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Acute and Chronic Toxicity Test Methods for *Nematostella vectensis* Stephenson

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The Starlet Sea Anemone, *Nematostella vectensis* Stephenson, is a small burrowing anemone in the family Edwardsiidae (Hand 1957). These anemones are found in shallow estuarine environments and are widely distributed in the United States and Europe. They are euryhaline, preferring salinities of 10–26 ppt, but tolerating salinities up to 51 ppt (Williams 1983). They are also eurythermal, tolerating temperatures from 5–28.5°C (Bleakney and Meyer 1979). Although they normally inhabit estuarine sediments, they do not require a sediment substrate and can be maintained for long periods in aquaria containing natural or synthetic seawater (Hand 1992). They reproduce asexually by transverse fission, and can be induced to develop eggs that begin division and gastrulation 12–15 hours following fertilization. Planula larvae emerge from the egg sac approximately 36–48 hours after fertilization, and swim by means of cilia. The planula larvae develop four tentacles after about 5 days, and settle after 1 week. A second set of four tentacles develops after 2–3 weeks; fully mature individuals develop a full complement of 16 tentacles within about 8 weeks. Sexual and asexual reproduction may occur at 10 weeks (Hand 1992).

Because of its ease of culture and ubiquitous distribution, *N. vectensis* seemed an excellent aquatic toxicity test organism. Our research objective was to determine the feasibility of using *N. vectensis* for acute and chronic toxicity tests. We selected $CdCl_2$ as the toxicant because it is widely used in aquatic toxicity bioassays and would allow comparison to the LC_{50} results for other common test organisms.

Although *N. vectensis* appears to be quite common along the Pacific and Atlantic coastlines, it is considered threatened in portions of its range, and was listed as "vulnerable" by the International Union for Conservation and placed on the "Red List" of threatened species (2002 IUCN Red List of Threatened Species, www.redlist.org). The causes for this listing were primarily habitat loss and environmental pollution. Being a sediment dweller, *N. vectensis* is quite vulnerable to pollutants that settle into its estuarine habitat. Accordingly, our second objective was to contribute to the general knowledge concerning culturing and maintaining these organisms, which will be essential in any reintroduction plans for the species.

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MATERIALS AND METHODS

Although *N. vectensis* is normally found in estuarine sediments, it thrives in culture on hard substrates as long as it is kept at the proper salinity and temperature (i.e., out of direct sunlight). It can be cultured in natural or artificial seawater under a range of salinities and does not require aeration as long as the culture dishes are wide and shallow to allow oxygen exchange. It may be cultured under ambient conditions on a laboratory bench or in an environmental chamber. *Nematostella* are sensitive to vibration, so fan motors such as those used in environmental chambers may cause some degree of stress (Harter 1997). Salinity transitions should be gradual (2-3 ppt per day). Once at the desired salinity, aquaria should be cleaned weekly by scraping the sides with a rubber spatula to dislodge debris, decanting the overlying water, and refilling the aquaria with fresh seawater.

Nematostella for our tests were collected from Padilla Bay, Washington (46° 27′ 30″ N, 122° 27′ 30 W″). The top 2–3 cm of sediment was skimmed using a plastic scoop to collect an equal volume of seawater and sediment. The sediment/seawater mixture was placed in a container for transport to the laboratory. At the same time, 1–2 L of overlying seawater was collected, and the salinity at the site was recorded. In the laboratory, the sediments were placed in large, shallow glass aquaria (9 × 12 inch glass casserole dish). The aquaria were half filled with sediment and approximately 1 L of seawater from the collection site was added. The sediments were mixed gently and allowed to settle for 10–30 minutes. A light directed through the side of the aquaria revealed small (<2 cm) anemones on top of the sediment. Using forceps, anemones were gently removed to shallow, glass culture dishes containing filtered seawater from the collection site. When all visible anemones were removed, the sediments were resuspended, allowed to settle, then examined for anemones. The mixing/settling routine continued until no additional anemones were found.

