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Sensitivity of Four Test Organisms (*Chironomus xanthus*, *Daphnia magna*, *Hydra attenuata* and *Pseudokirchneriella subcapitata*) to NaCl: an Alternative Reference Toxicant

M. A. P. F. SANTOS,* J. VICENSOTTI & R. T. R. MONTEIRO

Laboratório de Ecotoxicologia, Centro de Energia Nuclear na Agricultura,
Av. Centenário, 303, CEP 13416-000, Universidade de São Paulo, Piracicaba, SP, Brasil

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ABSTRACT

In order to establish a safer reference toxicant for use in monthly sensitivity tests at the Ecotoxicology Laboratory (CENA-USP, Brazil), sodium chloride (NaCl p.a.) was evaluated as a possible surrogate for metals during a period of two years with the following test organisms: *Chironomus xanthus*, *Daphnia magna*, *Hydra attenuata* and *Pseudokirchneriella subcapitata*. All tests were performed following protocols most commonly used for each species. Mean endpoint values obtained were: $LC_{50}(96\text{ h}) = 5.84\text{ g L}^{-1}$ ($426.9\text{ }\mu\text{S cm}^{-1}$) for *C. xanthus*, $LC_{50}(48\text{ h}) = 5.55\text{ g L}^{-1}$ ($461.5\text{ }\mu\text{S cm}^{-1}$) for *D. magna*, $LC_{50}(96\text{ h}) = 2.57\text{ g L}^{-1}$ ($245.6\text{ }\mu\text{S cm}^{-1}$) and $EC_{50}(96\text{ h}) = 1.44\text{ g L}^{-1}$ ($147.2\text{ }\mu\text{S cm}^{-1}$) for *H. attenuata*, and $IC_{50}(72\text{ h}) = 0.87\text{ g L}^{-1}$ ($85.3\text{ }\mu\text{S cm}^{-1}$) for *P. subcapitata*. Results indicate good reproducibility, as acceptable coefficients of variation were obtained: 17.03%, 15.02%, 14.84%, 15.28%, and 20.08%, respectively. These results agree with those obtained by other authors, for the same test organisms, using metals such as potassium dichromate. Based on these data, it was possible to conclude that sodium chloride constitutes an adequate alternative reference toxicant to metals, for routine sensitivity tests.

Key words: *Chironomus*, *Daphnia*, *Hydra*, NaCl, *Pseudokirchneriella*, sensitivity, toxicity, reference toxicant.

RESUMO

Sensibilidade ao NaCl de quatro organismos-teste (*Chironomus xanthus*, *Daphnia magna*, *Hydra attenuata* e *Pseudokirchneriella subcapitata*), como alternativa para substância de referência

Com o objetivo de estabelecer o uso de uma substância de referência mais segura em testes mensais de sensibilidade no Laboratório de Ecotoxicologia (CENA-USP, Brasil), avaliou-se o cloreto de sódio (NaCl p.a.) como possível substituto dos metais, em um período de dois anos, para os seguintes organismos-teste: *Chironomus xanthus*, *Daphnia magna*, *Hydra attenuata* e *Pseudokirchneriella subcapitata*. Todos os testes foram realizados seguindo os protocolos mais comumente usados para cada espécie. Os valores médios obtidos dos parâmetros foram: $CL_{50}(96\text{ h}) = 5,84\text{ g L}^{-1}$ ($426,9\text{ }\mu\text{S cm}^{-1}$) para *C. xanthus*, $CL_{50}(48\text{ h}) = 5,55\text{ g L}^{-1}$ ($461,5\text{ }\mu\text{S cm}^{-1}$) para *D. magna*, $CL_{50}(96\text{ h}) = 2,57\text{ g L}^{-1}$ ($245,6\text{ }\mu\text{S cm}^{-1}$) e $CE_{50}(96\text{ h}) = 1,44\text{ g L}^{-1}$ ($147,2\text{ }\mu\text{S cm}^{-1}$) para *H. attenuata*, e $CI_{50}(72\text{ h}) = 0,87\text{ g L}^{-1}$ ($85,3\text{ }\mu\text{S cm}^{-1}$) para *P. subcapitata*. Estes resultados apresentam boa reprodutibilidade, como indicam os valores dos coeficientes de variação, para cada valor acima: 17,03%, 15,02%, 14,84%, 15,28% e 20,08%, respectivamente. Os resultados estão de acordo com os obtidos por outros autores, que utilizaram metais como o dicromato de potássio para os mesmos organismos. A partir desses dados foi possível concluir que o cloreto de sódio constitui uma boa substância de referência, como alternativa para metais em testes de rotina para sensibilidade.

