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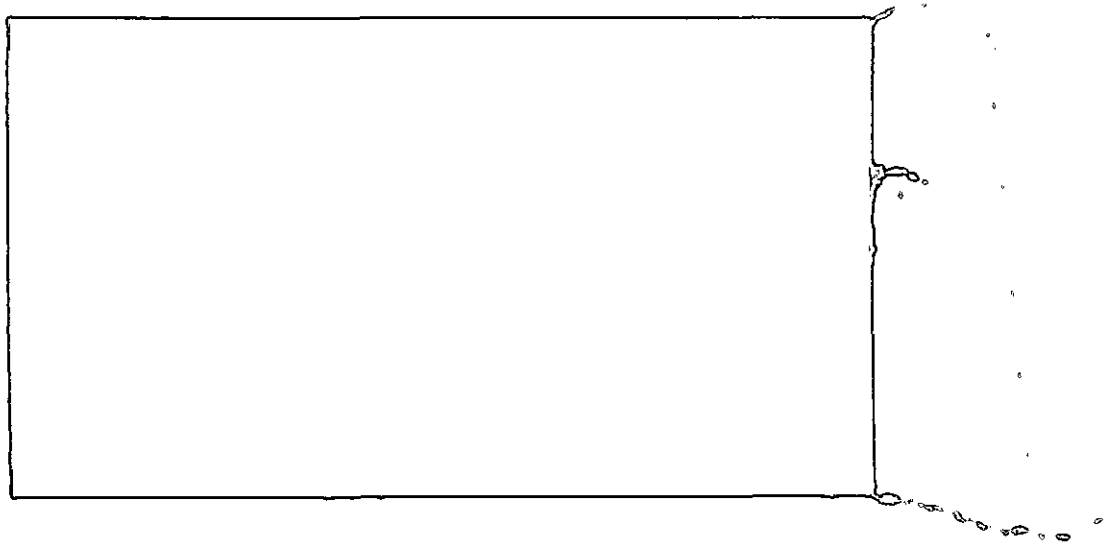

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Acute Toxicity Tests: General Description and Materials and Methods Manual II. Daphnia

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Acute Toxicity Tests: General Description and Material and Methods Manual

II. Daphnia

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I. ACKNOWLEDGEMENT

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II. EXECUTIVE SUMMARY

Aquatic toxicity can be assessed using a variety of test organisms including fish, algae, or invertebrates. The materials, methods and procedures used for conducting aquatic toxicity tests with daphnids, a macroinvertebrate, are presented.

Two macroinvertebrates, Daphnia magna and Daphnia pulex have been utilized in such toxicity testing because of their sensitivity to many chemical pollutants and ease in culturing. D. magna is recommended for toxicity testing of hard waters (>160 mg/l as $CaCO_3$) while D. pulex is used for testing softer waters (<100 mg/l as $CaCO_3$). Either species is suitable for waters of intermediate hardness although D. magna is hardier and larger than D. pulex and may therefore be preferred.

Daphnids are cultured in the laboratory in an unpolluted surface or groundwater source or in glass-distilled reconstituted water. Toxicity tests are conducted for 48 hours using replicate samples containing a dilution of the toxicant solution and ten daphnid first stage instars in one liter glass beakers. Mortality, as determined by cessation of antennal or leg movement after gentle prodding, is observed at frequent time intervals so that the LC50 (toxicant concentration causing 50 percent mortality) and ILC50 (incipient lethal concentration) can be calculated. The testing procedure can determine the acute toxicity of both known chemical toxicants and complex effluent samples. Methods for developing and maintaining a daphnid culture, obtaining first stage instars,

establishing the viability of the test organisms and conducting the toxicity test, are described. The persistence of the toxicity of a sample is evaluated by comparing the LC50 value determined immediately following sample collection with that determined on a second portion of the same sample after 96 hours of storage at ambient temperature. Transfer toxicity tests are described which are used to determine the effect of intermittent or varying exposure of a toxicant to daphnids. Such a procedure more closely reflects conditions encountered in an aquatic environment receiving intermittent or variable pollutant discharges. Methods used to determine LC50 and ILC50 toxicity values are based upon the observed mortality of the daphnids over time, at the various toxicant concentrations. The procedure is the same as for the determination of toxicity values using fish as the test organisms and has been described previously by Plotkin and Ram (1982b). The 1982 direct cost for establishing a daphnid toxicity testing laboratory, including equipment (capital expenses), glassware, and chemical supplies was about $13,000 (excluding the cost of a constant temperature room). Approximately 16 hours are needed to conduct a single daphnid bioassay, excluding sampling requirements and culture maintenance. The direct cost for each daphnid test is dependent upon the wages of the technician conducting the test and proportion of capital expenses assigned to each bioassay. The direct cost, including labor and supplies, ranges from about $280 to $160 per test (in 1982 dollars). The former figure includes one percent of

the capital expense per test (equal to $130) and the later figure represents labor and supply costs alone. A minimum of $1000 (1982 dollars) is required per year for chemical and glassware replacement. Daphnid bioassays are considerably cheaper and less time consuming than fish bioassays.

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V. *INTRODUCTION*

Determination of aquatic toxicity can be achieved using a variety of test organisms such as fish, algae, or invertebrates. The test organisms are exposed to a range of toxicant concentrations to determine the concentration causing 50 percent mortality after a specified exposure period (LC50) or the incipient lethal concentration, below which a mortality of no more than 50 percent is observed even upon prolonged periods of exposure (ILC50). Some of the test protocol and statistical methods used when conducting toxicity tests with these various test organisms are the same. For example, such water quality parameters as dissolved oxygen, pH, hardness, alkalinity, conductivity, and temperature influence the extent to which a particular toxicant can affect a test organism, whether it be a species of fish such as the fathead minnow (Pimephales promelas) or the invertebrate Daphnia magna. All of these water quality parameters, then, must be determined and reported when conducting a toxicity test. The degree of susceptibility to any given toxicant, however, may vary between different test organisms. It is therefore prudent, when determining the potential deleterious impact of a chemical toxicant on an aquatic ecosystem, to conduct several toxicity tests using different test organisms from various trophic levels. In this way the effect of the toxicant on a broader range of organisms in an ecosystem can be estimated. The materials and methods for conducting fish bioassays and algal assays have been presented previously (Plotkin

and Ram; 1982a,b). This report presents additional test procedures for toxicity testing using daphnids.

