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B.J. Dutka

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B.J. Dutka

Rivers Research Branch
National Water Research Institute
Burlington, Ontario L7R 4A6

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MANAGEMENT PERSPECTIVE

One of the simpler procedures used to estimate the bioavailability of toxicants/genotoxicants is to establish the response that water, sediment and water/sediment extracts produce in various bioassays. However, if these bioassays are to become our working tools then we must know the replicability and stability of these testing procedures. Knowledge of the reliability of bioassay methods is usually attained through the standardization of the method's protocols. This theoretically ensures that if all laboratories perform the same bioassay test with the same sample or chemical, they will all have the same or similar results. However, before we can establish whether or not the results are the same or similar we must know the innate variability of each test system that we use. In this report we provide information on the stability and replicability of many of the bioassays used in our battery of tests approach. These data are essential in establishing future QA/QC (quality assurance/quality control) protocols for biological toxicity screening tests.

SOMMAIRE À L'INTENTION DE LA DIRECTION

L'une des méthodes simples permettant d'évaluer la biodisponibilité des toxiques/génotoxiques consiste à déterminer la réaction que produit l'eau, les sédiments et des extraits eau/sédiments au cours de divers dosages biologiques. Toutefois, ces dosages ne seront utilisés réqulièrement que lorsque nous connaîtrons leur répétabilité et leur stabilité. Normalement, on détermine la fidélité d'une méthode de dosage en normalisant les protocoles. Ainsi, on s'assure que tous les laboratoires qui feront subir le même dosage biologique au même échantillon ou au même produit chimique obtiendront, théoriquement, des résultats identiques ou semblables. Toutefois, avant de déterminer si les résultats sont ou non identiques ou semblables, il faut connaître la variabilité propre de chaque système de dosage que nous utilisons. Dans ce rapport, nous fournissons des informations sur la stabilité et la répétabilité d'un bon nombre de dosages biologiques utilisés dans notre batterie de tests. Ces données sont essentielles pour établir les futurs protocoles d'AQ/CQ (assurance de la qualité/contrôle de la qualité) des dosages biologiques servant à déceler les produits toxiques.

ABSTRACT

Information on the reliability of a bioassay is usually attained through the standardization of the method's protocols. This theoretically ensures that all laboratories following the method will attain the same result with the same chemical or sample. However, before we can establish whether or not the results are the same or similar we must know the innate variability of each test system that we use. In this report are shown the stability over time and replicability of the various bioassays used in the battery of tests approach.

RÉSUMÉ

Normalement, on obtient des informations sur la fidélité d'un dosage bilogique en normalisant les protocoles. Ainsi, on s'assure que tous les laboratoires qui appliqueront le même dosage biologique au même échantillon ou au même produit chimique obtiendront, théoriquement, des résultats identiques ou semblables. Toutefois, avant de déterminer si les résultats sont ou non identiques ou semblables, il faut connaître la variabilité propre de chaque système de dosage que nous utilisons. On donne dans ce rapport la stabilité en fonction du temps, ainsi que la répétabilité des divers dosages biologiques utilisés dans cette batterie de tests.

INTRODUCTION

One of the simpler procedures to estimate the bioavailability of toxicants/genotoxicants is to establish the response that water, sediment and water/sediment extracts produce in various bioassays. A variety of short term bioassays has been developed to assess the ecological impact of industrial and domestic effluents, land wash and airborne contaminants on waters and sediments (Bitton and Dutka 1986, Dutka and Bitton 1986, Liu and Dutka 1984). However, the application of these short term bioassays to environmental samples revealed that there was no single test which was responsive to all contaminants or mixtures of contaminants (Bitton & Dutka 1986) and that there was great variability in responses from one type of sample to the next. This realization led to the concept of using a "battery of tests" to ascertain the bioavailability and impact of environmental contaminants.

The need for reliable methods to assess the bioavailability of the toxicants/genotoxicants in the aquatic ecosystem is well understood. If the "battery of tests" concept is to become a working tool then reliability and reproducibility of test results must be established.

