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Estimation of the critical effect level for pollution prevention based on oyster embryonic development toxicity test: The search for reliability

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Abstract

In spite of the consideration that toxicity testing is a reduced approach to measure the effects of pollutants on ecosystems, the early-life-stage (ELS) tests have evident ecological relevance because they reflect the possible reproductive impairment of the natural populations. The procedure and validation of *Crassostrea rhizophorae* embryonic development test have shown that it meets the same precision as other U.S. EPA tests, where EC_{50} is generally used as a toxicological endpoint. However, the recognition that EC_{50} is not the best endpoint to assess contaminant effects led U.S. EPA to recently suggest EC_{25} as an alternative to estimate xenobiotic effects for pollution prevention. To provide reliability to the toxicological test results on *C. rhizophorae* embryos, the present work aimed to establish the critical effect level for this test organism, based on its reaction to reference toxicants, by using the statistical method proposed by Norberg-King (Inhibition Concentration, version 2.0). Oyster embryos were exposed to graded series of reference toxicants ($ZnSO_4 \cdot 7H_2O$; $AgNO_3$; KCl; $CdCl_2H_2O$; phenol, 4-chlorophenol and dodecyl sodium sulphate). Based on the obtained results, the critical value for *C. rhizophorae* embryonic development test was estimated as EC_{15}. The present research enhances the emerging consensus that ELS tests data would be adequate for estimating the chronic safe concentrations of pollutants in the receiving waters. Based on recommended criteria and on the results of the present research, zinc sulphate and 4-chlorophenol have been pointed out, among the inorganic and organic compounds tested, as the best reference toxicants for *C. rhizophorae* ELS-test.

Keywords: Oyster embryo toxicity test; Critical effect level; Reference toxicants

1. Introduction

In spite of their limitations, aquatic toxicity tests play a crucial role in assessing the potential or actual impact of contaminants on the natural environment. They are used to estimate the "safe" concentration of effluents or pure compounds, which will permit "normal life" and propagation of organisms in the receiving waters. However, "normal life" is characterized by multiple and inter-related biological processes that can be altered by contaminants. The toxicity tests measure the integrated responses to the possible acute or chronic contaminant effects, on these processes (Weber, 1993; Klem et al., 1994).

The chronic toxicity tests data are generally more reliable, providing responses related to a complete or part of the test-species life cycle. Chronic or sub-chronic tests may have a higher ecological relevance when dealing with the embryonic development of a key species for the ecosystem at risk (Mckim, 1977; Macek and Sleight, 1977; Norberg-King, 1989; Nascimento et al., 2000a). This relevance is assured when the test results provide an estimate of an effluent or contaminant concentration that is unlikely to produce chronic toxic effects on the local biota. This estimated concentration is compared to toxicity permit limits or environmental exposure concentration to indicate the risk of environmental impact. The NOEC (no observed effect concentration), determined statistically by hypothesis testing based on chronic test results, is the effect level still currently used for environmental protection control. However,

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the NOEC value is dependent on the concentrations used in an experiment and may cause considerable biological effect. It actually means lack of statistical effects, not lack of biological effects at the determined concentration (Chapman, 1996). It was considered (Grothe et al., 1996) that IC*p*, a point estimate interpolated from contaminant concentrations, at which effects start, is far more useful and realistic than the "no effect concentration", considered very variable and susceptible to errors. The estimated effect level should be biologically significant; that is, it should protect a high proportion of all species and be predictive of an effluent or contaminant concentration that produces adverse effect in the receiving water. Point estimates, based on ELS-test results, can predict the effluent or contaminant concentration that will produce any particular level of effect.

The results of toxicity tests, analyzed by point estimates, have been usually expressed as the lethal, effective or inhibitory (LC_{50}, EC_{50} or IC_{50}) concentration causing, respectively, mortality, abnormalities and impairments in growth or reproduction to 50% of the test organisms. However, it was not clearly proved that this level of effect (50%) would protect the ecosystems from pollution. The recognition that EC_{50} or IC_{50} are not the best endpoints to assess contaminant effects, led U.S. EPA (1991) to suggest EC_{25} or IC_{25} derived by hypothesis testing procedure, as analogues of NOEC (no observed effect concentration). However, Denton et al. (1994), using a database involving responses of different organisms to toxicants, proved that the approximate concentration from where population effects start was below the value indicated as causing noxious effects to 25% of the exposed organisms, for all the test methods analyzed.

