9. ROTIFER INGESTION TEST FOR RAPID ASSESSMENT OF TOXICITY

TERRY W. SNELL
School of Biology
Georgia Institute of Technology
Atlanta, GA 30332-0230, USA
terry.snell@biology.gatech.edu

1. Objective and scope of test method

This method is intended as a screening tool for rapid toxicity assessment. The test is designed for use with fresh or marine waters, to evaluate chemicals, surface waters, effluents, pore waters, drinking waters, and contamination emergencies. Because of its speed and simplicity, this test could easily be integrated into a battery of tests representing several species. Rotifers are generally responsive to a wide variety of toxicants, including metals, organics, pesticides, and endocrine disruptors. The ingestion test is performed in 1 hour in 24-well microplates in volumes of 750 μL.

2. Summary of test procedure

Test animals are obtained by hatching resting eggs (cysts), encysted dormant embryos that remain viable for years when kept cold, dark, and dry. Resting eggs enable researchers to eliminate the pre-test culture that is required to obtain most test animals. No pre-culture eliminates a major source of variability in toxicity tests, reduces cost, and the expertise required of personnel to perform the test (Persoone, 1991). Since rotifer resting eggs hatch synchronously, physiologically uniform animals of similar age can be used for the test. Approximately 15 newborn rotifers are placed into each well containing 750 μL of test solution. The format of a 24-well plate allows for a control and five test concentrations, each with four replicates. Animals are exposed to the test solutions for 45 minutes and then 5 μm red microspheres are introduced into each well for 15 minutes. The rotifers readily ingest these microspheres in the absence of toxicant stress. Rotifer ingestion rate is a dose-dependent function of toxicant concentration, as toxicity increases rotifers feed less (Juchelka and Snell, 1994). The red microspheres accumulate in rotifer stomachs so that after 15 minutes, their guts appear bright red. This can be easily seen under a dissecting microscope at 25X magnification. Rotifers with red guts are scored as feeding and those with no visible red as non-feeding. The number of red

C. Blaise and J.-F. Férard (eds.), Small-scale Freshwater Toxicity Investigations, Vol. 1, 323-335.

microspheres in the gut is not counted, only the presence or absence of red color. This experimental design allows for the calculation of percent feeding in each of the four replicates. Statistical analysis can be performed with the same procedures as used in analyzing percent survival data in acute toxicity tests.

Table 1. Summary of the rotifer ingestion toxicity test.

Test animal	Freshwater: *Brachionus calyciflorus* Marine*: Brachionus plicatilis*
Test type	Rapid screening
Test format	24-well plate
Test volume	750 µL per well
Test duration	1 hour
Source of test animals	Hatching cysts, commercially available
Rotifers per replicate	10-15
Temperature	20-30°C
Salinity	*Brachionus calyciflorus* : 0-5 ppt *B. plicatilis :* 3-40 ppt
Light	No specific requirements
Dilution water	Artificial freshwater or seawater, natural surface water
Endpoint	Percent feeding
Reference toxicants	Copper ($CuSO_4$), pentachlorophenol
Ingestion in controls	Should exceed 80%

3. Overview of applications reported with rotifer toxicity tests

Rotifer cysts were introduced to ecotoxicology by Snell and Persoone (1989a,b) who described a 24-hour acute toxicity test conducted with hatchlings from cysts. This test has been validated and adopted as a standard method (ASTM 1440) and is commercially available as a test kit (Rotoxkit F) from Microbiotests, Inc. (see below). A method to estimate chronic toxicity using asexual reproduction has been developed (Snell and Moffat, 1992) and published as standard method 8420 in Standard Methods for the Examination of Water and Wastewater (2001). It also is commercially available as a test kit (Rotoxkit F chronic) from Microbiotests, Inc. (see below). A number of other endpoints have been developed using cyst hatchlings as starting material such as resting egg production (Preston et al., 2000; Preston and Snell, 2001), swimming (Charoy et al., 1995), enzyme activity (Burbank and Snell, 1994) and stress protein gene expression (Cochrane et al., 1994). Tests to estimate

toxicity based on rotifer ingestion rate were developed by Fernandez-Casalderry et al., (1992, 1993a and b) and Juchelka and Snell (1994), then expanded to cladocerans and ciliates (Juchelka and Snell, 1995). This latter work employed fluorescent microspheres and quantified fluorescence in rotifer guts using epifluorescent microscopy. Although useful in research, this method requires expensive equipment that is not widely available to quantify rotifer ingestion rate. The method described here simplifies the estimation of rotifer ingestion as an endpoint for toxicity tests. The use of rotifers in ecotoxicology has been reviewed by Snell and Janssen (1995; 1998).