Knowledge of the reliability of bioassay methods is usually attained through the standardization of the method's protocols. This theoretically ensures that if all laboratories perform the same bioassay test with the sample or chemical, they will all have the same or similar results.

However, before we can establish whether or not the results are the same or similar we must know the innate variability of each test system that we use.

In our studies on contaminants we use the battery of tests approach in any new area being evaluated in order to establish (a) priority areas of concern and (b) the core group of bioassay which are responsive to the samples. For the majority of the bioassays which are included in our battery of tests approach, we have accumulated data on the reliability or stability of the test responses. These data will be presented in this report.

Methods

Reliability or stability data will be presented for the following bioassays.

ATP-TOX System

The concentration of ATP per bacterial cell remains relatively constant and stable throughout all phases of growth (D'Eustachio and Johnson, 1968). Thus bacterial densities can be estimated by measuring the ATP content of the test system. When rapidly growing bacterial cells are exposed to toxicants, growth inhibition usually occurs. After several life cycles the toxic effect can be estimated by comparing sample cell growth to the control via ATP content. However, some toxicants not only inhibit bacterial growth but also affect the luciferase activity during ATP determinations. Therefore, the observed light output reduction of the test system is the net result of the inhibition of both bacterial growth and luciferase (called "total inhibition of the ATP-TOX System"). Luciferase activity inhibition can be determined by adding a standard ATP

solution, as enzyme substrate, to the sample and to a distilled water control and measuring the light emission of the enzyme. In our studies, we use E. coli K-12.PQ37 strain, although any bacterium or mixture of bacteria can be used in this technique (Xu and Dutka 1987).

Microtox

Microbics Corporation has devised a test for acute levels of toxicants in water or sediment extracts, in which specialized strains of luminescent bacteria (Photobacterium phosphoreum) are used as the bioassay organism. This test is functional because the metabolism of the luminescent bacteria is influenced by low levels of toxicants, and occasionally, stimulants. Any alteration of metabolism affects the intensity of the organism's light output. By sensing these changes in light output, the presence and relative concentration of toxicants can be obtained by establishing the EC_{50} levels from graphed data. The EC_{50} is that concentration of toxicant causing a 50% reduction in light from the baseline level (Bulich 1986, Dutka 1988).

SOS-Chromotest

This test is based on colorimetric assays of microbial enzymatic activities after incubating the bacterial tester strain (E. coli K12-PQ37) in the presence of water, sewage or sediment extracts. The E. coli strain is rfa, a rough mutant, which increases the permeability of the cell wall and it also lacks a functional genomic copy of Lac Z and contains an operon fusion (sfiA::lacZ) which places

the production of β-galactosidase under the express control of the SOS response pathway which is induced by DNA damaging agents by the action of the REC/A protein on the Lex A repressor (Little and Mount 1982). In the test an exponential growth phase culture of the E. coli is introduced into the cells of a microtitration plate containing samples and controls. After, a two hour incubation at 35°C for protein sythesis, β-galactosidase activity (SOS response activity) is measured by changes in the optical density of the sample at 615 nm in a microtitration plate reader (Xu et al. 1987). This measures the level of β-galactosidase via its effect on the indicator compound 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Thus the greater the amount of β-galactosidase produced by SfiA::lacZ the greater the SOS response pathway has been induced.

Mutatox

This is a relatively new test, developed by the Microbics Corporation and is based on the use of a dark mutant strain of Photobacterium phosphoreum, M169, to screen for the presence of genotoxic agents. This test will pick up chemicals which are (a) DNA damaging agents, (b) DNA intercalating agents, (c) direct mutagens which either cause base substitution or are frame shift agents and (d) DNA synthesis inhibitors (Kwan et al., 1989). The test procedures are similar to those followed in the Microtox test with incubation of M169 cells, cell media and sample being carried out at 22 ± 2°C. Light level is read after 18 ± 1 hour contact and compared to negative controls (dilution water, solvent concentration used and sodium azide).

