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Tolerance of the Nematode *Caenorhabditis elegans* to pH, Salinity, and Hardness in Aquatic Media

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Abstract. The toxicity of many chemicals depends on the physical conditions of the test environment, and any change or adjustment made to the tests can alter the results. Therefore it is important to establish the sensitivity of the test organism over a range of test conditions to determine when it is necessary to make adjustment and to what extent. In this study, we established the tolerance range of the nematode *Caenorhabditis elegans* for pH, salinity and hardness using 24- (without food source) and 96-h (with food source) aquatic toxicity tests. The tests were performed in two media: K-medium and moderately hard reconstituted water (MHRW). *C. elegans* has high tolerance under these test conditions. In K-medium worms survived a pH range of 3.1 to 11.9 for 24 h and 3.2 to 11.8 for 96 h without significant ($p > 0.05$) lethality. In MHRW the pH range was 3.4 to 11.9 for 24 h and 3.4 to 11.7 for 96 h. Salinity tolerance tests were approximated with NaCl and KCl individually. Up to 15.46 g/L NaCl and 11.51 g/L KCl were tolerated by *C. elegans* in K-medium without significant lethality ($p > 0.05$). In MHRW higher salt concentrations were tolerated; about 20.5 g/L NaCl and 18.85 g/L KCl did not show any adverse effect compared to control. Hardness tolerance was tested by adding $NaHCO_3$. The nematode could tolerate 0.236 to 0.246 g/L of $NaHCO_3$. The high tolerance of *C. elegans* to these test conditions (pH, salinity, and hardness) allows more versatility than other organisms commonly used in aquatic toxicity tests. It also allows the monitoring of effluents and receiving waters from freshwater or estuarine sources without dilution or adjustment.

Nematodes are the most numerous of all the multi-cellular organisms (Platt *et al*. 1984). Almost 500,000 species of nematodes are known worldwide (Hyman 1951). However, they are virtually unrepresented in standardized soil and water toxicity testing protocols. The nematode *C. elegans* is the most thoroughly studied and most completely understood metazoan in terms of molecular and classical genetics, development, behavior, and anatomy. *C. elegans* has been widely used in molecular biology studies because of its ease of use, short lifespan, cellular simplicity, and genetic manipulability. It is the only animal with an entirely known cell lineage from fertilized egg to all 810 cells in the somatic tissues of the adult (Sulston *et al*. 1988), and a completely mapped nervous system (Wood 1988). Williams and Dusenbery (1990a) first proposed using this organism for aquatic toxicity testing, and since then many other researchers have investigated its potential (Honda and Matsuo 1992; Kammenga *et al*. 1994; Stringham and Candido 1994; Donkin and Williams 1995; Donkin *et al*. 1995). Rapid toxicological bioassays using *C. elegans* also have been developed in agar medium (Williams and Dusenbery 1988, 1990b) and in soil (Donkin and Dusenbery 1993, 1994).

In spite of the wide research done on *C. elegans*, data on several physical parameters of the environment have not been investigated. Physical parameters such as pH, temperature, salinity, and hardness of the medium are important not only for the optimum growth and development of the nematode, but may also have a significant effect on the toxicity of certain chemicals (USEPA 1992). *C. elegans* grow over a temperature range of 16 to 28°C (Hedgecock and Russell 1975; Anderson 1978), and investigators commonly use 20°C. Tolerance of *C. elegans* to pH, salinity, and hardness has been qualitatively suggested by many researchers (Dusenbery 1974; Wood 1988; Donkin and Williams 1995; Donkin *et al*. 1995), but limited quantitative data can be found in the literature.

In this study the tolerance of *C. elegans* to pH, salinity, and hardness was determined for aquatic media. The data were collected for both acute (24 h without a food source) and chronic (96 h with a food source) exposures.

Material and Methods

Maintenance and Synchronization of Nematode Culture

Caenorhabditis elegans (N2, wild type strain) were maintained in the dauerlarval state in an M9 buffer at 20°C, and renewed monthly (Cox *et al*. 1981). Dauerlarvae occur in the life cycle of *C. elegans* when, in the absence of a food source, the worms enter an alternate life stage of arrested growth (Brenner 1974; Cassada and Russell 1975). The dauers were used to obtain adult worms by transferring them onto a K-agar p|late (Williams and Dusenbery 1988) with a mature lawn of a uracil

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deficient strain of *Escherichia coli* (OP50) as a food source (Brenner 1974). The plates were incubated at 20°C for two days to obtain gravid adults.