Palavras-chave: *Chironomus*, *Daphnia*, *Hydra*, NaCl, *Pseudokirchneriella*, sensibilidade, toxicidade, substância de referência.

*Corresponding author: Maria Alice P. F. dos Santos, e-mail: masantos@cena.usp.br.

INTRODUCTION

Environmental agencies around the world (OECD, 1998; USEPA, 2000; USEPA, 2002; ABNT, 2005; BRASIL, 2005) have been using ecotoxicity bioassay protocols both as a regulatory tool based on endpoints and for the selection of new test organisms. The first test organisms used were fish species (Rand, 1995), then crustacean species such as *Daphnia magna* and *Ceriodaphnia dubia* were established as test organisms (Adema, 1978). Presently there is a wide variety of standardized species, from different trophic levels, such as bacteria, algae, primary producers, and primary and secondary consumers (Dellamatrice & Monteiro, 2006). Geis *et al.* (2000) investigated the U.S. EPA database and found that the unicellular green algae *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* Printz and *Raphidocelis subcapitata* Korshikov) is more sensitive than other standard test organisms to many common compounds. Recently, many papers are addressing *Hydra attenuata* and other *Hydra* species as adequate and sensitive test organisms (Trottier *et al.*, 1997; Beach & Pascoe, 1998; Karntanut & Pascoe, 2000). *Daphnia* species (OECD, 1998; USEPA, 2002) and *Chironomus* species (Fonseca & Rocha, 2004; USEPA, 2000) are addressed as standardized test organisms as well.

In the field of Ecotoxicology, the reference toxicants employed to assess the health and culture conditions of test organisms from laboratory cultures are mainly metals, such as cadmium, chromium, copper, nickel and zinc (Fargašová, 1997; Karntanut & Pascoe, 2002; Buratini *et al.*, 2004; Bossuyt & Janssen, 2005). The sensitivities of test organisms to these substances have been extensively proven over the years, thus allowing their validation as test organisms. However, handling these materials can be hazardous to human health. Testing new substances to use as reference toxicants in routine sensitivity bioassays can be a safer alternative to both scientists and the environment itself, which ultimately receives these laboratory effluents in one way or another (Utz & Bohrer, 2001). For example, the waste of a *Chironomus* monthly sensitivity test consists of approximately 4.5 liters of water, contaminated with a metal such as potassium dichromate ($K_2Cr_2O_7$).

In order to determine a safer reference substance for routine monthly sensitivity bioassays, sodium chloride (NaCl, p.a.) was tested for a period of two years as reference toxicant, to obtain control charts for four test organisms: the midge *Chironomus xanthus*, the water flea *Daphnia magna*, the cnidarian *Hydra attenuata* and the freshwater green algae *Pseudokirchneriella subcapitata*.

MATERIALS AND METHODS

Test organisms

All test organisms used in these experiments had been successfully cultured and maintained in the Ecotoxicology Laboratory at CENA/USP for more than 3 years, following

the test protocols and guidelines for each species. Toxicity tests were conducted monthly, between April 2005 and March 2007, and acute toxicity of sodium chloride (NaCl, p.a.) was determined by the Trimmed-Spearman Karber method (Hamilton *et al.*, 1977). All reagents used were of analytical grade, and all culture media were prepared with glass-distilled water. All tests were conducted under static conditions (non-renewal).

