Designation: E 1366 – ~~96~~02

Standard Practice for
Standardized Aquatic Microcosms: Fresh Water[1]

1. Scope

1.1 This practice covers procedures for obtaining data concerning toxicity and other effects of a test material to a multi-trophic level freshwater community.

1.2 These procedures also might be useful for studying the fate of test materials and transformation products, although modifications and additional analytical procedures might be necessary.

1.3 Modification of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, results of tests conducted using unusual procedures are not likely to be comparable to results of many other tests. Comparison of results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting multi-trophic level tests.

1.4 This practice is arranged as follows:

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[1] This practice is under the jurisdiction of ASTM Committee E~~-47~~47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

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1.5 The values stated in SI units are to be regarded as the standard. The values given in parentheses are for information only.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 7.

2. Referenced Documents

2.1 *ASTM Standards:*

D 1193 Specification for Reagent Water[2]

D 3978 Practice for Algal Growth Potential Testing with *Selenastrum capricornutum* [3]

E 380 Practice for Use of the International System of Units (SI) (The Modernized Metric System)[4]

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians[3]

E 943 Terminology Relating to Biological Effects and Environmental Fate[3]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[3]

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians[3]

E 1193 Guide for Conducting Renewal Life-Cycle Toxicity Tests with *Daphnia magna*[3]

3. Terminology

3.1 The words "must," "should,"" may," "can," and "might" have very specific meanings in this practice. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specific condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see Section 17). "Should" is used to state that the specified condition is recommended and ought to be met in most tests. Although a violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to,"" can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus, the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 For definitions of other terms used in this practice, refer to Guide E 729, Terminology E 943, and Guide E 1023. For an explanation of units and symbols, refer to Practice E 380.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *algal biovolume*, *n*—an estimate of the total volume of algal cells ($\times 10^4$ μ^3/mL) (see 14.1.10).

3.3.2 *available algae*, *n*—an estimate of the volume of algae ($\times 10^4$ μ^3/mL) presumed available to the *Daphnia* (see 14.1.10).

3.3.2.1 *Discussion*—The estimate is calculated from the numerical abundance of each species of algae, its nominal volume, and an availability factor based on its size and growth characteristics (see 14.1.10). Small algal cells are presumed 100 % available and large, filamentous forms are presumed 1 to 20 % available. Species that attach to sediment or walls are presumed to be less available than planktonic forms.

3.3.3 *axenic*, *adj*—a culture of organisms growing without neighbors, that is, pure culture free from contaminant organisms (see gnotobiotic (**1–2**) [5]).

3.3.4 *community metabolism*, *n*—the oxygen or carbon balance of the entire community.

3.3.4.1 *Discussion*—In this microcosm, community metabolism is estimated by the gain in oxygen during the lighted period (an estimate of net photosynthesis—*P*) and the loss of oxygen during the dark period (an estimate of respiration—*R*). When expressed as a *P/R* ratio, a value of >1 indicates that autotrophic processes are dominant; a value of <1 indicates that heterotrophic processes are dominant. If the difference of *P* and *R* are considered (*P-R*), a positive number indicates autotrophic processes are dominant, and a negative number indicates heterotrophic processes are dominant. ~~A biological community cannot persist with a low *P/R* or negative value because it is consuming its biomass~~ Because P and R often change in the same direction and magnitude, P/R maybe less sensitive than P ~~or other storage supplies.~~ R considered separately.

3.3.5 *detritivore*, *n*—an organism that feeds on detritus, dead organic material.

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[2] *Annual Book of ASTM Standards*, Vol 11.01.
[3] *Annual Book of ASTM Standards*, Vol. 11.05.
[4] *Annual Book of ASTM Standards*, Vol 14.02; excerpts in gray pages of Vol 11.04.
[5] Boldface numbers in parentheses refer to the list of references at the end of this practice.

3.3.6 *ecosystem*, *n*—a system made up of a community of animals, plants, and bacteria and its interrelated physical and chemical environment **(3).**

3.3.7 *gnotobiotic*, *adj* ~~known biota, for example, an experimental biological~~ —a culture ~~system to~~ which the exact composition~~ly~~ of the organisms is known, down to the prese~~l~~nce or absence of bac~~te~~dria. Such cultures are develo~~p~~mped fro~~n~~m axenic cultures ~~(~~. The wor~~ga~~d implies know biota **(2)**. The microcosms described here are not gnotobiotic because of the bacteria and ~~nutrients)~~ other microbes are ~~introduced.~~ not known.

3.3.7.1 *Discussion*—An organism growing "without neighbors" is axenic (that is, free of all contaminants); growing with one organism is monoxenic (that is, the rotifers growing with one species of food bacteria); growing with two organisms is dixenic; growing with many organisms (provided the organisms are known) is gnotobiotic. A culture or community with many undefined organisms can be termed "xenic." The aquatic microcosms used in this practice are xenic because the bacterial component is undefined and contaminating organisms can enter. (Definitions are in accordance with **(1, 2)**).

3.3.8 *grazer*, *n*—an animal that grazes or feeds on growing plants; in these aquatic communities, organisms that feed on algae.

3.3.9 *herbivore*, *n*—an animal that feeds on plants, synonymous with grazer.

3.3.10 *medium*, *n*—the chemical solution (for example, T82MV) used in the microcosms.

3.3.11 *microcosm*, *n*—a small ecosystem that is regarded as miniature or epitome of a large world.

3.3.12 *primary producer, adj*, *n*—an organism capable of converting inorganic chemicals and energy into organic compounds.

3.3.12.1 *Discussion*—Primary producers are synonymous with autotrophs; in these microcosms they are the algae (including the blue-greens).

3.3.13 *secondary producer, adj*, *n*—an organism that requires organic chemicals for its energy source.

3.3.13.1 *Discussion*—Secondary producers are synonymous with heterotrophs; some researchers define grazers as secondary producers, and carnivores as tertiary producers. In these microcosms, all of the organisms with the exception of the algae can be considered secondary producers.

3.3.14 *semicontinuous culture, adj*, *n*—a culture that is partially harvested and that receives fresh nutrient from time to time.

3.3.14.1 *Discussion*—Most of the stock algal cultures are harvested daily to maintain them in active growth, and are thus semicontinuous cultures. A true continuous culture would require continuous harvesting and a nutrient renewal system.

3.3.15 *treatment*, *n*—the (usually) six replicate microcosms that have had the same (if any) chemical addition; the control is one treatment.

3.3.16 *trophic level, adj*, *n*—refers to position in food chain; useful in analyzing energy flow **(3).**

3.3.16.1 *Discussion*—The first trophic level encompasses the primary producers; second trophic level encompasses grazers or herbivores (sometimes referred to as primary consumers); third trophic level encompasses carnivores (sometimes referred to as secondary consumers); the fourth trophic level encompasses top carnivores. The detrital or recycling level is usually considered a trophic level, but not given a numerical term. These microcosms include the first and second trophic levels as well as a detrital (recycling) level.

3.3.17 *unialgal*, *adj*—refers to an algal culture that contains only one type (strain, species) of algae, although bacteria or other non-algal species might be present.

4. Summary of Practice

4.1 Replicate microcosms are synthesized from a chemically defined medium and sediment which are initially sterile. On Day 0, 10 species of algae are inoculated and allowed to grow in competition with each other. On Day 4, grazers and detritivores are introduced. On Day 7, an appropriate number of the microcosms are selected as being most similar and randomly assigned to treatments and to specific locations on the light table. Test material is added to microcosms in the appropriate treatments. If the test material is a potential source of nutrients, for example, nitrogen, phosphate, or organic carbon, another treatment should receive another material that would supply equivalent nutrients. A control treatment is established and sampled simultaneously with the other treatments. If a solvent is used, a solvent control is also established.

4.2 All measurements (see 11.5) are collected twice a week for the first 28 days (21 days after treatment). Thereafter, measurements are made twice a week for organism enumerations, 3-point oxygen concentrations, *in vivo* fluorescence, pH and absorbance until the end of the experiment, usually Day 63 (56 days after treatment). After Day 28, dissolved nutrients (nitrate, phosphate, nitrite, and ammonia) are measured once a week until the end of the experiment. Carbon uptake, alkalinity and extracted pigments (chlorophylls, phaeopigment) are measured if results are to be compared with field studies.

4.3 Organisms are reinoculated (in small numbers) each week to allow reestablishment of populations after temporary reductions (see 11.6).

4.4 The means of the variables are compared between the control(s) and other treatment(s) to assess the effects of the test material. A one-way analysis of variance of each variable with accompanying *a priori* *t*-tests is performed on data from each sampling day. All quantitative data are presented in tables of means, standard deviations, and statistical differences. Selected data are displayed in graphics showing the control mean bordered by the "Interval of Nonsignificance" (IND), and the treatment means. The findings should describe changes that have been shown on primary, secondary, and ecosystem variables, for example, see Annex A1.

5. Significance and Use

5.1 A microcosm test is conducted to obtain information concerning toxicity or other effects of a test material on the interactions among three trophic levels (primary, secondary, and detrital) and the competitive interactions within each trophic level. As with most natural aquatic ecosystems, the microcosms depend upon algal production (primary production) to support the grazer trophic level (secondary production), which along with the microbial community are primarily responsible for the nutrient recycling necessary to sustain primary production. Microcosm initial condition includes some detritus (chitin and cellulose) and additional detritus is produced by the system. The microcosms include ecologically important processes and organisms representative of ponds and lakes, but are non-site specific.

5.2 The species used are easy to culture in the laboratory and some are routinely used for single species toxicity tests (Guide E 729; Practice D 3978, Guides E 1192 and E 1193). Presumably acute toxicity test results with some of these species would be available prior to the decision to undertake the microcosm test. If available, single species toxicity results would aid in distinguishing between indirect and direct effects.

5.3 These procedures are based mostly on previously published methods (4-6), interlaboratory testing (7-10), intermediate studies (11-22), statistical studies (23-25) and mathematical simulation results (26-28)(26) . Newer studies on jet fuels have been reported (27)(See 15.1 for multivariate statistical analyses) and on the implications of multispecies testing for pesticide registration (28). Environmental Protection Agency, (EPA) and Food and Drug Administration, (FDA) published similar microcosm tests. (29). The methods described here were used to determine the criteria for Acceptable Tests (Section 16).

5.4 Concurrent to measuring the ecological effects, it is advisable to measure the concentration of the parent test chemical, and if possible, the transformation products ((30) see Section 12). The concentrations can be measured on either the same microcosms or on concurrent replicates. Information on the chemical concentrations of parent material and transformation products would aid in the assessment of chemical persistence, exposure, accumulation, and in interpreting, if recovery is associated with chemical degradation or biological adaptation. This protocol deals only with ecological effects, because the techniques for fate studies are in general usage.

5.5 In the microcosm, as in natural ecosystems, a population must be able to obtain its requirements from the products of other trophic levels, to maintain a birth rate equal to or greater than its death rate, and to support populations of organisms that will remove its waste products. As in natural ecosystems, several organisms might be capable of fulfilling the same function, and shifts in species dominance can occur without disruption of an ecological process. However, species that are "ecological equivalents" in one function might not be "equivalent" in other functions; for example, a filamentous alga and a single cell alga might equally produce O_2, remove NO_3, NH_3, and PO_4, but differ in the type of grazer populations they can sustain, for example, filamentous alga might support amphipods whereas unicellular algae might support *Daphnia*.

5.6 Results of these microcosm tests might be more likely to be indicative of natural ecosystem responses to chemicals than single species toxicity tests because microcosm tests can indicate the explosive population increases that might occur in a community when more sensitive competitors or predators are eliminated or the food supply is increased through competitive interactions. Also, microcosm tests are more likely to display the effects of chemical transformation or increased exposure to certain organisms by means of concentration of parent or degradation products in their food source or habitat.

5.7 A list of potential ecological effects is provided to serve as a summary (see Annex A1).

5.8 The microcosm test can also be used to obtain information on the toxicity or other effects of species or strains, not included in the control inocula (12). Additional modifications might be required.

5.9 *Explicit Limitations of the Aquatic Microcosm Protocol*:

5.9.1 The scope of the test is limited in the following respects:

5.9.1.1 No fish or other vertebrates are included,

5.9.1.2 Predation on *Daphnia* is extremely limited or absent,

5.9.1.3 The ecosystem becomes nutrient limited,

5.9.1.4 The inocula are not gnotobiotic and aseptic technique is not used (except in maintaining stock cultures of microorganisms). Contaminating microorganisms are likely to be introduced with the larger organisms and during sampling, and

5.9.1.5 Most detrital processing is carried out by the sediment microbial community, but this community is not clearly described or measured by this protocol.

5.9.2 Extrapolation to natural ecosystems should consider differences in community structure, limiting factors, and water chemistry (see Section 17).

6. Apparatus

6.1 *Facilities*:

6.1.1 *Temperature Control*—An incubator or temperature controlled room is required that provides an environment of 20 to 25°C with the minimal dimensions of 2.6 by 0.85 by 0.8 m high. Short periods of temperatures outside this range would not invalidate a test if controls behave normally (see Section 16). Temperature around microcosms should be continuously recorded with a device that will continue to function during a power failure.

6.1.2 *Work Surface*—The table should be at least 2.6 by 0.85 m (8 ft 9 in. by 2 ft 9 in.) and have a white or lightcolored top or covering.

6.1.3 *Illumination*—80 μE m^{-2} photosynthetically active radiation s^{-1}(850 to 1000 fc) of warm or cool white light should be provided at the top of the table.[6] A period of 2 to 3 weeks of use should be allowed after the installation of new tubes and ballasts to avoid the initially higher light output. Tubes usually are stable for about six months and ballasts for about two years. Declining light output might occur in older tubes and ballasts. Light intensity should be measured weekly and recorded. The light meter should be moved over the table top to establish a light isobar where values are ±10 %. The microcosm containers should be placed within this area in an oval configuration (see Fig. 1). A light cycle of 12 h OFF and 12 h ON should be established. Unless the table is enclosed care should be taken that other room lights are off when lights over the table are off.

6.2 *Containers*:

6.2.1 All containers that might contact stock solutions, test solutions, or any water into which test organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that can adversely effect aquatic organisms. In addition, equipment and facilities that contact stock solutions or test solutions should be chosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, and fluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption, except that stainless steel should not be used for tests on metals.

6.2.2 *One-gal (3.8-L) Glass Jars*—recommended for microcosms; soft glass is satisfactory if new containers are used for each test. The jars should measure approximately 16.0 cm wide at the shoulder and be 25 cm tall with a 10.6-cm opening. Jars should be rinsed with 10 % HCl and glass-distilled water before use.

6.3 *Major Equipment Items*:

6.3.1 *Autoclave*, (large enough to sterilize several microcosm containers, media carboys, glassware, and solutions).

6.3.2 *Standard Laboratory Facilities* , for preparing solutions, including balances for weighing to tenths and hundredths of a gram; volumetric flasks, pipettes, and graduated cylinders.

6.3.3 *Compound Microscope*, with a 40× water immersion objective and an 8× ocular are recommended.

6.3.4 *Stereomicroscope*, with magnification of 10× to 100×.

6.3.5 *Fluorometer*, (for in vivo fluorescence).

6.3.6 *Oxygen Meter*, with exchangeable electrodes. (New electrodes should be used with each new chemical; control electrodes from previous experiments can be reused.)

6.3.7 *Spectrophotometer*.