Five basic steps are required to conduct toxicity tests using daphnids:

i. choosing the test organism;

ii. procuring the daphnids;

iii. establishing and maintaining the daphnid culture;

iv. conducting the toxicity test (Toxicity Testing Procedures); and

v. evaluating toxicity data.

The first four topics are discussed in detail in this paper along with associated test protocol, procedures and recommendations. Evaluation of toxicity data has been described previously (Plotkin and Ram, 1982b).

VI. CHOOSING THE TEST ORGANISMS: D. MAGNA OR D. PULEX?

1. General description of D. magna and D. pulex

Two macroinvertebrates, Daphnia magna and Daphnia pulex, have frequently been used by investigators (Winner, 1976; Sherherban, 1977; and Maki, 1979) for toxicity testing because of their sensitivity to many chemicals. D. magna is principally a lake dweller and is restricted to waters in northern and western North America which exceed a hardness of 150 ppm as $CaCO_3$ (Pennak, 1978). D. pulex (Figure 1) occurs over most of the North American continent. It is principally a pond dweller, but also is found in lakes.

The life span of daphnids, from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the species and environmental conditions (Pennak, 1978). Generally the life span increases as temperature decreases, due to lower metabolic activity. The average life span of D. magna is about 40 days at 25° C, and about 56 days at 20°C. The average life span of D. pulex at 20° is approximately 50 days. Food availability additionally influences the life span of these organisms. Generally an inverse relationship is observed between life span and food availability, just short of starvation (Wetzel, 1975).

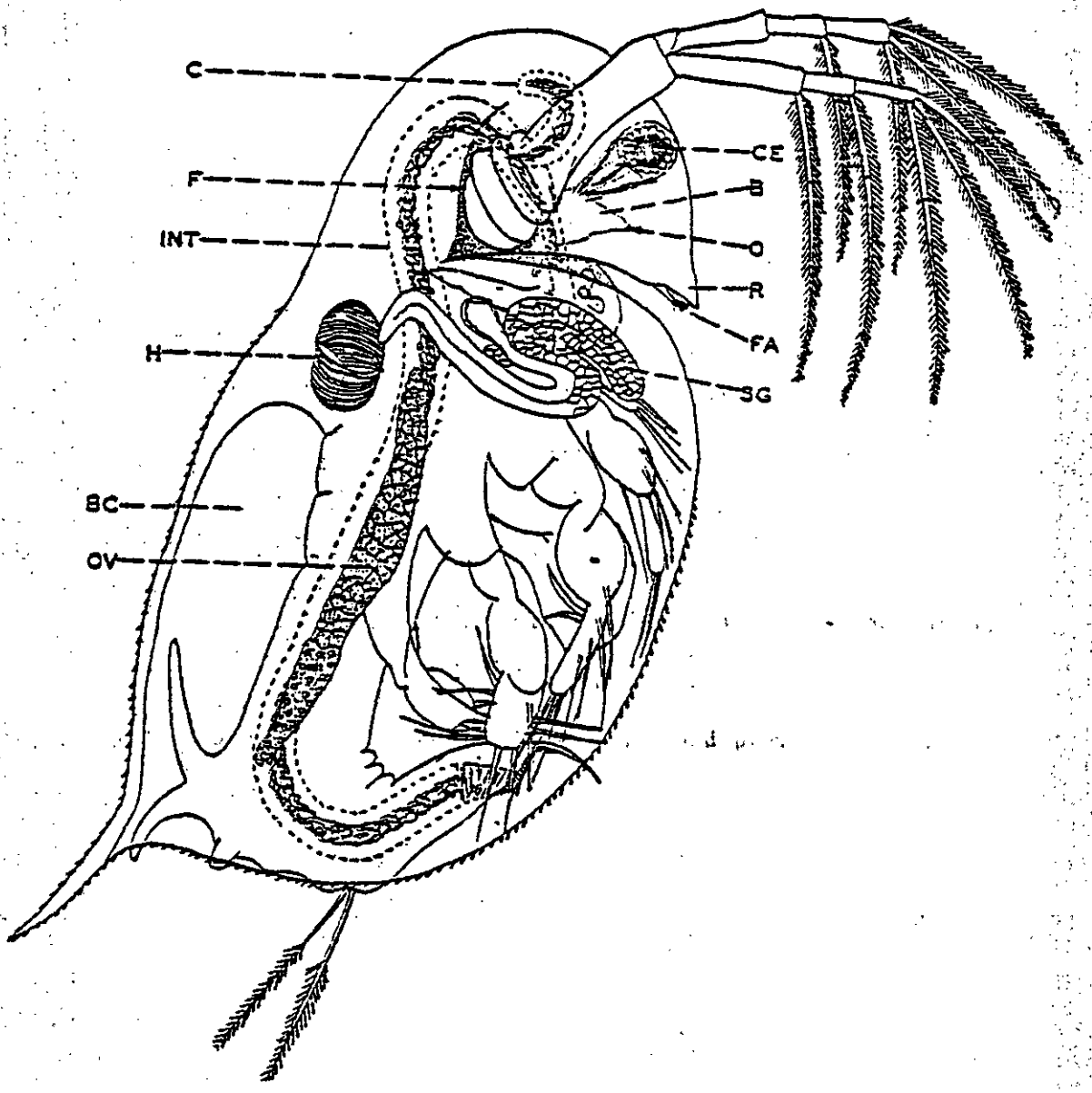

Figure 1. Anatomy of female Daphnia pulex (Magnification x70): B, brain; BC, brood chamber; C, digestive caecum; CE, compound eye; F, fornix; FA, first antenna (attennule); H, heart; INT, intestine; O, ocellus; OV, ovary; R, ros trumor beak; SG, shell gland. (From Pennak, 1978.)