Culturing conditions

Chironomus xanthus

The reconstituted soft water ($12 \text{ mg L}^{-1} \text{ CaSO}_4$, $0.8 \text{ mg L}^{-1} \text{ KCl}$, $19.2 \text{ g L}^{-1} \text{ NaHCO}_3$, $24.4 \text{ g L}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$; total alkalinity between 12 and 16 mg L^{-1} as CaCO_3 , and pH between 7.2 and 7.6) used for both culturing and toxicity testing (used as dilution water and for controls) of *C. xanthus* followed the protocol established in Fonseca & Rocha (2004) for *C. xanthus*, and recommendations of USEPA (2000) for *Chironomus tentans*. The temperature for both cultures and tests was $25 \pm 1^\circ\text{C}$. Stock cultures were initiated with three to five egg cases (200 to 400 larvae), maintained in a plastic tray ($45 \times 35 \times 6 \text{ cm}$) covered by a mesh cage (to trap emerging adults) with a control sand layer (1 cm, sieved through 0.5 mm mesh size and sterilized at 550°C for 2 h), and 4 liters of reconstituted water under constant aeration and 12 h/12 h light/dark photoperiod. Larvae were fed *P. subcapitata* on the first day after hatching ($10^5 \text{ cells mL}^{-1}$), and on subsequent days three times per week with a fish flake feed (Alcon Basic®) suspension (10 g L^{-1}).

Daphnia magna

Daphnia magna stock cultures were grown in ElenDt M-4 Medium (OECD, 1998), which has a total alkalinity of $250 \pm 25 \text{ mg L}^{-1}$ as CaCO_3 , and a pH between 7.0 and 8.0. Culturing and toxicity testing were conducted at a temperature of $22 \pm 1^\circ\text{C}$ and a photoperiod of 12 h/12 h light/dark. Females were reared up to one month old, and then discarded. Neonates from the first and second broods were discarded, and not used for toxicity tests. Culture medium was renewed three times per week, and females were fed an algal suspension of *P. subcapitata* ($1.5 \times 10^6 \text{ cells mL}^{-1}$), plus a solution of dry yeast (5 g L^{-1}) and trout flake feed (5 g L^{-1}), according to USEPA (2002) recommendations.

Hydra attenuata

Reconstituted water used for *Hydra* culturing ($147 \text{ mg L}^{-1} \text{ CaCl}_2$, $110 \text{ mg L}^{-1} \text{ TES buffer}$; pH of 7.0 ± 0.1) followed the protocol established by Trottier *et al.* (1997), without EDTA addition. Stock cultures were maintained in 20 cm diameter circular glass culture bowls filled with 500 mL culture medium. Individuals were fed hatched brine shrimp (*Artemia salina*) three times a week, and culture medium was renewed after feeding (2 h). Culturing and toxicity testing were conducted at a temperature of $22 \pm 1^\circ\text{C}$ and a photoperiod of 12 h/12 h light/dark.

Pseudokirchneriella subcapitata

Culturing of *P. subcapitata* followed the protocols established by Blaise *et al.* (2000), in which cultures were renewed weekly into fresh sterilized (autoclaved) medium, composed of both micro- and macronutrients. This procedure ensured a steady supply of logarithmic phase cells in 5-7 days after inoculations. *P. subcapitata* culture was obtained from American Type Culture Collection (ATCC n. 22622). The pH of algae medium was adjusted to 7.5 (± 0.01) with 0.1 N NaOH or 0.1 N HCl prior to sterilization. Algae inocula were incubated in Erlenmeyer flasks covered with transparent plastic sealing film under continuous lightning (4000 lux $\pm 10\%$) at 25 $\pm 1^\circ\text{C}$, and manually agitated three times daily by gently swirling the culture flask for a few seconds.

Sensitivity test conditions

For all tests, culture medium conductivity measurements were carried in the beginning of the incubation period in order to obtain a linear relationship where

$$\text{Conductivity } (\mu\text{S cm}^{-1}) = a + \text{salinity } (\text{g L}^{-1}) \cdot b \quad (1)$$

(*a* and *b* being constants), thus allowing a transformation of the NaCl concentration (g L^{-1}) endpoint values to conductivity ($\mu\text{S cm}^{-1}$) endpoint values (Table 1).