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[6] Fluorescent light tubes such as GE F96PG17WW, or equivalent, (8-ft, high intensity warm white "power groove") mounted 51 cm above the top of the table have proven satisfactory. If warm white tubes are not available, F96PG17–CW are satisfactory.
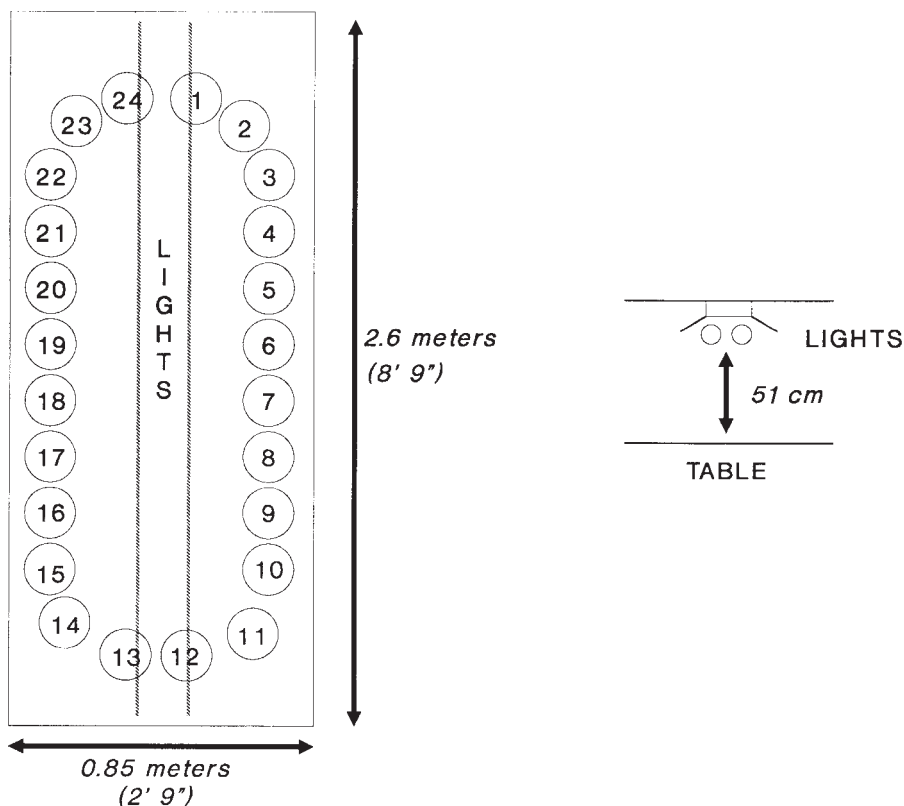

FIG. 1 Position of Microcosms under Lights (6.2.3 and 12.3.1)

6.3.8 *pH Meter*, with sensitivity to at least 0.1 pH units.

6.3.9 *Apparatus for Analysis of Nitrate, Nitrite, Ammonia, and Phosphate.*

6.3.10 *Refrigerator*, with freezer for storage of medium component solutions and samples.

6.3.11 *Computer*, to process the data.

7. Hazards

7.1 Material safety data sheets should be reviewed for test substances and reagents to evaluate the safety hazard. Appropriate protective clothing such as laboratory coats, aprons, and glasses and equipment should be used when conducting this test.

7.1.1 Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans (31), recommended handling procedures (32) and chemical and physical properties of the test material should be studied before a test is begun. Special procedures might be necessary with radio-labeled test materials (33) and with materials that are, or are suspected of being carcinogenic (34).

7.2 Although disposal of stock solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

7.3 If microorganisms are used as test material, precautions might need to be taken to prevent contamination of the laboratory and of the controls. If the organisms are genetically engineered, appropriate containment procedures should be used (12, 35). The microcosms can be autoclaved at the conclusion of the test.

7.4 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a well-ventilated area in which no smoking is allowed and no open flame, such as a pilot light, is present.

7.5 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and mixing it with water should be performed only in a fume hood.

7.6 Because test solutions are usually good conductors of electricity, use of ground fault systems and leak detectors should be considered to help avoid electrical shocks.

8. Microcosm Components

8.1 *Microcosm Medium*—Medium T82MV (Table 1), is recommended on the basis of interlaboratory testing (7-10). An alternative microcosm medium (T86MVK) with additional trace metals is also described (Appendix X1), but has not been as extensively tested. These media are designed to have low pH buffer and low metal chelation capacity. Media used in earlier studies are described in Appendix X1. Related media are recommended for maintenance of stock cultures (described in Section 10). All of these media can be made by adding various quantities of master solutions to distilled water, such as Type II or III (Specification D 1193).

8.2 *Medium Preparation*:

8.2.1 The medium should be prepared as follows:

(1) Read instructions through 8.2.5,

(2) Prepare master solutions (8.2.2); sterilize if so indicated,

(3) Prepare final basal medium (8.2.3), autoclave and cool,

(4) Add sterile solutions to final basal medium (8.2.4), and

(5) Adjust pH (8.2.5).

8.2.2 *Master Solutions*—Non-sterile master solutions can be prepared in 1-L bottles with ground glass stoppers and refrigerated prior to use. Sterile master solutions can be stored in serum-capped or screw-top containers in the refrigerator. Master solutions are stable and can be used for up to a year if prepared and stored satisfactorily. Cloudiness or precipitation indicates the need for replacement.

8.2.2.1 Each of the master solutions (A through K, MV, 10× Silicate and (optional) Keating's metals) should be prepared and stored separately (see Tables 2-4).

8.2.2.2 *Silicate Solution (10×)*— Add 45.95 g $Na_2SiO_3 \cdot 9H_2O$ to distilled water in a 1-L volumetric flask, filter through a 0.22-µ membrane filter, and store in a sterile nontoxic plastic bottle.

8.2.2.3 *HCl Solution*—Add 100 mL of concentrated HCl with 900 mL of distilled water in a volumetric flask, transfer the solution to a glass container and autoclave.

8.2.3 *Preparation and Sterilization of Final Basal Medium*:

8.2.3.1 Place 16 L of distilled water in a clean 20-L (5-gal) carboy. Add the solutions listed at the end of this paragraph and dilute with distilled water to 18 L. A nontoxic stopper or top equipped with a serum stopper and a clamped-off dispensing tube is added. Six carboys of medium are needed for a microcosm experiment (if 30 microcosms are initiated) (see X2.1 for data sheet).

Master Solution	Salt	mL/L	mL/18 L	Concentration mM (Final Solution)
A	$NaNO_3$	5	90	0.5
B	$MgSO_4 \cdot 7H_2O$	1	18	0.1
D	$CaCl_2 \cdot 2H_2O$	10	180	1.0
E	NaCl	15	270	1.5

H	Al$_2$(SO$_4$)$_3$·18H$_2$O	1	18	0.0048
I	Na$_2$SiO$_3$·9H$_2$O	5	90	0.080

8.2.3.2 The final basal medium should be dispensed into the microcosm jars and sterilized with the sediment and allowed to cool (see 8.4). Alternately, the final basal medium can be autoclaved in the carboys (121°C, 60 min), allowed to cool, and be dispensed aseptically into sterile microcosm jars. The final basal medium is stable and should not precipitate during autoclaving or storage. The final basal medium lacks phosphate, trace metals, and vitamins, which are added in the individual test chambers. The pH is also adjusted in the test containers.

8.2.3.3 If the medium is being used for the nutrient reservoir of the algal semicontinuous cultures, the final basal medium should be autoclaved in the carboy.

TABLE 1 Microcosm Medium (T82MV) and Sediment Composition (see 8.1)

NOTE 1—Microcosm composition is 3 L of medium and 200.1 g of sediment (see 8.2-8.4 for direction).
NOTE 2—pH adjusted to 7.0 with sterile 10 % HCl.

Medium T82MV Composition				
Compound	Molecular Weight	Concentration		
		mM	Element	mg/L
NaNO$_3$	85.0	0.5	N	7.0
MgSO$_4$·7H$_2$O	246.5	0.1	Mg	2.43
KH$_2$PO$_4$	136.0	0.04	P	1.23
NaOH	40.0	0.099	Na	2.27
CaCl$_2$·2H$_2$O	147.0	1.0	Ca	40.0
NaCl	58.5	1.5	Na	34.5
Al$_2$(SO$_4$)$_3$·18H$_2$O	666.5	0.0048	Al	0.26
Na$_2$SiO$_3$·9H$_2$O	284.0	0.80	Na	36.8
			Si	22.4
Trace Metals		µM		mg/L
FeSO$_4$·7H$_2$O	278.0	1.12	Fe	0.0625
EDTA	292.0	1.42	EDTA	0.4146
H$_3$BO$_3$	61.8	0.75	B	0.008
ZnSO$_4$·7H$_2$O	287.5	0.025	Zn	0.0015
MnCl$_2$·4H$_2$O	197.9	0.25	Mn	0.0135
Na$_2$MoO$_4$·2H$_2$O	242.0	0.025	Mo	0.0024
CuSO$_4$·5H$_2$O	249.7	0.005	Cu	0.00032
Co(NO$_3$)$_2$·6H$_2$O	291.0	0.0025	Co	0.00015
Murphy's Vitamins		µM		mg/L
Calcium pantothenate	476.5	1.47		0.70
Cyanocobalamin (B$_{12}$)	1355.4	0.000022		0.00003
Thiamin (B$_1$)	337.3	0.18		0.06
Riboflavin (B$_2$)	376.4	0.11		0.04
Nicotinamide	122.1	1.06		0.13
Folic acid	441.4	0.75		0.33
Biotin	244.3	0.12		0.03
Putrescine	161.1	0.19		0.03
Choline	181.7	2.75		0.50
Inositol	216.2	5.09		1.10
Pyridoxine monohydrochloride	205.7	2.43		0.50
Sediment		g/microcosm		
Silica sand		200.0		
Chitin		0.5		
Cellulose powder		0.5		

8.2.4 Addition of sterile solutions to the final basal medium to prepare the medium T86MV and medium T86MVK are as follows:

Sterile Master Solution	mL/L	mL/18 L
C	0.4	7.2
K	0.05	0.9
MV	1.0	18.0
Silicate Solution (10×)	5.0	90.0
Keating's Metal Solution	1[A]	18.0[A]
HCl	to pH 7	to pH 7

[A] Use only for medium T86MVK.

TABLE 2 Master Solutions

Master Solution	Salt	Molecular Weight	Concentration	
			g/L	M
A	$NaNO_3$	85.0	8.5	0.1
B	$MgSO_4 \cdot 7H_2O$	246.5	24.65	0.1
C^A	KH_2PO_4	136.0	13.6	0.1
	NaOH	40.0	3.2	0.035
D	$CaCl_2 \cdot 2H_2O$	147.0	14.7	0.1
E	NaCl	58.5	5.84	0.1
F^B	$FeSO_4 \cdot 7H_2O$	278.0	24.9	0.0895
	$EDTA^C$	292.0	26.1	0.0895
	NaOH	40.0	10.7	0.267
G^D	H_3BO_3	61.8	1.85	0.03
	$ZnSO_4 \cdot 7H_2O$	287.5	0.287	0.001
	$MnCl_2 \cdot 4H_2O$	197.9	1.98	0.01
	$Na_2MoO_4 \cdot 2H_2O$	242.0	0.242	0.001
	$CuSO_4 \cdot 5H_2O$	249.7	0.0499	0.0002
	$Co(NO_3)_2 \cdot 6H_2O$	291.0	0.0291	0.0001
H	$Al_2(SO_4)_3 \cdot 18H_2O$	666.5	3.2	0.0048
I	$Na_2SiO_3 \cdot 9H_2O$	284.0	4.55	0.016
J^E	EDTA	292.0	29.0	0.1
	NaOH	40.0	12.0	0.3
K^F	...	...	...	...

[A] Solution C should be filter-sterilized through 0.22-μ membrane filter or heat-sterilized and stored in a flask with a serum stopper in a refrigerator.

[B] Solution F is used to prepare Solution K.

[C] Ethylenedinitrolotetraacetic Acid. (Do not use di-sodium or tetra-sodium EDTA; use the ethylenedinitrolotetraacetic acid form.) EDTA is dissolved in 268 mL of 1*N* NaOH. The $FeSO_4 \cdot 7H_2O$ is added and the volume brought to 1 L. The solution is aerated overnight and stored in a 1-L bottle with ground glass stopper under refrigeration.

[D] Solution G is used to prepare Solution K.

[E] Solution J is used to prepare Solution K.

[F] Solution K is made from Solutions F, G, and J where F is 250 mL, G is 500 mL, J is 60 mL, and distilled H_2O is 190 mL.

Note 1—The specified amounts of the listed solutions are added to the final basal medium after autoclaving and cooling (see 8.2.3). This prevents precipitation prior to dispensing. The final medium without Keating's metals is termed T82MV; with Keating's metal solution, it is termed T86MVK (see Appendix X1 for the relationships among several similar media that were used in the development of the test or are used in organism cultures—see Section 10).

8.2.5 *pH Adjustment*—A known volume of medium should be removed and titrated with HCl to pH 7. Given the volume of the medium remaining, the volume of HCl necessary to adjust the pH to 7 should be added aseptically, and the final pH checked.

8.3 *Sediment*:

8.3.1 The sediment of each microcosm is composed of the silica sand (200 g), ground, crude chitin (0.5), and cellulose powder (0.5 g).

8.3.1.1 *Silica Sand*—Approximately 4 kg (four 2-lb bags) of sand are emptied into a large container, covered with 10 % concentrated HCl and mixed. After 2 h, the acid is decanted and the sand rinsed with distilled water until rinse water reaches pH 7. Sand is then oven-dried, cooled, and weighed.

8.3.1.2 *Chitin*—A small amount of crude chitin is rinsed well in distilled water and air dried. It is then ground for 10 min in a blender or grinder, then filtered through a 0.4-mm sieve. Larger pieces are reground.

8.3.1.3 *Cellulose Powder*—Weighed directly.

8.4 *Microcosm Assembly*:

8.4.1 To assemble microcosms, 200 g of silica sand are weighed into a beaker, 0.5 g of chitin and 0.5 g of cellulose powder are added, then the sediment is placed in the rinsed microcosm containers. At least 6 extra microcosms with sand, chitin, and cellulose should be prepared in case of breakage during autoclaving and to allow culling of outliers (see 11.3). Six carboys of unsterilized final basal medium (see 8.2.3) are made if 30 microcosms are to be prepared. Five hundred mL of media from each carboy are added to each container (for a total of 3 L per container); this ensures that each microcosm receives medium from each carboy to provide uniform initial conditions.

8.4.2 Containers are then covered with foil and autoclaved a few at a time at 121°C (15-lb steam pressure) for 45 min. When the medium is cool, sterile solutions (see 8.2.4) are added, and pH is adjusted to 7.0 with 10 % HCl, then foil covers are replaced with 150 by 15-mm plastic petri dishes. A laboratory worksheet, X2.1, documents the media preparation (see data sheets in Appendix X2.).

TABLE 3 Modified Murphy's Vitamin Solution[A]

Name	Molecular Weight	Concentration	
		mg/L	mM
Calcium pantothenate	476.5	700.0	1.47
Cyanocobalamin (B$_{12}$)	1355.4	0.03	0.000022
Thiamin (B$_1$)	337.3	60.0	0.18
Riboflavin (B$_2$)	376.4	40.0	0.11
Nicotinamide	122.1	130.0	1.06
Folic Acid	441.4	330.0	0.75
Biotin	244.3	30.0	0.12
Putrescine	161.1	30.0	0.19
Choline	181.7	500.0	2.75
Inositol	216.2	1100.0	5.09
Pyridoxine (B$_6$) monohydrochloride	205.7	500.0	2.43

[A] Ingredients are added to 1 L of an alkaline solution that can be made by adding 2 pellets (approximately 100 mg each) of NaOH to 1 L of distilled water, filter-sterilized through 0.22µ -filter and stored in a flask with a serum stopper in a refrigerator. This modification omits the calcium acetate, antibiotics, serum, and trace metal solution used by Murphy **(36)**; reduces the vitamins to 1/10 concentration in the final medium and substitutes pyridoxine (B$_6$) for the pyridoxal (listed by Murphy on a typed erratum).

9. Test Material

9.1 *General*—The test material should be reagent grade[7] or better, unless a test on an effluent, a formulation, commercial product, or technical-grade or use-grade material is specifically needed. Concentration should be stated as active ingredients when possible. Before a test is begun, the following should be known about the test material:

9.1.1 Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than 1 % of the material,

9.1.2 Solubility and stability in the water.

9.1.3 An estimate of the lowest concentration of test material that is acutely toxic to some of the microcosm species, for example, *D. magna* and *S. capricornutum*,

9.1.4 Accuracy and precision of the analytical method at planned test concentration(s), and

9.1.5 Estimate of toxicity to humans and recommended handling procedures (see 7.1).

9.2 *Stock Solution*:

9.2.1 In some cases the test material can be added directly to the microcosm, but usually it is dissolved in a solvent to form a stock solution that is then added to the microcosm. If a stock solution is used, the concentration and stability of the test material in it should be determined before the beginning of the test. If the test material is subject to photolysis, the stock solution should be shielded from light.

TABLE 4 Keating's Metal Solution (Optional, for use in T86MVK or T85MVK[A]**)**

Name	Molecular Weight	Concentration	
		mg/L	mM
NaBr	102.89	64.4	0.626
SrCl$_2$·6H$_2$O	266.52	304.00	1.141
RbCl	120.92	141.5	1.17
LiCl	42.39	611.0	14.41
KI	166.00	6.5	0.0392
SeO$_2$	110.96	1.41	0.0127
NH$_4$VO$_3$	116.94	1.15	0.00984

[A] Add ingredients and bring volume to 1 L with distilled water. Autoclave and store in a refrigerator in glass container. Modified from **(37).** This solution includes only those trace metals in Keating's medium that were not already in T82MV.