Eggs and adults were centrifuged at 2.5 K rpm for 3 min in a 15-ml centrifuge tube to make a pellet. The eggs were then isolated from worms by using a mild bleach solution of 1% NaClO and 0.013 M NaOH (Emmons *et al*. 1979), and rinsed 3 times with K-medium. Synchronized adult cultures were produced by transferring the eggs to a K-agar plate with an established lawn of OP50. Adult worms were obtained after three days of incubation at 20°C.

Experimental Design and Test Conditions

The concentrations of test solutions were made to include the entire tolerance ranges for pH, salinity, and hardness. Tests were performed by gradually increasing the parameter concentration until the next concentration of the test parameter resulted in lethality which was significantly different ($p < 0.05$) from the control. Control mortality of <10% (5% preferred) is considered acceptable in aquatic bioassays by the Water Pollution Control Federation and the American Public Health Association (1985). Lethality in each test was compared against lethality in the appropriate control. The tests were performed in two media: K-medium (2.36 g KCl + 3.0 g NaCl per L distilled water; Williams and Dusenbery 1990a) and moderately hard reconstituted water (MHRW, 96 mg $NaHCO_3$ + 60 mg $CaSO_4 \cdot 2H_2O$ + 60 mg $MgSO_4$ + 4 mg KCl per L distilled water; USEPA 1993). Two exposure durations were used: 24 h (without food) and 96 h (with food source). For solutions containing a food source, a stationary-phase suspension of OP50 grown in a volume of L-broth (3.0 g beef extract, 5.0 g peptone, 5.0 g lactose per L) equal to twice the final volume of test solution was centrifuged (Donkin and Williams 1995). The resulting pellet was washed 3 times with K-medium and resuspended in the test solution.

Aquatic toxicity tests were performed in 12-well tissue culture plates (Costar, 3512). Four experiments with 3 replicate wells per concentration were conducted for each factor (pH, salinity, hardness). Respective testing medium was used as a control for each test. Ten (± 1) adult worms were transferred into each well containing 1 ml of test solution. Tissue culture plates were incubated at 20°C and counts were taken after 24 or 96 h (± 1 h) and expressed as % lethality (dead/total worms × 100). Dead worms were identified by visual inspection along with gentle probing using a platinum wire.

Beginning at a pH of 7.0, range finding solutions at pH increments of 0.5 (Orion, 720A pH-meter) were prepared by adding 0.1 M HCl and 1 M NaOH. Solutions of pH 3.0 to 3.5 and 11.5 to 12.3 at 0.1 unit increments were used in the final pH tolerance experiments. Initial pH was checked before adding solutions to the wells and final pH was checked after counting lethality by pooling the contents of each test replicate.

Salinity test solutions were prepared by increasing concentrations of NaCl or KCl until 10% lethality was reached. Final test concentrations of each salt in 0.05 g/L increments were used in each medium. Nominal salinity values for each test solution are reported as the total of the specific salt (either NaCl or KCl) in the respective medium, which includes the NaCl or KCl content of medium itself and the amount added to the medium.

Tolerance ranges for hardness were determined by increasing hardness until 10% lethality was achieved. Hardness in K-medium and MHRW is expressed as total $NaHCO_3$ which includes the $NaHCO_3$ present in the medium plus the $NaHCO_3$ added. Worms were also tested for lethality in EPA's hard reconstituted medium (HRW, 192 mg $NaHCO_3$ + 120 mg $CaSO_4 \cdot 2H_2O$ + 120 mg $MgSO_4$ + 8 mg KCl per L distilled water, USEPA 1993) and very hard reconstituted water (VHRW, 384 mg $NaHCO_3$ + 240 mg $CaSO_4 \cdot 2H_2O$ + 240 mg $MgSO_4$ + 16 mg KCl per L distilled water, USEPA 1993) without additional $NaHCO_3$.