At the Pellston workshop, carried on 1996, it was suggested the gradual replacement of the NOEC value by an EC*p* estimation of the effect level. The use of EC*p* (effective concentration for a specified percentage effect) in place of NOEC, requires the value of *p* to be specified. The problem arises on how to select this percent effect level, which is still a critical decision in test data analysis. It means the proportion of organisms affected (*p*) in the estimated contaminant or effluent concentration that would cause the population to show an adverse effect (EC*p*), or the estimated concentration that would cause a percent inhibition in population growth or reproduction (IC*p*). Norberg-King (1995) suggests that, by using reference substances and calibrated bioassays, this sensibility level can be established for each test-organism. Although this level (*p*) may be set as a standard IC*p* value, a doubt remains if it is specific to a given test-species (Denton and Norberg-King, 1996).

There is an increasing utilization of the oyster (*C. rhizophorae*) embryonic development bioassay (as a sub-chronic test) for effluent toxicity determination and pollution risk prevention in Brazil. This research aimed to estimate the biologically relevant effect level for this test organism, based on its responses to different organic and inorganic reference toxicants, and to select among these toxicants, the ones that could provide higher precision and reliability to this test.

2. Material and methods

Ripe oysters *Crassostrea rhizophorae* were collected from mangrove trees in an area free of industrial or domestic wastes (Itaparica Island, Bahia, Brazil), cleaned and kept overnight in one aquarium containing filtered (20 μm) natural seawater, taken from the same area. The test followed the protocol for *C. rhizophorae* (Nascimento, 2002), developed according to American Society for Testing and Materials (1989) standard method for *Crassostrea virginica*. Immediately prior to each test, gametes were collected from mature oysters (3–6 individuals). Pooled eggs and sperm were suspended in filtered (GF/C1.2 μm) sterilized (129 °C; 1.5/cm^2) seawater. Fertilization was accomplished by transferring 2 ml of sperm to 1.0 L of a dense egg suspension. About 1 h later, embryos having undergone the first cellular division were counted in order to maintain a density of 1000 viable embryos per 100 mL in the test vessels (wide mouth glass container of 100 mL capacity). At the end of the test period (24 h), two 10-mL samples were removed, preserved in 5% buffered formalin and later examined under a compound microscope. The numbers of embryos that develop normally and abnormally were counted. Responses to the different treatments were recorded as the percentage of embryos failing to develop or are developing in an abnormal manner. Embryos, larvae without shells, and larvae with incompletely developed or with malformed shells were considered abnormal. Larvae with perfect "D" form shells were considered normal, irrespective of their size.

Dissolved oxygen, salinity, temperature and pH were maintained at >4.0 ppm, 28‰, 27±2 °C and 7.0 to 8.5, respectively, according to the test protocol (Nascimento et al., 1989; Nascimento et al., 2002).

Reagent-grade zinc sulphate, potassium chloride, cadmium chloride, silver sulphate, phenol and 4-chlorophenol were used as contaminants, while dodecyl sodium sulphate (DSS) was used as reference (positive control) for the tests. The used metals and organic compounds concentrations were based on logarithmic series (Table 1), determined after preliminary exploratory tests.

The metal–salts stock solutions have been prepared by weighing the necessary grams of the compound to yield one gram of pure metal, in a water solution of 10% HNO_3. The use of HNO_3 reduces the chance of alteration of the metal concentrations in the solution, by preventing to a certain extent, absorption to container walls or sequestration by any chelating agents that may be present in the seawater. Consequently, there was no analytical confirmation of the nominal exposure concentrations used in the test. Part of the stock solution, necessary to obtain the serial concentrations used in the tests, was diluted in sterilized seawater and distributed in adequate volumes to achieve the desired nominal concentrations in the test vials (100 mL capacity).