9.2.2 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is medium or distilled water. Sterilization of the stock solution might be necessary if the test material is subject to microbial transformation. Several techniques have been specifically developed for preparing aqueous stock solution of slightly soluble materials **(38)**. The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such reagents might affect the pH of test solutions appreciably. Use of a more soluble form of the test material, such as chloride or sulfate salts of

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[7] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Analar Standards for Laboratory U.K. Chemicals," BDH Ltd., Poole, Dorset, and the "United States Pharmacopeia."

organic amines, sodium or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH more than use of the necessary minimum amount of a strong acid or base.

9.2.3 If a solvent other than medium or distilled water is used, its concentration in test solutions should be kept to a minimum and should be low enough that it does not affect survival or reproduction of any species used in the microcosm. In spite of its low toxicity to aquatic animals, low volatility, and high ability to dissolve many organic chemicals, triethylene glycol must not be used because it has caused low pH after approximately 28 days **(5).** Other water-miscible organic solvents such as methanol, ethanol, and acetone might be used as solvents, but they might stimulate undesirable growth of microorganisms and acetone is quite volatile. If an organic solvent is used, it should be reagent grade or better. A surfactant should not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in test solutions.

9.2.4 If a solvent other than distilled water or medium is used, (*a*) at least one solvent control, using solvent from the same batch used to make the stock solution, must be included in the test and (*b*) a medium control must be included in the test. If no solvent other than medium or distilled water is used, only the medium control must be included in the test.

9.2.4.1 The concentration of solvent should be the same in all test solutions that contain test material and in the solvent control.

9.2.4.2 If the test contains both a medium control and a solvent control, the variables measured in the two controls should be compared (see Section 15, 16.2, 16.3, and Appendix X3). If statistically significant differences are detected between the two controls, only the solvent control may be used for assessing the effects of the test material. If no statistically significant differences are detected, the data from both controls should be used for assessing the effects of the test material.

9.3 *Nutrient Control*—If the test material might serve as a source of nutrient (*N, P,* or organic carbon), a similar concentration of nutrient, possibly as part of a nontoxic chemical, should be one of the treatment groups. Alternatively, the nutrient supply may be considered a direct effect of the test compound.

10. Test Organisms

10.1 Algae (added on Day 0 at initial concentration of 10^3 cells for each algae species) are as follows: (see Fig. 2).

10.1.1 *Anabaena cylindrica,*

10.1.2 *Ankistrodesmus* sp.,

10.1.3 *Chlamydomonas reinhardi* 90,

10.1.4 *Chlorella vulgaris,*

10.1.5 *Lyngbya* sp.,

10.1.6 *Nitzschia kutzigiana* (Diatom 216),

10.1.7 *Scenedesmus obliquus,*

10.1.8 *Selenastrum capricornutum* , (also know as, *Raphidocelis subcapitata* (Korsh.) Nygaard, Komarek et al.; and *Pseudokirchneriella subcapitata* (Korshikov) Hindak.

10.1.9 *Stigeoclonium* sp., and

10.1.10 *Ulothrix* sp.

10.2 Animals (added on Day 4 at the initial numbers indicated in parentheses) are as follows: (see Fig. 3).

10.2.1 *Daphnia magna* (16/microcosm),

10.2.2 *Hyalella azteca* (12/microcosm),

10.2.3 *Cypridopsis* or *Cyprinotus* incongruens or similar species (vidua) (6/microcosm),

10.2.4 Hypotrichs [protozoa] (0.1/mL) (optional), and

10.2.5 *Philodina* acuticornis (rotifer) (0.03/mL).

10.3 Whenever possible, the species just listed should be used. These species were selected on the basis of past successful use. The scientific name of the species used should be verified using an appropriate taxonomic key. Cultures of algae may be obtained from the Starr Culture Collection;[8] *Daphnia magna* may be obtained from EPA.[9]

10.4 Stock cultures should be examined periodically to verify that contamination has not occurred. Stock cultures should be maintained in more than one room to minimize the risk of a total loss due to such events as a temperature control malfunction. This can be done with least effort by transferring the older culture to an alternate culture room after the new cultures have been inoculated. Stock cultures should not be maintained solely in rooms where tests are conducted, equipment is cleaned, or toxic materials are handled. Use of volatile chemicals should be avoided, but if paint fumes or other chemicals spread from other areas, this fact should be noted. Stock cultures should be protected against exposure to materials to be tested to prevent adaptation or genetic selection.

10.5 *Algal Culture Maintenance*—Algal cultures should be maintained on T82-LowSi agar slants under lights and transferred at appropriate intervals. Aseptic technique should be used with the maintenance of the stock cultures.

10.5.1 *T82-LowSi Agar Slants*:

Note 2—This differs from the microcosm medium T82MV by (1) the omission Murphy's vitamins, (2) the silicate concentration is 0.08 m*M* instead of 0.8 m*M*, and (3) the pH adjustment is unnecessary (see Table 5).

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[8] Cultures of algae may be obtained from the Starr Culture Collection, Culture Collection of Algae, Department of Botany, University of Texas, Austin, TX 78712.

[9] *Daphnia magna* may be obtained from EPA (Corvellis, OR; Duluth, MN).

10.5.2 The mixture (complete with solutions C and K) is heated in a steamer or on a heater with stirring to boiling, dispensed in aliquots of 10-mL into 150 by 16-mm screw-cap culture tubes, capped, and autoclaved at 121°C (15-lb steam pressure) for 15 min. ~~After autoclaving, but before the tubes have cooled, 0.004 mL Solution C and 0.01 mL Solution K are added by syringe.~~ Tubes are then laid at a slant and allowed to solidify. ~~Alternately, the C and K solutions can be added to the other solutions before autoclaving but prior to dispensing, provided that the mixture is well mixed during the dispensing processes.~~ The phosphate (Solution C) is likely to precipitate with the calcium (Solution D) when warmed and autoclaved. Provided that the precipitate is dispensed into the tubes, it will be available for algal growth.

10.5.3 Monthly transfers are usually adequate; the health can usually be estimated by the color of the colonies. Occasional microscopic checks should be made to ensure that cultures are unialgal. If cultures are suspected of being contaminated with another algae, they should be streaked on agar plates made from T82-LowSi Agar.

10.5.4 *Culture of Algae for Inoculation into Microcosm* —A separate culture should be established for each species. *Anabaena cylindrica, Ankistrodesmus* sp., *Selenastrum capricornutum, Lyngbya* sp., *Chlamydomonas reinhardi* 90, *Chlorella vulgaris, Scenedesmus obliquus* sp., *Stigeoclonium* sp., *Ulothrix* sp., and *Nitzchia kutzingiana* (D216) are grown in semicontinuous culture. *Anabaena cylindrica, Lyngbya* and *Nitzschia* are not aerated. *Nitzchia kutzingiana* grows best on agar plates, transferred to liquid culture in T82MV 1 week prior to use. Temperature and light conditions should be similar to those used for the microcosms (6.1.3). *Lyngbya* grows best with about 25 % of the light the other cultures receive.

10.5.4.1 *Container for Mass Cultures*—A 2-L bottle, stopper, tubing, drying tube, aerating tube, media inlet, and vent (see Fig. 4) is autoclaved with 1 L of final basal medium (see 8.2.3). Solutions C, K, and Murphy's Vitamins are added after autoclaving (8.2.4).

10.5.4.2 *Semicontinuous Culture*—To start cultures, algae from the slant are inoculated into 10 mL of T82MV in a test tube on a light table. The 10-mL culture is allowed to grow for 3 to 5 days prior to addition to the culture container (Fig. 3). The cultures are aerated except *Anabaena, Lyngbya* and *Nitzschia* (noted previously). Aeration with 2 % CO_2 is either by 100 % CO_2(cylinder) mixed with room air using an air-CO_2 mixer, or purchased cylinders of 98 % breathing air and 2 % CO_2.

10.5.4.3 When cell number (as determined by Palmer cell counter) reaches 10^6 cells/mL, 500 mL is drained out of the culture container and 500 mL new medium is added. This is done 2 to 3 times weekly or at intervals that will maintain the culture at 10^{4-5} cells per mL or exponential growth as indicated by graphed counts (see X2.2, suggested record form).

10.5.4.4 The slower growing species (*Nitzschia kutzigiana, Stigeoclonium, Ulothrix, Anabaena* and *Lyngbya*) will require somewhat longer (about a week) between draining and replenishment than the more rapidly growing species.

10.6 *Animal Culture Maintenance*—Stock cultures should be started at least 3 to 4 weeks before the microcosm test.

10.6.1 *Daphnia Cultures—Daphnia* used in the microcosms should be the third or fourth generation started from at least 4 to 6 females. All animals should come from healthy stocks that have received sufficient food to prevent ephippia formation and carapace abnormalities. Guide E 1193 has additional information on rearing.

10.6.1.1 Containers are 3.5-L bottles (1-gal jars) half filled with medium T85MV (see Table 6) or a satisfactory natural water such as autoclaved lake water or well water. The quality of the water is important in producing *Daphnia* that fulfill the quality control criteria (Section 17). *Daphnia* reared in inadequate water do not survive and reproduce adequately. *Daphnia* reared in the microcosm medium T82MV are not as healthy as those reared in lakewater or reared in a medium to which Keating's trace metals are added such as T85MVK. *Daphnia* rearing medium should have low algal nutrients or be maintained in relatively low light to prevent excess photosynthesis and high pH.

10.6.1.2 To begin a culture, 4 to 6 females with eggs, are added to a container. *Daphnia* are fed approximately 250 mL of unicellular algae (10^{4-5} cell/mL) in log phase from semicontinuous culture apparatus about every other day. When a *Daphnia* culture becomes densely populated, it is subcultured. Subculture is recommended if few adults are carrying parthenogentic eggs or if ephippia are present. It is recommended that medium replacement not be more than 50 %. To obtain enough animals to initiate a test, weekly subcultures are recommended.

10.6.1.3 If a satisfactory water source is not available, T85MVK may be used to culture *Daphnia*. It differs from T82MV (microcosm medium) by having 1/10 the nitrate and phosphate and the addition of some of Keating's trace metals **(37).** Only differences from T82MV are noted (see Table 6).

10.6.1.4 The major salt solution should be prepared as in Table 7.

10.6.2 *Amphipods Hyalella azteca*—Containers may be 3.5-L (1-gal) glass jars containing medium T82MV and the ten algal species inoculated approximately 14 days before the amphipods. (Old control microcosms—the complete culture—from experiments may make excellent amphipod cultures.) Amphipods do best when not disturbed. New amphipod cultures (3 to 5 containers) may be started with 3 or more pairs every 3 months. Cultures may be kept in diffused light and every week fed 100 mL *Ulothrix* sp. or *Stigeoclonium*, or both, from continuous culture described in 10.5.4. The algal culture including medium may be poured into the containers. For tests, amphipod cultures may be maintained as usual and test organisms may be removed from cultures using wide-mouth bulb suction devices or small nets, or filters.

10.6.2.1 Alternative rearing methods may be used. Amphipods may be reared in aquaria with sediments similar to the microcosm. If amphipods have been recently collected from a natural environment, gradual exchange of their water with the microcosm medium (T82MV) over a period of weeks may be necessary for survival. Bottom-feeding fish food may be used as an alternative to algae as food. Light levels of 5000 to 7500 lux with a 18:6 light:dark cycle may be used for rearing. Weekly exchange
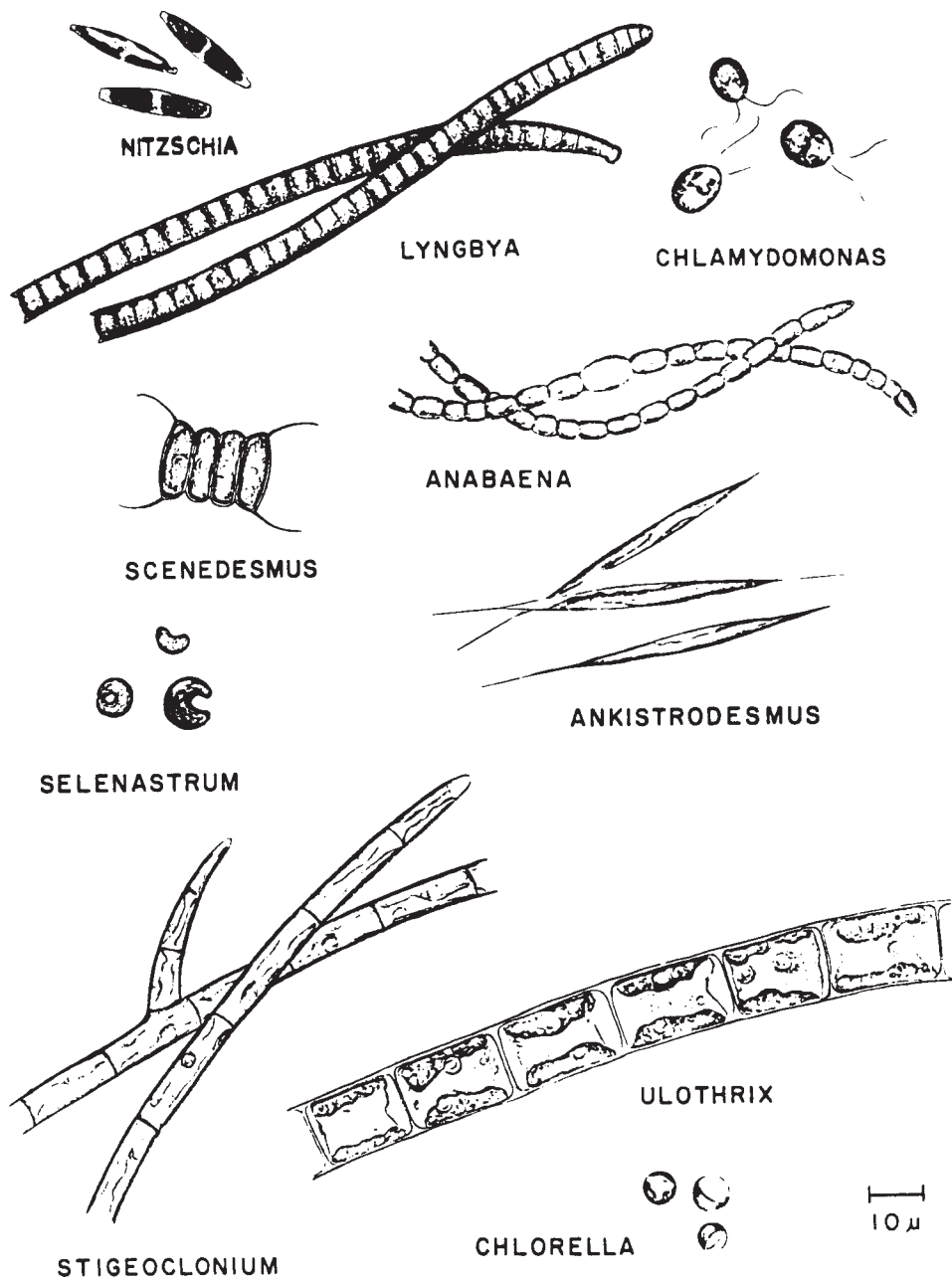
FIG. 2 Algae Used in the Standardized Aquatic Microcosm (10.1)

of medium may be necessary. Co-culture with ostracods (10.6.3) may be feasible.

10.6.3 *Ostracods (Cypridopsis sp. or Cyprinotus sp.)* —Containers should be 3.5-L (1-gal jars) as per the amphipod cultures with 50 to 100 mL of any algae from semicontinuous culture added weekly. (Old control microcosms made excellent ostracod cultures.) No special culture is required for tests. Ostracods for microcosms should be removed using a pipet or syringe. Alternatively, the ostracods may be reared with the amphipods as in 10.6.2.1.

10.6.4 *Protozoa Hypotrichs*—Protozoa should be maintained in 2 to 7-day cultures of *Enterobacter aerogenes* , that have been grown in wheat grass medium[10] (see Table 8). Other types of protozoa may be substituted.

10.6.4.1 To prepare 1 L of culture medium, add 2.5 g wheat grass powder (or substitute) and the volumes of stock solutions (see Table 8) to 1 L of distilled water, swirl, and bring to a full boil for at least 5 min. Filter through high-porosity filter paper to remove large particles and then filter twice through glass fiber filters. Make up volume to 1 L with distilled water and dispense 30 mL into large test tubes then autoclave.

