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Comparison Between Four and Seven-day *Ceriodaphnia dubia* Survival and Reproduction Test Protocols Using Oil Refinery Effluent Samples

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ABSTRACT

The aim of this study was to compare the 7-day Ceriodaphnia dubia survival and reproduction test with a 4 day test to evaluate the toxicity of oil refinery effluents. Both shown identical NOECs with three of 6 tested samples. With one of the tested samples, the 4-day NOEC was one tested-concentration lower, and with the remaining two samples, it was one tested-concentration higher than those obtained using the 7-day protocol. Then results suggested that 4 and 7-day protocols were comparable to detect toxic effects.

Key words: Aquatic toxicology, water pollution, ecotoxicology, oil refinery effluents, reproduction, aquatic chronic tests

INTRODUCTION

Acute tests with a variety of freshwater organisms have been widely used to investigate the toxicity of chemicals and industrial effluents discharged into water bodies. Acute assays are, as a rule, relatively cheap and easy to perform but, on the other hand, they detect only lethal or immobilization effects. Chronic assays evaluate other physiological parameters in addition to lethality, but they are more expensive and time consuming and thus they have seldom been employed in routine monitoring programs.

The need of regulatory agencies for rapid screening of effluents chronic toxicity took the United States Environmental Protection Agency (USEPA) to develop short-term chronic tests to substitute the traditional longer chronic assays (Cooney, 1995). In this field, the 7-day fathead minnow (*Pimephales promelas*) larval survival and teratogenicity assay (Norberg and Mount, 1985), and the 7-day *Ceriodaphnia* survival and reproduction test (Mount and Norberg, 1984), both recommended by USEPA guidelines (USEPA, 2002), are today's most frequently conducted short-term chronic assays. In Brazil, the 7-day *Ceriodaphnia* survival and reproduction test has been included in test batteries used for safety evaluation and registration of new pesticides. It has also been used for evaluating chronic toxicity of effluents discharged into Brazilian water bodies. Brazilian criteria for water classification (Brasil, 2005) suggest the performance of aquatic toxicity

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assays including the 7-day *Ceriodaphnia* chronic assay for assessment of water quality. Though, at the beginning of 1990's, an even shorter (4-day) test has been proposed as a feasible alternative to the 7-day *C. dubia* survival and reproduction assay (Oris et al, 1991). The present study was designed to compare the standard 7-day and the proposed 4-day *C. dubia* survival and reproduction test protocols, evaluating the aquatic toxicity of oil refinery effluents.

MATERIALS AND METHODS

Toxicity assays with *C. dubia* were performed essentially as recommended by ABNT (2005) and USEPA (2002) guidelines. All the organisms were from stock cultures maintained at the Laboratory of Environmental Toxicology (ENSP-FIOCRUZ). The tests were carried out in environmental chambers at 25 ± 1°C on a 16-h light/8-h dark photoperiod.

Acute toxicity tests with sodium chloride (NaCl)

NaCl was used as a reference chemical. Acute tests with NaCl were run in parallel with effluent samples testing. Sodium chloride 24-h and 48-h EC_{50}s (immobilization) were added to *C. dubia* culture historical control records.

Acute toxicity test with oil refinery effluents

For six months (September – 1999 to February – 2000), a sample/month of oil refinery effluents was collected, (always at the same point). All the samples were received at the laboratory within 24 h after sampling and were firstly tested for their acute toxicity. Oil refinery effluent samples and sodium chloride (positive control chemical) were diluted in a previously aerated synthetic soft water ($CaSO_4.2H_2O$ 30 mg/L, KCl 0,2 mg/L $NaHCO_3$ 48 mg/L, $MgSO_4.7H_2O$ 61 mg/L) with hardness = 42 to 45 mg/L as $CaCO_3$, pH = 7,2 ± 0.2, and saturating oxygen levels (ABNT, 2005).