Statistical Methods

Four replicate experiments with 3 replicate wells per concentration and 10 (± 1) worms/well produced a total of 120 exposed worms for each test concentration. Average lethality was calculated for each concentration tested. This resulted in 4 estimates of lethality for each treatment level. All data sets were checked for normality using the chi-squared test and for homogeneity of variance using Barlett's test. All data sets passed the tests of normality and homogeneity of variance at $\alpha = 0.01$. The data were statistically analyzed using a one-way ANOVA with 3 treatment levels for pH, salinity and hardness. Tukey's tests was used to determine significance difference ($\alpha = 0.05$) from the controls. All analysis was conducted using Toxstat® 3.4 (1994) PC software.

Results

pH

C. elegans shows a wide pH tolerance range (see Table 1). pH tolerance ranges that showed no significant difference ($p>0.05$) from the controls were obtained for K-medium and MHRW. The pH tolerance range for K-medium was 3.1 to 11.9 for 24 h, and 3.2 to 11.8 for 96 h. In MHRW the pH range was 3.4 to 11.9 for 24 h, and 3.4 to 11.7 for 96 h. The pH of the test solutions did not change over the test period.

Salinity

C. elegans can tolerate up to 15.46 (24 h) to 15.50 (96 h) g/L total NaCl in K-medium (Table 2a), and 20.50 (24 h) to 20.95 (96 h) g/L NaCl in MHRW without mortality significantly different ($p > 0.05$) from the controls. The tolerance of *C. elegans* to KCl is somewhat lower, 11.51 g/L total KCl in K-medium (24 and 96 h) and 18.85 (24 h) to 18.90 (96 h) g/ L of KCl in MHRW (Table 2b).

Water Hardness

C. elegans tolerated $NaHCO_3$ up to 0.236 g/L (in MHRW) and 0.241 g/L (in K-medium) with lethality not significantly different ($p > 0.05$) from the controls. Testing worms in HRW (hardness: 0.160 to 0.180 g/L $CaCO_3$) and VHRW (hardness: 0.280 to 0.320 g/L $CaCO_3$) did not produce lethality significantly different from the controls ($p>0.05$) and was less than 10% for both 24- and 96-h tests (Table 3).

Discussion

The results of the study suggest a wide tolerance range for *C. elegans* to the physical parameters pH, salinity and hardness in aquatic media. Changes in pH effects the chemical form (and consequently the toxicity) of many toxicants (cyanide, NH_3, heavy metals). Standard methods for the examination of water and waste water require a description of the test conditions (water quality) when reporting aquatic toxicity results. Small differences between laboratory test conditions (pH, temperature) and those in the environment can affect test results

Table 1. Effect of pH on percent lethality of C. elegans for 24 h and 96 h exposures in K-medium and MHRW. Same letter in same column indicates no significant difference ($\alpha = 0.05$)

K-medium[a]			MHRW[b]		
Duration	pH	% Lethality	Duration	pH	% Lethality
24 h	control[c]	1.76A	24 h	control[d]	1.25A
	3.0	11.70B		3.3	19.75B
	3.1	4.53A		3.4	3.30A
	11.9	3.13A		11.9	3.95A
	12.0	11.63B		12.0	10.90B
96 h	control[c]	1.00A	96 h	control[d]	2.30A
	3.0	11.18C		3.3	14.53B
	3.1	7.73B		3.4	5.75A
	3.2	1.25A		11.8	3.75A
	11.8	4.38A		11.9	11.63B
	11.9	11.89C			

[a] K-medium = 3.0 g/L NaCl + 2.36 KCl g/L per L distilled water.
[b] MHRW = 96 mg $NaHCO_3$ + 60 mg $CaSO_4 \cdot 2H_2O$ + 60 mg $MgSO_4$ + 4 mg KCl per L distilled water.
[c] pH 5.1–5.4.
[d] pH 7.3–7.5.
Note: Range finding tests were performed in the pH range of 3.5 to 11.5 at increments of 0.5 without significant ($p > 0.05$) lethality.

Table 2a. Effect of NaCl (as approximations of salinity) on % lethality of C. elegans for 24 h and 96 h exposures in K-medium and MHRW. Same letter in same column indicates no significant difference ($\alpha = 0.05$)

K-medium[a]			MHRW[b]		
Duration	NaCl g/L[c]	% Lethality	Duration	NaCl g/L	% Lethality
24 h	control	2.28A	24 h	control	2.70A
	15.46	7.38A		20.50	6.05A
	15.50	11.75B		20.55	12.28B
96 h	control	1.88A	96 h	control	2.30A
	15.50	5.55A		20.95	6.07A
	15.55	10.95B		21.00	13.15B

[a] K-medium = 3.0 g/L NaCl + 2.36 KCl g/L per L distilled water.
[b] MHRW = 96 mg $NaHCO_3$ + 60 mg $CaSO_4 \cdot 2H_2O$ + 60 mg $MgSO_4$ + 4 mg KCl per L distilled water.
[c] In addition to NaCl, the medium contains 2.36 g/L of KCL.