10.6.4.2 For tests, bottles containing 200 mL of wheat grass medium are inoculated with *E. aerogenes* and after 48 h are

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[10] Wheat Grass Powder, available from Cerophyl Labs, 4722 Broadway, Suite 259, Kansas City, MO 64112, or its equivalent, has been found suitable for this purpose.
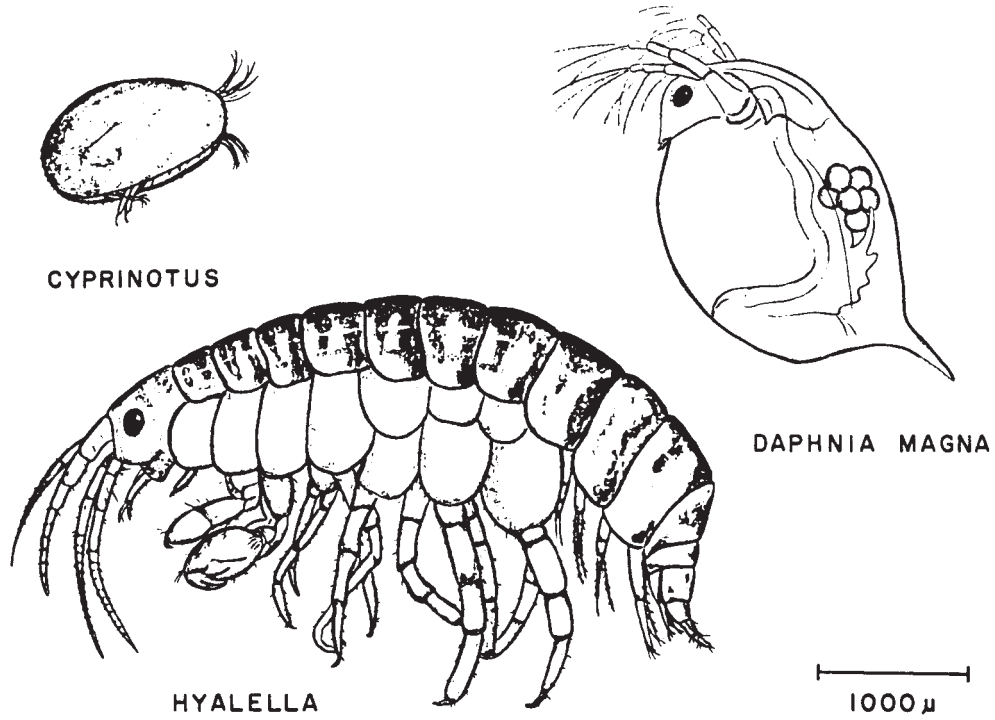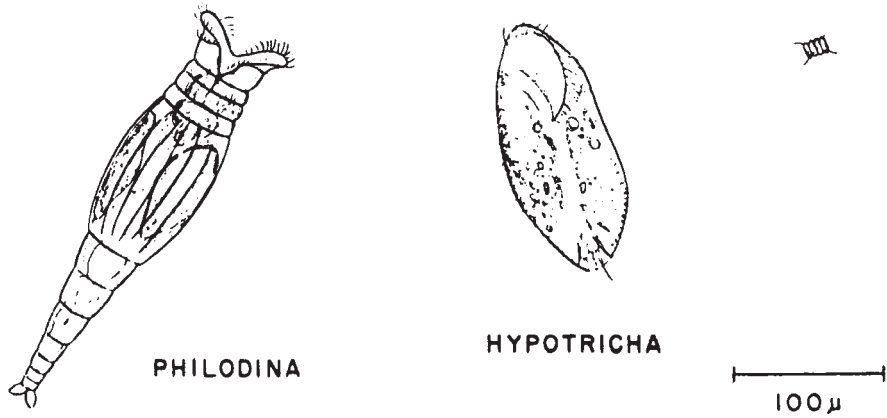
FIG. 3 Animals Used in the Standardized Aquatic Microcosm (10.2)

TABLE 5 T82-Low Si Solid Medium for Algal Cultures

Master Solution	Salt	mL/L	Concentration
(defined in 8.2.2.3)			(Final Solution)
A	NaNO$_3$	5	0.5 mM
B	MgSO$_4$·7H$_2$O	1	0.1 mM
C	KH2PO4	0.4	0.04mM
	NaOH		0.099 mM
D	CaCl$_2$·2H$_2$O	10	1.0 mM
E	NaCl	15	1.5 mM
H	Al$_2$(SO$_4$)$_3$·18H$_2$O	1	0.0048 mM
I	Na$_2$SiO$_3$·9H$_2$O	5	0.080 mM
K	Trace metal mixture	0.05	same as T82
	Distilled Water		to 1000 mL
Bacto-Agar			15 g

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Semi-continous Culture Apparatus for Algae
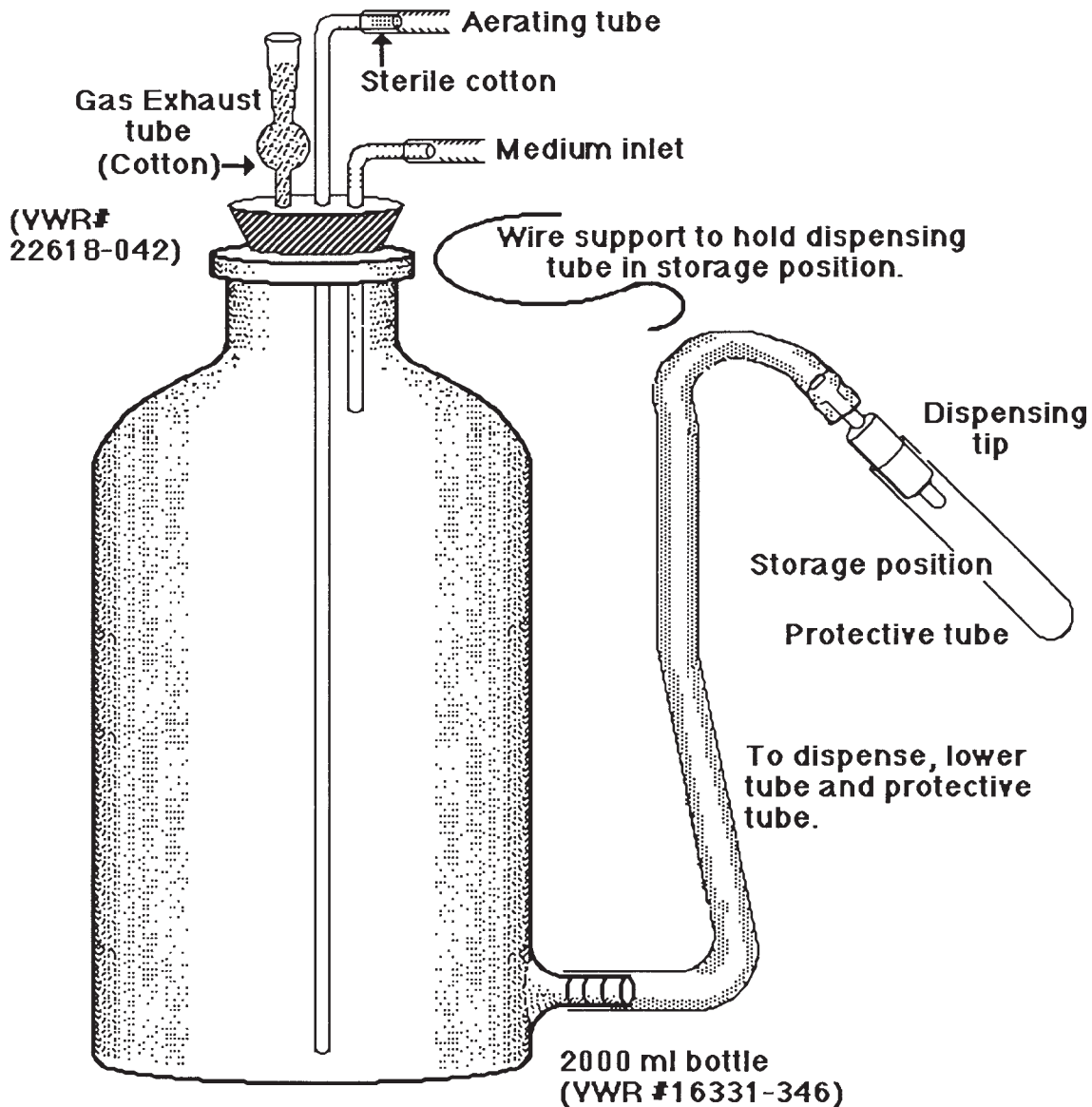
FIG. 4 Semicontinuous Algal Culture Unit (10.5.4.1)

inoculated with a few millilitres of Hypotrich culture. Five bottles should be inoculated about 72-h before addition of the organisms to the microcosms.

10.6.5 *Rotifers Philodina*—Cultures of *E. aerogenes* are grown for 48-h in 30 mL of wheat grass medium (see Table 8). Then a few rotifers are added to the bacterial culture using a Pasteur pipet. New cultures are started every seven to 10 days.

10.6.5.1 For tests, 2 to 3 large (about 200-mL) bottles of wheat grass medium are inoculated with *E. aerogenes* and 48-h later with *Philodina* about 30 days before addition to the microcosms.

11. Procedure

11.1 *Experimental Design*—Decisions concerning such aspects of experimental design as the number of treatments and number of test chambers per treatment should be based on the purpose of the test and the statistical procedure that is to be used to calculate results (see Section 15 and Appendix X3). The minimum desirable number of test chambers per treatment should be calculated from (*a*) the expected variance between test chambers within a treatment and (*b*) either the minimum difference that is desired to be detectable using hypothesis testing or the maximum acceptable confidence interval on a point estimate **(39).** If such calculations are not made, 24 microcosms (6 test chambers for each of 4 treatments) are recommended **(4-9, 22-24),** although 25

TABLE 6 Medium T85MVK Composition—Differences from T82MV[A]

Compound	Molecular Weight	Concentration		
		mM	Element	mg/L
NaNO$_3$	85.0	0.05	Nitrogen	0.70
KH$_2$PO$_4$	136.0	0.004	Phosphorus	0.123
Added (Keating's Metals)		µM		µg/L
NaBr	102.89	0.626	Bromine	50
SrCl$_2$·6H$_2$O	266.52	1.141	Strontium	100
RbCl	120.92	1.170	Rubidum	100
LiCl	42.39	14.41	Lithium	100
KI	166.00	0.0392	Iodine	5.0
SeO$_2$	110.96	0.0127	Selenium	1.0
NH$_4$VO$_3$	116.93	0.00983	Vanadium	0.5

[A] Basal medium is prepared as for T82MV (Table 1, Section 8) except that ¹⁄₁₀ the volume of Solution A (nitrate) is added.

TABLE 7 T85MVK—Daphnia Culturing Medium

Master Solution	Salt	mL/L	mL/18 L	Concentration/mM
				(Final Solution)
A	NaNO$_3$	0.5	9.0	0.05
B	MgSO$_4$·7H$_2$O	1.0	18.0	0.1
D	CaCl$_2$·H$_2$O	10.0	180.0	1.0
E	NaCl	15.0	270.0	1.5
H	Al$_2$(SO$_4$)$_3$·18H$_2$O	1.0	18.0	0.0048
I	Na$_2$SiO$_3$·9H$_2$O	5.0	90.0	0.080
C[A]		0.04	0.72	...
K[A]		0.05	0.9	...
Vitamins[A]		1.0	18.0	...
Si-10X[A]		5.0	90.0	...
Keatings[A]		1.0	18.0	...
HCl[A]		to pH 7	to pH 7	...

[A] The specified amounts of these solutions should be added after the major salt solution is autoclaved and cooled. This delays precipitation prior to dispensing.

TABLE 8 Wheat Grass Medium[10] **for Culture of Protozoa and Rotifers**

	I. Inorganic Stock Solutions		
Solution Number	Solution	Stock Concentration	mL/L Culture Medium
1	Na$_2$HPO$_4$	70.9825gL^{-1}(0.5M)	10 mL
	or	or	
	Na$_2$HPO$_4$7H$_2$O	134.04 g/L (0.5 M)	10 mL
2	KH$_2$PO$_4$	68.04 g/L (0.5 M)	10 mL
3	NaOH	4.0 g/L (0.1 M)	20 mL
4	KCl	7.48 g/L	5 mL
	NaCl	23.40	...
	MgSO$_4$7H$_2$O	26.64	...
	CaCl$_2$2H$_2$O	2.78	...
	MnCl$_2$4H$_2$O	0.40	...
	NH$_4$Cl	38.22	...
II. Dried Grass or Wheat Grass Powder[10]			2.5 g/L

microcosms (5 test chambers for each of 5 treatments) have also been used satisfactorily. The microcosms can receive equal lighting in an oval (Fig. 1). Typical designs are as follows:

Design A

11.1.1 Control,

11.1.2 Low concentration (single addition),

11.1.3 Medium concentration (single addition),

11.1.4 High concentration (single addition),

Design B

11.1.5 Control,

11.1.6 Solvent control,

11.1.7 Solvent and low concentration (single addition),

11.1.8 Solvent and high concentration (single addition),

Design C

11.1.9 Control,

11.1.10 Low concentration (single addition),

11.1.11 High concentration (single addition), and

11.1.12 Low concentration (multiple additions).

11.2 *Inoculation of Microcosms* (See X2.3 Data Sheet)—Sufficient microcosms should be prepared and inoculated to provide about 6 more than required for the experimental design so that elimination by breakage or culling on Day 7 leaves enough for the test. For most tests, 30 microcosms are adequate.

11.2.1 *Algae*—The day algae are added is designated Day 0. If a single test is being conducted, Day 0 should be a Friday to avoid weekend work. Microcosms are numbered 1 through X. Each alga is inoculated into microcosms to provide an initial concentration of 10^3 cells/mL. Stocks of algal cultures should be removed from the continuous culture apparatus and their concentration is determined by Palmer cell counts (see 11.5.6.2(*b*)); the necessary volume to be added to each microcosm is calculated. An automatic pipettor with replaceable tips should be used to ensure accurate dispensing. It is recommended that clumping or filamentous forms be vigorously shaken in sterile jars with sterile glass beads, and the resultant uniform suspension after settling be used for inoculation. The addition of clumps will cause over-inoculation.

11.2.2 *Microscopic Animals (Protozoa and Rotifers)*—On Day 4, Hypotrichs should be inoculated to an initial density of 0.1/mL, and *Philodina* to 0.03/mL. Stock cultures should be counted on counting plates. The necessary inoculum volume is calculated and pipetted directly from the culture into the microcosm. Note that wheat grass medium (see Table 8) contains high amounts of phosphate, and excessive volume additions of the medium with Hypotrichs and *Philodina* should be avoided. Organisms should be concentrated by carefully pipetting from the top of the inoculum cultures, where they concentrate themselves.

11.2.3 *Larger Organisms (Daphnia, Amphipods and Ostracods)* —On Day 4, *Daphnia*, amphipods, and ostracods should be rinsed in medium before they are added to the microcosm. It is convenient to place the appropriate number of animals in small beakers of medium prior to addition to each microcosm. For each microcosm, add 16 *Daphnia*: six large (>1.8 mm), three large with eggs, three without eggs, and ten small (0.7 mm); 12 amphipods (six adults and six small), and six ostracods.

11.2.4 *Stock Cultures*—Stock cultures should be maintained for reinoculation during the test (11.6).

11.3 *Culling and Assignment*—On Day 7, the necessary number of microcosms should be selected for the test. Any microcosms in cracked jars should be eliminated because cracks tend to grow and leak. Data through Day 7 on the following measurements (see 12.5) should be used to determine culls: change in dissolved oxygen from a.m. to p.m., pH, number of *Daphnia*, the number of *Selenastrum* and *Chlamydomonas*, and the dominant algae early in microcosm development. The necessary number of test systems with the least variation from one another in these measurements should be saved and the others discarded or used for other purposes (for example, as amphipod and ostracod cultures).

11.3.1 *Treatment Assignment*—The selected microcosms should be numbered and a random number table used to assign each microcosm to a treatment and to a position separately on the light table (Fig. 1). For table position the microcosms are assigned to 6 blocks, and randomly assigned to each. Treatments must be randomly assigned to individual test chamber locations. A randomized block design (with each treatment being present in each block, which may be a row or a rectangle) is preferable to a completely randomized design. Microcosms are then numbered consecutively as in Fig. 1.

Note 3—Microcosms that crack while the experiment is in progress may be transferred to new sterile containers after a thorough scraping. Occasionally this give rise to outliers; examination of the data determines their eventual inclusion or exclusion.

11.4 *Addition of Test Material*:

11.4.1 *Single Addition*—Test material should be added on Day 7 after treatment assignment. Each microcosm should be stirred with a glass rod after test chemical or solvent, or both, are added.