Chronic toxicity tests with oil refinery effluents

For comparative purposes, all the oil refinery effluent samples were concurrently submitted to 4 and 7-day *C. dubia* chronic assay protocols so that 4 as well as 7-day-NOECs ("No Observed Effect Concentrations") were determined for each sample. In both cases, tests began by placing less than 8-h-old neonates into 30 mL beakers (one neonate per beaker) containing 15 mL of synthetic soft water or diluted effluent samples. Exposure of *C. dubia* to oil refinery effluent samples started on the first day of life for the 7-day protocol and on the fourth day of life for the 4-day protocol. In the 4-day as well as in the 7-day-exposure protocol, however, the organisms were individually maintained, food was provided and all the solutions were renewed on a daily basis from the first day of life onwards. Every day, at the time of solution renewal, cladocerans were fed with 0.15 mL of a mixed diet consisting of Fleishmann® yeast and Purina® trout chow, prepared as described elsewhere (ABNT, 2005), plus 0.1 mL of a suspension of the microalga (*Selenastrum capricornutum*) culture with approximately 10^7 cells/mL. Cultures of *S. capricornutum* were grown at the laboratory as recommended by USEPA (2002) and ABNT (2005) guidelines.

Statistical Analysis

EC_{50} (Immobilization) values and their respective 95% confidence limits (95% CL) were determined by the Trimmed Spearman-Karber method (Hamilton et al., 1977). NOECs (no-observed) and LOECs (lowest-observed-effect concentrations for inhibition of reproduction) were calculated by the Dunnett's procedure (Dunnett, 1955; USEPA, 2002) and IC_{50} (50% Inhibitory Concentration for Reproduction) were calculated by the Linear Interpolation Method (Norberg-King, 1993).

RESULTS AND DISCUSSION

Acute toxicity of oil refinery effluents

As shown in Table 1, *C. dubia* used in the chronic tests assays was susceptible to the positive control substance (NaCl). All the six positive control (NaCl) EC_{50}s were within the limits of variability (mean ± 2 Standard Deviation), considered as acceptable by current guidelines. Monitoring of chronic toxicity of the oil refinery effluent samples was performed for the six months and only one (month 5) was not acutely toxic. The acute toxicities (48-h EC_{50}) of the other five effluent samples were between 84% v/v (less toxic) and 44% v/v (more toxic) (Table 1).

Table 1 - Acute toxicity of oil refinery effluent samples to *Ceriodaphnia dubia*. Results are shown as 48-hours 50% Effective Concentrations (48-h EC_{50}; immobilization) and respective 95% Confidence Intervals. Oil refinery effluent concentrations are expressed as dilutions (%, v/v) of collected samples in the assay water (undiluted sample = 100 %).

Month	**48-h EC_{50}**	
	NaCl (g/L)	**Effluent Samples (%, v/v)**
1	0.73 (0.61-0.88)	58.77 (50.17-68.86)
2	0.88 (0.73-1.07)	44.05 (36.02-53.86)
3	0.8 (0.67-0.96)	70.71 (64.61-77.39)
4	1.21 (1.06-1.41)	49.18 (36.01-67.17)
5	1.01 (0.87-1.18)	NT*
6	1.33 (1.08-1.66)	84.09 (58.95-119.94)

*NT – not toxic, i.e. undiluted sample was not acutely toxic.

Chronic tests and comparison between 4 and 7-day exposure protocols

All the six oil refinery effluent samples were toxic to *C. dubia* after 4 or 7-day exposures and their NOECs ranged from 50% v/v (less toxic) to 10% v/v (more toxic) (Tables 2 and 3). NOECs/LOECs and IC_{50}s for effects on reproductive performance in both chronic assays (4 and 7-day exposure protocols) were lower than 48-h EC_{50}s obtained for the same samples in acute exposure tests. These findings are consistent with the view that life-cycle toxicity assays are more sensitive to detect adverse effects of chemicals on aquatic organisms than acute lethality tests.