Nematostella were cultured as described above and fed one drop of brine shrimp every other day. The brine shrimp volume was adjusted depending on the number and size of anemones and the concentration of brine shrimp to avoid fouling the water with uneaten, decomposed food. To induce sexual reproduction, the anemones were fed minced (1–2 mm), frozen, mussel gonad weekly. Females were cultured separately from males. (Males are uncommon, and comprised only ~7% of the individuals collected from Padilla Bay.) Mature females were identified by egg production, which was apparent about four weeks after collection. Females were segregated into female-only aquaria once positively identified. Positively identified males and questionable individuals were placed in separate aquaria.

A variety of testing conditions are possible with this anemone, including acute and chronic tests using sediments or purely aqueous solutions. Because *N. vectensis* can burrow into sediments, they can stay anchored in continuous flow or static-renewal tests. Our methods describe development of a 96-hr acute aqueous (sediment-free) test and a 21-day chronic aqueous (with renewal) test to measure $CdCl_2$ toxicity. Cadmium chloride was selected as a toxicant because data are available to compare

N. vectensis results with other commonly used marine and estuarine test organisms. Nominal cadmium exposures were calculated for each test, and water and tissue samples from the 21-day chronic tests were sent to a certified laboratory (AmTest, Inc., Kirkland, WA, USA) for analysis. These analyses indicated that approximately 20% of the cadmium dose was lost from the water column, probably due to adsorption onto glass surfaces (Harter 1997). Cadmium concentrations in anemone tissue samples increased according to dose, but there was no way to determine whether this represented external adsorption or actual uptake of cadmium.

The 96-hr acute toxicity tests were conducted by placing mature, acclimated female anemones into acid-cleaned glass beakers containing 100 mL filtered seawater diluted with distilled, deionized water to 10 ppt. Depending on the test, 8 or 10 individuals of similar size were randomly assigned to each of 3 or 4 replicate treatment groups. All individuals were cultured in the laboratory for at least 3 months. Toxicant concentrations ranged from 0.000–2.200 mg $CdCl_2$/L (0.000–1.349 mg Cd/L). Beakers were covered loosely to prevent contamination, but allow air exchange, and placed in a controlled environment room at 20° C on a 12 hr light/dark cycle (163–216 lux). Test beakers were checked daily and mortality was recorded (mortality was defined as the absence of reaction to gentle prodding). Dead individuals were removed daily. No feeding or water exchanges were done during the acute tests. Dissolved oxygen, pH, salinity, and water temperature were monitored daily.

The 21-day chronic toxicity tests were conducted by placing mature, acclimated female anemones into acid-cleaned glass culture bowls (110 mm diameter) containing 200 mL filtered seawater diluted to 12 ppt. Eight female anemones were used for each replicate; one male anemone was included in each replicate during the second chronic test to fertilize egg masses (fertilization endpoints are discussed in Harter 1997). All individuals were cultured in the laboratory 7–7.5 months. Toxicant concentrations ranged from 0.000–2.200 mg $CdCl_2$/L (0.00–1.349 mg Cd/L). Bowls were covered loosely and placed in a controlled environment room at 20° C on a 12 hr light/dark cycle (163–216 lux). Anemones were fed brine shrimp every other day and cadmium solutions were changed once a week to coincide with feeding of mussel gonad. Response variables were measured daily (egg production, mortality) or weekly (weight). Dead individuals and egg masses were removed daily. Dissolved oxygen, pH, salinity, and water temperatures were measured in all dishes at the beginning of the test, following each water exchange, at the end of the test, and daily in one replicate from each test group.

Statistical analyses were conducted using SPSS (version 11.0.1) or R (www.r-project.org). Parametric assumptions were tested using Shapiro-Wilk's and Bartlett's tests (Zar 1996). Analysis of variance tests with paired student's t-tests and Holm's p-value adjustments (parametric data) or Kruskal-Wallis rank sum tests with pair-wise Wilcox test and Holm's p-value adjustments (nonparametric data) were used to test for significant differences between treatments and to determine whether within-treatment replicates could be pooled (Holm 1979; Zar 1996). The LC_{50} values and 95% confidence intervals were estimated using probit (Zar 1996).