Chironomus xanthus

The acute toxicity (96 h) of sodium chloride (LC_{50}) to *C. xanthus* was determined by following the protocol of Fonseca (1997). Larvae at third- to fourth-instars (about 10 to 12 days old) were placed in 160 mL reconstituted water containing 0, 1.25, 2.5, 5.0, 10, and 15 g L^{-1} NaCl, with three replicates per concentration, and 6 larvae per replicate. Test flasks were covered with a sealing film and kept at 25 $\pm 1^\circ\text{C}$, under a 12 h/12 h light/dark photoperiod. Larvae were fed only on the first day of experiment, with 1 mL of fish flake feed (Alcon Basic®) suspension (10 g L^{-1}). After 96h, the mortality of individuals was visually determined and the LC_{50} estimated by the Trimmed-Spearman Karber method (Hamilton *et al.*, 1977).

Daphnia magna

Elendt M4 medium was used both as dilution water and for controls. *D. magna* neonates up to 24 h-old were exposed to the following concentrations of NaCl (g L^{-1}): 0 (control),

1, 3, 5, 7 and 9, with four replicates per concentration, and five neonates per replicate. Test vials were kept at 22 $\pm 1^\circ\text{C}$, under a 12 h/12 h light/dark photoperiod for 48 h. Neonates were not fed during the test. After 48 h, the mortality of individuals was determined and the LC_{50} estimated by the Trimmed-Spearman Karber method (Hamilton *et al.*, 1977).

Hydra attenuata

Hydra test procedures followed those of Trottier *et al.* (1997) and Blaise & Kusui (1997). Adult animals were exposed to the following concentrations of NaCl (g L^{-1}): 0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5, with three replicates per concentration, and three individuals per replicate. Tests were conducted in 12-well microplates (Corning, #25815) at 22 $\pm 1^\circ\text{C}$, under a 12 h/12 h light/dark photoperiod for 96 h. Animals were not fed during the test. Adult individuals without buds or any morphological damage were chosen from stock cultures and transferred with a plastic pipette into 35 mm polystyrene Petri dishes (Corning, #430165), containing the same concentration of NaCl as the test solutions. These Petri dishes act as a "Hydra transfer wells", with the purpose of minimizing the dilution of the prepared NaCl concentrations that would occur if hydras were transferred directly to microplates from stock cultures. From the transfer wells, hydras were redistributed to the corresponding NaCl concentration.

In *Hydra* tests the endpoint value determinations are based on changes in morphology as the toxicity intensifies. Animals undergo drastic morphological changes during exposure periods. From their normal appearance, they will progressively exhibit "clubbed" tentacles as a first sign of intoxication, followed by the shortening of the tentacles, and then the so-called "tulip stage" when all the tentacles disappear. Beyond this point regeneration is not possible. The post-tulip stage terminates with the disintegration of animal body. Thus, the tulip stage is also considered a stage corresponding to acute lethal effects (Figure 1).

Changes in *Hydra* morphology were observed after 96 h of exposure with a stereomicroscope (Leica, model Zoom 2000) at 10.5x magnification. Two endpoints were observed in *Hydra* tests: for estimates of LC_{50} (96 h), only lethal stages (tulip and disintegrated stages) were considered; and for EC_{50} (96 h), any observed stage different than the normal appearance is considered, thus constituting a more sensitive endpoint.