The rotifer *Brachionus calyciflorus* was chosen for this test because it is an herbivore with a broad diet, feeding non-selectively on particles in the size range of 2-15 μm (Starkweather, 1987). Ingestion is an ecologically important process which is incorporated into most bioenergetics models (Starkweather, 1987). Energy ingested is directly linked to reproductive output, a key element of fitness and long-term survival of a population. Ingestion rate, therefore, should be a good estimator of chronic toxicity. Ingestion tests to estimate toxicity in other zooplankters have been described. CerioFAST is a method to measure ingestion rate of *Ceriodaphnia dubia* which is based in ingestion of fluorescently labeled yeast (Jung and Bitton, 1997).

4. Advantages of conducting the rotifer ingestion test

One of the main advantages of the rotifer ingestion test is its speed. The test can be conducted in one hour on five test solutions plus a control, each with four replicates. The test also requires little technical expertise since it is initiated with rotifers hatched from cysts and no difficult manipulations are required. After a few practice sessions, even inexperienced people should be able to conduct the rotifer ingestion test and produce useable data. Small volumes of test material are required, so this test is well suited for testing pore waters, incorporating into a battery of tests, or guiding the bioassay-directed fractionations of toxicity identification evaluations. No expensive equipment is required to perform the test, so it is of particular interest for performance in the field or in developing countries. The cost per sample for estimating toxicity is attractive compared to other toxicity tests. The sensitivity of the rotifer ingestion test compares favorably to other endpoints (Juchelka and Snell, 1994; 1995; Preston and Snell, 2001) and other species (Snell and Janssen, 1998). The disadvantages of this test include the short exposure time which may not be long enough for slow acting toxicants to have an effect, the small size of rotifers which requires a good quality microscope to clearly see them, the small exposure chamber which may increase sorption of test compounds, and the fact that this test is currently not approved as a standard method.

5. Test species

Rotifers are classified in the phylum Rotifera, one of several phyla of lower invertebrates. There are approximately 2000 rotifer species named; they are divided

into two classes, Digononta and Monogononta (Nogrady et al., 1993). Monogononts reproduce parthenogenetically, but in response to specific environmental cues, they reproduce sexually yielding dormant embryos called cysts (resting eggs) which have been used in toxicity testing (Snell and Janssen, 1995). Most rotifer species inhabit fresh and brackish waters (Wallace and Snell, 2001), but there are some genera, like *Synchaeta*, where the majority of species are marine (Nogrady, 1982). In coastal marine habitats, rotifers sometimes comprise the dominant portion of the zooplankton biomass (Egloff, 1988). They are also abundant in marine interstitial habitats, the interstitial water of soils (Pourriot, 1979), and in water clinging to mosses, liverworts and lichens (Ricci, 1983). In freshwater lake plankton (Stemberger, 1990) and in river sediments (Schimd-Araya, 1995), rotifers often are abundant with high species diversity.

Rotifers play an important role in the ecological processes of many aquatic communities (Pace and Orcutt, 1981). As suspension feeders, planktonic rotifers influence algal species composition through selective grazing (Bogdan and Gilbert 1987; Starkweather, 1987; Arndt, 1993). Rotifers often compete with cladocera and copepods for phytoplankton in the 2 to 18 µm size range. Along with crustaceans, rotifers contribute substantially to nutrient recycling (Esjmont-Karabin, 1983). Rotifers are food for many fish larvae (Lubzens et al., 1997).

The genus *Brachionus* is large with over 25 species distributed in marine and freshwater habitats all over the world (Nogrady et al., 1993). The species *Brachionus calyciflorus* and *B. plicatilis* are a complex of cryptic species with many distinct populations (Gomez et al., 2002). The geographical strain of *B. calyciflorus* typically used in toxicity testing was collected in Gainesville, Florida, in 1983 (Snell et al., 1991) and has been used to produce cysts in the laboratory ever since. The *B. plicatilis* strain was originally collected in the Azov Sea, Russia, in 1983 (Snell and Persoone, 1989b) and likewise has been a source of cysts. These strains were selected because of their ability to produce cysts, not because of their extraordinary sensitivity to toxicants. Rotifer cysts for toxicity testing can be purchased from Microbiotests, Inc., Venecoweg 19, 9810 Nazareth, Belgium, tel. 3293808545, fax 3293808546, e-mail microbiotests@skynet.be (contact the company for distributors in various countries). Rotifer cysts should be stored in a freezer (-20°C).

