenol, which is indicated in OECD and Environment Canada protocols (Canada, 2007; OECD, 2006), allows one to conclude that *L. minor* is more sensitive to the effects posed by this last compound. Results from reference tests performed in the EU Waste Ring Test 2006/2007 for 3,5-dichlorophenol in *L. minor* growth inhibition tests ranged from 2.20 to 3.50 mg L^{-1}, with mean value of 2.77 ± 0.44 mg L^{-1} (Pattard et al., 2009). Thus, the required range for the EC_{50} values determined in tests with *L. minor* using this reference toxicant is 1.8 to 3.6 mg L^{-1} (Pattard et al., 2009). ISO 20079 (ISO, 2006) establishes a narrower range as validity criteria for this compound, of an EC_{50} (frond number) of 2.2 - 3.8 mg L^{-1}. Based on an international ring-test including laboratories from several global regions, 3,5-dichlorophenol has been recommended for use in sensitivity tests with *L. minor* as it showed the best result (OECD, 2002). However, the important criteria of safety to user should have been also observed before recommending this reference substance. Chlorophenol compounds can cause severe human toxic effects, including cancer (NLM, 2015). Furthermore, disposal of its residues is another problem to be considered, since 3,5-dichlrorophenol can bioconcentrate in aquatic organisms, being toxic to them with possible long-term adverse effects (Zagorc-Koncan et al., 2002; NLM, 2015).

Canada (2007) recommends using reagent-grade nickel (Ni), in the form of nickel sulphate ($NiSO_4.6H_2O$), in reference toxicity tests with *L. minor*. According to that protocol, Ni was considered favorable due to the relatively steep dose response curve produced in reference toxicity tests performed with the macrophyte. Visual toxicity signs caused by Ni at concentrations > 3.0 mg L^{-1} include chlorosis, necrosis and frond disconnection (Khellaf and Zerdaoui, 2009). Those phytotoxicity signs are similar to the ones observed in this study in the macrophytes exposed to concentrations ≥ 4,500 mg L^{-1} of NaCl (Figure 2) (A, B, C, D, E).