RESULTS AND DISCUSSION

Three 96-hr acute and two 21-day chronic toxicity tests were conducted (Table 1). The first 96-hr test was used for range-finding; 96-hr tests 2 and 3 produced LC_{50} values of 2.11 and 1.78 mg $CdCl_2$/L (1.284 and 1.092 mg Cd/L), respectively. These results were comparable to 96-hr acute toxicity results for marine and estuarine amphipods (Table 2). The first 21-day chronic test was used to measure chronic mortality. All but the lowest dose produced mortalities that were significantly different from the control, and resulted in a 21-day LC_{50} of 0.31 mg $CdCl_2$/L (0.190 mg Cd/L).

The second 21-day chronic test was designed to measure nonlethal endpoints (egg production and weight gain/loss), so the maximum dose was reduced to 0.500 mg $CdCl_2$/L. Anemone weight and egg production data did not meet assumptions for parametric testing, and conventional transformations did not solve the problem, so nonparametric statistics were used for descriptive and confirmative statistical tests.

Table 1. *Nematostella vectensis* 96-hr acute and 21-day chronic $CdCl_2$ toxicity results. Doses are in mg $CdCl_2$/L. No LC_{50} calculated for range-finding tests.

Test	Salinity (ppt)	Time in Culture	Reps/ Dose (#/Rep)	Dose ($CdCl_2$ mg/L)	Mort/ Rep	Avg Pct Mort	LC_{50} (95% CI)
96-hr	10	3 mo	3(10)	0.0004	0/0/0	0	na
#1				0.0041	0/0/0	0	
(range-				0.0450	0/0/0	0	
finding)				0.5000	9/9/8	86.7	
96-hr	10	3.5 mo	3(10)	0.000	0/0/0	0	2.11
#2				0.038	0/0/0	0	(1.60-
				0.413	2/1/3	46	2.84)
				4.545	10/10/9	96.7	
				50.00	10/10/10	100	
96-hr	12	7 mo	4(8)	0.000	0/0/0/0	0	1.78
#3				0.050	0/0/0/0	0	(1.55-
				0.250	0/0/0/0	0	2.02)
				0.750	0/0/0/1	2.5	
				2.200	7/4/6/8	65.8	
21-day	12	7 mo	4(8)	0.000	0/0/0/0	0	0.31
#1				0.050	0/0/0/0	0	(0.26-
(lethal				0.250	2/4/3/3	37.5	0.45)
endpts)				0.750	8/8/8/8	100	
				2.200	8/8/8/8	100	
21-day	12	7.5 mo	4(8)[a]	0.000	0/0/0/0	0	na
#2				0.075	0/0/0/0	0	
(nonlethal				0.250	0/0/0/0	0	
endpts)				0.500	3/2/1/0	18.8	

[a]Test chambers contained 8 females and 1 male. Toxicity data are for females.