Table 1
Estimated EC_{50} and EC_{15}, NOEC and LOEC values obtained as results from oyster embryonic development-toxicity-test, using inorganic and organic reference substances

Reference toxicants and used concentrations	Effect levels		NOEC (mean±S.D.)	LOEC (mean±S.D.)
	EC_{50} (mean±S.D.)	EC_{15} (mean±S.D.)		
$ZnSO_4 \cdot 7H_2O$ (4.6; 10; 22; 46; 100 μg/L)	14.97±02.46	04.05±01.22	05.68±02.23	04.60±0.00
$CdCl_2 \cdot H_2O$ (24; 32; 42; 56; 75 mg/L)	282.50±36.90	114.03±30.45	159.33±24.63	150.00±0.00
$AgNO_3$ (150; 220; 320; 450; 680 μg/L)	30.30±01.28	01.47±00.74	01.46±00.62	01.11±0.30
KCl (1.0; 1.8; 3.2; 5.6; 10 μg/L)	35.56±02.93	25.13±04.47	28.26±04.13	24.00±0.00
4-Chlorophenol (19; 27; 37; 52; 72 mg/L)	20.97±04.03	12.06±03.42	13.13±03.50	10.62±1.76
Phenol (0.32; 0.56; 1.0; 1.8; 3.2 mg/L)	55.38±09.14	28.75±11.84	30.86±10.96	25.18±6.95
SDS (10; 15; 22; 32; 46 mg/L)	01.36±00.29	00.69±00.32	01.02±00.64	00.63±0.30

Table 2
Ranked MSD values obtained from oyster embryo toxicity tests, using organic and inorganic reference toxicants

Rank	Substance	MSD (% of control)	Rank	Substance	MSD (% of control)
1	Zinc sulphate	3.6	1	Dodecyl sodium sulphate	2.3
2	Cadmium chloride	3.8	2	Dodecyl sodium sulphate	3.8
3	Cadmium chloride	4.1	3	Dodecyl sodium sulphate	6.1
4	Zinc sulphate	4.7	4	Dodecyl sodium sulphate	7.1
5	Zinc sulphate	4.8	5	Phenol	7.4
6	Zinc sulphate	5.0	6	Dodecyl sodium sulphate	7.5
7	Cadmium chloride	5.4	7	Dodecyl sodium sulphate	7.8
8	Zinc sulphate	5.6	8	Dodecyl sodium sulphate	7.9
9	Zinc sulphate	5.7	9	Dodecyl sodium sulphate	8.1
10	Zinc sulphate	5.8	10	4-Chlorophenol	8.5
11	Zinc sulphate	5.8	11	Phenol	8.6
12	Potassium chloride	5.8	12	4-Chlorophenol	8.8
13	Zinc sulphate	6.0	13	Phenol	8.9
14	Cadmium chloride	6.3	14	Dodecyl sodium sulphate	9.5
15	Zinc sulphate	6.5	15	4-Chlorophenol	9.8
16	Cadmium chloride	6.6	16	4-Chlorophenol	10.0
17	Silver nitrate	7.4	17	Dodecyl sodium sulphate	10.0
18	Cadmium chloride	7.4	18	Dodecyl sodium sulphate	10.3
19	Cadmium chloride	7.5	19	4-Chlorophenol	10.6
20	Potassium chloride	8.1	20	Dodecyl sodium sulphate	11.4
21	Cadmium chloride	8.1	21	4-Chlorophenol	11.7
22	Silver nitrate	8.4	22	Phenol	11.7
23	Zinc sulphate	9.2	23	4-Chlorophenol	12.0
24	Silver nitrate	9.6	24	4-Chlorophenol	12.0
25	Silver nitrate	10.0	25	Phenol	12.3
26	Zinc sulphate	10.1	26	Phenol	12.4
27	Silver nitrate	10.1	27	Dodecyl sodium sulphate	12.5
28	Silver nitrate	10.3	28	Dodecyl sodium sulphate	13.0
29	Potassium chloride	10.4	29	Phenol	13.1
30	Silver nitrate	10.5	30	4-Chlorophenol	13.6
31	Silver nitrate	10.7	31	4-Chlorophenol	14.6
32	Zinc sulphate	10.8	32	Phenol	14.6
33	Silver nitrate	11.2	33	Phenol	14.8
34	Potassium chloride	11.3	34	4-Chlorophenol	16.2
35	Potassium chloride	11.3	35	Phenol	16.7
36	Silver nitrate	11.5	36	Phenol	16.7
37	Potassium chloride	11.6	37	4-Chlorophenol	17.0
38	Potassium chloride	12.5	38	Phenol	17.5
39	Potassium chloride	12.9	39	4-Chlorophenol	18.4
40	Potassium chloride	13.0	40	Phenol	18.8
41	Zinc sulphate	13.1	41	4-Chlorophenol	19.9
42	Potassium chloride	13.5	42	4-Chlorophenol	20.0
43	Potassium chloride	14.0	43	Phenol	20.8
44	Cadmium chloride	14.6	44	Phenol	24.6
45	Silver nitrate	15.8	45	Dodecyl sodium sulphate	28.3
46	Cadmium chloride	16.0			
47	Silver nitrate	16.1			
48	Silver nitrate	16.7			
49	Potassium chloride	16.7			
50	Potassium chloride	17.0			
51	Silver nitrate	17.3			
52	Potassium chloride	17.5			
53	Potassium chloride	17.7			
54	Cadmium chloride	18.0			
55	Silver nitrate	19.1			
56	Cadmium chloride	19.1			
57	Cadmium chloride	19.7			
58	Zinc sulphate	20.2			
59	Cadmium chloride	21.4			
60	Cadmium chloride	27.5			