The presence of food is expected to reduce the concentration of free toxicants in assay water thereby decreasing their bioavailability (Sprague, 1995; Oliveira-Filho et al, 1999). Owing to this fact, it seemed fair to conclude that differences would have been even more marked if acute tests had also been conducted in the presence of food. The two test protocols (4 and 7-day exposure tests) gave rise to identical NOECs for three out of six evaluated samples, with one of the tested samples the 4-day NOEC was one-tested-concentration lower, and with the remaining two samples the 4-day exposure protocol resulted in NOECs that were one-tested-concentration higher than that obtained with the longer exposure test. Another result could be observed by IC_{50}, a calculated point estimative that could be more precise and comparable by the confidence intervals (CI) and coefficient of variation (CV). In three samples (1, 2 and 4) the ICs_{50} and ICs_{25} analysis of 4 and 7 days were comparable with the NOEC results (Table 3). Data from ICs_{25} were more comparable to NOECs. USEPA (2002) reported that the ICs_{25} for *C. dubia* were comparable to the NOECs for a set of tests with single chemicals, but these comparisons could be very limited, especially when derived from effluent toxicity test data. Table 3 shows that only two NOECs of 4-day tests (1 and 3) were included in the confidence intervals of ICs_{25}. On the other hand, four NOECs of 7-day tests (1, 2, 5 and 6) were included. The results from this comparative study on the sensitivity of 4 and 7-day test protocols using six oil refinery effluent samples (a rather complex mixture of chemicals) were consistent with those previously provided by Oris et al (1991) for twelve chemically-defined toxicants. Comparisons between 4-day test versus 7-day test performed by Oris et al (1991) showed that the two protocols either yielded in most cases identical toxicity indices or, in a few cases, the 4-day assay produced slightly lower chronic values (i.e. geometric means between NOECs and LOECs). Oris et al (1991) concluded that the 4-day *C. dubia* survival and reproduction test provide an acceptable level of sensitivity in the toxicity testing of single chemical compounds. Masters et al (1991) also compared results of 4 and 7-day *C. dubia* survival and reproduction tests, conducted concurrently, for a municipal effluent and a variety of substances such as metals, PCP, ethylene glycol, phenol and surfactants. According to the authors, chronic values based on survival and young production were within a factor of two in approximately 70% of all tests conducted in their study and in 100% of tests performed with a municipal effluent. Based on the foregoing results, Masters et al (1991) concluded that the 4-day test protocol was a technically valid alternative to the 7-day chronic assay. In the present work the similarity observed between CVs confirmed the better precision of point estimation. ICs_{50} CVs ranged between 2.9 to 17% and the overall means were 7.3 and 7.1% for 4 and 7 days respectively. For IC_{25} CVs ranged between 4.8 to 36.7% and the overall means were 10.2 and 15.9%.

Other studies have investigated the influence of the age on the sensitivity of *C. dubia* 7-day survival and reproduction test. All of them have pointed that there were not significant differences for the ages tested (Cooney et al, 1992; Lasier et al, 2000; Aragão and Pereira, 2003). These data supported the 4-days test proposed and based on the present results seemed to have no significant difference between the sensitivity of organisms < 24 h and with 72 h old. Thus, the results of the present study with oil refinery effluent samples supported the view that the 4-day assay could be an acceptable sensitivity in the toxicity testing of chemicals and industrial effluents.

Table 2 – Toxicity of oil refinery effluent samples to *Ceriodaphnia dubia* as evaluated by four and seven-day survival and reproduction test protocols. Results are shown as the number of young/female (mean ± SD) and, between brackets, the percentage of exposed females that survived until the end of the test (%). Oil refinery effluent concentrations are expressed as dilutions (%, v/v) of the collected sample (undiluted sample = 100 %)

Month	**Exposure**	**Effluent sample concentration**						
		0	**2.5**	**5**	**10**	**25**	**50**	**100**
1	4 days	11.5 ± 6.1 (89)	11.6 ± 5.7 (100)	11.7 ± 2.1 (100)	11.7 ± 2.0 (89)	12.2 ± 2.8* (100)	3.4 ± 3.7 (100)	0
	7 days	18.8 ± 4.6 (100)	16.8 ± 4.2 (90)	16.0 ± 6.8 (80)	16.2 ± 4.8* (90)	12.5 ± 6.0 (100)	0.6 ± 0.5 (50)	0
2	4 days	14.0 ± 6.0 (90)	19.1 ± 3.2 (100)	15.4 ± 6.9 (100)	20.3 ± 6.9 (100)	20.8 ± 6.4* (100)	6.0 ± 4.4 (78)	0
	7 days	15.3 ± 3.7 (90)	19.3 ± 4.8 (90)	20.3 ± 5.3 (100)	23.9 ± 3.9 (100)	17.2 ± 6.9* (100)	8.9 ± 7.1 (90)	0
3	4 days	14.4 ± 5.4 (80)	-	-	14.7 ± 7.5 (100)	16.5 ± 7.4* (90)	5.6 ± 2.0 (70)	0
	7 days	16.7 ± 3.2 (100)	-	-	27.1 ± 7.4 (90)	23.4 ± 10.3 (90)	10.8 ± 5.3* (90)	0
4	4 days	22.5 ± 2.4 (100)	-	25.3 ± 6.0 (100)	22.1 ± 4.8 (100)	23.4 ± 3.2* (100)	8.0 ± 2.8 (80)	0
	7 days	23.6 ± 4.2 (100)	-	30.2 ± 8.4 (90)	27.7 ± 5.1* (90)	16.4 ± 5.3 (100)	0 (90)	0
5	4 days	17.8 ± 3.1 (100)	-	-	-	19.9 ± 2.8 (100)	17.9 ± 5.7* (90)	5.3 ± 1.8 (90)
	7 days	18.4 ± 3.9 (100)		22.8 ± 1.7 (100)	26.5 ± 10.5 (90)	25.0 ± 5.1 (100)	18.4 ± 5.7* (80)	0.7 ± 1.3 (100)
6	4 days	17.0 ± 3.9 (90)	-	-	17.3 ± 3.3* (100)	11.4 ± 4.9 (100)	7.6 ± 2.4 (100)	4.0 ± 0.9 (70)
	7 days	22.0 ± 5.9 (90)	-	-	19.3 ± 2.1* (100)	5.6 ± 3.4 (90)	0 (90)	0 (90)