2. Life cycle

Four distinct periods may be recognized in the life history of daphnids: (1) egg, (2) juvenile, (3) adolescent, and (4) adult (Pennak, 1978). Typically, a clutch of 6-10 eggs is released into the brood chamber. The eggs hatch in the brood chamber and the juveniles, which are already similar in form to the adults, are released in approximately two days when the female molts (casts off her exoskeleton). The time required to reach maturity (defined as the stage in which the first offspring are produced) varies from six to ten days and also appears to be dependent on body size. The growth rate of the organism is greatest during its juvenile stages (early instars), and the body biovolume may double during each of these stages. D. pulex has 3-4 juvenile instars, whereas D. magna has 3-5 instars. Each stage is terminated by a molt. Growth occurs immediately after each molt while the new exoskeleton is still elastic.

Following the juvenile stages, the adolescent period is very short, and consists of a single instar. It is during the adolescent instar that the first clutch of eggs reaches full development in the ovary. Generally, eggs are deposited in the brood chamber within minutes after molting and the young which develop are released just before the next molt.

D. magna usually has 6 to 22 adult instars, while D. pulex has 18 to 25. In general, the duration of instars increases with age, but also depends on environmental conditions. A given instar

generally lasts approximately two days under favorable conditions, but when conditions are unfavorable, it may last as long as a week.

Four events take place in a matter of a few minutes at the end of each adult instar in females: (1) release of young from the brood chamber to the outside; (2) molting; (3) increase in size; and (4) release of a new clutch of eggs into the brood chamber. The number of young per brood is highly variable for daphnids, and depends primarily on food availability and environmental conditions. D. magna and D. pulex may both produce as many as 30 young during each adult instar, but more commonly the number is 6-10. The number of young released during the adult instars of D. pulex reaches a maximum at the tenth instar, after which there is a gradual decrease (Anderson and Zupancic, 1937). The maximum number of young produced by D. magna occurs at the fifth adult instar, after which it decreases (Anderson and Jenkins, 1942).

3. Considerations regarding choice of the test organism

When conducting a toxicity test using macroinvertebrates as the test organism, one must first decide whether to use indigenous organisms or a particular daphnid species.

D. magna and D. pulex are often preferable to using indigenous species in toxicity testing because:

1. data rarely exist as to which species indigenous to a given receiving water is the most sensitive;
2. pollution may have eliminated sensitive species, leaving only extremely tolerant species behind;

3. sensitive indigenous species may not be commercially available (daphnids are easily obtained) and culturing requirements may not be known;

4. the history of indigenous organisms from a receiving water may be unknown. Animals previously exposed to a pollutant might give erroneous toxicity test results because of possible acclimation to the toxicant.

If the investigator has decided to use either D. magna or D. pulex as the test organism he must then decide which of these two species is preferable for the purpose of the particular study. An important distinction between D. magna and D. pulex is their different tolerances to water hardness. Water with a hardness of 160-180 mg/L as $CaCO_3$ is recommended for D. magna while a hardness of 80-90 mg/1 as $CaCO_3$ is most suitable for D. pulex. Both species can be readily cultured in the laboratory, although D. magna has been found to be a hardier organism (Weber, 1980). Ideally, the water quality characteristics used for laboratory toxicity testing should closely parallel the field conditions under investigation. Since Massachusetts inland waters are generally soft with associated alkalinity values of less then 34 mg $CaCO_3$/L (Frey, 1963), D. pulex might seem like the logical test organism as it is more tolerant than D. magna to softer waters. However, the small size of D. pulex makes observing their mortality during a toxicity test very difficult and tedious. Since D. magna are hardier and larger than

D. pulex the former species may be preferred in conducting invertebrate toxicity tests.

VII. PROCUREMENT OF DAPHNIDS

1. Sources of organisms

Daphnids may be obtained from the U. S. Environmental Protection Agency, Lexington, MA laboratory, or from a biological supply house such as Carolina Biological or Berkshire Biological (see Appendix A). Only a small number (50-100) of organisms are needed to start a culture. The organisms are generally shipped in 200 milliter plastic jars. Transport time should not exceed two days. This is in order to ensure the maintenance of a healthy culture. Upon their receipt, the organisms should be acclimated to the temperature of the room used for conducting the toxicity tests ($20^{\circ}C$ is optimal). Sudden changes in temperature may cause death in some portion of the daphnid population. A temperature change of less than $2^{\circ}C$ per 12 hours is therefore recommended (Pucke, unpublished). Following temperature acclimination, daphnids should be immediately transferred to a freshly prepared culture medium and then fed.

VIII. ESTABLISHMENT AND MAINTENANCE OF THE DAPHNID CULTURE

1. Culture media

Daphnids may be cultured in either an 'unpolluted' surface or groundwater source, or in glass-distilled 'reconstituted' water. The factors which one considers in choosing the appropriateness of these two water sources for a given toxicity test application has been discussed previously (Plotkin and Ram, 1982b). The chemical constituents and concentration used for making up reconstituted water are shown in Table 1. Glass-distilled water or purified water obtained by reverse osmosis is preferred over that obtained by carbon absorption or ion exchange methods of purification. The latter procedure is known to leach constitutents from the carbon or exchange resin which might interfere with the toxicity test.

2. Container

Daphnids may be cultured in 2 to 4 liter widemouth glass jars. Alternatively, five gallon glass aquaria may be used. The latter has the advantage of being able to contain a larger volume of culture media and permits daphnids to avoid air bubbles being introduced into the vessel by the required aeration procedure. It is recommended that several culture vessels be maintained to avoid the loss of an entire culture resulting from a daphnid population crash contained in a single vessel.