6. Culture/maintenance of rotifers in the laboratory

There is no culture required for the rotifer ingestion test. Test animals are obtained by hatching cryptobiotic stages (cysts) that are commercially available (see Section 5). Because the duration of the test is only one hour, there also is no food required to feed test animals. Disposable plastic 24-well plates are used, so there is no glassware to wash.

Water to dilute test solutions may be prepared from high quality deionized or distilled water. Artificial freshwater may be used for *Brachionus calyciflorus* and artificial seawater for *B. plicatilis*.

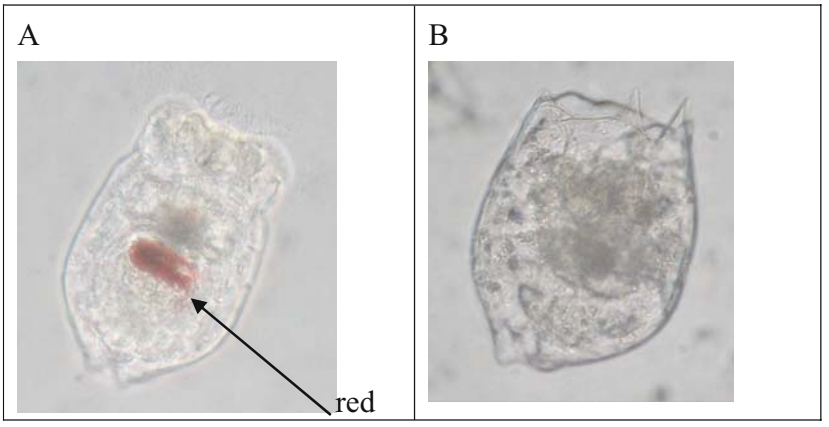

Figure 1. A photomicrograph at 400X magnification of a B. calyciflorus *which has ingested red carmine particles (A) and one which has not ingested (B). The key trait is the dark gut which would appear red in color.*

6.1 ARTIFICIAL FRESHWATER

Prepare standard synthetic freshwater by adding 96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot 2H_2O$, 60 mg $MgSO_4$, and 4 mg KCl to 1 L of deionized or distilled water. Mix well on a magnetic stirrer and adjust pH to 7.5 with 10 M KOH or HCl. Use within one week. This is a moderately hard standard freshwater, with hardness of 80-100 mg $CaCO_3$ per liter and alkalinity of 60-70 mg per liter.

6.2 ARTIFICIAL SEAWATER

Prepare standard synthetic seawater with a salinity of 15 parts per thousand (ppt) by adding: 11.31 g NaCl, 0.36 g KCl, 0.54 g $CaCl_2$, 1.97 g $MgCl_2 \cdot 6H_2O$, 2.39 g $MgSO_4 \cdot 7H_2O$, 0.17 g $NaHCO_3$ to 1 L of deionized or distilled water. Mix well on a magnetic stirrer and adjust pH to 8.0 with 10 M KOH or HCl. Use within one week.

Other waters: bottled mineral water (no gas), dechlorinated tap water, surface water, well water, natural seawater, and waters from other sources can be used as dilution water in rotifer toxicity tests. Prior to their use, ingestion studies should be conducted to ensure acceptable levels of feeding can be obtained in the negative control (*e.g.*, $\geq$ 80% feeding).

7. Preparation of rotifers for toxicity testing

Rotifers are prepared for the ingestion test by hatching cysts. Hatchlings are collected within a few hours of their birth so they all are of similar age and physiological condition. They need no feeding in their first day since they are well

provisioned with energy by their mothers. There is no need for acclimation and there are no nutritional issues since there is no feeding during the test. Ingestion in the test is quantified by uptake of inert microspheres.