11.4.2 *Multiple Addition*—The test material may be added biweekly or weekly after sampling. The volume of each microcosm should be measured again before test material addition and the addition calculated to provide the appropriate concentration. If residual test material in the microcosm is to be measured, the samples should be removed prior to the new addition. ~~No~~ It is recommended that medium T82MV not be added to compensat~~ione~~ for ~~the volume removed by sampling is made because~~ sample removal, any medium addition would serve as a source of nutrients and eliminate the need for nutrient recycling to occur for continued photosynthesis. However, medium addition may be necessary if large samples must be removed for test chemical analyses (See 11.5.6.2(e)) Distilled water is not added because it could cause osmotic imbalances.

11.5 *Measurements*:

Note 4—Each task should be assigned to a person (see Appendix Fig. X2.4). All data should be written on computer data sheets as they are collected. Data sheet formats are suggested in Appendix, Figs. X2.1-X2.9. Microcosms should be grouped by treatments on the data sheets. When a microcosm breaks or data are missing for a measurement, an entry should still be made that includes experiment designation, subcode, microcosm number and test day number, and the data portion left blank.

Note 5—Separate probes, and other equipment (containers, scrapers, stirrers, and syringes) should be used for each treatment. It is recommended that where possible all sampling devices be autoclaved or otherwise sterilized prior to the beginning of each test. Although aseptic techniques are not required for most of the sampling procedures, care should be exercised to avoid contamination with other organisms; for example, none of the sampling devices should be used with lake or sediment samples. Care should be taken so that mixing of even small amounts of test and control microcosms is avoided.

11.5.1 *Sampling Frequency*—Sampling and measurement of organism abundances and physical factors should be done twice each week until the end of the test, usually Day 63. Measurements should be done on the same test day for each test if it is desirable to compare data between tests. Tuesdays and Fridays are the most convenient days because the first two oxygen measurements (of the 3-point method) can be taken Monday and Thursday; thus, no weekend work is required on this schedule.

11.5.2 *Turbidity*—Prior to dissolved oxygen determinations, approximately 5 mL should be withdrawn from each unstirred microcosm for measurement of turbidity. If the instrument does not provide a digital output of absorbance, *t*, the percent transmittance should be recorded, and converted to optical density during the computerized data processing (14.1.7).

11.5.3 *Volume*—An external calibrated rod may be used to estimate volume. A30-cm rod may be calibrated to a sample microcosm jar containing 200 g of microcosm sediment sand and 500 mL of medium or water. The first gradation is marked next to the liquid level and designated 500 mL. Aliquots of 100 mL of water are added and the stick marked each time until 3500 mL is reached. On sampling days, volume can be measured by holding the calibrated rod against a microcosm and noting on a data sheet which gradation is closest to the microcosm water level.

11.5.4 *Dissolved Oxygen*—Dissolved oxygen concentrations should be measured 3 times: before lights-on (8:00 to 9:00 a.m.) and late afternoon (4:00 to 5:30 p.m.) before a sampling day, and the predawn before sampling (8:00 to 9:00 a.m.). Separate probes, marked with tape or symbol should be used for each test material. They are calibrated and stored in the mouth of reagent bottles, unplugged from the meter. For use, probes should be plugged into the meter, standardized and readings taken. If the test involves a control group and more than one concentration of a test material, all replicates with test material are read using the same probe, the group with the lowest concentration of test material being measured first. The probe is rinsed with distilled water between groups. Control replicates are always measured only with the control probe. The a.m. measurements are taken before the lights are on using a flashlight to observe meter readings and microcosms. The p.m. measurements are taken late in the afternoon after maximum photosynthetic activity has been achieved. Dissolved oxygen is measured while moving the probe in a circular motion. A self-stirring probe may be used.

11.5.5 *pH*—pH values should be measured before lights-on each sampling day. Separate probes are used for the control and treatments, as specified for dissolved oxygen probes.

11.5.6 *Sampling*.

11.5.6.1 *Method of Mixing*—The sides and bottom of the microcosm jars should be scraped as completely as possible and stirred vigorously with a prerinsed and autoclaved rubber scrapper (rubber policeman,[11] see Fig. 5) attached to a glass rod.

11.5.6.2 *Sampling and Organism Enumeration* :

(a) (*a*) *Counts of Daphnia, Amphipods and Ostracods*—A visual count of the larger organisms in the entire microcosm is performed before sample removal if there are very few organisms. If there are too many organisms to count directly, subsamples of 100 mL are removed to the holding container until a total of at least 20 organisms of each species have been removed. See Fig. 6 for sampling progression. The sampling devices used, one for each treatment, are 41-mm diameter glass tubes into which No. 8 rubber stoppers attached to long glass rods are inserted (see Fig. 5). The microcosm contents are stirred vigorously with the glass rod, then the tube is lowered tightly onto the stopper, sealing the lower end of the tube and capturing the water column sample that is then removed. The sampler is held upright over a holding container which has 100, 200, and 300-mL markings and the stopper is loosened by pushing the glass rod slightly allowing the liquid out the bottom of the tube (Fig. 6). Stir vigorously enough to obtain samples of amphipods and algal mat. The holding container is marked with the designation color or symbol for its treatment but has no number since in the course of sampling, it will be used to hold liquid from each replicate in the treatment. To count the organisms, a small portion from the holding jar is poured into a clean 100 by 15-mm petri dish. The liquid is examined and the number of organisms totaled on the laboratory counter. Small organisms are newborn, approximately 0.7 mm, medium are up to 1.8 mm, large are greater than 1.8 mm. Amphipods are small if <4 mm, large if >4 mm. Ostracods are not sized. The portion is then poured into the empty numbered subsample jar and another small sample poured into the petri dish and counted. This is done until the holding container is empty, that is, all of the large organisms in the 100-mL subsamples have been counted. The number of organisms and the number of subsamples are entered on the data sheet, and the subsample returned to the microcosm. The holding container and petri dish are rinsed with distilled water prior to sampling the next microcosm in the treatment. Data collection forms are shown in Appendix X2, Fig. X2.5.

(b) (*b*) *Algal Counts*—Samples are removed by Pasteur pipet from the stirred microcosm or the subsample jars. Algae are counted using a Palmer Cell algal counting slide. A magnification of 320× is used; 8× ocular and 40× water emergent objective provides adequate magnification to see all algal species and it provides adequate depth of field. Cells of each genus and species and number of fields counted are recorded. An inverse sampling procedure is utilized, in which a greater number of fields are counted for the rarer algal species. Once the count for any single species exceeds 50, the cell count and number of fields for that species should be recorded, with additional fields being examined for only those species still lacking counts of 50. The upper limit of fields counted is determined by the area of the Palmer Cell, and ranges from 55 to 65. Care should be taken to avoid counting fields excessively close to the cell boundaries, where debris typically accumulates and the cell dispersal was found to be nonrandom. The Palmer Cell should be rinsed and dried between counts. The diameter of the microscope field should be measured

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[11] This practice is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

with an ocular micrometer or hemocytometer. Given that the depth of the Palmer Cell is 0.4 mm, the volume of each field can be calculated (see 14.1.1 for calculations).

Note 6—The well of a Palmer Cell is 0.4 mm in depth; a hemocytometer slide is not satisfactory because it is too shallow (0.1 mm) for the large algae and contains too small a volume to count rare cells (see Appendix Fig. X2.6 for data form).

(a) (*c*) *Protozoan and Rotifer Counts*—For protozoa and rotifers 1 or 2 mL from the stirred microcosm or subsample container are dispensed in 0.1-mL or in 0.2-mL aliquots on a counting plate. The aliquots are counted if there are fewer than 100 organisms/mL (that is, 10 organisms/0.1-mL sample). If densities are >100 organisms/mL, 0.1 mL in 0.01-mL drops are counted on the plates. A measured volume containing at least 30 Hypotrichs is an acceptable lower limit for counting. Two mL is an acceptable maximum volume for counting, regardless of densities. All drops are counted at 6 to 12× total magnification using a stereomicroscope (see Fig. X2.7 for data form).

(b) (*d*) *In vivo Fluorescence*—Samples for *in vivo* fluorescence **(40)** can be removed from the stirred microcosm or subsample container. The tubes are stirred and immediately inserted into the fluorometer.[12] The power (door window) is adjusted so that the readings fall within the 20 to 80 range on the fluorometer scale. Door factors should be accurately determined prior to use of the fluorometer. The scale reading, as well as the power used (1×, 3×, 10× or 30×), must be recorded (see Fig. X2.5 for data form).

(c) (*e*) *Nutrient Analysis Samples*—Samples for nutrient analysis are removed from the stirred microcosms or subsample container using a 10 or 20-mL plastic syringe without needle. The container is swirled clockwise and counterclockwise and the sample withdrawn. A filter unit with a 25-mm 0.45-μm membrane filter is attached and approximately 7 mL dispensed into a distilled-water-rinsed 50-mL plastic bottle, and frozen. Care should be taken that bottles are dry before adding filtrate. For micro-analyses, removal of excessive liquid has not a problem for 63 day tests. If greater quantities of liquid are removed for chemical analyses. T82MV medium may be added after samples have been removed for chemical analyses. Analyses for levels of nitrate, nitrite, ammonia, and phosphate, are performed using standardized techniques **(41, 42)** (see Fig. X2.8 for data form).

(d) (*f*) Temperature should be recorded continuously (6.1.1). The range should be recorded for each week if the temperature control apparatus maintains the temperature within 20 to 25°C. If a temperature occurs outside that range, the duration and temperature extremes must be reported. If continuous temperature recordings have been made, the data should be stored with the original data.

(e) (*g*) Light intensity should be measured weekly and recorded (6.1.3).

(f) (*h*) *Estimation of Extracted Pigments (optional)*—If this technique is used, appropriate references should be consulted, for example, **(41-46).**

(g) (*i*) *Estimation of Carbon Uptake, Calculated from ^{14}C Uptake and Alkalinity (optional).* If these measurements are to be done, appropriate references should be consulted **(41, 42, 46).**

11.6 *Reinoculation*—Reinoculation with the larger organisms should take place once per week, usually on Friday after sampling. The number of large organisms is brought up to three per microcosm by adding daphnids, amphipods and ostracods rinsed in T82MV. One drop each (circa 0.05 mL) of the hypotrich and *Philodina* cultures are added to each microcosm. For the algae, one drop (circa 0.05 mL) is added to each microcosm from a mix of the ten species. If algae are at their maximum possible concentrations in stock culture this will result in 5×10^2 cells of each alga added per microcosm. These reinoculations are noted on the data sheets stored in a microcosm data notebook (see Fig. X2.9 for data form).

Note 7—These concentrations of algae and animals are below the detectable limits and will not affect counts unless reproduction occurs. The purpose of the reinoculation is to reduce the potential for random extinctions leading to increased variance among replicates, and to allow the recovery of populations if the toxicity is temporary. The ecological equivalent is immigration.

12. Analytical Methodology

12.1 The methods used to analyze water, sediment, and organisms for test material might aid in determining if recovery (should it occur) is due to degradation of the parent compound or adaptation of the organisms. If the analytical method distinguishes reaction or biodegradation products from the parent test material, it may be possible to assess the effects of the degradation products separately from effects of the parent material. Measurement of major products, in addition to parent material, is usually desirable in water, sediment, and organisms. If the analytical method measures any impurities, reaction, or degradation products along with the parent test material, results can be calculated only for the whole group of materials, and not for the parent material itself, unless it is demonstrated that such impurities and products are not present.

12.2 If samples cannot be analyzed immediately, they should be handled and stored appropriately to minimize loss of test material by such things as microbial degradation, hydrolysis, oxidation, reduction, photodegradation, volatilization, and sorption.

12.3 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources.

12.4 The precision and bias of each analytical method used should be determined in appropriate matrices, that is, in the

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[12] Fluorometer such as, Turner Model #111 with 931A photomultiplier, dark-blue Corning CS-5-60 primary filter and dark-red Corning CS-2-64 secondary filter, or equivalent available from Unipath, 250 Maude Ave., Mountain View, CA 94043, has been found suitable for this purpose.

(Dip) Sampler

Scraper

One liter cylinder

Glass rod

Distilled water

Glass sampling tube

Glass rod

100

Rubber policeman

No. 8 stopper
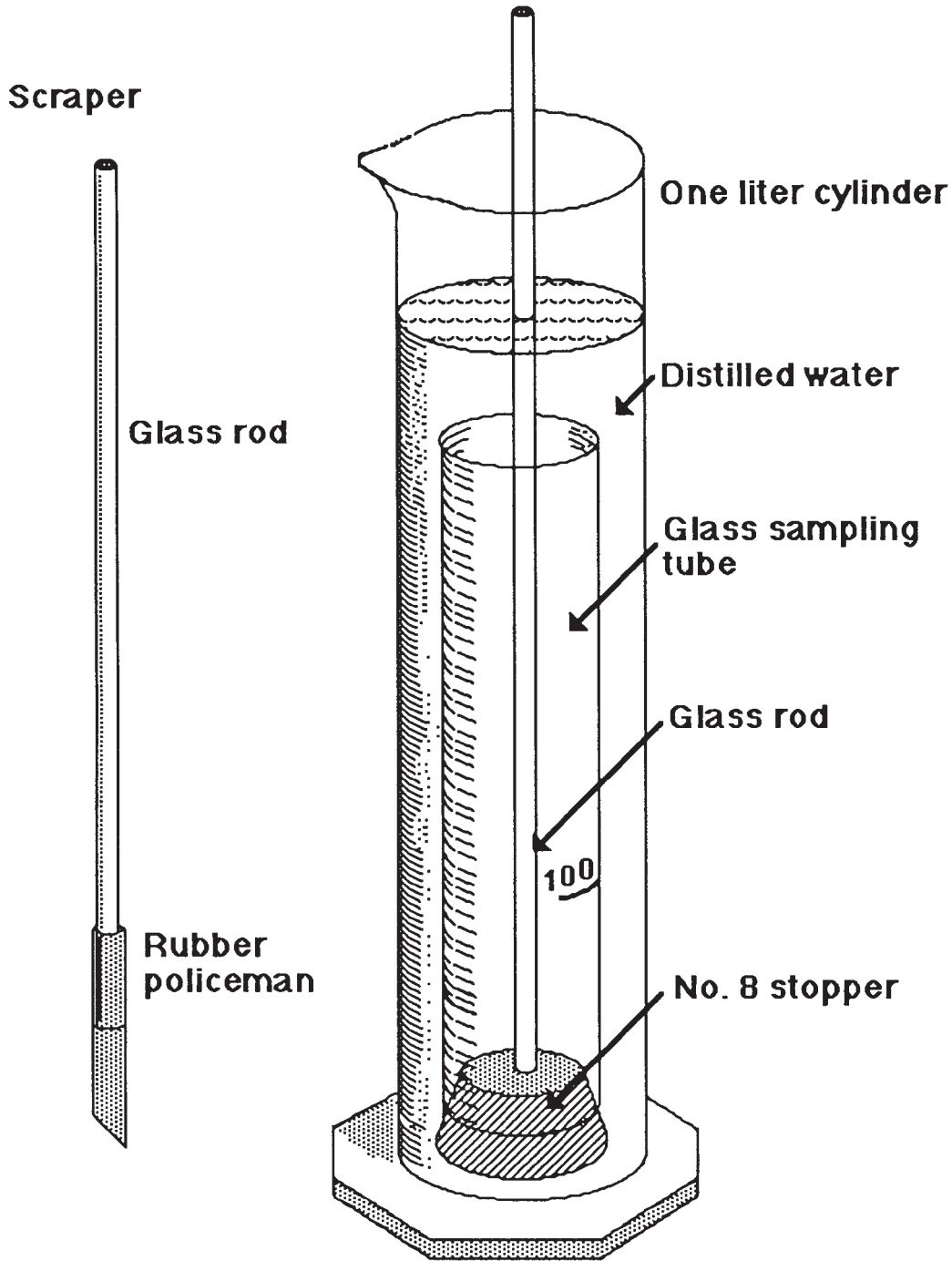
FIG. 5 Devices for Scraping, Mixing, and Subsampling Microcosms (11.1 and 11.2.1)

organisms, sediment, and water. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed.

12.5 When tests are conducted using a radiolabeled material, separate microcosms might be desirable to measure biological effects using nonradiolabeled material to prevent worker or laboratory contamination during measurements. Gross visual comparisons should be made to assess that the labeled and unlabeled microcosms are behaving similarly.

13. Data Processing

13.1 Data, as measured, should be entered into a spreadsheet or Data Handling/Statistics/Graphics Program that can be used for data sorting, log transformations, one- or two-way analyses of variance, t tests, and other statistical analyses that might be useful. The graphics program should permit at least six lines per graphic. All calculations should be done by computer; data should be

Sample Progression

Number label ——

Tape denoting
test chemical

Numbered microcosm container
(1 gallon)

Direct Daphnia, amphipod, ostracod
counts depending if low density.