Table 1

Components of Reconstituted Water Used in Culturing Daphnids

Water Type	Species Suitability	Chemical Components (mg/L) $NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	Resulting Hardness (mg $CaCO_3$/L)	Resulting Alkalinity (mg $CaCo_3$/L)
Moderately hard	D. pulex	96.0	60.0	60.0	4.0	85	57
Hard	D. magna	192.0	120.0	120.0	8.0	170	114

3. Aeration

The culture solution should be continuously and gently aerated by placing an air stone in one corner of the culture vessel. Care should be taken not to aerate too vigorously as this might result in super saturation of the water and/or the entrapment of air under the daphnids carpaces. Super saturation has occurred if small bubbles form on the sides of the culture vessel. Compressed air of unknown purity should be scrubbed through polyester or cotton batting to remove oil and particulate material.

4. Temperature

Daphnids can be cultured successfully over a wide temperature range (16-25°C). The optimum temperature is approximately 20°C, and if ambient laboratory temperatures remain in the range of 21 + 2°C, normal growth and reproduction of daphnids can be maintained without special temperature control equipment.

5. Illumination

The variations in ambient light intensities (50-100 ft candles) and prevailing day/night cycles in most laboratories do not seem to significantly affect daphnid growth and reproduction. However, a day/night cycle of approximately 12 hours light/12 hours dark is recommended.

6. Feeding

Appropriate food preparation and feeding are very important in maintaining daphnid cultures. Weber (1980) recommends a suspension of trout chow, alfalfa and yeast. Personnel at the EPA lab in

Lexington, MA have determined that viable cultures can be maintained without the yeast portion of the suspension (Davis, personal communication). The omission of yeast in the food being used to maintain daphnid cultures at the UMASS/Amherst bioassay laboratory, however, was suspected to have caused frequent population crashes. The inclusion of yeast in the daphnid food, therefore, is highly recommended. It is also recommended that the food solution be prepared by homogenizing it in an electric blender as described below. Mixing, utilizing a mortar and pestle, does not result in a sufficiently homogenized solution.

The food is prepared as follows:

a. Place 6.3 grams of trout chow pellets, 2.6 grams of dried yeast and 0.5 grams of dried alfalfa into a blender.

   NOTE: The trout chow must conform to United States Fish and Wildlife Service specifications PR(11)-78 and can be obtained through livestock feed stores. Dried alfalfa and yeast can usually be obtained at health food stores.

b. Add 500 ml of distilled water.

c. Blend at high speed for five minutes.

d. Place mixture in a refrigerator and allow to settle for one hour.

e. Remove from the refrigerator and decant 300 ml of the supernatant into a beaker.

f. Place 30-50 ml aliquots in 100 ml polyethylene bottles with screw-on tops and freeze.

g. Portions should be thawed as needed. After thawing the food solution should be refrigerated and then discarded after one week if not used.

Approximately 1 to 1.5 ml of daphnid food per 1000 ml of culture solution should be added three times per week (Monday, Wednesday and Friday, for example). Cultures will crash if they are fed over less frequent time intervals. Small amounts of excess food do not pose any problem if the solution is continuously aerated and replaced every two weeks, as stipulated by the culture maintenance protocol.

7. Culture maintenance

Careful culture maintenance is essential to ensure a viable daphnid population of sufficient size for bioassay testing needs. The solution in each stock culture vessel should be replaced every two weeks with freshly prepared reconstituted water or unpolluted ground or surface water. Concurrently, the daphnid culture should be thinned to prevent overcrowding. This is best accomplished as follows:

a. Using tygon tubing (5/16 in), gently syphon out about 10-15 adult daphnids per liter of culture medium from each culture vessel and place into a 1000 ml beaker containing about 100 ml of freshly prepared medium. The tip of the syphon should be kept under the surface of the water to

avoid air entrapment under the daphnid carpaces. Discard the remaining daphnids in the old culture solution.

b. Clean the culture vessel with 20 percent hydrochloric acid, allowing the acid to remain in contact with the vessel inner surface for about four minutes. The vessel should then be rinsed thoroughly with tap water followed by five rinses with distilled water.

c. Fill the cleaned vessel with freshly prepared culture media.

d. Gently pour the daphnids from the 1000 ml beaker into the cleaned vessel containing the freshly prepared culture media.

e. Cover each culture vessel with clear plastic film such as 'Saran Wrap', Parafilm, or a plexiglass plate to prevent dust and dirt from entering into the culture media.

Infrequent replacement of the culture media will result in the accumulation of waste products, which may lead to a population crash with over-production of males and sexual eggs.

Sexual eggs called ephippia or 'resting eggs' are produced in daphnid cultures during prolonged periods of stress. During these times the relative population of male daphnids increases. Ephippia are then produced by the females. The ephippia are resistant to environmental stress (e.g. overcrowding, insufficient food, and media dessication). These eggs produce viable daphnids when

environmental conditions improve. Cultures that contain ephippia should not be used for assays.

Occasionally a daphnid population will crash, for no apparent reason, shortly after the culture is thinned. This phenomenon has been observed in this and other laboratories with no satisfactory explanation offered. To avoid the loss of an entire daphnid culture, the investigator should maintain several cultures and thin them on a staggered basis. Alternatively a new culture can be initiated immediately following a crash by obtaining a new test population as described in Section VII.

IX. TOXICITY TESTING PROCEDURES

1. Establishing the viability of the test organisms

Once a viable daphnid population has been established, it is possible to begin toxicity testing using these organisms. To ensure that a healthy and normal daphnid culture has been established in the laboratory, a toxicity test on a known standard toxicant, such as sodium dodecyl sulfate (SDS) should be conducted. The 48LC50 value of this toxicant for D. magna should fall in the range of 5-10 mg/L for a water hardness of 160-180 mg/L as $CaCO_3$ (Weber, 1980). A minimum of three reference toxicant concentrations should be employed: one above, one equal to, and one below the expected LC50. For daphnids, use SDS concentrations of 5, 10 and 15 mg/L.

If the LC50 of SDS does not fall in the recommended range for the test organisms, the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects, and a different batch of test organisms should be employed in repeating the reference toxicant and effluent toxicity tests.

A large deviation from the expected 10 mg/L LC50 value may be indicative of an overly stressed, or otherwise unhealthy daphnid culture.