7.1 ROTIFER CYST HATCHING

Hatching should be initiated 18 hours before the start of a test for *B. calyciflorus*. Place about 30 ml of artificial freshwater or mineral water into a clean Petri dish, empty the contents of one vial of rotifer cysts (a few thousand cysts) into the water and rinse the vial to remove all cysts (Snell et al., 1991). Incubate the Petri dish at 25°C in the light of one or two 20 Watt fluorescent tubes (1000-4000 lux) for 16-18 hours. Make sure that the cysts are submerged during the incubation by rinsing the sides of the hatching dish using a pipette. Hatching should start after about 15 hours and 1-2 hours later the rotifers can be transferred to the 24-well test plate. Cooler temperatures, low or high pH, elevated hardness and alkalinity can delay hatching. When hatching is delayed the cause often is low temperature or poor water quality. The problem is usually corrected by bringing temperature to 25°C or switching to a different water source. If hatching is delayed, check cysts hourly to insure collecting test animals within a few hours of hatching. Hatching of *B. plicatilis* cysts should be initiated 24 hours before a test in 15 ppt seawater in conditions as described above. Unused cysts should be stored in a freezer (-20°C).

8. Testing procedure

8.1 HANDLING SAMPLES

Water samples should be collected and handled according to standard procedures. Surface waters, pore waters, and effluents should be transported at cool temperatures in containers that protect them from light. The rotifer test should be performed within 24 hours of sample collection. Because the rotifer ingestion test requires only 750 µL per test well, usually 50 mL per sample is plenty to perform the test. When testing pure chemicals that have low solubility in water, a carrier solvent such as acetone can be used. This requires a solvent control to be included in the experimental design.

8.2 PREPARING A DILUTION SERIES

As the rotifers are hatching, prepare a dilution series of the test compound or effluent according to standard methods. If the sample contains debris or large floating and/or suspended solids it may be necessary to first coarse-filter it through a sieve that has 2-4 mm mesh openings. If the sample contains organisms, it should be filtered through a sieve with 60 µm mesh openings. Centrifugation (2000-4000 rpm for 3 minutes) is effective for removing small suspended particles. CAUTION: filtration or centrifugation may remove some toxicants if they are bound to particles.

It is advisable to measure pH, conductivity or salinity, total alkalinity, total hardness, and total residual chlorine in the undiluted effluent or surface water. If these water chemistry parameters are very different from the dilution water, this can reduce rotifer ingestion in the absence of toxicity.

A concentration-response test on effluent or pore water consists of a control and a minimum of five concentrations commonly selected to approximate a geometric series, such as 100%, 50%, 25%, 12.5% and 6.25%. One method of preparing a dilution series is as follows: pipette 10 mL of effluent or pore water sample into a test tube (**NEVER** pipette by mouth). Label this as the 100% test solution. Pipette 5 mL of the 100% sample into a second test tube and add 5 mL of dilution water, mix thoroughly and label this tube as the 50% test solution. Pipette 2.5 mL of the 50% solution into a third test tube and add 7.5 mL of dilution water, mix and label as the 25% test solution. Repeat this procedure for the 12.5%, and 6.25% test solutions. If 100% mortality has occurred in the higher concentrations after the 45 minute exposure, lower concentrations should be tested such as 3.1%, 1.6%, and 0.8%.

When testing a single chemical of unknown toxicity, it is best to do a range-finding test first. This is accomplished by creating a log series (0.01, 0.1, 1, 10, 100 mg/L) and identifying the lowest concentration where effects are observed. This is used as the highest concentration in a second, definitive test with a log concentration series spanning the two log concentrations. For example, if effects were observed at 10 mg/L in the range-finding test, a concentration series of 1.6, 2.5, 4.0, 6.3, 10 mg/L could be used in the definitive test. This series was calculated by subtracting 0.2 from the log 10 and calculating 10^x (antilog) to give the five test concentrations.

8.3 FILLING THE TEST WELLS

The rotifer ingestion test is conducted in 24-well polystyrene plates (Corning 25820 or equivalent) and consists of a negative control and five test concentrations. Notice that these plates are labeled as columns 1-6 across and rows A-D down. Pipette 0.75 mL of dilution water into well A in column 1 (A1, the upper left most well) of the test plate. This well is dilution water without toxicant and will serve as the negative control. Fill wells B1, C1, and D1 with dilution water in a similar fashion. This experimental design provides four replicates for each treatment. Working from the lowest concentration, pipette 0.75 mL of the first test concentration into wells A2, B2, C2, and D2 of column 2 of the test plate. Repeat this procedure for the wells in columns 3-6.