Table 2b. Effect of KCl (as approximations of salinity) on % lethality of C. elegans for 24 h and 96 h exposures in K-medium and MHRW. Same letter in same column indicates no significant difference ($\alpha = 0.05$)

K-medium[a]			MHRW[b]		
Duration	KCl g/L[c]	% Lethality	Duration	KCl g/L	% Lethality
24 h	control	2.90A	24 h	control	2.70A
	11.51	5.63A		18.85	4.15A
	11.56	14.45B		18.90	16.90B
96 h	control	2.93A	96 h	control	3.55A
	11.51	4.80A		18.90	7.85A
	11.56	10.95B		18.95	13.00B

[a] K-medium = 3.0 g/L NaCl + 2.36 KCl g/L per L distilled water.
[b] MHRW = 96 mg $NaHCO_3$ + 60 mg $CaSO_4 \cdot 2H_2O$ + 60 mg $MgSO_4$ + 4 mg KCl per L distilled water.
[c] In addition to KCl, the medium contains 3.0 g/L of NaCL.

Table 3. Effect of hardness (g/L $NaHCO_3$) on % lethality of C. elegans for 24 h and 96 h exposures in K-medium and MHRW. Same letter in same column indicates no significant difference ($\alpha = 0.05$)

K-medium[a]			MHRW[b]		
Duration	$NaHCO_3$ (g/L)	% Lethality	Duration	$NaHCO_3$ (g/L)	% Lethality
24 h	control	2.62A	24 h	control	2.70A
	0.241	5.13A		0.236	1.80A
	0.246	11.90B		0.241	11.25B
96 h	control	1.25A	96 h	control	2.30A
	0.241	3.33A		0.236	3.10A
	0.246	12.27B		0.241	11.00B

[a] K-medium = 3.0 g/L NaCl + 2.36 KCl g/L per L distilled water.
[b] MHRW = 96 mg $NaHCO_3$ + 60 mg $CaSO_4 \cdot 2H_2O$ + 60 mg $MgSO_4$ + 4 mg KCl per L distilled water.

substantially and therefore may decrease the utility of the test results. In such cases, a test organism that tolerates wide ranges of pH can be extremely useful. As a result, the testing procedure would not require pH adjustment, and the toxicant could be presented to the organism in its environmental state.

The results show that *C. elegans* can withstand a range of pH that is unmatched by commonly used test organisms such as daphnids. When testing daphnids and other fresh water and marine organisms, it has been a practice to adjust pH within the range 6.0 to 9.0 or to run parallel tests comparing effluent with pH adjusted to 7.0 with unadjusted effluent (USEPA 1985). *C. dubia* and *D. magna* both can survive at a pH of 9.3 without significant mortality (American Public Health Association, American Water Works Association, and Water Environment Federation 1992). It has been reported that *D. magna* has a pH range of 5.0 to 9.0 and that no mortality was observed within this range for 6 days (Lewis and Weber 1985). They also reported that all the organisms died within 24 h when the pH range was lowered to 3.6 and raised to 11.0. Another daphnia species, *D. pulex*, has been shown to survive a low pH of 4.3 for 5 days, but only in the absence of high levels of free CO_2 (France 1982). However, at an exposure to pH 3.7 the survival time was only 3 h. *C. elegans*, with a pH tolerance range of 3.2 to 11.8 for K-medium and 3.4 to 11.7 for MHRW, is an excellent test organism for conducting bioassays with environmental samples of wide pH range, especially industrial wastes and receiving waters.

The toxicity observed in the pH experiments can be strictly attributed to H^+ and OH^-, due to the fact that the amount of Na^+ and Cl^- added during pH manipulations was negligible compared to the amount of Na^+ and Cl^- tolerated by *C. elegans* in the salinity experiments. Approximately 2.91 g/L and 5.44 g/L of Cl^- (0.1 M HCL) was used to adjust pH to the desired acidic range for K-medium and MHRW, respectively. While, approximately 0.46 g/L and 0.35 g/L of Na^+ (1 M NaOH) was required to adjust pH to the desired basic range for K-medium and MHRW, respectively.