Absorbance

110 ml (approx.)

Three 100 ml subsample "dips"

14C and alkalinity
(optional)

Temporary
container

Tape denoting
toxicant

300
200
100

Petri dish count
of Daphnia, amphi-
pods and ostracods

Numbered
subsample
container

Number
label

Tape
denoting
toxicant

Algae, protoza,
rotifer counts

in vivo fluoresence
chlorophyll (optional)
pH
nutrient sample

Alternately, samples maybe
taken directly from the mixed
microcosms.

FIG. 6 Subsampling Progression for Counting *Daphnia* , Amphipods, and Ostracods (11.5.6.2(*a*))

entered in the formats provided in the appendices, organism densities and chemical concentrations should be calculated by the equations in Section 14, prior to statistical analyses. The data should be sorted into treatments; control should be Treatment 1, solvent control (if used), Treatment 2, and the other treatments from low to high concentration. The treatments should be clearly labelled in the report.

13.2 All statistics and graphics should be prepared from the spreadsheet or Data Handling/Statistics/Graphics Program. In addition to the printed report (Section 18), it is recommended that the complete data and statistical analyses be available on computer disk for potential checking by an independent laboratory or regulatory agency.

14. Calculations of Variables from Measurements

14.1 *Immediate Data* (that is, those available on sampling day):

14.1.1

$$\text{Algal cells/mL} = \frac{\text{algal cells}}{\text{number of fields} \times (\text{volume (mL) per field})} \tag{1}$$

where:
volume of field is calculated from Palmer Cell depth (0.4 mm) and field diameter for the microscope used to count algae (see 11.2.2 and 11.5.6.2 (*b*)).

14.1.2

$$\text{Rotifer or protozoan densities/mL} = \frac{\text{protozoans found}}{\text{volume (mL) examined}} \tag{2}$$

14.1.3 *Large Zooplankton (Daphnia, Amphipods, Ostrocods Abundances/100 mL)*—If data sheet entry for each 100-mL subsample (dip) is zero, (that is, the entire microcosm has been counted for large organisms): abundance must be converted to abundance/100 mL as follows:

$$\text{number of } (100-\text{mL}) \text{ samples in the microcosm} = \frac{\text{volume in microcosm (mL)}}{100 \text{ mL}} \tag{3}$$

then,

$$\text{abundance/100 mL} = \frac{\text{number of each zooplankton species}}{\text{number of } (100-\text{mL}) \text{ samples in the microcosm}} \tag{4}$$

14.1.3.1 If dips is greater than zero (that is, one or more 100-mL subsamples (dips) were counted):

$$\text{abundance/100 mL} = \frac{\text{number of each zooplankton species}}{\text{number of } (100-\text{mL}) \text{ subsamples}} \tag{5}$$

14.1.4 *Total Daphnia* /100 mL = small + medium + large *Daphnia* as calculated in 14.1.3.

14.1.5 *Percent Small Daphnia* = small/total (optional).

14.1.6 *Total Amphipods* /100 mL = small + large amphipods as calculated in 14.1.3.

14.1.7 *Absorbance* = $2 - \log_{10}$ (percent transmission). This calculation is necessary only if the instrument does not provide absorbance in digital form.

14.1.8 *Changes in Dissolved Oxygen* (see 3.3.4, 17.1.5, and 17.2)

where:
DO1 = first a.m. measurement (before lights on), ppm,
DO2 = first p.m. measurement (before lights off), ppm,
DO3 = second a.m. measurement (before lights on), ppm,

then:

$$\text{DO p.m.} = \text{DO2} - \text{DO3} \tag{6}$$

Loss in O_2 during dark period = R, an estimate of respiration.

$$\text{DO a.m.} = \text{DO2} - \text{DO1} \tag{7}$$

Gain of O_2 during the lighted period = P, an estimate of net photosynthesis.

$$\text{DO} = \text{DO3} - \text{DO1} \ (24-\text{h change}) \tag{8}$$

then:

$$P/R = \text{net photosynthesis/respiration ratio}$$

$$= (\text{DO2} - \text{DO1})/(\text{DO2} - \text{DO3}) \ . \tag{9}$$

14.1.9 *Fluorometry (In vivo)*—Adjust fluorometry measurement for amplification (power), the area of the light path. Conversion units may be machine specific. The value is for 1-cm diameter tube.

14.1.10 *Algal Biovolume and Available Algae*—The factor equals biomass × feeding availability (to *Daphnia*). The factor is an estimate of the relative food value of each species of algae to *Daphnia*, assuming that volume and feeding availability are the only aspects of importance and are as follows:

$$\text{Total Algae} = \sum_{i=1}^{n} \text{Algae}_i \ (10^4 \text{ cells mL}^{-1}) \tag{10}$$

$$\text{Algal Biovolume} = \sum_{i=1}^{n} \text{Algae}_i \times \text{Cell Volume}_i \, (10^4 \ \mu m^3 \, mL^{-1})$$

$$\text{Available Algae} = \sum_{i=1}^{n} \text{Algae}_i \times \text{Factor}_i \, (10^4 \, \mu m^3 \, mL^{-1})$$

where:

Algae$_i$	Factor$_i$ μm^3/cell	=	Cell Volume$_i$ μm^3/cell	×	Feeding Availability
Selenastrum	22.0		22		1.0
Chlamydomonas	22.0		22		1.0
Scenedesmus	66.0		133		0.5
Ankistrodesmus	34.0		43		0.8
Chlorella	22.0		22		1.0
Nitzschia	35.7		51		0.7
Anabaena	2.0		10		0.2
Lyngbya	0.1		10		0.01
Stigeoclonium	1.0		20		0.05
Ulothrix	2.0		185		0.01

14.1.10.1 The feeding availability is an estimate of the availability of that type of algal cell to *Daphnia magna* . Small, unicellular species are assumed to be 100 % available. Filamentous cells, such as *Ulothrix, Stigeoclonium, Anabaena* and *Lyngbya* are assumed to be too large for *Daphnia* and only 1 to 20 % available. The diatoms, although small, are judged to be 70 % available to the *Daphnia* because they tend to grow attached to the glass and sand surfaces. *Scenedesmus* is assumed to be 50 % available because it is in colonies of four cells, and *Ankistrodesmus* is assumed to be 80 % available because the cells are long, and often in loose clumps. These availability factors are provisional, and subject to change as more information is gathered. The factor is the product of the cell volume × feeding availability.

14.1.11 *Algal Species Diversity*

14.1.11.1 *Diversity of (All) Algal Species, (DVRSALG)*

where:

$$DVRSALG = - \sum_{i=1}^{n} (\text{algae}_i/\text{total algae}) \times \ln(\text{algae}_i/\text{total algae}) \tag{11}$$

only for terms where algae$_i$ > 0

14.1.11.2 *Diversity of (Size-Weighted) Algal Species*, (DVRSFD), (This estimates the diversity of algal cells that are available to *Daphnia*) as in the following equation:

$$DVRSFD = - \sum_{i=1}^{n} ((\text{algae}_i \times \text{factor}_i)/\text{available algae})$$
$$\times \ln ((\text{algae}_i \times \text{factor}_i)/\text{available algae}) \tag{12}$$

only for terms where algae$_i$ > 0

14.2 *Nutrients*:

14.2.1 NUT equals the nutrient measurement as reported by the analyzer, μg-atm/L

where:

$$DIL = \text{dilution factor} = \frac{\text{total diluted volume (mL)}}{\text{sample volume (mL)}} \tag{13}$$

if (NUT < 0) NUT = 0,
then NUT = NUT × DIL,
therefore:

$$NUT = PO_4, SIO_4, NO_3, NO_2, NH_3. \tag{14}$$

15. Statistical Analyses (See also Appendix X3, Statistical Guidance)

15.1 *Preface*—The statistical techniques described in this section were specifically developed for performing hypothesis tests. These procedures have been used to analyze data from Standardized Aquatic Microcosm (SAM) tests and have been shown to reflect ecological changes between a control treatment and the chemical treatments (4, 7-9, 23). Appendix X3 contains hypothesis tests and other methods that might be suitable for analysis of data from microcosm tests, depending upon the experimental design and the behavior of the response variables through time, that is, the dynamics of the response. (50-71) Multivariate statistical techniques have been used with microcosm data (72) . Since mesocosms inherently deal with multiple species and multiple chemical endpoints, multivariate techiques allow the detection of subtle interactions among species, long-term persistence of effects, and allow the visualization of the experiment at a community and ecosystem level.

15.2 *Analyses of Variance* (ANOVA):

15.2.1 Enumeration data (population densities and variables calculated from them) should be log transformed, either ($\log_{10}(X + 1)$ or $\ln(X + 1)$)). It is well established that the statistical behavior of certain variables follows the requirements (normality, equal variances) more closely if one uses the logarithm of the variable **(23, 70)**.

15.2.2 A one-way ANOVA of each variable with accompanying *t* tests should be performed for each sampling day. The technique compares results from the control (or solvent control) to each chemical treatment. Variances are pooled over all treatment for these comparisons. Some treatment designs may be analyzed in a two-way analyses of variance; for example, if two treatments have a solvent and two do not. It is not recommended that many unplanned *a posteriori* t-tests be done on means after looking at the data. (The number of *a posteriori* t-tests is limited by the degrees of freedom between treatments; that is, one t-test per degree of freedom.)

15.2.2.1 It is recommended that the probability values be summarized into tables showing *t*-tests to display the significant differences from *t*-tests between the control and each chemical treatment. This can be achieved by using symbols to demonstrate significant differences for each variable for each day, indicating the direction of the change, and the level of significance (<0.01 and <0.05). Examples of an "arrow diagram" are shown in Refs. **(4)** and **(21)**. These significance levels (0.01 and 0.05) have been chosen based on tradition and experience with the observed within-treatment variability and the differences between group means **(23).** Coefficients of variation for different measurements have ranged from 10 to 150 %, significant differences between group means therefore also experience a large range (15 to 226 %). Microcosm data pose a problem by the presence of correlated observations through time, which raises the overall probability of at least one observed difference showing up significantly when in fact the null hypothesis is true. If 18 time points were completely independent (which they are not), the calculated probability of at least one stray significant result appearing is 0.60. While the exact probability of at least one stray significant result for dependent time points cannot be calculated without investigating the autocorrelation structure of the data, stray significant results could occur. Such stray results would not be likely to exhibit any logical pattern. Therefore, interpretation of data should emphasize sequences of significant differences for a variable, and should corroborate observed differences by comparison with biologically related variables (see 17.1.2).

15.2.2.2 Choosing critical values based on experiment-wise error rates might reduce the incidence of stray significant results **(64).** An experiment-wise comparison on a given sampling day will take into account the fact that more than one comparison is being made; the corresponding 0.05 level of significance is an umbrella level of significance for that sampling day. Carmer and Walker **(68)** have presented further arguments based on comparison-wise error rates as to why a least significant difference approach is often in order when doing *a priori* multiple comparisons. Discussions on experiment-wise and comparison-wise error rates and critical values may be found in **(50, 55, 59, 68, 69, 71).**

15.2.2.3 The statistical program should also tabulate the data into individual microcosm values, means, standard deviations, and sample sizes. Calculation of the coefficients of variation is optional.

15.2.2.4 These tables are required for the Report (see Section 18) and are also used to generate the graphical displays of the data.

15.3 *Calculation of Intervals of Nonsignificant Difference*:

15.3.1 To graphically summarize treatment means and significance for one variable over the course of the experiment, the interval of nonsignificant difference (IND) should be calculated (see Fig. 7). This IND about the control mean indicates the area within which treatment means are not significantly different from the control mean. Outside this boundary, differences are significant. The IND is similar to the least significant difference based on pooled variances. This IND is calculated as follows:

$$\bar{x}_c \pm t_{df}\sqrt{s^2(1/n_t + 1/n_c)} \tag{15}$$

where:
x_c = control mean,
t_{df} = Students *t*-value for the degrees of freedom associated with the Mean Square (MS) error term from the ANOVA procedure,
s^2 = MS error from the ANOVA,
n_t = number of treatment replicates. (If this varies over the treatment groups, use the geometric mean of the sample sizes), and
n_c = number of control replicates.

15.3.2 For transformed data, use an interval calculation based upon an ANOVA of the transformed data, and calculated about a transformed control mean, and then back transformed. The back-transformed interval will not be symmetric about its mean.

15.3.3 For balanced designs (equal sample sizes), parametric techniques like *t*-tests and ANOVA are highly robust to deviations from normal distributions and inequality of variances **(50, 55, 57, 69, 71).** In fact, the statistical expression for the variance of the difference between means is algebraically the same whether one is using pooled or unpooled variances, when the sample sizes are equal.

15.4 *Elimination of Data*:

15.4.1 Elimination of data from the statistical analyses should only be done when justified by a known or highly probable laboratory accident. For example, if a container cracks and the contents are placed into another container, the microcosm might not behave as the other replicates. Laboratory accidents do occur and a microcosm might become contaminated or otherwise

compromised. In such events data might be eliminated from the statistical evaluation, but the data should be retained as part of the data set, or reported separately (see Section 18).

16. Acceptability of Tests

16.1 Two categories are of concern in establishing criteria for a valid experiment: (1) was the biological performance of the control microcosms adequate (that is, were the organisms healthy enough that effects of a test material could be demonstrated) and (2) did the magnitude of the within-treatment variable excessively reduce the sensitivity of the test. If the controls do not meet these acceptable ranges, it is likely that the medium was inadequate or toxic, or that the organisms were impaired. Unless this level of activity was present, it is unlikely that negative effects could be expressed. It is possible for a test to achieve the mean values, but have variances of such magnitude that treatment means will have to exceed normal biological ranges in order to demonstrate statistically significant differences.
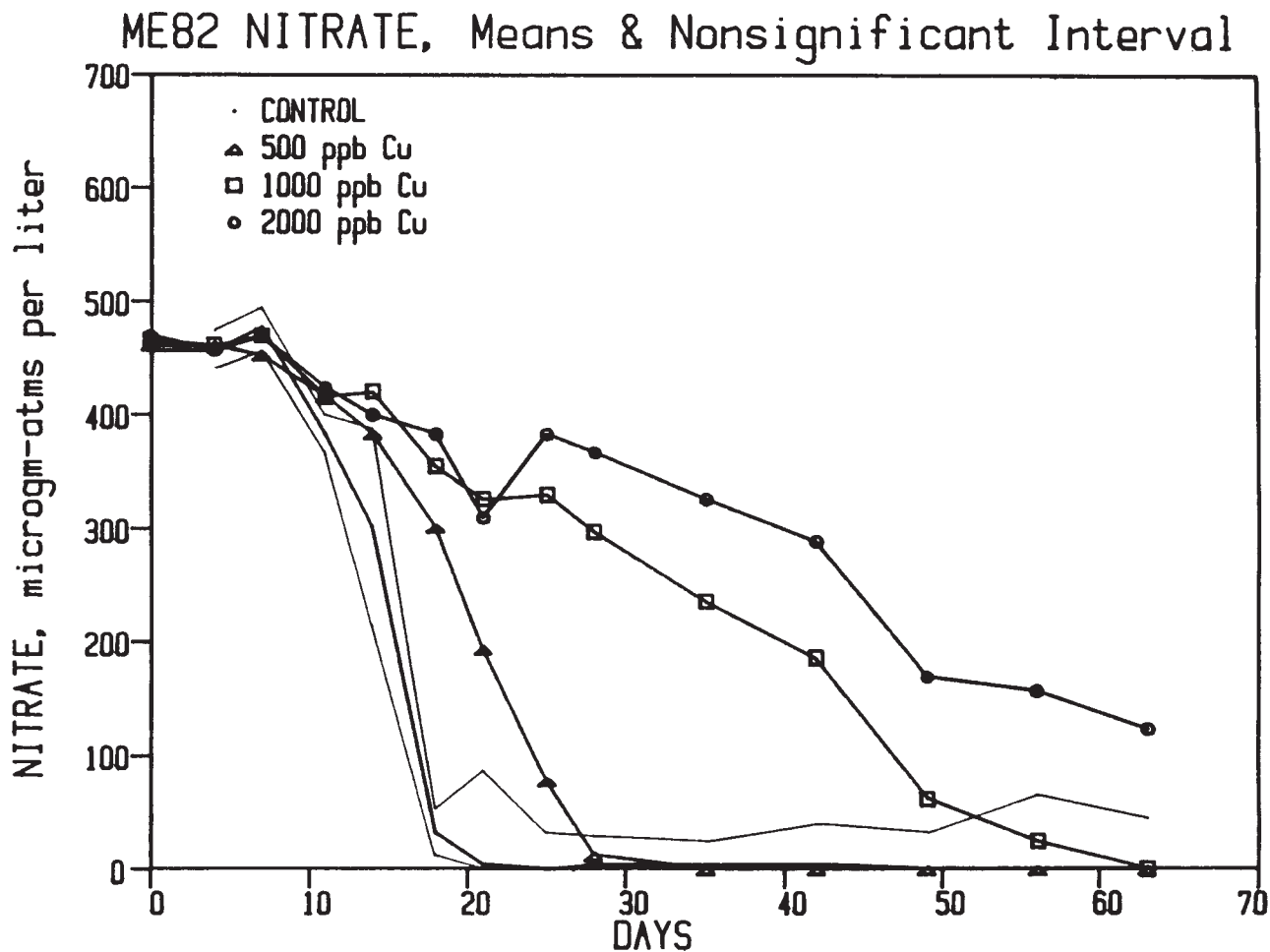
FIG. 7 Graphic of Treatment Means and Interval of Nonsignificance (4.4, 17.3, and 19.4)

16.2 *Biological Performance*:

16.2.1 The interactions among the components in the microcosms are more critical than the absolute timing **(9, 10).** For example, it is critical that the nitrate (limiting nutrient) be depleted as it is converted into algal biovolume, and that the algal biovolume be converted into *Daphnia* abundance fairly early in the development of the microcosms. Thereafter, it is to be expected that the *Daphnia* will overgraze the algal food supply, and that both the algal biovolume and the *Daphnia* will be at lower abundances after their initial peaks. Available algae and *Daphnia* should persist throughout the 63-day experiment. On the other hand, it is not important on which days the peak algal or *Daphnia* populations occur. The timing can be expected to differ by one or two sampling days (3 to 7 days real time) in different tests, and differences between tests do not compromise the ability to detect effects of a test material within tests. Because means of different tests show the same sequences, but are slightly out of phase relative to each other, each test must have a simultaneous control treatment.