8.4 ADDING THE ROTIFERS

Beginning with the control, use a small bore micropipette to transfer about 15 rotifers from the hatching dish into well A1 of the test plate. Rotifers can be concentrated in the hatching dish by shining a light from one side. Repeat this transfer for the remaining wells, adding about 15 rotifers to each well. The exact number of rotifers added is not important at this point because they will be counted at the end of the test. Minimize the transfer of water along with the rotifers. For best results, rotifers should be 2-6 hours old. Rotifers 0-1 hour old may not feed.

8.5 INCUBATION AND SCORING OF THE TEST PLATE

Incubate the test plate at about 25°C in darkness for 45 minutes. After incubation, place the plate under a dissecting microscope and observe the rotifers at about 10X magnification, recording whether most rotifers are swimming in each well. If the sample is so toxic that it has killed the rotifers, there will obviously be no ingestion. In this case, lower concentrations should be tested. Pipette 0.01 mL of a concentrated suspension of 5 µm diameter red microspheres (Bangs Laboratories, www.bangslabs.com; similar products may be available from other suppliers*) into 6 mL of dilution water and shake to mix well. Add 0.03 mL of this microsphere suspension to each test well. The final microsphere concentration in the test wells should be about 250,000/mL. Allow the rotifers to feed for 15 minutes. At the end of the feeding period, animals should be killed by adding one drop (~50 µL) of 10% formalin solution to each well. This does not affect the red color of the beads, so the test can be scored at a later time. This allows, for example, the test to be conducted in the field and scored back in the lab. The rotifers in each well should be observed under the microscope at 25X magnification and the number of feeding and non-feeding in each concentration should be counted. Your data can be recorded in a table (Box 1) that looks like this:

Box 1. Example of a table used to report test data.

Test concentration	***Rep.***	***Well***	***# feeding***	***# not feeding***	***Percent feeding***	***Swimming after 45 minutes?***
0 (control)	1	A1	10	0	100	yes
	2	B1	12	1	92	yes
	3	C1	13	1	93	yes
	4	D1	11	0	100	yes
1	1	A2	12	2	86	yes
	2	B2	14	1	93	yes
	3	C2	11	3	79	yes
	4	D2	10	2	83	yes
2	1	A3	13	4	76	yes
	2	B3	11	3	79	yes
	3	C3	9	2	82	yes
	4	D3	14	5	74	yes

* Carmine can be substituted for the microspheres and is cheaper. A carmine suspension is a mixture of particles of various sizes, and can be prepared by adding 1 mg carmine to 2 mL dilution water. Mix well to suspend the fine particles. Add 10 µL of this suspension to each well after the 45 minute exposure. Rotifers will accumulate red color in their guts after a few minutes of feeding. Carmine can be obtained from several suppliers. We have purchased it from Fisher Scientific (https://www.fishersci.com/index.jsp, product number AC19020-0050).

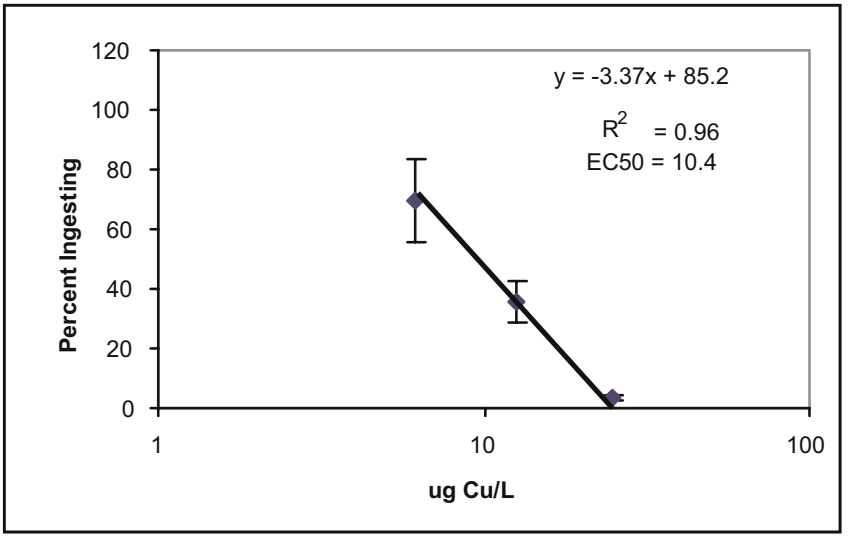

Figure 2. Example of an ingestion concentration-response curve for B. calyciflorus *exposed to copper. Vertical lines equal one standard deviation. The 0 concentration (control) ingestion was 90.1%, but cannot be plotted on this log scale.*

9. Post-exposure observations/measurements and endpoint determinations

9.1 CRITERIA FOR TEST VALIDITY

For this test to be valid, a red color should be observed in > 80% of the guts of control animals. Since this is a sublethal assay, test concentrations should not immobilize (kill) the rotifers after 45 minutes exposure. Ideally, only the highest test concentration should have 0% ingestion and there should be intermediate values between this and the negative control.