Toxi-chromotest

This is a fairly rapid (2-3 hr) bacterial colourimetric assay in kit form which can be used to test for toxicant activity in water and sediment extracts. The assay is based on the ability of substances (toxicants) to inhibit the de novo synthesis of an inducible enzyme, β-galactosidase, in a highly permeable mutant of E. coli. The sensitivity of the test is enchanced by exposing the bacteria to stressing conditions (provided by kit materials) after which they are rehydrated in a cocktail containing a specific inducer of βeta galactosidase and essential factors required for the recovery of the bacteria from their stressed condition. The activity of the induced enzyme is detected by the hydrolysis of a chromogenic substrate. Toxic materials interfere with the recovery process and thus with the synthesis of the enzyme and the colour reaction (Organics 1985).

Spirillum volutans

The organism S. volutans is a large aquatic bacterium which is readily visible under low magnification. It has a fascicle of flagella at each end which, under normal conditions, form oriented revolving cones allowing the bacterium to move forward and reverse directions at will. During the reversing process the polar fascicles reorient simultaneously. To perform the test, S. volutans is added to a volume of the sample and the mobility of the organisms is observed with a microscope. If the sample is toxic but contains non-lethal levels of toxicants, S. volutans loses coordination, as both fascicles try to assume the head or tail orientation, thus preventing normal bacterial motion. (Bowdre and Krieg, 1974, Moore, 1984, Dutka, 1988).

Daphnia magna

The cladocern Daphnia magna used in our tests is the largest of the Daphnia, often reaching 5 mm in size. The neonates (first-instar young) are 0.8 and 1.0 mm long and can be observed by eye. This stage is the one most commonly used for tolerance studies. Tests are performed on neonate Daphnia that have been released from the mothers brood chamber during the previous 24 hours. In the test, 10 neonates are used for each dilution of sample (usually 100, 75, 50, 25, 10%) to be tested (APHA, 1985). The neonate Daphnia are observed at 1 hour, 4 hour, 24 hour, and 48 hour, and the number of dead animals are recorded. A 24 hour or 48 hour LC_{50} or EC_{50} value is then derived from the pattern of deaths observed. Daphnia are less tolerant of toxic substances than are fish (Kemp et al., (1971).

Ceriodaphnia dubia

The cladoceran, Ceriodaphnia dubia is used to evaluate the chronic toxicity of a sample. In this test four beakers of approximately 30 mL volume are used for each sample dilution and control with one animal per beaker. Tests are performed with young animals that are as similar in age as possible (8 hrs. maximum). On the 3rd, 5th and 7th day of the test, as these young cladocerans mature into adults and produce offspring, these offspring are counted and discarded. During the test period the animals are fed daily and the sample solutions replaced with fresh samples. At the end of the test the number of young per original test cladoceran and the number of broods per adult are established against that obtained in the

control sample. An average of 2.5 broods per adult in the controls has been used as the end point in some testing procedures (Mount and Norberg, 1984, Rao, 1988).

Agar Spot Plate

This is a simple, rapid procedure for determining the toxicity of both water-soluble and -insoluble compounds by use of a direct agar-diffusion assay. The procedure involves the use of a non-toxic carrier system for extracted samples (DMSO-glycerol) and a 30°C incubation temperature and zones of inhibition are noted. The bacterial species used to provide the background lawn is Bacillus cereus and the sample (water or DMSO-glycerol extract) is spotted onto the seeded agar plate. Results can be obtained in 3-4 hr. or overnight as required (Dutka 1988, Liu et al., 1989,).

ECHA Biocide Monitor

This is an extremely simple inexpensive kit test for screening waters, effluents and sediments for the presence of toxicants. The test is based on the use of a small absorbent pad impregnated with sensitive test organisms (Bacillus species) and a growth indicator dye (tetrazolium) to detect the presence of microbial growth. In the test, the absorbent pad is dipped into the sample for 10 to 60 seconds. The strip is then incubated at 35 - 37°C for 18-24 hours and the results are interpreted according to the colour card enclosed in the kit. (Dutka and Gorrie 1989, Hill et al., 1989).

Nematode Test

The nematode test based on the use of Panagrellus redivivus (Samoiloff 1986, 1990) was a new addition to our battery of bioassay tests. This nematode gives live birth to nematodes designated as second stage juveniles (J2). In the test 100 J2 animals, in groups of 10, are monitored over a 96 hour period and their reactions, growth, lack of growth or death, can provide a measure of both lethal and sublethal effects of the sample being tested. Lethal effects are noted by the number of animals that die.