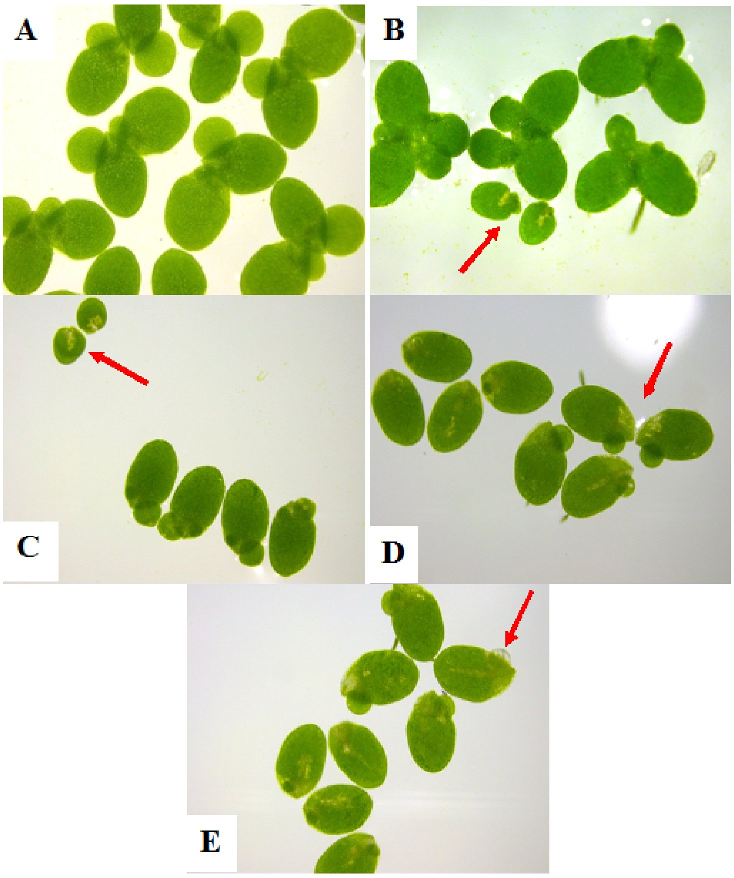

Figure 2. Macroscopic signs of phytotoxicity of *L. minor* fronds exposed to several concentrations of NaCl: (A) Plants exposed to the control showing fronds without visual phytotoxicity signs; (B) Beginning of frond disconnection and of chlorosis signs in plants exposed to the concentration of 4,500 mg L^{-1}; (C) and (D) Total frond disconnection and chlorosis signs in plants exposed to the concentrations of 5,500 mg L^{-1} and 6,500 mg L^{-1} respectively; (E) Necrosis observed in frond exposed to the concentration of 7,500 mg L^{-1}.

Therefore, similar phytotoxicity signs can be observed for the reference toxicant Ni in lower concentrations compared to the ones observed for NaCl. However, two major problems can be highlighted regarding Ni. First, the requirement that the culture medium used in tests with Ni compounds contains no EDTA restricts the choice of the dilution water to the modified APHA medium, whereas the use of other recommended and commonly used test medium such as Steinberg and Swedish Standard (OECD, 2002) is not possible. Moreover, Ni compounds are considered carcinogenic to humans (IARC, 1990).

Considering the important human safety issue, Canada (2007) suggests potassium chloride (KCl) as another possible reference substance for using in *L. minor* routine toxicity tests. ISO 20079 (ISO, 2006) suggested that EC_{50} based on frond number should be in a range between 5,500 and 10,000 mg L^{-1} for KCl in reference toxicity tests with *L. minor*. Canada (2007) reported a lower mean EC_{50} value of 4,770 mg L^{-1} (n = 18) and a % C.V. of 15.9 % for this compound in tests with this macrophyte. Therefore, mean EC_{50} value of 3,981 mg L^{-1} obtained in this study for NaCl (considering the same endpoint frond number) is slightly lower than the mean EC_{50} value reported for KCl in the Environment Canada protocol (2007). Moreover, it must be highlighted that NaCl presents the same advantages desired in a reference substance of stability in storage, stability in solution and safety to the user as observed for KCl (Sigma Aldrich, 2016).

NaCl was found to be suitable for evaluating the sensitivity based on growth endpoints other than frond number, which is required by OECD 221 (OECD, 2006). Regarding the evaluation of the other growth endpoints, the total frond area was the most sensitive one according to the Scott-Knott's multiple comparison tests performed. As it occurs with frond number, total frond area also is relatively easy to perform *in vivo* and allow measurements over all the time of the experiment. Moreover, evaluation of total frond area overcomes the problem pointed out by Wang (1990), who stated that frond count is irrelevant to frond size or biomass and therefore it can roughly underestimate the toxic effect to duckweed.

4. CONCLUSION

To the best of our knowledge, this is the first study to generate reference data for the NaCl toxic effects by measuring simultaneously three growth endpoints recommended in *Lemna* sp. guidelines. Therefore, we have expanded the results published in the literature regarding the NaCl toxicity for *L. minor*, since we have evaluated additional endpoints neglected in routine tests, such as total frond area and fresh weight.

The results presented in this study show that NaCl can be a suitable alternative reference substance for routine toxicity tests using *L. minor* since the tests performed with this salt presented acceptable sensitivity and reproducibility, measured in multiple growth endpoints. Mean EC_{50} value obtained for NaCl based on frond number were somewhat lower than the mean EC_{50} reported in standardized protocols for KCl. Moreover, this compound has the advantages of being less hazardous to both human health and the environment than other commonly used reference substances recommended by standardized protocols, such as 3,5-dichlorophenol and Ni compounds.

Moreover, we were able to demonstrate that there are significant differences in the sensitivity among *L. minor* growth endpoints for the NaCl effects. Our results clearly showed that the *L. minor* total frond area is a more sensitive endpoint for the NaCl effects than the commonly used endpoint frond number. Therefore, considering the advantage of higher sensitivity compared to frond number, we recommend that total frond area should be actually the endpoint of choice when assessing *L. minor* growth inhibition in routine sensitivity tests using NaCl as an alternative reference substance.

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