9.2 DATA ANALYSIS

Percent feeding may be arcsine transformed and then a one-way analysis of variance can be performed on the data according to standard methods. A Dunnett's test can be used to compare all treatments to the control. This analysis will produce a no observed effect concentration (NOEC) and a lowest observed effect concentration (LOEC). Alternatively, a probit or trimmed Spearman-Karber test may be performed to estimate NOECs. An EC50 can be estimated from percent feeding data by calculating a linear regression of log concentration versus percent ingesting (Fig. 2). It is advisable to maintain a cumulative record of control performance so that the range of results expected under your conditions can be characterized.

10. Factors influencing the rotifer ingestion test

Most problems with the rotifer ingestion test center around the dilution water. A typical symptom is markedly reduced ingestion in the controls. Even carefully prepared dilution water can be unusable if it is too old or if deionization was inadequate. Rather that performing experiments to determine the cause of these problems, it is usually more prudent to simply change water sources. For example, bottled mineral water (no gas) is usually a good source. Often problems with dilution water quality are seasonal, disappearing as mysteriously as they arrived.

A second source of problems could involve rotifer cyst hatching. This often occurs due to poor dilution water quality. Changing water sources usually alleviates the problem. A second possibility is storage of cysts in poor conditions. It is recommended that cysts be stored in a freezer at –20°C. Cysts will age more rapidly at room temperature and at high humidity. Hatching also can be delayed by low temperatures, low or high pH, or inadequate lighting. This can be avoided by following the guidelines provided.

A third source of problems could be using rotifers very soon after hatching. For about the first hour after birth rotifers do not feed, so it is important to collect hatchlings that are at least two hours old. Hatchlings older than about six hours may have reduced feeding due to starvation. Rotifer ingestion may also be suppressed by a heavy load of suspended particles in the test medium. These samples should be filtered or centrifuged to reduce this effect.

11. Applications of the rotifer ingestion test

The sensitivity of the rotifer ingestion test has been compared to reproduction and mortality endpoints for five organics, three metals, and two insecticides (Juchelka and Snell, 1994). The 48-h reproduction NOEC was more than two-fold lower than the 1-h ingestion NOEC for phenol, dimethylphenol, cadmium, copper, mercury and diazinon. Similar reproduction and ingestion NOECs were observed for pentachlorophenol, naphthol, and chlorpyrifos. Rotifer ingestion NOEC was a more sensitive endpoint than 24-h mortality by at least two-fold for all ten toxicants tested except copper. Rotifer ingestion as an endpoint can be more or less sensitive than mortality, depending on the toxicant (Fernandez-Casalderry et al., 1992; 1993a and b). The response of *Brachionus calyciflorus* ingestion rate to toxicants has been compared to that of *B. plicatilis*, *Ceriodaphnia dubia*, and *Paramecium aurelia* by Juchelka and Snell (1995). Ingestion rate was used to investigate the effects of UV-B exposure on *B. calyciflorus* (Preston et al., 1999).

The rotifer ingestion test has been used to assess the toxicity of pore waters collected from 13 urban creeks in the Atlanta area (Juchelka and Snell, 1995). The *B. calyciflorus* ingestion test was compared to ingestion by *Ceriodaphnia dubia*, and *Paramecium aurelia* and esterase enzyme activity by the yeast *Candida tropicalis* and the bacterium *Bacillus subtilis*. The *B. calyciflorus* test detected toxicity in pore water from 9 of the 13 sites. The *P. aurelia* test detected toxicity at 7 sites and the *C. dubia* and *B. subtilis* tests detected toxicity at 3 sites. No toxicity was detected by

C. tropicalis at any site. The rotifer test found two sites to be toxic that were not toxic in any of the other tests and one site was non-toxic in all five of the tests.