The condition and sensitivity of each population of new test organisms to the reference toxicant should be determined. No population of test organisms should be used in a toxicity test unless its condition has been determined against the reference

toxicant. If preferred, this sensitivity test may be run concurrently with an effluent toxicity test.

2. Test vessels

The vessels used for toxicity testing should be made of material that will not adsorb chemical constituents from the test solution. Furthermore, they should not be comprised of substances which might leach into the test solution. Glass is preferable, but other materials, such as stainless steel, teflon, polyethylene and polypropylene are acceptable. One liter glass beakers are an ideal size and are relatively inexpensive. They have the additional advantages of being transparent to permit the observations of daphnid mortality during a toxicity test.

3. Test conditions

A summary of the test conditions used for conducting bioassays using either D. magna or D. pulex are shown in Table 2.

4. Obtaining first stage instars for the toxicity test

First instar D. magna or D. pulex (24 hours or less in age) are used in conducting the toxicity tests. To obtain the required number of these instars, 50 adult females bearing eggs in their brood pouches are pipeted from the culture vessels 24 hours preceding the initiation of a toxicity test. These daphnids are carefully transferred into five 400 ml beakers (10 per beaker) containing 300 ml of the reconstituted water or other unpolluted water and 0.5 ml of the prepared food solution.

Table 2

Summary of Test Conditions for D. magna and D. pulex

a.	Temperature (^{o}C):	16-25^{o}C (20^{o}is optimal)
b.	Light quality:	Ambient laboratory light
c.	Light intensity:	50-100 foot candles (ambient light levels)
d.	Photoperiod:	1-16 hours light/photoperiod with a minimum total of 8 hours of light per 24 hour period (12 hours light/12 hours dark is recommended)
e.	Test vessel:	1 liter glass beakers, covered with plastic film to preclude entry of dust or other airborne particulates
f.	Test solution volume:	200-400 ml
g.	Age of test animals:	*0-24 hours (first instar)*
h.	Number of animals/beaker:	10
i.	Number of replicate test vessels per concentration:	2

Table 2, Continued

j.	Feeding regime:	Begin the test with test medium containing 0.3 ml food/200 ml as described previously: no further feeding is required.
k.	Aeration:	None, unless dissolved oxygen falls below 40 percent saturation, at which time gentle single-bubble aeration should be started.
l.	Dilution water:	Reconstituted water: Hard water for D. magna moderately hard water for D. pulex. Alternatively an unpolluted ground or surface water may be used.
m.	Test duration:	48 hours
n.	Observed effect:	Mortality as evidenced by the cessation of antennal or leg movement.

The daphnids should be transferred using a 20 ml pipet with the delivery end removed and fire polished. The inner diameter of the tube should be approximately 5 mm. The pipet tip is submerged into the culture solution, and adult daphnids are carefully removed using suction from a pipet bulb or propipet. The daphnids are then gently released into the beakers taking care to avoid the introduction of air bubbles which could become entrapped under their carapaces. This is best accomplished by placing the tip of the pipet below the water surface before releasing the daphnids.

The young that are found in the beakers on the following day are then used for the toxicity test. Five beakers, each containing 10 adults, usually will supply enough first instars for one toxicity test.

Male daphnids should not be used for testing. During most of the year the population of daphnids consists nearly exclusively of females. Males become abundant only during the spring and autumn or during periods of high population densities with subsequent accumulation of excretory metabolites or when food is unavailable. Ephippia are then produced by females. Male daphnids are distinguishable from females in many ways. The males are are smaller in size than females. They also have a modified abdomen, larger antennules than females and first legs armed with a stout clasping hook. Unless ephippia are observed in the culture vessels, all the daphnids will be females, and it will not be necessary to check morphology.

5. Test procedures

Two types of aqueous samples can be subjected to toxicity testing: a) a known chemical toxicant or mixture of known chemicals or, b) a complex effluent of unknown content. As in fish bioassays, there are two steps required to evaluate the toxicity of these types of samples:

1. determination of the range of toxicant dosage which results in an observable response (Screening Test); and
2. determination of acute toxicity using toxicant dosage applied over a narrower range (Definitive Test).

a. Known chemical toxicant: Screening Toxicity Test

A 24 hour ranging toxicity test is first performed on a toxicant solution of known chemical composition to determine the overall toxicity range. This is performed using a ten-fold dilution series of the toxicant and observing 100 percent mortality and 100 percent survival of two to four test organisms per vessel exposed to different toxicant levels after 24 hours of exposure. The finer incremental toxicant dosage range to be used for determining the 48LC50 is then evaluated. This range occurs between the highest toxicant concentration resulting in 100 percent survival and the lowest concentration resulting in 100 percent mortality. The procedure for the 24 hours screening toxicity test is as follows:

i) Prepare a dilution series of the toxicant solution by dissolving a known quantity of the material to be tested in an appropriate dilution water, and then

making ten-fold dilutions of this solution using additional dilution water. (CAUTION: Do not over aerate water as supersaturation may lead to air bubbles being trapped under the carpaces of the daphnids. The formation of small bubbles on the sides of the dilution vessel is an indication of supersaturation.)

ii) Pour 200 ml of each dilution into one liter glass beakers. Additionally pour 200 ml of dilution water containing no toxicant into a one liter glass beaker to serve as a control.

iii) Add 0.3 ml food/200 ml of test medium.

iv) Carefully transfer 2 to 4 first stage daphnid instars into each test vessel. The daphnids are transferred using a wide-mouthed eye dropper or pipet as described previously. Care must be taken to preclude the introduction of air bubbles during the transfer.

v) Cover each test container with plastic wrap to preclude entry of air borne particulate material.

vi) Observe daphnid mortality after 24 hours. First instar daphnids are very small and somewhat difficult to see. A light table and magnifying glass aid in determining mortality. An inexpensive light table can be readily constructed using clear or frosted plexiglass placed over a fluorescent light bulb.