Table 1 – Linear regression parameters (conductivity = a + b . salinity), obtained based on conductivity measurements, for each test organism.

	a	b	R
<i>C. xanthus</i>	51.43	64.29	0.99174
<i>D. magna</i>	49.38	74.25	0.99869
<i>H. attenuata</i>	21.70	87.13	0.99899
<i>P. subcapitata</i>	8.94	87.75	0.99975

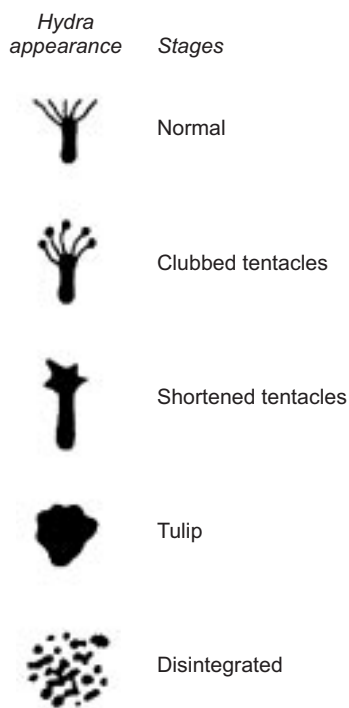


Figure 1 – Progressive morphological changes in *Hydra attenuata* exposed to a toxic agent. Sublethal and lethal toxicity endpoints are determined by the clubbed tentacles and tulip stages, respectively. (Adapted from Blaise & Kusui, 1997).

Pseudokirchneriella subcapitata

Test flasks (scintillation vials) were inoculated with 5- to 7-days old *P. subcapitata* culture to achieve an initial concentration of 1×10^4 cells mL^{-1} , and covered with sealing transparent plastic film. Algae were exposed to the following concentrations of NaCl (g L^{-1}): 0 (control), 0.2, 0.4, 0.8, 1.0 and 1.6, with three replicates for each concentration, except the control, which was conducted with five replicates (C_1 - C_5). The dilution water used was buffered water, composed of 15 mg L^{-1} NaHCO_3 . Flasks were manually agitated three times daily, and randomized daily, under continuous lighting ($4000 \text{ lux} \pm 10\%$) and temperature ($25 \pm 1^\circ\text{C}$). Tests were terminated after 72 h, when growth was measured in each test flask with an improved Neubauer chamber. For the obtained results to be acceptable, control flasks must reach a concentration of 1.6×10^5 cells mL^{-1} and the coefficient of variation (C.V. %) of the five replicates must be less than 40%. Cell counts of each flask were converted to cells mL^{-1} and then to %Inhibition, with the formula:

$$\% \text{Inhibition} = 100 - \left[\left(\frac{\text{cell count}}{\text{average}(C_1 + C_2 + C_3 + C_4 + C_5)} \right) * 100 \right] \quad (2)$$

Thus the values converted in this way were used to estimate IC_{50} values by the Trimmed-Spearman Karber method (Hamilton *et al.*, 1977).

RESULTS AND DISCUSSION

The sensitivity test results conducted from April 2005 to March 2007 are illustrated in control-chart format, for *C. xanthus* (LC_{50} (96 h)) in Figure 2, for *D. magna* (LC_{50} (48 h)) in Figure 3, for *H. attenuata* (EC_{50} (96 h) and LC_{50} (96 h)) in Figures 4-5, and for *P. subcapitata* (IC_{50} (72 h)) in Figure 6. In Figures 2-6, endpoint values, mean values, standard deviations, and coefficients of variation (%) are depicted. All organisms exposed to NaCl as a reference toxicant had good sensitivity responses, having

acceptable C.V. (%) values: 17.03% for *C. xanthus*; 15.02% for *D. magna*; 15.28% and 14.84% for *H. attenuata* EC₅₀ and LC₅₀ values, respectively; and 20.09% for *P. subcapitata*.

Mean endpoint and standard deviation values of all test organisms (g L⁻¹) were also transformed to conductivity values, according to linear regressions obtained for each species (Table 2).

End point values obtained in this study are comparable to the results of other authors, for the exposure of *P. subcapitata*

and *D. magna* to NaCl. Geis *et al.* (2000), exposing *P. subcapitata* to NaCl, obtained an IC₅₀ (96 h) of 2.5 g L⁻¹. The most common results found in the literature were those testing *D. magna* with this reference toxicant. Mean end point values found in the present study for *D. magna* are among those found in the literature (LC₅₀ (48 h)): Cowgill & Milazzo (1990): 6.03 g L⁻¹; Cowgill & Milazzo (1991): 7.7 g L⁻¹; Hong *et al.* (2004): 7.3 g L⁻¹; and Mount *et al.* (1997): 4.77 g L⁻¹. Results for the other organisms tested were not found in the literature.