12. Conclusions/Prospects

The rotifer ingestion test allows investigators to estimate toxicity in surface water, effluent, and pore water samples as well as to characterize toxicity in solutions of pure chemicals. With this test, toxicity can be quantified rapidly, inexpensively, and with minimal training of personnel. The method would benefit from application to a wide variety of environmental problems so that its usefulness and limitations can be more fully understood.

Acknowledgements

The author is grateful for the insightful comments of Don Versteeg, Guido Persoone, and Roberto Rico-Martinez which have improved this paper.

References

ASTM (American Society for testing and Materials) (2002) Standard Guide for Acute Toxicity Test with the Rotifer *Brachionus*, ASTM, Vol. 11.05, method E 1440, pp. 806-813.

Arndt, H. (1993) Rotifers as predators on components of the microbial web (bacteria, heterotrophic flagellates, ciliates)-a review, *Hydrobiologia* **255/256**, 231-246.

Bogdan, K.G. and Gilbert, J.J. (1987) Quantitative comparison of food niches in some freshwater zooplankton, *Oecologia* **72**, 331-340.

Burbank, S.E. and Snell, T.W. (1994) Rapid toxicity assessment using esterase biomarkers in *Brachionus calyciflorus* (Rotifera), *Environmental Toxicology Water Quality* **9**, 171-178.

Charoy, C.P., Janssen, C.R., Persoone, G. and Clement, P. (1995) The swimming behavior of *Brachionus calyciflorus* (Rotifera) under toxic stress: I. The use of automated trajectometry for determining sublethal effects of chemicals, *Aquatic Toxicology* **32**, 271-282.

Cochrane, B.J., De Lama, Y.D. and Snell , T.W. (1994) Polymerase chain reaction as a tool for developing stress protein probes, *Environmental Toxicology and Chemistry* **13**, 1221- 1229.

Egloff, D.A. (1988) Food and growth relations of the marine zooplankter, *Synchaeta cecelia* (Rotifera), *Hydrobiologia* **157**, 129-141.

Ejsmont-Karabin, J. (1983) Ammonia nitrogen and inorganic phosphorus excretion by the planktonic rotifers, *Hydrobiologia* **104**, 231-236.

Fernandez-Casalderry, A., Ferrando, M.D. and Andreu-Moliner, E. (1992) Filtration and ingestion rates of *Brachionus calyciflorus* after exposure to endosulfan and diazinon, *Comparative Biochemistry Physiology* **103C**, 357-361.

Fernandez-Casalderry, A., Ferrando, M.D. and Andreu-Moliner, E. (1993a) Effect of the insecticide methylparathion on filtration and ingestion rates of *Brachionus calyciflorus* and *Daphnia magna*, *Science of the Total Environment* **suppl,** 867-876.

Fernandez-Casalderry, A., Ferrando, M.D. and Andreu-Moliner, E. (1993b) Feeding behavior as an index of copper stress in *Daphnia magna* and *Brachionus calyciflorus*, *Comparative Biochemistry and Physiology* **106C**, 327-331.

Gomez, A., Serra, M., Carvalho, G.R. and Lunt, D.H. (2002) Speciation in ancient cryptic species complexes: Evidence from the molecular phylogeny of *Brachionus plicatilis* (Rotifera), *Evolution* **56**, 1431-1444.

Juchelka, C.M. and Snell, T.W. (1994) Rapid toxicity assessment using rotifer ingestion rate, *Archives* of *Environmental Contamination and Toxicology* **26**, 549-554.

Juchelka, C.M. and Snell, T.W. (1995) Rapid toxicity assessment using ingestion rate of cladocerans and ciliates, *Archives* of *Environmental Contamination and Toxicology* **28**, 508-512.

Jung, K. and Bitton, G. (1997) Use of Ceriofast[tm] for monitoring the toxicity of industrial effluents – comparison with the 48-h acute *Ceriodaphnia* toxicity test and Microtox[R], *Environmental Toxicology and Chemistry* **16**, 2264-2267.

Lubzens, E., Minkoff, G., Barr, Y. and Zmora, O. (1997) Mariculture in Israel - past achievements and future directions in raising rotifers as food for marine fish larvae, *Hydrobiologia* **358**, 13-20.

Nogrady, T. (1982) Rotifera, in S.P. Parker (ed.), *Synopsis and Classification of Living Organisms*, McGraw-Hill, pp. 865-872.

Nogrady, T., Wallace, R.L. and Snell, T.W. (1993) Rotifera, *Biology, Ecology and Systematics*, Vol. 1, The Hague, Belgium, SPB Academica Publishing bv.