Growth from J2 to J3 or from J3 to J4 requires very little gene activity, while growth from J4 to adult requires extensive gene activity. Many known mutagens will selectively inhibit the J4 to adult molt and specific inhibition of the growth of J4 animals to adults can be used as an indicator of potential mutagenicity in the test sample (Samoiloff 1990). The test as used here has two end points, one being the ability to survive in the sample for 96 hours and the other is whether or not the survivors can reach the adult stage in 96 hours.

Seed Germination and Root Elongation

This test is based on the ability of the test seeds, Buttercrunch Lettuce (or any fast growing seed) to germinate and produce a root when placed in contact with the testing solution. In the test, 20 seeds per sample are placed on an absorbent paper (Whatman #3 filter paper) in a 100 x 50 mm petri dish to which 5.5 mL of the testing solution is added. The petri dish is incubated at 22°C in the dark

for 4-6 days (we prefer 4) and at the end of the incubation the number of seeds germinating are counted and their root lengths are measured. These counts and measurements are compared to controls (aerated dechlorinated tap water, distilled water or diluted solvent).

RESULTS

Microtox

The Microtox test is the oldest kit type of bioassay and as a result we have the greatest data base on this test. The data obtained from positive controls over various time period has provided us with a good history on the stability of this test. The positive controls used were solutions containing Zn^{++} 20 ppm, Hg^{++} 20 ppm, phenol 100 ppm and 3, 5 dichlorophenol 100 ppm.

The stability of the Microtox test as indicated by the concentrations of the control chemicals producing EC_{50} values of the various time periods can be seen in the Table below.

Table 1. Microtox stability

Chemical	No of Tests	Time	Mean ppm	S.D.
Zn^{++}	95	36 months	6.40	2.88
Zn^{++}	32	15 months	5.38	1.87
Phenol	68	24 months	22.28	3.56
Phenol	32	15 months	22.88	3.81
Hg^{++}	14	10 months	0.045	0.011
2,4 dichloro-phenol	21	10 months	30.01	4.70

Replicability of the Microtox test can be seen in the data from five replicates using Zn^{++} and phenol.

Table 1a. Microtox replicability

Chemical	No of Tests	Mean ppm	S.D.
Zn^{++}	5	5.37	.827
Phenol	5	21.41	2.123

The above data suggest that the Microtox test is a very stable procedure with excellent replicability for a biological assay.

ATP-TOX System

The ATP-TOX System has proven over the years to be our most sensitive test. Rarely do we find a sample not producing a reaction in this test. However, the ATP-TOX System has great variability between replicates and thus three replicates are taken to establish a mean value.

Over a 12 month period various positive (toxic) controls have been used as quality control guides. These positive controls were solutions of $ZnSO_4$, $HgCl_2$ and 2,4 dichlorophenol. A summary of these positive mean values are shown in Table 2 below. These results indicate the stability of the testing procedure using the same E. coli strain.

Table 2. Stability of ATP-TOX System over 12 month period.

Agent and Concentration		Mean % Inhibition	S.D.	No. of Tests	Background Inhibition without chemical Mean % Inhibition	S.D.
$ZnSO_4$	10 ppm	93.58	16.71	23	4.51	5.82
$ZnSO_4$	1 ppm	37.45	22.76	23	3.25	3.48
$HgCl_2$	.25 ppm	33.72	23.45	12	2.35	3.03
$HgCl_2$	.1 ppm	15.89	13.78	12	1.59	1.83
2,4 - dichloro-phenol	100 ppm	68.97	12.28	19	9.69	6.16
2,4 - dichloro-phenol	1 ppm	40.53	19.59	19	2.61	3.92

Short term replicability of results are shown in Table 2a. The results were obtained from a sediment pore water sample which had four separate aliquots tested in triplicates. Mean and standard deviations of each set are presented along with the summarized mean and S.D.

Table 2a. Replicability of ATP-TOX System using sediment pore water. Four subsamples of one extraction.