Table 2 – Mean end point and standard deviation values of all test organisms, both on NaCl concentration (g L⁻¹) and conductivity (μS cm⁻¹).

	Mean end point		Standard deviation	
	g L ⁻¹	μS cm ⁻¹	g L ⁻¹	μS cm ⁻¹
<i>C. xanthus</i> (LC ₅₀)	5.84	426.86	0.99	115.08
<i>D. magna</i> (LC ₅₀)	5.55	461.46	0.83	111.01
<i>H. attenuata</i> (LC ₅₀)	2.57	245.61	0.38	54.84
<i>H. attenuata</i> (EC ₅₀)	1.44	147.23	0.22	40.93
<i>P. subcapitata</i> (IC ₅₀)	0.87	85.32	0.17	23.85

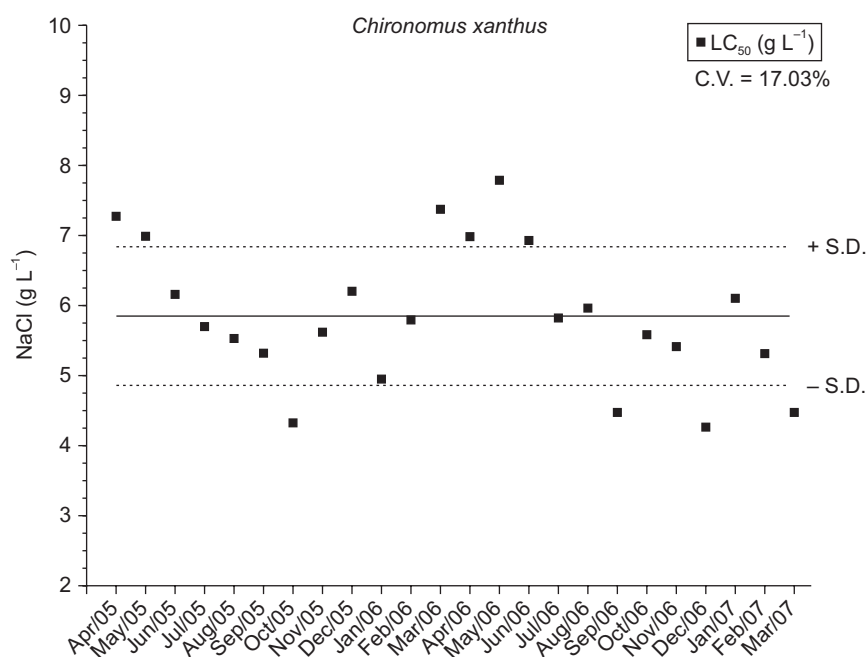


Figure 2 – Control-chart showing LC₅₀(96 h) of *Chironomus xanthus* exposed to NaCl in sensitivity tests. The solid line indicates the mean value of LC₅₀, and dotted lines represent ± standard deviation.