Responses of organisms to chemical compounds toxicity, were expressed as percent net risk of abnormal, calculated with Abbot's formula (Finney, 1971) and analyzed by the computer statistical method Trimmed Spearman Karber (Hamilton et al., 1977, 1978) to provide EC_{50} values, equivalent to the metal concentration that may cause abnormalities to 50% of the exposed embryos. Means of the EC_{50} results, standard deviation of the means and coefficient of variation (CV) among test results have been calculated using the Graphpad Instat, version 3.0 (Graphpad Software Inc., 1997).

The results have also been analyzed by hypothesis test method, using the software Toxstat 3.3 (Gulley et al., 1991) to estimate the minimum significant difference (MSD), calculated as a percentage of the obtained response in the control (% MSD = MSD / Control mean × 100) for each test. The data have been arc–sin transformed, analyzed by ANOVA and Dunnett's test, after passing the tests for normality (Shapiro–Wilks and χ^2) and homogeneity (Hartley and Bartlett). Using Excel, the values have been ranked to determine which

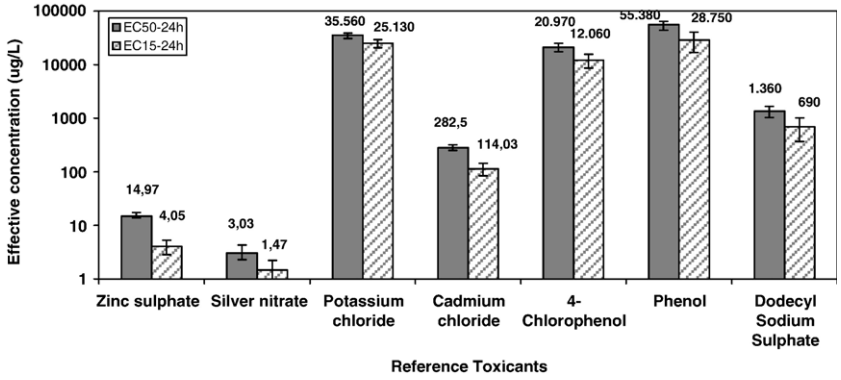

Fig. 1. Comparison between EC_{15} and EC_{50} mean values ($n=15$) and standard deviation resulting from oyster embryos exposed to inorganic and organic reference toxicants.

correspond to the 75th percentile. The biologically relevant effect concentration has been estimated using the Inhibition Concentration method (IC*p* Version 2.0, edited by Norberg-King, 1993); this method provides, by interpolation, a point estimate of a single concentration causing the specified percentage effect (Environment Canada, 1992). According to Shukla et al. (2000), the definition of this biologically acceptable percent effect may be carried out on historic data. In the present research it was applied to 105 different test results (Table 2).