Sample No.	Mean % Inhibition	S.D.
Set 1	31.5	18.6
Set 2	36.2	13.5
Set 3	40.8	9.97
Set 4	37.6	5.44
All subsamples	36.5	10.35

SOS Chromotest

To ensure the reliability of the SOS Chromotest, two positive standards are used as controls in the test procedure, 2-aminoanthracene (2AA) and 4- nitroquinoline-1-oxide (4NQO). 2AA is activated by S-9 addition while 4 NQO is not.

The reporting unit for the SOS Chromotest is the Induction Factor. An induction factor of 1.3 or greater is considered a positive for the presence of genotoxicants. The induction factor is obtained from the ratio of β-galactosidase units to alkaline phosphatase units with corrections for spontaneous induction of the testing bacterium, E. coli (Xu et al. 1987).

Over a 12 month period, 18 sets of samples were run using 100 ppm 4NQO as a positive control and 16 sets of samples using 10 ppm 2AA with S-9 as controls.

The stability of these test as indicated by the control chemical concentrations to produce induction factors of 1.3 over this time period can be seen in the data below:

2AA - N = 16, mean 0.5825 ppm and S.D. = .360

4NQO - N = 18, mean .0203 ppm and S.D. = .013

Mutatox Test

The Mutatox test is still in the field testing and developmental stage and is not available commercially at this time. With this test we have noted, as well as other researchers, great variations from one batch of organisms to another. This has been acknowledge as a problem by the developers of the test (Microbics) and the problem is still being worked on.

In our studies we have found daily replicability to be reasonable; however, week to week or month to month comparisons are difficult due to batch to batch variations. We are not certain whether the variations are due to differences in bacterial numbers or due to variations in the bacteria.

As an indication of the type of results we have been finding, the positive controls 2AA (with S-9) and 4NQO are presented in close response mode in the following Tables 3 and 3a. In each Table the mean and standard deviation (S.D.) of four typical sets of daily duplicated positive controls are shown as well as the mean and S.D. for 22 sets of data over a 12 month period. Results are recorded as light output as measured by the modified Beckman Microtox reader Model 2055.

Our studies strongly suggest that Mutatox data can only be compared within the daily set and not with previous or forthcoming data.

Table 3. Mutatox Test Replicability and Stability of 2AA in DMSO with S-9.

Concentration ppm 2AA	Daily Results								12 month results - 22 sets	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
100	153.5	47.37	190.0	98.99	100.5	28.99	473.0	45.25	212.00	175.18
50	73.0	14.14	500.0	84.85	17.5	2.12	343.5	43.13	182.13	150.63
25	55.5	9.19	720.0	42.40	6.50	2.12	169.0	5.65	158.45	181.05
12.5	40.0	1.41	505.0	63.60	5.0	1.41	112.5	10.6	121.68	166.09
6.25	36.0	5.65	206.5	4.94	3.50	0.70	96.0	4.24	81.77	95.69
3.125	24.5	.707	66.5	3.53	4.50	0.70	70.0	5.65	57.00	35.48
1.5625	16.5	2.12	25.5	6.36	3.50	2.12	44.0	0.00	32.00	24.32
.7813	10.5	2.12	7.0	2.82	1.50	0.70	19.0	0.00	16.68	12.24

Table 3a. Mutatox Test Replicability and Stability of 4NQO in MeOH

Concentration ppm 4NQO	Daily Results								12 month results - 22 sets	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0.6250	.5	.707	3.0	0.00	4.5	.707	6.5	3.53	1.75	2.38
0.3125	16.0	8.48	233.0	97.58	86.0	1.41	203.0	1.41	67.20	82.04
0.1563	48.0	2.82	210.0	28.28	17.5	3.53	27.0	0.00	47.40	57.49
0.0781	52.0	7.07	103.5	26.1	7.5	6.36	9.0	4.24	30.00	32.49

Toxi-Chromotest

The Toxi-Chromotest is based on the ability of a toxicant(s) to inhibit the de novo synthesis of an inducible enzyme (βeta-galactosidase) in a specially mutated strain of E. coli (Orgenics 1985). In carrying out this test all samples and positive controls are compared to the yellow colour produced by the non-toxic (negative) control (sample diluent supplied with the kit).