Salinity was measured in g/L and expressed as parts per thousand (ppt). The concentrations of NaCl and KCl used in this experiment are only approximations of salinity. However, the ability of *C. elegans* to withstand the osmotic stress of these salt solutions suggests that *C. elegans* may be successfully used in testing samples with salinity less than 20 ppt. The high salt concentrations used with *C. elegans* are unlikely to be found in

fresh water or wastewater samples, but are likely to occur in estuarine samples. Therefore, *C. elegans* may be used for samples with salinity (up to 20 ppt) without affecting the quality of the results or displaying control mortality beyond the desired limit of 10%. The salinity tolerance of *C. elegans* is also unmatched by daphnids. Cowgill *et al*. (1991) found that the EC50 for NaCl, based on progeny, for *D. magna* and *C. dubia* averaged 4.205 and 2.160 ppt, respectively. Higher concentrations of NaCl resulted in a pronounced effect on both progeny and brood size of *Ceriodaphnia*, with no broods produced at 10.0 g/L NaCl.

Tolerance to salt concentration was much higher in the case of MHRW compared to that in K-medium. In K-medium, the initial salt concentration was 2.36 g/L KCl and 3.0 g/L NaCl, while MHRW contained only 0.004 g/L KCl. Also, the combined effect of KCl and NaCl in K-medium with added salt might be causing higher sensitivity. Summing initial NaCl and KCl concentrations and the added salt concentration (of either NaCl or KCl) brings the tolerable salinity range for K-medium closer to that of MHRW.

C. elegans was also tested for hardness tolerance using $NaHCO_3$. The importance of hardness as a water quality variable in the case of two cladocerans has been investigated by Cowgill *et al*. (1991). They suggested that in the toxicity testing of effluents on cladocerans, toxic responses are often attributed to toxicants in the test effluent, when in fact the response is due to a water quality parameter, such as alkalinity, being outside the tolerance limits of the test organism. Also, it has been reported that the variation in hardness of the medium can alter the toxicity of certain chemicals. Belanger *et al*. (1989) have tested the interaction of hardness on copper toxicity to *C. dubia* and found toxicity varied with hardness. The tolerance of *C. elegans* to hardness was higher than that reported for *C. dubia*.

C. elegans was found to tolerate up to 0.236 to 0.241 g/L $NaHCO_3$ with lethality not significantly different ($p > 0.05$) from the control. Testing worms in hard (0.160 to 0.180 g/L $CaCO_3$) and very hard (0.280 to 0.320 g/L $CaCO_3$) water produced no statistically significant lethality for both 24- and 96-h exposures. LC50 values for *D. magna* and *C. dubia* are 0.730 (ranging from 0.592 to 1.006) g/L $CaCO_3$ and 0.237 (ranging from 0.183 to 0.306) g/L $CaCO_3$ as reported by Cowgill *et al*. (1991). The no-observable-effect-levels (NOEL) reported were 0.129 and 0.122 g/L $CaCO_3$, respectively.

Since the desired hardness levels were prepared with $NaHCO_3$, it may be argued that the contribution of sodium may be involved in the observed toxicity. However, at the observed tolerance levels, the contribution of sodium in the test solution was 0.038 to 0.039 g/L, which is considerably lower than the amount of sodium in salinity tests with NaCl where approximately 5 g/L of sodium was involved.

This study has established tolerance ranges of *C. elegans* for pH, salinity, and hardness in aquatic media. The high tolerance of *C. elegans* to these parameters allows its use in testing a wide variety of environmental samples without adjustment of the sample's physical parameters. This improves the validity of the test results, so that it can be claimed that observed toxicity is due to the chemical under consideration and not the physical conditions of the test medium. This study also indicates the potential of using *C. elegans* for testing waters and effluents of intermediate salinity. *C. elegans*, due to the extensive biological and molecular information available, can substitute for other commonly used test organisms, and also help gain insight into the mechanism of action of chemicals at the molecular or cellular level. In spite of its tolerance to the wide range of water quality parameters, previous testing does not indicate that *C. elegans* is less sensitive than other organisms to toxicants (Williams and Dusenbery 1990a).

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