3. Results and discussion

Due to the ease of obtaining "in vitro" fertilization, bivalves have been used world-wide to provide biological material for embryo–larval bioassay (Calabrese et al., 1973; Robert and His, 1985; Beiras and His, 1995; His et al., 1997; Nascimento et al., 2000a,b).The oyster early-life-stage

Table 3
EC_{50} and EC_{15} values and their respective confidence intervals (95% CI) obtained from oyster (*C. rhizophorae*) embryos toxicity test, using inorganic reference substances

Potassium chloride			Cadmium chloride			Silver nitrate			Zinc sulphate		
Test number	EC_{50} (mg/L)	CI (95%)	Test number	EC_{50} (µg/L)	CI (95%)	Test number	EC_{50} (µg/L)	CI (95%)	Test number	EC_{50} (µg/L)	CI (95%)
1	38.03	35.81–40.40	1	264.59	245.28–285.42	1	2.73	2.56–2.92	1	14.25	13.00–15.63
2	30.89	29.67–32.16	2	286.96	264.55–311.27	2	2.63	2.43–2.85	2	12.11	11.50–12.75
3	32.96	31.89–34.06	3	316.23	285.77–349.95	3	2.73	2.52–2.95	3	12.43	9.95–15.52
4	40.57	38.81–42.40	4	300.65	278.33–324.76	4	1.34	1.22–1.47	4	12.55	10.13–15.54
5	41.40	39.75–43.11	5	292.07	261.10–326.71	5	1.31	1.05–1.64	5	17.31	14.60–20.52
6	36.77	34.79–38.85	6	370.53	321.72–426.75	6	2.85	2.52–3.22	6	11.24	8.74–14.47
7	38.06	36.73–39.43	7	262.66	242.43–284.58	7	1.43	1.08–1.88	7	18.18	15.20–21.73
8	33.00	31.80–34.23	8	262.51	198.08–264.82	8	5.69	5.27–6.15	8	15.26	12.54–18.56
9	33.48	32.35–34.64	9	246.79	220.47–276.24	9	3.94	3.63–4.27	9	11.87	9.55–14.74
10	33.98	32.86–35.10	10	291.99	271.37–314.18	10	3.36	2.46–4.60	10	15.77	13.45–18.48
11	35.36	33.34–37.48	11	247.11	223.26–273.51	11	2.59	2.38–2.82	11	19.09	16.36–22.28
12	34.97	32.94–37.13	12	211.77	177.53–252.62	12	2.92	2.65–3.22	12	15.10	13.23–17.25
13	35.65	34.18–37.16	13	276.56	255.19–299.71	13	5.07	4.49–5.71	13	16.82	14.69–19.26
14	34.84	33.27–36.47	14	309.71	284.31–337.37	14	2.61	2.44–2.80	14	16.12	13.07–19.89
15	33.50	32.24–34.80	15	297.38	275.28–321.26	15	4.34	3.88–4.86	15	16.54	14.03–19.51

Potassium chloride			Cadmium chloride			Silver nitrate			Zinc sulphate		
Test number	EC_{15} (mg/L)	CI (95%)	Test number	EC_{15} (µg/L)	CI (95%)	Test number	EC_{15} (µg/L)	CI (95%)	Test number	EC_{15} (µg/L)	CI (95%)
1	24.53	21.06–27.68	1	126.73	83.05–171.87	1	1.97	1.88–2.05	1	3.34	3.18–3.49
2	24.20	18.93–25.51	2	121.50	95.19–167.77	2	1.83	1.41–2.00	2	3.75	3.55–4.13
3	26.47	25.00–28.17	3	94.63	88.17–104.87	3	1.91	1.59–2.08	3	2.99	2.40–4.21
4	32.17	28.28–33.19	4	143.50	109.12–169.38	4	0.42	0.28–0.64	4	2.49	2.34–2.67
5	33.33	23.31–34.77	5	122.51	107.01–174.16	5	0.33	0.27–0.51	5	4.29	3.08–5.77
6	15.92	12.05–28.91	6	118.43	82.80–132.80	6	1.06	0.72–1.27	6	2.10	1.91–2.38
7	22.00	15.54–32.57	7	145.28	114.45–157.72	7	0.35	0.26–0.57	7	4.30	3.23–6.32
8	26.09	26.01–26.16	8	67.20	54.78–78.75	8	2.94	2.58–3.68	8	4.12	3.18–5.31
9	26.60	24.74–27.60	9	82.01	55.19–158.36	9	2.03	1.37–2.51	9	2.82	2.64–3.20
10	26.94	25.72–27.19	10	173.77	104.12–204.83	10	0.86	0.01–2.00	10	4.30	3.12–5.67
11	20.43	14.71–25.27	11	92.34	72.94–142.25	11	1.65	0.92–1.96	11	5.76	5.37–6.25
12	19.45	14.52–25.13	12	59.89	43.24–79.77	12	1.33	1.23–1.46	12	5.77	4.19–7.09
13	26.67	25.06–28.74	13	115.47	83.52–167.32	13	1.81	0.80–2.48	13	6.09	5.19–7.53
14	25.36	24.67–26.20	14	117.40	110.59–126.90	14	1.90	1.56–2.04	14	3.65	2.82–5.09
15	26.86	25.83–28.48	15	129.84	107.45–157.21	15	1.80	1.32–3.25	15	5.05	3.84–6.00