*Mean not significantly different (α=0.05) when compared to the respective control group by Dunnett's Procedure, (USEPA, 2002).

Table 3 - Toxicity of oil refinery effluent samples to *Ceriodaphnia dubia* as evaluated by four and seven-day survival and reproduction test protocols. Results are shown as 50% and 25% Inhibitory Concentrations (IC_{50}, IC_{25}) and respective 95% Confidence Interval, Coefficient of Variation – CV (%), No-Observed-Effect (NOEC) and Lowest-Observed-Effect (LOEC) concentrations for reproductive performance. Oil refinery effluent concentrations are expressed as dilutions (%, v/v) of the collected sample (undiluted sample = 100 %).

Month	Exposure	IC_{50}	CV 4 days	CV 7 days	IC_{25}	CV 4 days	CV 7 days	NOEC	LOEC
1	4 days	42.69 (36.62-52.79)	9.9	-	33.85 (25.74-36.57)	13.6	-	25	50
	7 days	32.35 (24.07-36.96)	-	11.0	20.85 (4.60-29.85)	-	36.7	10	25
2	4 days	43.79 (40.20-49.58)	6.1	-	34.39 (32.56-36.62)	3.7	-	25	50
	7 days	47.46 (39.95-49.93)	-	7.2	32.83 (22.32-40.12)	-	16.8	25	50
3	4 days	44.79 (38.66-47.78)	4.4	-	34.91 (24.21-36.54)	10.4	-	25	50
	7 days	49.20 (43.52-49.82)	-	4.4	37.10 25.89-40.29)	-	10.3	50	-
4	4 days	43.30 (41.07-45.47)	2.9	-	33.18 (29.22-35.09)	4.8	-	25	50
	7 days	29.31 (24.75-33.12)	-	7.5	19.48 (15.86-24.47)	-	10.3	10	25
5	4 days	83.63 (75.43-88.01)	3.5	-	64.93 (53.83-68.81)	6.9	-	50	100
	7 days	69.20 (62.86-73.95)	-	5.1	52.81 (43.49-60.46)	-	10.4	50	100
6	4 days	44.57 (24.68-52.81)	17.0	-	21.59 (15.65-30.55)	21.6	-	10	25
	7 days	19.08 (16.35-21.82)	-	7.6	13.07 (9.16-15.73)	-	10.7	10	25
Overall Means C.V.		NA	7.3	7.1	NA	10.2	15.9	NA	NA

NA – Not Applied

However, it was not possible to skip working on weekends by shortening the exposure period from 7 to 4 consecutive days. In a preliminary experiment, it was noted that the absence of water and food renewal during weekends increased the mortality of test organisms maintained individually. Since to avoid working on Saturdays and Sundays food for the weekend had to be provided on Friday, the increased *C. dubia* lethality was probably due to enhanced fermentation of food. Moreover, it was found that if water and food were not renewed on daily basis (i.e. encompassing week ends), the first brood was delayed and hence, the reproductive output during the assay was substantially reduced among untreated survivors (control group). Therefore, although exposure to the test substance was shorter with 4-day protocol, both (4 and 7-day) protocols required a change of assay water and food on daily basis for seven consecutive days.