Mortality can be determined by cessation of antennal or leg movement after gentle prodding.

vii) Determine the maximum toxicant concentration in which 100 percent survival is observed and the minimum toxicant concentration in which 100 percent mortality is observed. These will be the upper and lower toxicant concentrations, respectively, used in the Definitive Toxicity Test.

b. Known chemical toxicant: LC50 value determination (Definitive Test)

The second stage of evaluation is the determination of the 48LC50 value and ILC50 value. This Definitive Toxicity Test is essentially the same as the ranging toxicity test with the exception that the test is conducted for 48 hours using ten first stage daphnid instars per vessel. Five to seven toxicant concentrations in exponential series are used in addition to a control containing no toxicant. The test is performed using two replicates per concentration. To determine the acute toxicity of a toxicant solution with reasonable accuracy a definitive test must meet both of the following criteria:

i. Each concentration of the toxicant must be at least 50 percent of the preceding concentration.

ii. One concentration must have killed (or affected) more than 65 percent of the organisms exposed to it, and

one concentration must have killed (or affected) less than 35 percent of the organisms.

The control consists of test organisms exposed to the same dilution water, conditions, and procedures, used in testing the toxicant solution. A test is not acceptable if more than ten percent mortality occurs in the control.

For the definitive test, more frequent observations of daphnid mortality should be made to achieve more reliable and statistically significant 48LC50 and ILC50 values. The test vessels should therefore be checked after 1, 2, 4, 8, 16, 24 and 48 hours for both mortality and decline in dissolved oxygen. If dissolved oxygen falls below 4 mg/L O_2/L, then gentle bubble aeration should be initiated in the test vessels as previously described.

c. Complex effluent: Screening Toxicity Test.

Complex effluents such as those produced from industrial or municipal wastewater treatment processes are a second group of aqueous samples which can be subjected to bioassays. A preliminary toxicity test is again required to determine the range of effluent dilution resulting in an observable response. Weber (1980), presented a protocol for the preliminary toxicity screening of effluents. A somewhat modified procedure developed by the UMASS Environmental Engineering Laboratory follows:

1. Measure the pH and DO of the (100 percent) effluent and control water. If the pH falls outside the range of 6.0-8.0, set up two parallel tests, using pH-adjusted

(pH = 7.0) and unadjusted 100 percent effluent. If the DO is less than 4 mg O_2/L, the test solutions should be aerated, as previously described, before use.

2. For each test, ten first stage daphnid instars are carefully transferred into each of two test vessels containing 200 ml of 100 percent effluent plus 0.3 ml of the food solution.

3. Ten first stage daphnid instars are also immediately placed in each of two vessels containing control medium consisting of dilution water.

4. Cover each container with plastic wrap to preclude entry of air-borne particulates.

5. The test vessels are checked after 1, 2, 4, and 8 hours for early mortality of the daphnids and dissolved oxygen concentrations. Gentle aeration should be initiated if the DO levels fall below 4 mg O_2/L.

6. Upon observing mortality over these prescribed time intervals the following action is taken:

   i. If the mortality of the daphnids exposed to 100 percent effluent exceeds 20 percent at anytime during the first eight hours, and the mortality in the controls has not exceeded ten percent, the effluent is considered to be toxic. The test is immediately terminated, and a definitive test is initiated. If the initial pH of the effluent falls outside of the

range, 6.0-8.0, adjust the pH to 7.0 before running the definitive test.

ii. If the mortality of the daphnids in the control beakers exceeds ten percent at any time during the first eight hours or at any time later in the test, the preliminary screening test is immediately terminated and repeated.

iii. If the mortality in the effluent is less than 20 percent after eight hours, the test is continued until the 20 percent level is exceeded, or until the test period reaches 24 hours, whichever occurs first.

7. If the test runs for the full 24 hour period, the results are interpreted as follows:

i. If the mortality in the effluent was less than 20 percent, the effluent is considered not toxic and no further tests are conducted, <u>unless</u> the mortality in the controls exceeded ten percent or the reference toxicant data were abnormal.

ii. If the mortality exceeded 20 percent in the effluent, but was less than ten percent in the controls, the effluent is considered toxic and a definitive test is conducted.

iii. If the mortality exceeded ten percent in the controls, or if the reference toxicity data are

abnormal, the preliminary screening test must be repeated.

d. Complex effluent: Definitive Test.

The definitive test is carried out to determine the LC50 of the effluent and must employ controls and five-to-seven concentrations of effluent in a dilution series. The duration is 48 hours.

The definitive test is usually run at effluent concentrations of 12.5, 25, 50, 75 and 100 percent by volume. However, if the toxicity of the effluent is such that the preliminary screening test is terminated in the first eight hours, it may be necessary to modify the dilution series to provide lower effluent concentrations. The following suggestions are offered:

i. If, in the preliminary test, the eight hour (and 24-hour) mortality in 100 percent effluent is less than 50 percent, the definitive test is run at the concentrations of 12.5, 25, 50, 75 and 100 percent by volume.

ii. If, in the preliminary test, the eight hour (or 24-hour) mortality in 100 percent effluent is greater than 50 percent, the range of dilutions is changed to provide lower concentrations of effluent, such as 1.5, 3.1, 6.25, 12.5, 25, 50 and 75 percent.

iii. The test vessels should be checked after at least 1, 2, 4, 8, 12, 24 and 48 hours for mortality and decline in DO. Additional effluent dilutions may be required if the effluent is highly toxic. Gentle aeration, as previously

described, should be initiated if DO concentrations fall below 4 mg O_2/L.

e. Persistence of effluent toxicity.

The persistence of the toxicity of the effluent may be a factor in estimating the effect of a discharge on aquatic life in the receiving water, and in establishing the toxicity limits of an National Pollutant Discharge Elimination System (NPDES) permit. The persistence of toxicity is examined by determining the 24-hour LC50 value of the sample immediately following collection, and then repeating the test on the remaining portion of the sample after 96 hours. The portion of the effluent sample used in the second test must be held at ambient temperature (20°C) and out of direct sunlight. The sample should be held in a glass container covered partially with plastic sheeting to permit gas exchange, and the DO should be checked after 1, 2, 4 and 8 hours, and daily thereafter to determine if aeration is required to prevent oxygen depletion.