Table 4
EC_{50} and EC_{15} values and their respective confidence intervals (95% CI) obtained from oyster (*C. rhizophorae*) embryos toxicity test, using organic reference substances

Phenol			4-Chlorophenol			Dodecyl sodium sulphate		
Test number	EC_{50} (mg/L)	CI (95%)	Test number	EC_{50} (mg/L)	CI (95%)	Test number	EC_{50} (mg/L)	CI (95%)
1	70.21	65.27–75.53	1	23.75	22.44–25.13	1	2.03	1.81–2.28
2	67.57	63.05–72.41	2	18.64	17.84–19.47	2	1.15	0.99–1.34
3	44.41	40.58–48.60	3	30.27	28.28–32.39	3	13.29	1.20–1.38
4	50.45	48.27–52.73	4	14.37	13.81–14.95	4	1.38	1.30–1.48
5	54.61	51.29–58.15	5	19.59	18.29–20.99	5	1.33	1.24–1.43
6	41.93	37.78–46.55	6	21.90	20.54–23.35	6	1.33	1.23–1.44
7	52.13	49.70–54.67	7	20.05	17.12–23.47	7	1.13	1.05–1.20
8	48.10	41.77–55.39	8	20.91	16.92–25.84	8	1.13	10.6–1.21
9	49.93	45.88–54.34	9	22.55	21.42–23.73	9	1.22	1.16–1.28
10	66.20	61.96–70.74	10	24.11	22.42–25.93	10	1.10	1.02–1.18
11	55.69	51.29–60.47	11	22.58	21.44–23.78	11	1.29	1.18–1.41
12	44.18	38.07–51.26	12	19.24	17.34–21.35	12	1.76	1.68–1.85
13	58.41	48.78–69.93	13	16.46	15.88–22.31	13	1.89	1.77–2.02
14	63.70	61.05–66.47	14	15.58	10.88–22.31	14	1.34	1.25–1.44
15	63.31	62.79–65.94	15	24.61	23.10–26.23	15	1.11	0.92–1.33

Phenol			4-Chlorophenol			Dodecyl sodium sulphate		
Test number	EC_{15} (mg/L)	CI (95%)	Test number	EC_{15} (mg/L)	CI (95%)	Test number	EC_{15} (mg/L)	CI (95%)
1	40.50	38.93–42.12	1	16.39	15.70–16.95	1	0.31	0.21–0.64
2	22.66	20.50–24.89	2	11.26	7.73–14.42	2	0.19	0.16–0.21
3	24.21	23.40–25.68	3	16.34	15.16–17.29	3	0.89	0.77–1.00
4	37.19	24.42–52.34	4	11.22	11.01–11.43	4	1.02	0.86–1.10
5	12.38	10.97–15.48	5	11.38	7.56–12.46	5	0.98	0.89–1.03
6	20.72	14.03–25.65	6	13.06	11.64–16.16	6	1.03	0.97–1.08
7	42.88	32.65–54.33	7	7.29	6.73–8.40	7	0.59	0.50–0.74
8	34.60	28.90–52.28	8	8.62	7.75–10.41	8	0.61	0.48–0.81
9	27.52	15.87–39.21	9	17.26	15.70–20.19	9	0.89	0.72–1.03
10	15.09	13.13–18.24	10	13.29	11.93–15.49	10	0.55	0.46–0.77
11	13.02	11.86–19.02	11	14.82	13.04–16.41	11	0.85	0.66–1.10
12	53.38	48.56–55.44	12	6.42	4.66–6.42	12	0.70	0.48–0.79
13	25.14	20.20–36.97	13	11.48	10.84–13.10	13	0.24	0.05–0.25
14	25.32	13.95–53.44	14	7.95	5.68–12.72	14	1.0	0.84–1.09
15	36.65	33.75–40.31	15	14.21	10.68–16.48	15	0.17	0.16–0.18

test has been developed by Woelke (1972) for *Crassostrea gigas* and standardized by ASTM (1989) for *C. virginica*. Nascimento et al. (1989) has established the protocol for *C. rhizophorae* based on ASTM recommended standard method. This protocol has been revised lately (Nascimento et al., 2002). Intro- and inter-laboratory validation of the test procedure have been performed (Araújo et al., 2003) and showed that it meets the precision of current U.S. EPA tests (acceptable CV <40%).