The main advantage of the 4-day test over the 7-day test seemed to be the fact that the former protocol required a smaller volume of the testing sample (e.g. an effluent or an environmental sample). The volume of effluent needed for the 4-day test was approximately half of the volume required for the 7-day protocol. If there is a large number of samples to be tested and all samples have to be transported to a laboratory facility far from the sampling site, then sample volume could become a critical variable. Therefore, unless sample volume comes to be a limitation for the test, there was not clear advantage in performing the 4-day test instead of the 7-day *C. dubia* survival and reproduction assay.

RESUMO

O objetivo do presente estudo foi comparar os protocolos de sobrevivência e reprodução de *Ceriodaphnia dubia* com 7 e 4 dias de duração, avaliando a toxicidade de amostras de efluentes de refinaria de petróleo. Ambos os protocolos apresentaram NOECs idênticos para 3 das 6 amostras testadas. Com uma das amostras o NOEC no ensaio de 4 dias ficou numa concentração testada menor, e nas duas amostras restantes ele ficou numa concentração testada maior do que os NOECs obtidos no ensaio de 7 dias. Esses resultados sugerem que os protocolos de ensaio com 4 e 7 dias são comparáveis quanto a sensibilidade para detectar o efeito tóxico crônico de efluentes de refinaria de petróleo.

REFERENCES

ABNT (Associação Brasileira de Normas Técnicas), (2005), *Ecotoxicologia aquática – Toxicidade crônica – Método de ensaio com Ceriodaphnia* spp. *(Crustacea, Cladocera).* NBR 13373, Rio de Janeiro, ABNT, 12p.

Aragão, M. A. and Pereira, E. V. (2003), Sensitivity of *Ceriodaphnia dubia* of different ages to sodium chloride. *Bull Environ Contam Toxicol*., **70**, 1247-1250.

Brasil (2005), CONAMA directive n^{o} 357, March, 17th, 2005.

Cooney, J. D. (1995), Freshwater tests. In- *Fundamentals of aquatic toxicology: effects, environmental fate, and risk assessment*, ed. G. M. Hand. Taylor and Francis, Washington, pp. 71-102.

Cooney, J. D.; DeGraeve, G. M.; Moore, E. L.; Lenoble, B. J.; Pollock, T. L. and Smith, G. J. (1992), Effects of environmental and experimental design factors on culturing and toxicity testing of *Ceriodaphnia dubia. Environ Toxicol Chem*., **11**, 839-850.

Dunnett, C. W. (1955), Multiple comparison procedure for comparing several treatments with a control. *J Am Stat Assoc.*, **50**, 1096-1121.

Hamilton, M. A.; Russo, R. C. and Thurston, R. V. (1977), Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol.*, **11**, 714-719.

Lasier, P. J.; Winger, P. V. and Bogenrieder, K. J. (2000), Toxicity of manganese to *Ceriodaphnia dubia* and *Hyalella azteca*. *Arch Environ Contam Toxicol*., **38**, 298-304.

Mount, D. I. and Norberg, T. J. (1984), A seven-day life cycle cladoceran toxicity test. *Environ Toxicol Chem.*, **3**, 425-434.

Norberg-King, T. J. (1993), A linear interpolation method for sublethal toxicity: the inhibition concentration (Icp) approach. (version 2.0). U.S. Environmental Protection Agency. Environmental Research Laboratory. Duluth, Minnesota.

Oliveira-Filho, E. C.; De-Carvalho, R. R. and Paumgartten, F. J. R. (1999), The influence of environmental factors on the molluscicidal activity of *Euphorbia milii* latex. *J Environ Sci Health*., **B34**, 289-303.

Oris, J. T.; Winner, R. W. and Moore, M. V. (1991), A four-day survival and reproduction toxicity test for *Ceriodaphnia dubia*. *Environ Toxicol Chem.,* **10**, 217-224.

Norberg, T. J. and Mount, D. I. (1985), A new fathead minnow (*Pimephales promelas*) subchronic toxicity test. *Environ Toxicol Chem*., **4**, 711-718.

Sprague, J. B. (1995), Factors that modify toxicity. In- *Fundamentals of aquatic toxicology: effects, environmental fate, and risk assessment*, ed. G. M. Hand. Taylor and Francis, Washington, pp. 1012-1051.

USEPA (United States Environmental Protection Agency), (2002), Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth Edition EPA-821-R-02-013, 350p.

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