16.2.2 *Criteria for Microcosm Performance through Day 28*—If the behavior of the controls has not conformed to the criteria of this section by Day 28, it is recommended that the microcosm test be discontinued, because it is probably too flawed to be successful. The following criteria refer to the means of the control treatment for each sampling day:

16.2.2.1 Mean nitrate should have been reduced from the initial concentration of 500 μM to <50 μm by Day 28.

16.2.2.2 The mean oxygen gain should reach at least 4 mg l^{-1} sometime during this period.

16.2.2.3 The mean *Daphnia* populations should have reached at least 80 animals/100 mL sometime during this period.

16.2.2.4 The algal biovolume should exceed 2000×10^4 μm^3 mL^{-1} sometime during this period. If this criterion is not met, the test might be acceptable if the *Daphnia* population has exceeded 100 animals/100 mL. (Low algal abundance might occur in a satisfactory experiment if the grazers eat the algal cells as fast as they grow.)

16.3 *Biological and Low Variance Performance*:

16.3.1 *Criteria for Total Microcosm Performance*—In addition to meeting the criteria for the first 28 days, the following criteria should be met for the control treatment:

16.3.1.1 Control mean oxygen gain must be positive throughout the experiment; the lower bound of the interval of nonsignificance should be >0 for more than 50 % of the sampling periods after addition of the test material.

16.3.2 Control mean *Daphnia* populations must have greater than 15 animals/100 mL, and the lower bound of the interval of nonsignificance must be >0 for more than 50 % of the sampling periods after the addition of the test material.

16.4 *Reference Toxicant*:

16.4.1 To prepare a concentrated stock solution, dissolve 1.1789 g $CuSO_4 \cdot 5H_2O$ in 150 mL of distilled water. The solution should be analyzed to determine the actual concentration.

16.4.2 To obtain 500, 1000, and 2000 ppb of copper, add 0.25, 0.5 and 1.0 mL of the concentrated stock solution/L of microcosm medium. The volume of the microcosm should be estimated. Since the microcosms contain approximately 3 L, this approximates 0.75, 1.5, and 3.0 mL/microcosm.

16.4.3 Response to these concentrations of copper can be compared to the responses obtained in the interlaboratory tests **(5-9)** to determine if the microcosm responses have similar sensitivity. It is suggested that a laboratory conducting the procedure for the first time perform this experiment.

17. Interpretation of Results

17.1 *Interpretation of Microcosm Data (see Section* 15*, Statistics)*:

17.1.1 The summarized statistical analyses (15.2.2.1) should be examined to rapidly identify variables that are statistically different as a result of the treatment. If solvent control variables are different from the medium controls, the *t*-tests between the test plus solvent and the solvent control should be examined. These analyses will allow the effects of the solvent to be separated from the effects of the test chemical.

17.1.2 The magnitude of the differences should be examined to determine its biological importance (Fig. 7). Occasionally some statistical differences (or failures to show statistical differences) might appear to be artifacts caused by temporary phase differences. If an effect is suspected of being an artifact, examination of related variables might clarify the tissue. For example, if one primary production variable is reduced, check to see if it is corroborated by reductions in oxygen gain, algal biovolume, *in vivo* fluorescence, or rate of dissolved nutrient depletion. If there are changes in total *Daphnia*, they are likely to be associated with changes in the size distribution. Although these measurements are not entirely redundant, there should be some obvious correspondence between them.

17.1.3 Although alterations in species abundance provide useful information, they might not necessarily indicate damage. For example, if one or more species of algae are significantly reduced, are others increased? If a change from small algal cells (presumably available to *Daphnia*) to filamentous algae (presumably not available) occurred, this would be indicated by the variable "available algae" (see 14.1.10). Similarly, if there have been changes in algal species abundances, the algal biovolume and species diversity (of total algae) and the species diversity (of available algae) variables should be examined to determine if the changes in species abundance have been compensatory (that is, one algal species has replaced another without altering the algal biovolume or species diversity) or not.

17.1.4 The variables associated with the grazers should be checked in a similar manner. If the algal biovolume or available algae has been reduced, grazer abundance might have been reduced or altered; these changes often occur after a 1 to 2-week lag, especially among the *Daphnia* or amphipods. If any of the grazer populations have been reduced, others might have increased in compensation; for example, if the *Daphnia* have been reduced, rotifers or protozoa might have increased. If the *Daphnia* have decreased, algal abundances, especially of small species, might have increased. If the amphipods have decreased, filamentous algal abundance might have increased.

17.1.5 The variables associated with community responses may indicate alterations, for example, pH, daytime oxygen gain, nighttime oxygen loss, turbidity, and dissolved algal nutrients. Many chemicals simultaneously alter the community production (oxygen gain) and community respiration (oxygen loss) and these should be examined separately as well as in the ratio (P/R) or 24-h difference. Mortality of obligate aerobes might follow very low oxygen concentrations. Similarly, mortalities might be associated with high ammonia concentrations, especially at high pH values. High ammonia concentrations usually indicate impaired algal nutrient uptake, either indirectly by overgrazing of algal populations, or by toxic effects on the algae. Except prior to the initial algal growth, the presence of high levels of algal nutrients (nitrate, nitrite, ammonia or phosphate) usually indicates the inhibition of algal growth. Failure of primary production without the accumulation of algal nutrients probably indicates the blockage of nutrient recycling or generally toxic conditions, for example, excessive pH values.

17.2 *Interpretation of Ecological Data—General* —These microcosms have been designed to include interactions between three

trophic levels (primary, secondary and detrital) and to include competitive interactions within each trophic level. The ecological interactions must be inferred from the ecological relationships among the components. For example, primary production includes the conversion of the inorganic nutrients (nitrate and phosphate) into algal cells; these are consumed by the grazers. Primary production is accompanied by the production of oxygen and the consumption of carbon dioxide (indicated by increased pH) during the lighted period. Thus, an inhibitor of photosynthesis will delay the depletion of algal nutrients, reduce oxygen gain during the lit period, not increase the pH, as well as reduce the algal cell abundances (5, 17). Algal cell counts and the other variables associated with primary production will also respond to inhibition or mortality on the grazer level. Nutrient recycling is mediated by the grazers and detritivores as shown by increases in ammonia and phosphate. These nutrients are used by algae to maintain populations in spite of the losses from grazing. Within each trophic level, species compete. Any test material is likely to be more toxic to some species than others, and the selectivity will be displayed as changes in species, dominance, for example, the change from *Ankistrodesmus* to *Scendesmus* (5). Changes in the algal dominance may also change the grazer food chain that can be supported; small cells can be eaten by protozoa, rotifers, ostracods and *Daphnia*; filamentous algae can be eaten by amphipods. Thus, all of the components are interactive; this differs from multiple single-species toxicity tests.

17.3 *Extrapolation to Natural Communities* :

17.3.1 *General Extrapolation:* The aquatic microcosm test has been designed to screen chemicals and their degradation products for the effects they might have on ecologically important processes such as photosynthesis, grazing, detrital processing, and nutrient recycling. The microcosms were not designed to represent a specific naturally occurring community, although their components make them most similar to a pond. Extrapolations from microcosm results to predictions of effects in natural environments must be made with the same cautions as extrapolations between different natural environments. Extrapolations between different communities must consider differences in water quality and community structure. The microcosms provide responses that are different from single species toxicity tests, although in some cases the microcosm results could be predicted if results of tests with individual species are applied to the trophic relationships of the microcosm. In other cases, the results could not be predicted from results of tests with single species. Subject to the limitations (5.9), the following types of extrapolations are suggested:

17.3.1.1 If a test material decreases primary production and alters algal species dominance, it is almost certain to have similar effects in a natural community, but it is unlikely that the microcosm results will predict the species that will become the new competitive dominant. The microcosm contains only a small subset of all possible species, and the outcome of competitive dominance depends on the species present and the balance of many complex relationships. The microcosm results might be more predictive of the taxonomic group of the species that will be most supressed; for example, streptomycin inhibits blue-green algae (Cyanophyta or Cyanobacteria) to a greater extent than green algae in the microcosms and in samples from natural communities.

17.3.1.2 If the test material has little direct effect on primary production, but is selectively toxic to grazers, it will probably have a similar effect in natural systems, but again, the microcosms will not be able to predict which species will dominate. Microcosm results are more likely to indicate which taxonomic groups might be most sensitive and therefore most reduced. *Daphnia* might be an adequate representative of Cladocera, but a poor representative of Copepoda.

17.3.1.3 Indirect effects that are observed in the microcosms, for example, algal blooms if grazers are eliminated, are also likely to occur in natural communities if algal nutrients are available. Given the uncertainties of the species dominance of the direct effects, the exact species involved in the indirect effects are not likely to be predicted.

17.3.2 *Water Quality:* Extrapolation between the microcosm and a specific body of water must consider water quality characteristics such as nutrient level, pH, hardness, alkalinity, TDS, and chelation. The microcosms are designed to support dense populations of algae if the grazers are supressed; less intense algal blooms would occur in natural communities with lesser nutrient supplies. The medium used in the microcosms has little buffering capacity and therefore shows pH shifts with carbon dioxide exchanges; the microcosms are also sensitive to acidic or basic degradation products. These responses would be reduced in natural waters that have higher buffering capacity. The potential exists to increase alkalinity to mimic the buffering capacity of specific natural waters. The chelation level of the natural community should be considered in extrapolating ecological effects, especially of metals.

17.3.3 *Community Structure*:

17.3.3.1 The ability to predict the effects of the test material on natural communities can be expected to vary with the degree to which processes measured here are controlling processes in a specific natural community. Differences in community structure and controlling processes can also be expected to limit the degree to which responses in one natural system will be predictive of changes in another natural system. For example, the microcosms developed algal blooms in response to the temporary elimination of grazers by Malathion and Dimilin (5, 17) and it is likely that natural communities with algal populations held in check by zooplankton grazing would behave in the same way. Pond communities treated with the insecticide Dursban were shown to do so (47). In contrast, a natural community whose grazer population was at low density because of fish or invertebrate predation would not be likely to have an algal bloom as a result of an insecticide application (14, 47). If the herbivorous grazer population was controlled by an invertebrate predator (for example, *Chaoborus* larvae) that was even more sensitive to the insecticide than the grazers, the grazer population might have temporary decreases, and then increase to higher densities than the control.

17.3.3.2 The presence of competitors of differing sensitivities would also be expected to modify the response of a specific community to a test material. For example, a mathematical model was used to contrast the responses of two communities to a

selective toxicant that induced a 20 % mortality of a grazer **(21, 25).** If Grazer No. 1 had no competitor, an induced mortality of 20 % had relatively little effect other than to change the phasing of the population cycles; however, in the presence of a competitor with only a small feeding overlap, the same induced mortality caused extinction of Grazer No. 1.

17.3.3.3 Thus, higher level interactions would have to be taken into consideration in using the microcosm results to predict effects on a specific natural community. Specifically, the controlling factors or processes must be known in order to extrapolate between communities, be they extrapolations between natural communities or between microcosms and natural communities.

17.3.3.4 There is growing awareness that not all natural communities respond to a test material in the same manner. For example, when a saltwater community was treated with copper during the spring, several measures of abundance and photosynthesis were reduced; when the summer community at the same location was treated, the same algal measures were increased concurrent with the reduction in grazers **(48).** Natural communities, although they share trophic level relationships, differ in their limiting factors and controlling processes.

18. Report

18.1 The record of the results should include the following information either directly or by reference to available documents:

18.1.1 Names of test and investigator(s), name and location of laboratory, and dates of initiation and termination of test,

18.1.2 Source of test material, its lot number, composition (identities and concentrations of major ingredients and major impurities), known chemical and physical properties, and the identity and concentration(s) of any carrier used,

18.1.3 Description of the experimental design, and

18.1.4 A concise summary of the findings (see Annex A1).

18.2 Variable values for each microcosm shall be shown either as illustrations or tables. Means, standard deviations, and sample number for each treatment group shall be shown on the table with the replicates, or on a separate table.

18.3 Statistical differences between control and treatment groups shall be summarized in a concise fashion to display all significant differences **(4, 21)** . It is suggested that arrows be used to indicate if the treatment is greater than control (up arrow) or less than control (down arrow). Plus or minus signs may be used. It is not necessary to distinguish between $P < 0.01$ and $P < 0.05$.

18.4 Estimate of interval of nonsignificance (about the control) and treatment means must be plotted for (*a*) nitrate, (*b*), algal biovolume, (*c*) *Daphnia*, (*d*) pH and (*e*) oxygen gain, and for variables having statistical differences (Fig. 7).

18.5 A table shall present any data that have been excluded from the statistical analyses with the justification for the exclusion; any restrictions of qualifications for the statistical analyses, for example, assumptions not satisfied; all laboratory accidents or events that might compromise the results, for example, malfunctioning equipment, lost samples, questionable data, should be listed.

18.6 Any alterations in the medium, species, duration or frequency of sampling or types of measurement must be reported. If the medium is altered from T82MV or T86MVK, the effects on the alteration on pH buffering, chelation or hardness must be specified.

18.7 Temperature and light conditions throughout the experiment should be described.

ANNEX

(Mandatory Information)

A1. SUMMARY OF POTENTIAL ECOLOGICAL EFFECTS (see 4.4 and 18.1.4).

A1.1 *Summary of Ecological Effects*—Fill in the following information by stating briefly if a variable has been significantly different, or if an effect is considered biologically important; and if temporary or permanent. Provide probability tables for all variables, and graphics for those that show significant differences (whether judged biologically important or not). Provide additional text to explain the effects observed.

CHEMICAL TESTED   ACTIVE CONCENTRATION
CARRIER   CARRIER CONCENTRATION
DATE   LABORATORY
INVESTIGATOR   Phone Number

A1.1.1 *Limitations or Qualifications on Experiment*:

A1.1.1.1 Effects have been noted on Primary (   ), Secondary (   ), Ecosystem (   ) Variables, as follows:

(1) Primary Production:

(*a*) List of changes of the abundance   (*b*) Effect on the following:
  of an algal species:
(1)   .   (1) Algal biovolume,
(2)   .   (2) Available algae,
(3)   .   (3) Algae species diversity,

(4) Species diversity of available algae,
(5) In vivo fluorescence,
(6) Oxygen gain (net photosynthesis),
(7) Extracted chlorophyll (optional),
(8) Extracted phaeopigment (optional),
(9) Carbon uptake (optional), and
(10) Photosynthetic efficiency.

(2) *Secondary Production:*

(*a*) List of changes of the abundance of
any animal (grazer or detritivore):
(1)   .
(2)   .
(3)   .