The primary objective of the toxicity tests is to insure that contaminants or effluents discharged into receiving water systems do not adversely affect aquatic life (Klem et al., 1994). The search to fulfill this aim relied mostly on analysis of test results, based on hypothesis test procedures (e.g., NOEC) or point estimation techniques (e.g., EC*p*). Although the hypothesis test method has been largely used to support regulatory actions, there are criticisms to its use for this purpose, due to

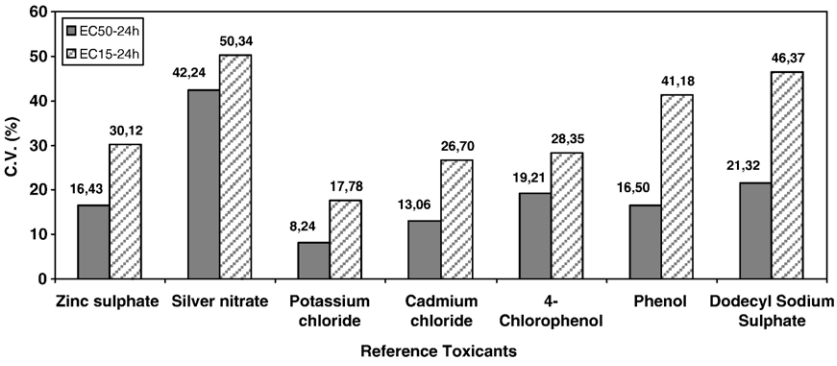

Fig. 2. Coefficient of variation between oyster embryonic development tests based on EC_{15} and EC_{50} endpoints.

the method's weaknesses (Stephan and Rogers, 1985; Chapman, 1996; Bailer and Oris, 1999). Some improvements addressing the disadvantages of the method have been proposed (Oris and Bailer, 1993; Erickson and McDonald, 1995; Thrusby et al., 1997), while, at the same time, there were suggestions for its substitution by other more powerful and precise statistical methods (Bruce and Versteeg, 1992; Hoekstra and Vanewijk, 1993; Moore and Caux, 1997; Bailer and Oris, 1997).

Results of ELS tests are generally expressed as EC_{50}, a value determined by point estimation that is used for risk assessment, as an alternative to NOEC. The comparison between the estimated value of EC_{50} and the environmental exposure concentration of a contaminant or effluent is used to assess the risk of pollution. However, it is already accepted that EC_{50} values do not guarantee the environmental safeguard (Denton et al., 1994), even though this point estimate can be useful for comparing the toxicity of different chemicals and effluents. Statistically, it represents the lowest variability surrounding a toxicity value; however, it may not correspond to the biologically safe concentration of the tested contaminant or effluent.

The present research compares the classical toxic effect values (NOEC, LOEC and EC_{50}) to an alternative level of effect (EC_{15}) determined for the first time, specifically for the oyster embryonic toxicity test (Table 1). Fig. 1 shows clearly the differences between EC_{50} and EC_{15}. This alternative level of effect, representing a concentration where the noxious effects start, was determine based on results of 60 tests (using inorganic compounds), and 45 tests (involving organic compounds), indicated as reference toxicants (Environment Canada, 1990).

The lack of knowledge about the level of adverse effect, which would have biological significance for the ecosystem at risk, has been the main difficulty in substituting the hypothesis test by the adoption of the point estimate technique. For the determination of the acceptable effluent or contaminant concentrations derived from toxicity tests data, the adoption of EC*p* approach requires a dose–response model and the selection of *p* value, which could be equivalent to the threshold chronic effect level. In the present study, it has been possible to estimate the appropriate biological level of effect for the EC*p* method, by calculating the minimum significant difference (% MSD) between one treatment and the control, for each assay (Chapman, 1996; Denton and Norberg-King, 1996). Chapman (1996) recommended the utilization of the MSD critical, corresponding to a value indicated as the equivalent to the 75th percentile of a series of test results as the critical level to be considered, either by using hypothesis test method or point estimate techniques.