Toxicant activity inhibits beta galactosidase production which results in a decrease in the depth of the yellow colour reaction. A 20% decrease in colour reaction is considered to be an indication of the presence of a toxicant in the sample.

The stability of this test has been established over an 18 month period during which positive controls for 27 samples were dilutions of 4 ppm Hg^{++}.

The stability of this test as indicated by the concentration of Hg^{++} solution to produce a 20% reduction in colour reaction can be seen in the data below.

N = 27 Mean = .407 ppm Hg^{++}, S.D. = .149

ECHA Dip Stick

The ECHA biocide monitor (Dip Stick) is a simple colorimetric test with four possible end points. If the sample is toxic, all the bacteria are killed, no colour is produced and the dip stick remains white (-). If the sample is not toxic then the dip stick pad turns red due to the reduction of the dye (+). In between these two responses there are ± and ∓ responses. In the ± response there is

more red colour than white indicating that there are more surviving organisms than dead organisms. The $\mp$ response suggests there are more dead organisms than there are viable ones. These are judgement calls.

Over a three year period 10, Hg^{++}, dechlorinated tap water and 2,4-dinitrophenol controls have been performed as part of laboratory sample testing. These controls were to assure that the dip sticks were working. The results were:

Tap water	+	(10)
Hg^{++} 2 ppm	+	(10)
Hg^{++} 10 ppm	$\mp$	(10)
2,4-dinitrophenol 200 ppm	-	(10)
2,4-dinitrophenol 50 ppm	+	(10)

Replicability in the test was evaluated by testing five replicates of four concentrations of Hg^{++}.

The results were:	25 ppm	Hg^{++}	-	(5)
	12.5 ppm	Hg^{++}	$\mp$	(5)
	6.25 ppm	Hg^{++}	$\pm$	(5)
	3.12 ppm	Hg^{++}	+	(5)

Spirillum volutans Test

In the 2hr Spirillum volutans test, toxicant effect, is based on the physical observation that 90% (EC_{90}) of the test organisms are disoriented or dead. The test can be made more sensitive by (a) increasing the length of the test and (b) changing the end point to "50% of the test organisms must be disoriented or dead" (EC_{50}). The EC_{50} endpoint requires a higher degree of judgement to make this decision and has been found difficult to duplicate by two observers.

The samples and controls are viewed, in special studies, every 15 minutes, otherwise under normal operating conditions they are viewed at time 0, 60 minutes and at the end of the test, 120 minutes.

Over a two year period 1989-1990, 19 negative and positive control tests were performed as part of environmental assessments. The negative control was dechlorinated tap water and the positive (toxic) controls were Hg^{++} and 2,4-dinitrophenol. The results observed were:

Tap water - 19 negative after 120 min. contact

Hg^{++} .2 ppm - EC_{90} reached within 60 minutes

Hg^{++} .1 ppm - EC_{90} not reached within 120 minutes

2,4-dinitrophenol 50 ppm - EC_{90} reached within 120 minutes

2,4-dinitrophenol 25 ppm - EC_{90} not reached within 120 minutes

In October of 1990, five replicates of four concentrations of Cd^{++} were carried out with the following results:

Cd^{++} 10 ppm - EC_{90} not reached in 120 minutes

Cd^{++} 20 ppm - EC_{90} reached in 105 minutes

Cd^{++} 30 ppm - EC_{90} reached in 45 minutes

Cd^{++} 40 ppm - EC_{90} reached in 30 minutes

Daphnia magna

Three control solutions (1% DMSO, dechlorinated tap water and $K_2Cr_2O_7$ - .00147 g/L) were routinely used when performing the Daphnia magna acute toxicity test. Over an 18 month period, 21 sets of samples were analyzed, with dechlorinated tap water and 1% DMSO being used as negative controls. With these two controls the results were exactly the same, no test animals (10 per test) died after 48 hours of contact.

Similarly over this 18 month period, 16 sets of samples were analyzed with $K_2Cr_2O_7$ being used as a positive control. These results are presented in the Table 4 below.