A decrease in toxicity will result in an increase in the LC50 value. Therefore, if the second toxicity test results in an increase of 100 percent of the LC50, the toxicity of the effluent is considered to be 'non-persistent'. If the LC50 has not increased 100 percent or more after 96 hours, the toxicity is considered to be 'persistent'.

f. Transfer toxicity tests using daphnids.

Transfer tests can be used to determine the effect of intermittent or varying exposure of a toxicant to daphnids (or other

test organisms). Such conditions are typically encountered in a stream or other aquatic environment receiving intermittent or variable pollutant discharges. Such variations may increase toxicity by excursion into very lethal toxicant concentrations, even for short periods, or may decrease toxicity via homeostatic rejuvenation during low toxicant level recovery periods. One would expect certain combatative biological functions, weakened by high concentrations of some toxicants, to be capable of replenishment during periods of low concentration. Bioassay results obtained from a toxicity test involving the intermittent exposure of daphnids or some other test organism to a toxicant might, therefore, result in different LC50 and ILC50 values than those obtained in a normal steady-state toxicant concentration bioassay. This may, therefore, necessitate varying standards for intermittent exposure, based on the capacity for homeostatic reserve rejuvenation.

Transfer assays are easily conducted with fish by dip netting. Such studies, however, are much more difficult using daphnids owing to their extremely small size. We have developed a procedure which does enable the transfering of these organisms between varying toxicant solutions. A daphnid transfer toxicity test can be conducted using 10 cm (O.D.) glass cylinders equipped with 145-175 μm porosity glass frits at one end of the cylinder to retain the daphnids while simultaneously allowing a test solution to be drained through the cylinder. The cylinders are placed within one

liter beakers containing the appropriate toxicant concentration in dilution water. The test is conducted as follows:

1. Use the general procedure previously outlined for the Definitive Toxicity Test with the exception that glass cylinders equipped with glass frits at one end are first placed within each of the one liter beakers followed by the addition of the 200 ml test solution and ten first stage daphnid instars to the cylinders.
2. After some prescribed time interval (2, 4, 8 or 12 hours) lift the cylinder out of the beaker allowing all but about 10-20 ml of the test solution to drain through the glass frit. Care should be taken not to allow all of the test solution to drain out of the cylinder as this might result in air entrapment under the daphnid carpaces.
3. Quickly insert the cylinder containing the daphnids into a second beaker containing some dilution water, and fill it with dilution water to rinse out the first toxicant solution.
4. Drain the cylinder again and quickly transfer it to a third beaker filled with 200 ml of the fresh toxicant solution. Care should be used in refilling the cylinders to minimize the formation of air bubbles in the test water.
5. Dilution water containing no toxicant should be used when transferring the daphnids from a high to lower toxicant

solution. When transferring the daphnids from a low to higher toxicant solution, the more concentrated solution should be used for rinsing. Several rinsing steps may be necessary to maintain a constant toxicant concentration in each of the two test solutions.

6. All of the beakers and cylinders should be placed in a large tray.

7. The cylinders should be loosely covered with plastic wrap during the test to preclude contamination by airborne particulates.

8. Methods for observing mortality have been described previously.

X. DETERMINATION OF LC50 AND ILC50 VALUES

The methods used to determine LC50 and ILC50 toxicity values are based upon the observed daphnid mortality over time, at the various toxicant concentrations. The procedure is the same as for the determination of toxicity values using fish as the test organism and has been described previously by Plotkin and Ram (1981b). It should be emphasized that frequent observations of daphnid mortality result in an easier determination of these toxicity values.

XI. REFERENCES

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XII. APPENDICES

APPENDIX A

EQUIPMENT, SUPPLIES AND LABOR REQUIREMENTS

Table 3 provides a detailed list of the equipment and chemical supplies needed to perform toxicity tests using daphnids as the test organism. The number of toxicity tests which can be performed is dependent upon labor availability. Table 4 presents approximate time requirements for conducting a single daphnid bioassay. Approximately 16 person hours are needed to conduct a single daphnid bioassay. This figure excludes both sampling requirements, which are dependent on the site location, and culture maintenance, which involves food preparation, feeding, cleaning aquariums, reconstituted water preparation, and population thinning. It is estimated, then, that a single laboratory technician would be able to conduct two bioassays per week, inclusive of data analysis and report preparation. This contrasts to the much greater labor requirements to conduct fish bioassays (approximately 24 person hours per fish bioassay). The decreased labor and space requirements to conduct toxicity tests using daphnids as the test organism as compared to fish represent a significant time and monetary savings in both the establishment of a bioassay laboratory and performance of toxicity tests. The direct cost for establishing a daphnid toxicity testing laboratory and supplying it for one year was about $13,000 in 1982 dollars. This figures does not include the cost of a constant temperature room which is required for

Table 3

Supplies and Equipment Required for Conducting Static Zooplankton Toxicity Tests

Item	Specifications or Usage	Cost[1] ($)	Vendor[2]
Equipment			
Culture flasks	4-five gallon glass aquaria	60	Pet store
Water still	produces 2 liter/hour	3,000	Fisher Sci.
pH meter and probe	range 0-14 pH unit ± .1 unit	500	Fisher Sci.
Thermometer	0°-100°C Hg thermometer	10	Fisher Sci.
Lighting	must provide about 50-100 ft-C	100	Hardware store
Light Timer	must automatically turn off on light at specified intervals (e.g. 12 hours light and 12 hours darkness	40	Hardware store
Analytical balance	capable of reading to fourth decimal point	3,000	Fisher Sci.
Oven	capable of reaching 120°C	800	Fisher Sci.
Dessicator	used for oven drying chemicals	100	Fisher Sci.
Filtering apparatus	for use with 47 mm filters	200	Millipore Corp.
Constant temperature room	to provide 16-25°C	-	-
Light table	for observing and counting Daphnid during a test	10	Materials found at a hardware store