Pace, M.L. and Orcutt, J.D. (1981) The relative importance of protozoans, rotifers and crustaceans in freshwater zooplankton communities, *Limnology and Oceanography* **26**, 822-830.

Persoone, G. (1991) Cyst-based toxicity tests: I. A promising new tool for rapid and cost-effective screening of chemicals and effluents, *Zeitschrift fur Angewandte Zoologie* **78**, 235-241.

Pourriot, R. (1979) Rotifères du sol, *Revue d'Écologie et de Biologie du Sol* **16**, 279-312.

Preston, B.L. and Snell, T.W. (2001) Full life-cycle toxicity assessment using rotifer resting egg production: implications for ecological risk assessment, *Environmental Pollution* **114**, 399-406.

Preston, B.L., Snell, T.W. and Kneisel, R. (1999) UV-B exposure increases acute toxicity of pentachlorophenol and mercury to the rotifer *Brachionus calyciflorus*, *Environmental Pollution* **106**, 23-31.

Preston, B.L., Snell, T.W., Robinson, T.L. and Dingmann B.J. (2000) Use of the freshwater rotifer *Brachionus calyciflorus* in a screening assay for potential endocrine disruptors, *Environmental Toxicology and Chemistry* **19**, 2923-2928.

Ricci, C. (1983) Life histories of some species of Rotifera Bdelloidea, *Hydrobiologia* **104**, 175-180.

Schmid-Araya, J.M. (1995) Disturbance and population dynamics of rotifers in bed sediments, *Hydrobiologia* **313/314**, 279-301.

Snell, T.W. and Persoone G. (1989a) Acute toxicity bioassays using rotifers. II. A freshwater test with *Brachionus rubens*, *Aquatic Toxicology* **14**, 81-92.

Snell, T.W. and Moffat, B.D. (1992) A two day life cycle test with the rotifer *Brachionus calyciflorus*, *Environmental Toxicology and Chemistry* **11**, 1249-1257.

Snell, T.W. and Janssen, C.R. (1995) Rotifers in Ecotoxicology: A review, *Hydrobiologia* **313/314**, 231-247.

Snell, T.W. and Janssen, C.R. (1998) Microscale toxicity testing with rotifers, in P.G. Wells, K. Lee and C. Blaise (eds.), *Microscale Aquatic Toxicology - Advances, Techniques and Practice*, CRC Lewis Publishers, Florida, pp. 409-422.

Snell, T.W. and Persoone, G. (1989b) Acute toxicity bioassays using rotifers. I. A test for marine and brackish water with *Brachionus plicatilis*, *Aquatic Toxicology* **14**, 65-80.

Snell, T.W., Moffat, B.D., Janssen, C. and Persoone, G. (1991) Acute toxicity tests using rotifers: IV. Effects of cyst age, temperature, and salinity on the sensitivity of *Brachionus calyciflorus, Ecotoxicology and Environmental Safety* **21**, 308-317.

Standard Method 8420 (2001) Rotifers, in *Standard Methods for the Examination of Water and Wastewater*, pp. 8-62 – 8-65.

Starkweather, P.L. (1987) Rotifera, in T.J. Pandian and F.J. Vernberg, (eds.) *Animal Energetics,*Vol. 1, Protozoa through Insecta, Academic Press, Orlando, FL.

Stemberger, R.S. (1990) An inventory of rotifer species diversity of northern Michigan inland lakes, *Archives fur Hydrobiologia* **118**, 283-302.

Wallace, R.L. and Snell, T.W. (2001) Rotifera, in J.H. Thorp and A.P. Covich (eds.) *Ecology and Classification of North American Freshwater Invertebrates*, Academic Press, New York, pp. 187-248.

Abbreviations

$CaCl_2$	calcium chloride
$CaCO_3$	calcium carbonate
$CaSO_4 \cdot 2H_2O$	calcium sulfate
HCl	hydrogen chloride
IC50	interference concentration where 50% of individuals are affected
KCl	potassium chloride
KOH	potassium hydroxide
LOEC	lowest observed effect concentration
M	molar
mM	millimolar
$MgCl_2 \cdot 6H_2O$	magnesium chloride
$MgSO_4$	magnesium sulfate
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NOEC	no observed effect concentration
ppt	parts per thousand
rpm	revolutions per minute.