Table 4. Stability data showing results of positive control $K_2Cr_2O_7$ (.00147 g/L) applied to Daphnia magna.

Testing chemical -	Date	% of Solution Producing EC_{50}
	Oct. 14, 1989	12.5
	Oct. 16, 1989	15.0
	Oct. 24, 1989	16.0
	Nov. 1, 1989	12.5
	Nov. 21, 1989	18.0
	Dec. 6, 1989	15.0
	Jan. 1990	12.0
	Feb. 1990	15.0
	March 1990	15.0
	July 1990	13.0
	Aug. 1990	12.0
	Sept. 1990	15.0
	Oct. 1990	15.0
	Jan. 1991	12.0
	Feb. 1991	15.5
	March 1991	15.0
	Mean	14.28
	S.D.	1.741

These three controls studies indicate the stability of the testing procedure over time.

Seven replicate tests using $ZnSO_4.7H_2O$ (.0044 g/L) were conducted to establish short term replicability. Results of this study are presented in the Table 4a below.

Table 4a. Replicability of Daphnia magna test.

Sample	% solution of $ZnSO_4.7H_2O$ producing EC_{50}
1	15
2	13
3	15
4	18
5	18
6	15
7	20
Mean	16.28
S.D.	2.43

The results of these long term and short term tests suggest that this procedure is very stable with reproducible results.

Ceriodaphnia dubia

In using Ceriodaphnia dubia to evaluate potential chronic effects of a chemical or sample, we believe is is essential to only consider those dilutions in which all four original test animals have survived. For instance if a 100% sample dilution produces 12 offspring but only three of the original four Ceriodaphnia dubia have survived, then the next dilution (1%) would be used in the calculations of chronic toxicity if the original four adults have survived.

Over a 12 month period, 11 sets of positive sample control data using $K_2Cr_2O_7$ (1.47 mg/L) and phenol (100 ppm) were collected. A negative control, dechlorinated aerated tap water was also performed with each positive (toxic) control and set of samples.

The observations on test stability are presented in the Tables 5 and 5a below.

Table 5. Stability of chronic toxicity testing procedure (C. dubia) using dilutions of $K_2Cr_2O_7$ (1.47 mg/L). Eleven sets of data over 12 months.

	100% $K_2Cr_2O_7$	50% $K_2Cr_2O_7$	10% $K_2Cr_2O_7$	1% $K_2Cr_2O_7$	.1% $K_2Cr_2O_7$	Tap Water Control
Mean no. offspring produced	-	-	14.94	16.01	15.71	17.72
S.D.	-	-	9.56	6.97	9.56	9.00

Table 5a. Stability of chronic toxicity testing procedure (C. dubia) using dilutions of phenol (100 ppm). Eleven sets of data over 12 months.

	100% phenol	50% phenol	10% phenol	1% phenol	.1% phenol	Tap Water Control
Mean no. offspring produced	-	.76	11.67	15.28	15.94	17.59
S.D.	-	2.20	9.11	9.45	10.48	8.51

In the performance of this test, we have arbitrarily selected an end point where if in any sample or dilution there is greater than 25% fewer offspring produced than the control, then the sample is deemed to have a toxic impact at that dilution.

Spot Plate Test

Any bacterial species can be used in this test; however, if long time comparisons are required, one specific organism should be selected and maintained. In most of our testing we use Bacillus cereus. The test can be made more sensitive by stressing the testing organism.

We use two versions of this test. In one version 10 µl of sample is carefully placed, directly on the bacterial lawn. The other version requires disks cut from Millipore membrane filter pads (or other similar absorbent paper/fibre pads) to be briefly immersed in the testing solution and the pad then placed directly on the prepared bacterial lawn. The zones of inhibition after 24 hours (or less) incubation are measured in mm.

The replicability of both these versions has been ascertained. In the direct drop method four workshop attendees prepared their own plates and tested Cd^{++} 12.5 ppm using six drops per plate. The following Table summarizes inhibition zone measurements in mm.