Table 3, Continued

Item	Specifications or Usage	Cost[1] ($)	Vendor[2]
Equipment, Continued			
Magnifying glass	aids in observing Daphnid	5	Hardware store
Conductivity meter and probe	measures conductivity	500	Yellow Springs Instruments
Dissolved oxygen meter and probe	measures oxygen concentration in water	1,000	Yellow Springs Instruments
Spectro-photometer	measures absorbance and transmittance	2,000	Perkin Elmer
SUBTOTAL.		11,325	
Supplies			
Polyester fiber	used for filtering oil and sand particles from air lines	10/lb	Pet store
Filters	0.45 µm membrane filter	18.50/ 100	Fisher Sci.
Assorted glassware	2 each 25 and 10 ml burets 5 each 100 ml volumetric flasks 3 each 1 liter volumetric flasks 4 each of 0.5,1.0,2.0,3.0, 5.0 ml volumetric pipets 20 each one liter glass beakers	450	Fisher Sci.
Disposable pipets	10 ml borosilicate glass pipets used for daphnid transfers	80	Fisher Sci.

Table 3, Continued

Item	Specifications or Usage	Cost[1] ($)	Vendor[2]
Supplies, Continued			
Pipet bulbs	1 needed to supply suction to pipets	4	VWR
Glass cylinders[4]	20-10 cm OD cylinders 10 cm high	150	Glass supply store
Glass frits[4]	20-9 cm diam. frits with pore size 145-175 μm	286	Ace Glass
Air stones	for aerating dilution water 3/$1.00	1	Pet store
Air line	for aerating dilution water (2 meters/$1.00)	1	Pet store
Polyethylene jars	4-100 ml containers for daphnid food	4	Fisher Sci.
Daphnid food	yeast, trout pellets and alfalfa (1 year supply)	20	Pet stores and health food stores
SUB-TOTAL.		1024.50	
Chemicals (one yr supply)	Certified ASC reagent grade to make up reconstituted water and performing chemical tests: Reconsituted water: $NaHCO_3$, $CaSO_4 \cdot 2H_2O$, $MgSO_4$, KCl	100	Fisher Sci.
	Hardness: NaOH, NH_4Cl, Mg·EDTA, Na_2EDTA, $CaCO_3$, eriochrome		

Table 3, Continued

Item	Specifications or Usage	Cost[1] ($)	Vendor[2]
Chemicals, Continued			
	black T indicator, H_2SO_4;	75	Fisher Sci.
	Alkalinity: Na_2CO_3, H_2SO_4	35	Fisher Sci.
SUB-TOTAL.		210	
GRAND TOTAL.		12,559.50[3]	

1. Costs are in 1982 dollars.
2. Vendor addresses are presented in Appendix A. Equipment and supplies may be obtained from other scientific vendors as well.
3. Grand total does not include cost of constant temperature room.
4. For conducting transfer toxicity tests.

Table 4

Labor Requirements for Conducting a Single Daphnid Bioassay

Procedure	Time Required (Hours)
1. Sampling	dependent on site location
2. Preparation of dilution water and toxicant dilution series	2
3. chemical analyses (pH, hardness, alkalinity, D.O.)	1
4. Observation of daphnid mortality over the 48 hour test duration	3
5. Termination of test and glassware washing	2
6. Data analysis and report preparation	8
Total time required[1] (excluding sampling)	16

1. In addition to labor requirements for conducting a single daphnid bioassay, approximately four person hours per week are needed for culture maintenance and thinning.

toxicity testing (Table 3). Additional yearly supply costs are dependent upon the number of chemical analyses performed and toxicity tests conducted each year. A minimum of about $1,000 (1982 dollars) is required per year for chemical and glassware replacement. The actual cost for each daphnid test will be dependent upon the wages of the technician conducting the test and proportion of capital expenses assigned to each bioassay. Some estimated cost figures are shown in Table 5. Vendor addresses are listed in Appendix A.

Table 5

Estimated Direct Costs (1982) Dollars to Conduct a Single Daphnid Bioassay[1]

Item		Quantity
A.	Capital cost to establish bioassay laboratory	$13,000
B.	Technican, annual salary	$15,000
C.	Number of assays conducted by one technican per year[2]	100
D.	Yearly supply cost	$1,000
E.	Cost per test, assuming capital expense is repaid during first year [(A+B)/C]	$280
F.	Cost per test after capital expense is repaid [(B+D)/C]	$160

1. Cost per test would be less if proportion of capital expenses assigned to each bioassay was distributed over more years. Cost excludes sampling.

2. Assumes two test per week for one year.

APPENDIX B

DEALER ADDRESSES

Ace Glass Inc.
P. O. Box 688
1430 NW Boulevard
Vineland, NJ 08360
609-692-3333

Berkshire Biological
210 Florence Road
P. O. Box 404
Florence, MA 01060
413-586-6149

Carolina Biological Supply Company
2700 Yorr Road
Burlington, NC 27215
919-584-0381

US Environmental Protection Agency Laboratory
60 Westview Street
Lexington, MA 02173
617-861-6700

Fisher Scientific
461 Riverside Avenue
P. O. Box 379
Medford, MA 02155
617-391-6110

Millipore Corporation
Bedford, MA 01730
800-225-1380

VWR Scientific Incorporated
P. O. Box 232
Boston, MA 02101
617-964-0900

Yellow Springs Instrument Company
Box 279
Yellow Springs, OH 45387
513-767-7241

APPENDIX C

Laboratory Worksheet

BIOASSAY/TOXICITY TEST DATA

DATE: _____

TEST METHOD:

TEST ORGANISM: _____ STARTING TIME: _____

WATER SOURCE
AND HARDNESS: _____ FINAL TIME: _____

TEMPERATURE: _____

DISSOLVED O_2: _____

pH: _____

24, 48 or 96 HOUR TEST

# SURVIVING/% MORTALITY																
TIME INTERVAL		2h		4h		8h		12h		24h		36h	48h		72h	96h
ACTUAL TIME																
TIME INTERVAL																
TOXICANT CONC.																
CONTROL	A															
	B															
	A															
	B															
	A															
	B															
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