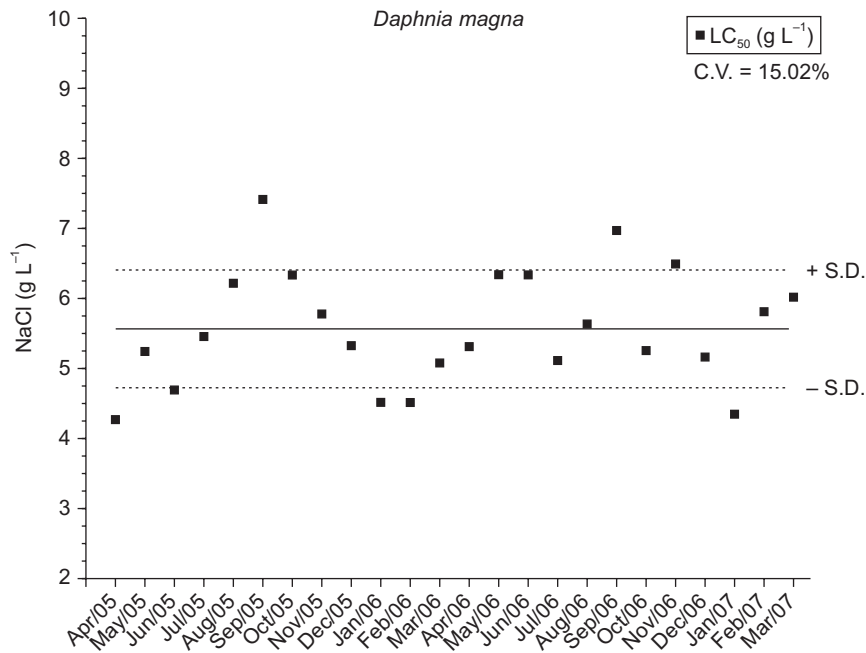


Figure 3 – Control-chart showing LC_{50} (48 h) of *Daphnia magna* exposed to NaCl in sensitivity tests. The solid line indicates the mean value of LC_{50} , and dotted lines represent \pm standard deviation.

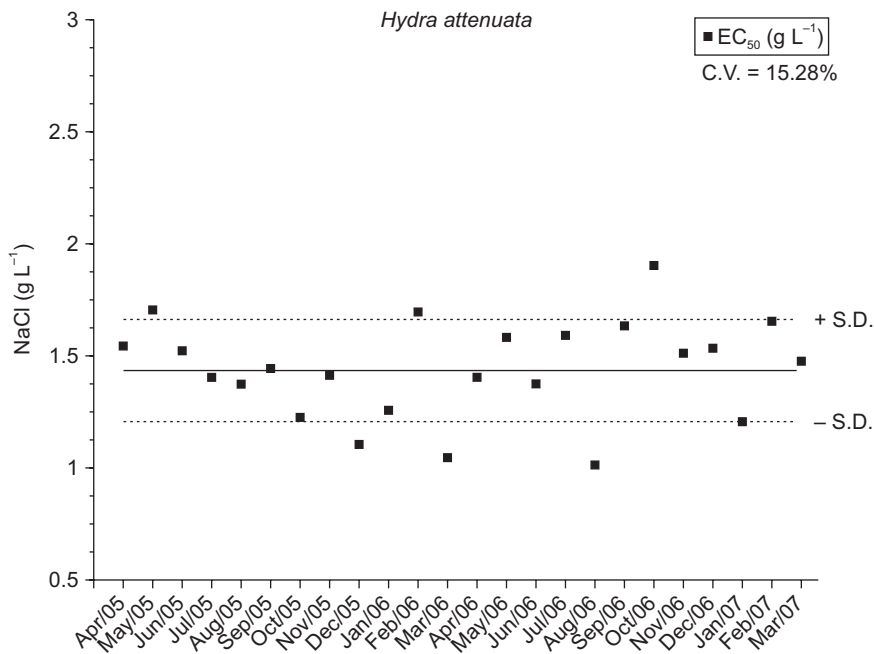


Figure 4 – Control-chart showing EC_{50} (96 h) of *Hydra attenuata* exposed to NaCl in sensitivity tests. The solid line indicates the mean value of EC_{50} , and dotted lines represent \pm standard deviation.