	Set 1	Set 2	Set 3	Set 4	Summary
Mean	11.0	11.08	11.1	11.11	11.08
S.D.	.663	.581	.438	.499	.508

For the disk method, three workshop attendees prepared their own plates and tested a solution containing Cd^{++} 200 ppm with four disks per plate. The following Table summarizes inhibition zone measurements in mm.

	Set 1	Set 2	Set 3	Summary
Mean	12.7	12.6	12.4	12.59
S.D.	.326	.294	.386	.320

Nematode (<u>Panagrellus redivivus</u>) Test

Routinely with the nematode bioassay we do not use a positive control, only negative controls. The negative controls are used to gauge the health of the organisms and the effects, if any, of the sample diluting solution. At present we have only one sample with three replicates to illustrate replicability of the test. We also have 8 control data points over a 14 month period for dechlorinated tap water and six control data points for 1% DMSO. The two end points reported in this test are; (a) % organisms surviving after four days and (b) % of organisms which have reached maturity compared to the organisms which have reached the J3 and J4 stages.

Results are presented in the Tables 6 and 6a.

Table 6. Stability of Nematode Test over 14 month period.

Water Control			1 % DMSO Control		
Sample No.	% Surviving	% Maturation	Sample No.	% Surviving	% Maturation
1	99	94.9	1	96	93.8
2	100	96.0	2	100	97.9
3	99	94.9	3	99	97.9
4	100	95.0	4	99	98
5	100	96.0	5	97	89.8
6	99	98.0	6	98	88.8
7	100	89.8	Mean	98.1	94.3
8	100	94.7	S.D.	1.47	4.26
	99.6	94.9			
	.51	2.33			

Table 6a. Replicability of Nematode test using sediment pore water.

Replicate	% Survival	% Maturation
1	94	96.7
2	96	96.8
3	98	93.8
Mean	96	95.7
S.D.	2.0	1.70

The results suggest that this is a very stable test with good replicability.

Seed Germination and Root Elongation Test

Buttercrunch lettuce seeds, purchased in bulk (500 gm) in the fall of 1989 and maintained in the dark at 4°C, were used in all seed germination and root elongation bioassay tests from that time until the present, March 1991.

In October 1990, two replicate studies were carried out to assess the stability of these seeds. In one, five sets of randomly selected seeds (10 seeds per set) were tested for their germination and root length using declorinated tap water (control). In the other, three sets of 10 seeds were tested using a solution containing 25 ppm 2,4 dinitrophenol. Incubation was at 22°C for 4 days.

The following Tables (7, 7a and 7b) summarize the results of these evaluations:

Table 7. Buttercrunch Lettuce seed replicability test using dechlorinated tap water.

	Set 1	Set 2	Set 3	Set 4	Set 5	Summary
Mean Root Length	30.2	31.3	32.2	31.9	32.5	31.6
S.D. Root Length	4.13	3.83	3.96	3.54	5.87	4.20
No. Seeds Germinating	10	10	10	10	9	9.8 mean

Table 7a. Buttercrunch lettuce seed replicability test using 2,4-dinitrophenol, 25 ppm

	Set 1	Set 2	Set 3	Summary
Mean Root Length	18.2	18.1	17.75	18.08
S.D. Root Length	3.64	3.48	3.01	3.26
No. Seeds Germinating	9	8	8	8.33 mean

Over an eight month period in 1990, six sets of 10 seeds were evaluated for their germination and root length stability. Results of this eight month survey are presented in the table 7b below.

Table 7b. Stability of seed germination and root elongation test over eight ninth period using Buttercrunch lettuce seeds and dechlorinated tap water.

	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6	Summary
Mean Root Length	31.2	31.5	29.7	31.5	31.7	30.7	31.05
S.D. Root Length	5.15	3.86	4.39	4.30	4.23	6.58	4.64
No. Seeds Germinating	10	10	10	9	9	10	9.66 mean

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NATIONAL WATER RESEARCH INSTITUTE
P.O. BOX 5050, BURLINGTON, ONTARIO L7R 4A6

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INSTITUT NATIONAL DE RECHERCHE SUR LES EAUX
C.P. 5050, BURLINGTON (ONTARIO) L7R 4A6