In the present research, the critical MSD values (Table 2), calculated by the IC*p* computer program, for both organic ($p=16.2\%$) and inorganic ($p=15.85\%$) reference toxicants, pointed out EC_{15} as the alternative effect level to EC_{50}, for the oyster early-life-stage test. According to Denton and Norberg-King (1996), the EC*p* approach is advantageous especially because the precision can be quantified and the confidence intervals may be calculated. These intervals indicate, with a probability of 95%, the range where the estimated effect value can be found, resulting from the experimental data. As a consequence, Chapman (1996) admit the usage of this range as an acceptable dispersion for the estimation of impacts. For regression methods, it is then important that the confidence interval be limited to values near the punctual estimated effect level, what indicates the fitting to the used statistical model. This was achieved in the present research (Tables 3 and 4), which involved 15 test results for each reference toxicant.

Reference toxicant is normally used to assess, under standardized test conditions, the relative sensitivity of the organisms used as test-species and the precision of data produced for this substance. The test precision has been defined as a general measure of test reproducibility in a single laboratory, over time (Environment Canada, 1990). However the use of reference toxicants is also essential for inter-laboratory comparison to establish a more secure protocol, which can provide reliability and reproducibility to the test.

Test precision is a requested characteristic to establish the chronic effect level, which is, for the first time, determined for *C. rhizophorae* embryonic development test. Generally the coefficient of variation (CV) is one indicator of test-precision. Environment Canada (1990) tentatively suggested a coefficient of variation of 20% or 30% as a limit. The present research showed that, except for silver, all the others metals tested provided results (Fig. 2) that are below the suggested maximum limit, both for the classical (EC_{50}) and the alternative (EC_{15}) effect concentrations. However, those proposed limits have been surpassed by the organic reference toxicants DSS and phenol, whose CV values among tests have been higher (>40).

The test precision achieved in the present research certifies the reliability of the obtained data, even though it would be valuable a possible comparison with data previously obtained for related species. Available data, referring specifically to the toxicity of metals for oysters embryos and larvae, relate mostly to species from a temperate climate (Calabrese et al., 1973; MacInnes and Calabrese, 1978; MacInnes, 1981), making it difficult comparing with the obtained data for *C. rhizophorae*, a tropical species. The temperature and salinity ranges, required by the different species, maintained during the tests, provide differences in the toxicity responses (MacInnes and Calabrese, 1979; Pereira et al., 1998). However, where the comparison was possible, the data showed that *C. rhizophorae*, in general terms, is more sensitive to metals than *C. gigas* (Woelke, 1972) and still more sensitive to zinc and silver than *C. virginica* (Calabrese et al., 1973). On the other hand, few data are available on the use of reference organic toxicants for the precision control of the oyster embryonic test (Araújo et al., 2003). The present research provided data to enhance the knowledge in this field and yielded results that could contribute to point out the best reference substance to be utilized in this type of test. Based on different criteria such as detection of abnormal organisms, established toxicity database, solubility, stability in solution, availability in pure form and limited intro-laboratory water quality effects (Environment Canada, 1990), zinc sulphate was selected as the best reference contaminant among the inorganic substances tested. Among the organic reference toxicants, 4-chlorophenol showed the best performance.

4. Conclusions

This research pointed out the EC_{15} as an alternative effect level, which can be equivalent to the chronic value for the early-life-stage test on *C. rhizophorae* embryos. Based on the assumption that it represents a point estimate interpolated from contaminant concentration, at which effects start, this concentration will permit the normal propagation and development of the exposed population, therefore being a highly relevant biological endpoint.

Zinc, as $ZnSO_4 \cdot 7H_2O$ was pointed out as the best reference substance for the *C. rhizophorae* embryonic development test, based on a series of criteria, which elected also the 4-chlorophenol as the one having the better performance between the tested organic reference toxicants.

The low-test variability and high reproducibility of the results have showed the analytical precision of the tests. Except for Ag, phenol and DSS, whose CV values were above 40%, all the others reference toxicants achieved the Environment Canada precision standards.

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