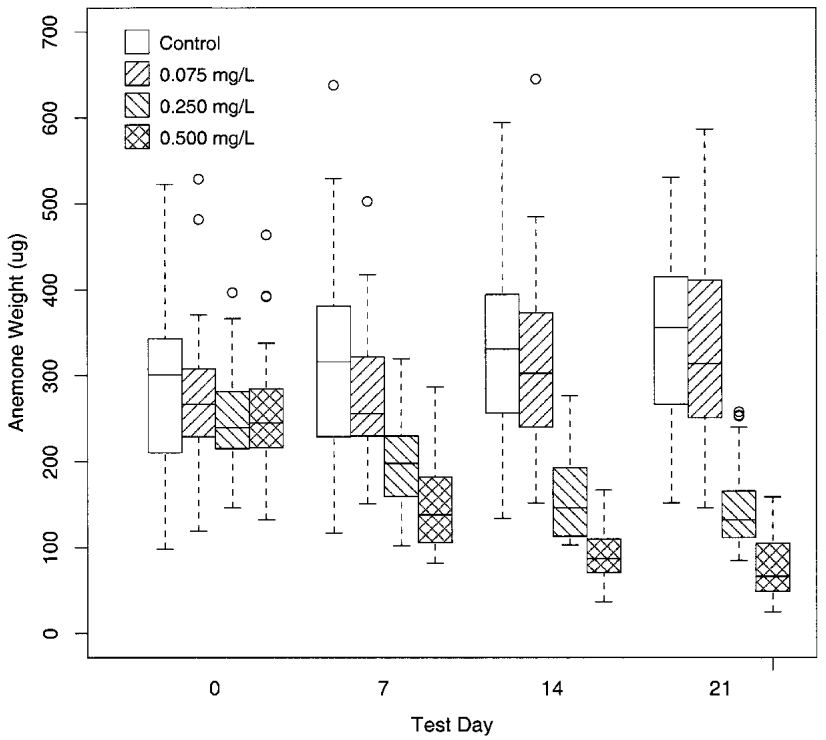

Figure 1. Boxplots of *N. vectensis* weights from the second 21-day chronic toxicity test. Boxes show weekly median, inner quartile range (IQR = 25% above and below median), whiskers (min/max values $\leq$1.5 x IQR), and outliers (>1.5 x IQR) for each treatment group consisting of 32 anemones.

Table 3. *Nematostella vectensis* egg production during the second 21-day chronic toxicity test. All doses are reported as mg-$CdCl_2$/L.

Dose (mg/L)	Min.	Median	Mean	Max.	Total	N
			Egg Counts, Days 1–21			
0.000	0	4	516	3159	43328	84[a]
0.075	0	0	554	4902	46551	84
0.250	0	0	433	4667	36381	84
0.500	0	38	265	2086	22253	84
			Reproducing Females Only, Days 1–21			
0.000	9	622	1032	3159	43328	42 (50%)[b]
0.075	8	580	1225	4902	46551	38 (45%)
0.250	4	3420	910	4667	36381	40 (48%)
0.500	2	96	365	2086	22253	61 (73%)

[a]Cumulative daily egg production from 8 females per test chamber.
[b]Cumulative daily egg production from reproducing females.

Table 2. Comparison of *N. vectensis* $CdCl_2$ 96-hr LC_{50} to amphipod data.[a]

Species	LC_{50} (mg/L)	Salinity (ppt)	Range USA, W. Coast	Range USA, E. Coast	Other
Ampelisca abdita	0.33	20–35	X	X	
Repoxynius abronius	0.92	25–28	X		
Leptocheirus plumulosus	1.06	2–32		X	
Grandidierella japonica	1.17	16–34	X		Japan
Nematostella vectensis	1.78–2.11	10–12	X	X	Europe
Eohaustorius estuarius	9.33	2–28	X		

[a]Amphipod toxicity data from ASTM (1995).

There were no significant differences in the initial anemone weights (Figure 1, day 0) and there were no significant differences between the control and 0.075 mg $CdCl_2$/L treatment groups on days 7, 14, and 21. By day 7, the 0.250 and 0.500 mg/L treatment groups were significantly lower than the control and 0.075 mg/L groups ($p < 0.05$, pair-wise Wilcox test with Holm's p-value adjustment). This difference persisted through the end of the experiment.

There were no significant differences between daily egg counts in the treatment groups, but this was due to a large number of "zero" counts in all treatments due to nonreproducing females (Table 3). Typically, only about half of the test chambers contained eggs (Table 3, Reproducing Females Only). When the data from reproducing females were analyzed, there were significantly fewer eggs in the 0.500 mg/L treatment group ($p < 0.05$, pair-wise Wilcox test with Holm's p-value adjustment). There appeared to be a stress response to cadmium, resulting in an increase in reproductive frequency but an inhibition of total egg production. By the end of the test, the control group produced a total of 43,328 eggs, with eggs present in 50% of the test chambers. By comparison, the 0.500 mg $CdCl_2$/L treatment group produced only 22,253 eggs, but eggs were present in 73% of the chambers.

In our preliminary tests, *N. vectensis* exhibited similar sensitivity to cadmium as other marine and estuarine test organisms. As a test species, *N. vectensis* offers a number of advantages. The species is indigenous to many regions in the United States and Europe, can be cultured easily over a wide range of temperatures and salinities, and can be induced to produce eggs year-round. The species can reproduce asexually, which allows cloning if a genetically uniform population is required. Because *N. vectensis* is now a species of concern in Europe, it will be important to continue developing culturing and testing protocols for working with this organism.

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