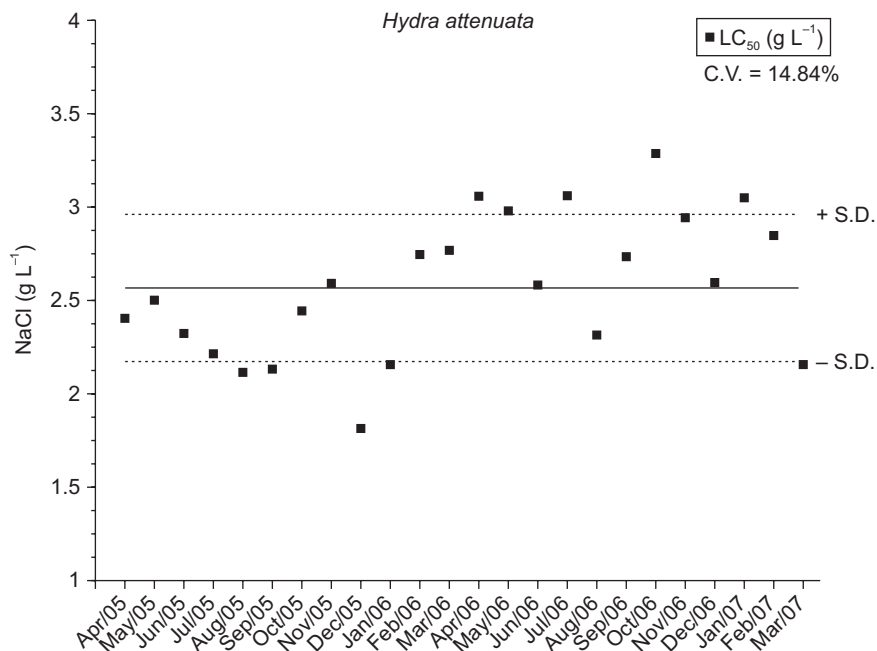


Figure 5 – Control-chart showing LC_{50} (96 h) of *Hydra attenuata* exposed to NaCl in sensitivity tests. The solid line indicates the mean value of LC_{50} , and dotted lines represent \pm standard deviation.

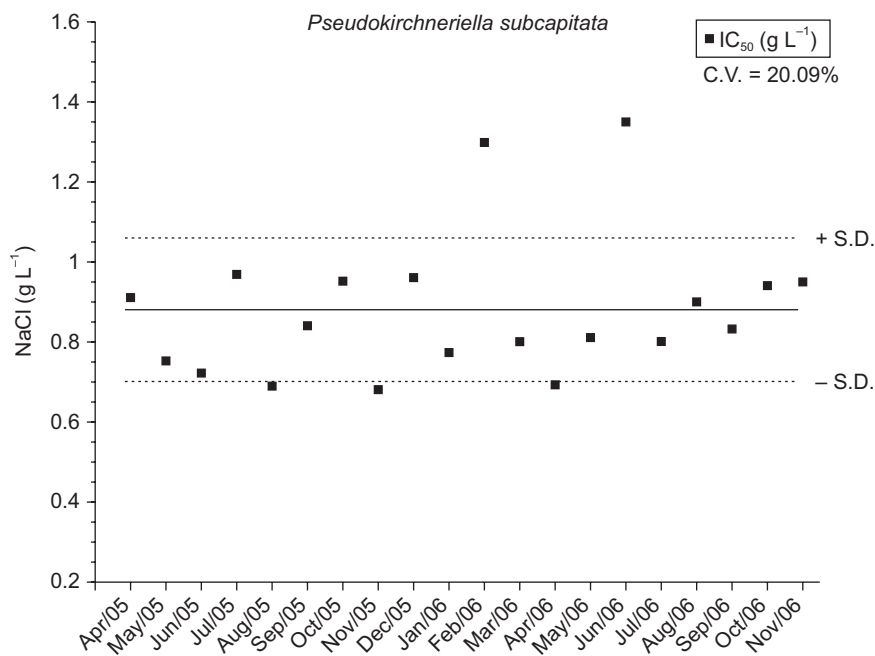


Figure 6 – Control-chart showing IC_{50} (72 h) of *Pseudokirchneriella subcapitata* exposed to NaCl in sensitivity tests. The solid line indicates the mean value of IC_{50} , and dotted lines represent \pm standard deviation.

CONCLUSIONS

This two-year study showed that NaCl can be used as a reference toxicant, meeting two proposed requirements: (i) it is less hazardous to both human handling and the environment than metals such as potassium dichromate and others, and (ii) it has acceptable reproducibility in routine laboratory tests.

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