(*b*) Effect on the following:

(1) Size distribution among the
    Daphnia, and
(2) Shift in timing of cycles.

NOTE A1.1—If a change in grazer populations has been noted, check to see if it is associated with a change in algal variables.

(3) *Ecosystem Variables:*

(*a*) Effect on the following:
(1) Oxygen loss (net respiration),
(2) P/R ratio,
(3) The 24-h O_2 balance,
(4) DO1 (predawn),
(5) DO2 (afternoon),
(6) DO3 (predawn),
(7) pH,
(8) Optical density,
(9) Nutrient concentrations such as nitrate, nitrite, ammonia, phosphate, and
(10) Other comments or observations.

NOTE A1.2—If there were effects in any community variables, check if they were associated with changes in primary or secondary production variables.

APPENDIXES

(Nonmandatory Information)

X1. RELATIONSHIP OF MEDIA USED IN RELATED MICROCOSM STUDIES AND ORGANISMS CULTURE MEDIA

X1.1 *T82MV* is recommended as the medium for standard microcosm use; it is the medium used for the interlaboratory test **(8, 9, and 13)** to define the Criteria for Acceptable Tests (Section 13). Compared to earlier media, it contains less EDTA (1.42 μM) and therefore allows greater sensitivity to metal effects; it also provides more silicate (0.8 mM) required for diatom growth in the presence of other algae.

X1.2 *T86MVK* is equivalent of T82MV with the addition of selenium and other trace metals that might be essential for long-term culture of *Daphnia* **(37).** Preliminary studies have suggested that it might be suitable as a medium for microcosm use, but has not been adequately tested.

X1.3 *T85MVK* is recommended for culturing *Daphnia magna* if a laboratory does not have a satisfactory water source, such as well or lake water. This medium varies from T82MV by having ¹⁄₁₀ the concentration of nitrate and phosphate (to avoid excessive photosynthesis and high pH), and by the addition of selenium and other trace metals, which might be required by *Daphnia* **(37),** and which are not present in T82MV.

X1.4 Medium *T82-LowSi* lacks the vitamins and has a lower concentration of silicate (0.08 mM). It is used, with 1.5 % agar, for maintaining the algal cultures on slants. In unialgal culture, diatoms grow well on it. It does not require pH adjustment (see 10.5 for directions).

X1.5 *T81MV* is similar to T82MV, but has 20× the concentration of EDTA and trace metals; microcosms with this medium are less sensitive to metal toxicity. It was used prior to the interlaboratory tests **(10, 15, and 17).**

X1.6 *T63MV* is similar to T81MV but has ¹⁄₁₀ the concentration of silicate (0.08 mM); diatoms tend to be outcompeted in it. Having high EDTA, microcosms with this medium are less sensitive to metal toxicity. It was used in numerous microcosm experiments **(18-22).** This medium is equivalent to ½ strength Medium 36, used in some other microcosm methods **(49).**

X2. DATA SHEETS

DATE_____

ME_____

AS ASSIGNED VOLUMES ARE ADDED
APPROPRIATE SPACE IS INITIALED.

			\multicolumn{6}{c}{Carboy Number}		\multicolumn{3}{c}{Solutions}
Microcosm	Prep		1	2	3	4	5	6		C	K	MV
Solutions		A B D E H I										

Microcosms — 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

FIG. X2.1 Microcosm Preparation Data Sheet (see 8.4.2)

ALGAE:_____

SEMI-CONTINUOUS CULTURE MONITOR

DAY	DATE	OD	NO. CELLS	NO. FIELD	CELLS PER ML	MLS REMOVED	MLS ADDED	REMARKS

FIG. X2.2 Semicontinuous Culture Monitor Data Sheet (see 10.5.4.3)

SPECIES	DENSITY	VOL. REQUIRED PER MICROCOSM AT 10^3 ml^{-1}	TOTAL INOCULATION VOLUME REQUIRED	ADDED BY
Anabaena				
Ankistrodemus				
Chlamydomonas				
Chlorella				
Lyngbya				
Scenedesmus				
Selanastrum				
Stigeoclonium				
Ulothrix				
Nitzschia				

Daphnia	3 with eggs	3 without eggs	10 young	
Amphipods		3 mating pairs	6 young	
Ostracods	6			
Philodina	initial concentration 0.03/mL			
Hypotrichs	initial concentration 0.1/mL			

Example

```
ANA
ANK
CHLAM
CHLO
LYNG
SCEN
SEL
STIG
ULO
NIT
```

(Tag form)

FIG. X2.3 Microcosm Inoculation Sheet (see 11.2)

EXPERIMENT: _____

	DATE											
	EXPERIMENT DAY											
Palmer cell algae counts												
Protozoan counts (1.0, 0.1 ml)												
Mat examination (0,1,2,3 scale)												
SDAPHNIA	SMALL DAPHNIA PER 100 ML/											
MDAPHNIA	MEDIUM DAPHNIA PER 100 ML/											
LDAPHNIA	LARGE DAPHNIA PER 100 ML/											
	EPHIPPIA											
DELDO	DISSOLVED OXYGEN											
FLUOR	FLUOROMETRY/											
PH	PH/											
SAMPH	SMALL AMPHIPODS PER 100 ML/											
LAMPH	LARGE AMPHIPODS PER 100 ML/											
AMPH	AMPHIPODS PER 100 ML/											
OSTRACOD	OSTRACODS PER 100 ML/											
BACT	BACTERIA COUNTS/											
C14L	C14 LIGHT UPTAKE DARK UPTAKE											
CHLA	CHLOROPHYLL											
ODMIX	MIXED OPTICAL DENSITY/											
CLARITY	WATER CLARITY/											
FILTERED NUTRIENT SAMPLES												
MICROCOSM VOLUME												
REINOCULATE												
MEASURE LIGHT INTENSITY												
MONITOR TEMPERATURE RECORDER												
DATA CARD VERIFICATION AND RELEASE												
DATA HANDLING PROGRAM VERIFICATION												

A14.2

FIG. X2.4 Job Assignments (Initials of Persons Performing Tasks) (see 11.5)

LARGE ORGANISM AND CHEMICAL MEASUREMENT DATA SHEET

FIG. X2.5 Large Organism and Chemical-Physical Data Sheet (see 11.5.2, 11.5.6.2(*a*), and 11.5.6.2(*d*))

ALGAL COUNT DATA SHEET

FIG. X2.6 Algal Count Data Sheet (see 11.5.6.2(*b*))

PROTOZAN COUNT SHEET

FIG. X2.7 Protozoa Count Data Sheet (see 11.5.6.2(c))

NUTRIENT DATA SHEET

ENTER ALL DATA IN µg-atml $^{-1}$

FIG. X2.8 Nutrient Data Sheet (see 11.5.6.2(e))

REINOCULATION

FIG. X2.9 Reinoculation Data Sheet (see 11.6)

X3. STATISTICAL GUIDANCE

X3.1 *Introduction*—The goals of statistical analysis are to summarize, display, quantify, and provide objective yardsticks for assessing the structure, relations, and anomalies in data. The data display and statistical techniques most commonly used to achieve these goals are: (*a*) preliminary and diagnostic graphical displays, (*b*) pair-wise comparison techniques such as *t*-tests and 2 by 2 contingency table tests, (*c*) ANOVA and corresponding contingency table tests, (*d*) multiple comparison techniques for simultaneous pair-wise comparison of treatment groups with control groups, (*e*) regression analysis and (*f*) concentration-effect curve analyses. If used correctly, each of these techniques can provide useful information.

X3.1.1 The three kinds of data obtained from toxicity tests are dichotomous or categorical (for example, mortality), count or enumeration (for example, number of young), and continuous (for example, weight). Statistical methods for analyzing dichotomous and other categorical data are directly analogous to those for analyzing count and continuous data. However, for technical reasons and because they arose from different application areas, different terminology and computing tools were developed for analyzing the various kinds of data. The corresponding procedures are considered together herein.

X3.2 *Endpoint*—The endpoint for sublethal tests generally has been defined in terms of whether differences from control organisms were statistically significant at the 5 % level. One of the main conceptual problems associated with such a definition of the endpoints is that the notions of biological importance and statistical significance are logically distinct. Effects of considerable biological importance might not be statistically significant if sample sizes are small, effects are extremely variable, or both. Conversely, biologically trivial effects might be statistically significant if sample sizes are large or effects are reproducible. An endpoint based solely on statistical significance might depend as much or more on sample sizes as on the magnitude of the effect.

X3.2.1 An alternative is to define the endpoint in terms of a specified absolute or relative amount of difference in a biological variable from control treatment. A regression-type model would be fitted to the data and a concentration associated with a specified amount of difference from the control(s) would be estimated using the model. For example, the concentration resulting in a specified percent decrease in number of live offspring might be estimated along with confidence limits on the estimated concentration. Results at a single time would then be reported as point estimates, with confidence limits, of the concentration expected to cause an amount of effect that was preselected as being unacceptable. However, no consensus currently exists concerning what constitutes unacceptable preselected biological effects **(9).**

X3.2.2 In general, an endpoint defined in terms of a statistically significant difference is calculated using analysis of variance, contingency tables, or other hypothesis testing procedures. An endpoint defined in terms of a specific amount of effect is calculated using regression analysis, concentration-effect curve analysis, or other point estimation procedures. Regardless of the procedure used, sufficient data should be present in reports (see 18.1) to permit calculation of endpoints other than those chosen by the authors

and to allow other uses of the data, such as modelling. For long experiments (for example, 63 days) with complex dynamic changes, analyses at several sampling days may be more appropriate than a single endpoint for each experimental unit.

X3.3 *Graphical Displays*—These should be an intergral part of every data analysis (51) and should be performed every time data are analyzed using either regression analysis or hypothesis testing. Preliminary scatterplots are desirable because they might provide insights into the structure of the data and reveal the presence of unanticipated relations or anomalies. Every time a regression-type model is fitted to the data, a graph of predicted and observed values should be examined to assess the goodness of fit of the model. A graph of the residuals from the fit should be examined to assess departures from the model. Histograms are useful for examining the distribution of data before hypothesis testing. The advent of modern computers and statistical computing packages, for example, Minitab, SAS, BMDP, SPSS (52), has made the inspection of data patterns both easy and inexpensive. Feder and Collins (53) illustrate the use of various types of preliminary and diagnostic graphical displays in analysis of data from chronic toxicity tests.

X3.4 *Outlier Detection Procedures*—Data that do not appear to be in conformance with the substantial majority are often referred to as outliers. Outliers might be due to random variation or to clerical or experimental errors. Statistical outlier detection procedures are screening procedures that indicate whether a datum is extreme enough to be considered not due just to random variation. Barnett and Lewis (54) describe many outlier detection procedures, and Feder and Collins (53) illustrate the use of several outlier detection procedures with aquatic toxicological data. If outliers can be shown to be due to clerical or experimental error, they should be either corrected or deleted from the data prior to analysis. If outliers are not known to be erroneous values, the question of how to deal with them is a matter of judgment. Data analysis should be performed with and without questionable values in order to assess their importance, because one or a few extreme outliers can sometimes greatly affect the outcome of an analysis.

X3.5 *Data Transformations*—Many standard statistical procedures such as regression analysis and ANOVA are based on the assumption that experimental variability is homogeneous across treatments. This assumption typically does not hold for certain kinds of data. If graphed data or tests of heterogeneity demonstrate that variability is not homogeneous across treatments, variance stabilizing transformations of the data might be necessary. The arc sine, square root, and logarithmic transformations are often used on dichotomous, count, and continuous data, respectively (55) . The question of whether to transform raw data should be decided on a case-by-case basis after studying data displays, tests of heterogeneity, and similar data from previous tests. For balanced designs, ANOVA and regression are not very sensitive to departures from normality and small deviations from this assumption are not prohibitive. Nonparametric procedures might be important aids in analyzing heterogeneous data.

X3.6 *Comparison of Solvent Control and Dilution-Water Control*—If both solvent and dilution-water controls are included in the test, they should be compared (for example, using a *t*-test for count and continuous data and Fisher's Exact Test of a 2 by 2 contingency table test for categorical data (56)). Adjustments for chamber-to-chamber heterogeneity might be necessary. The use of a large alpha level (for example, 0.25) will make it more difficult to accept the null hypothesis when it should not be accepted. The test statistic, its significance level, the minimum detectable difference, and the power of the test should be reported.

X3.7 *Analysis of Variance and Contingency Table Analyses*—ANOVA tests are often appropriate for untransformed continuous data and for transformed categorical and count data. Contingency table tests are usually appropriate for untransformed categorical data. If evidence of chamber-to-chamber heterogeneity is found, standard contingency table analyses might be inappropriate for categorical data. In this case, it might be appropriate to apply an arc-sine variance stabilizing transformation to the proportion dead within each experimental unit, and perform an ANOVA on the transformed proportions. Feder and Collins (53) illustrate transformation of data before use of a contingency table test.

X3.7.1 Both contingency table tests and ANOVA *F*-tests are overall tests that do not assume any particular form for the relation between effects and concentrations. They are thus not designed to be particularly sensitive to one-sided, monotone trends characteristically observed in toxicity tests. Specialized tests have been designed to be more sensitive to relations of this type. Some such tests are the one-sided measure of association tests, the Cochran-Armitage Test for categorical data, and tests based on linear or polynomial regression models for continuous data (57).

X3.7.2 ANOVA tests are based on normal distribution theory and assume (*a*) that the data within treatments are a random sample from an approximately normal distribution, and (*b*) that error variance is constant among treatments. As a part of the ANOVA, statistical tests for the assumption of normality and homoscedasticity should be performed to determine whether there are any obvious violations of these assumptions. When results of an ANOVA are reported, the ANOVA model and table, the *F* statistic and its significance level, the minimum detectable differences, and the power of the test should be presented.

X3.8 *Multiple Comparison Procedures*—The usual approach to analyzing data from sublethal tests is to compare data for each concentration of the test material to data for the control(s). In Fisher's Protected Test, which is only used if the ANOVA *F*-test is significant (58), each concentration of test material is compared to the control(s) using the *t*-test. If the investigator desires to set the experiment-wise, rather than a comparison-wise alpha (see (68) for a discussion of the two), Dunnett's procedure (58, 59) can be used without the ANOVA *F*-test. William's procedure (58, 60) also tests the control(s) versus each concentration, but makes

the additional assumption that the true mean follows a monotonic relation with increasing concentration. The latter procedure is more powerful if the assumption is correct. Alternatively, Tukey's **(61)** No Statistical Significance of Trend (NOSTASOT) test can be used with the same assumptions as William's procedure. Shirley **(62)** has developed a nonparametric equivalent for William's test and Williams **(63)** has modified and corrected Shirley's procedure to increase its power to detect the alternative hypothesis. Care must be taken when using any of these procedures that an appropriate estimate of variability is used, incorporating any chamber-to-chamber variation that is present. Presentation of results of each comparison should include the test statistic, its significance level, the minimum detectable difference and the power of the test.

X3.9 *Regression Analysis and Concentration-Effect Curve Estimation*—An alternative to tests for statistically significant differences is to fit concentration-effect models or regression models to the data and estimate the concentration that corresponds to a specified amount of difference from the control treatment **(64).** Concentration-effect curve models, such as probit and logit, are commonly used to describe trends in dichotomous data on survival. Linear and quadratic polynomial regression models are commonly used to describe trends in quantitative data on growth and reproduction. Toxicity tests should be designed to avoid the need for extrapolation, which can introduce biases into the estimates.

X3.9.1 Point estimates, such as the EC10, EC25, and EC50, are examples of endpoints calculated using regression analysis. Whenever a point estimate is calculated, its 95 % confidence interval should also be calculated. Finney **(66)** discusses the probit model in considerable detail, and Draper and Smith **(66)** and Neter, Wasserman, and Kutner **(67)** discuss most practical aspects of regression analysis. Feder and Collins **(53)** discuss the use of these techniques in aquatic toxicology.

X3.9.2 When a regression model or concentration-effect curve model is fitted, data for each experimental unit are plotted against concentration **(23).** If necessary, transformation of the effect data or concentration data, or both, should be performed to stabilize variability across treatments and to produce a smooth trend. For example, if effects or concentrations cover a range of one or more orders of magnitude, a logarithmic transformation of either concentration or effect, or both, might be appropriate. On the basis of preliminary graphs, a regression model should be postulated and fitted to the data using a linear or nonlinear regression fitting technique. Residuals from the model should be calculated and plotted against appropriate variables. Any systematic structure in the residuals indicates lack of fit of the model and the model should be modified and the procedure repeated. This cycling should continue until there is no further structure in the residuals to be explained. Presentation of results of regression or concentration-effect curve analysis should include the entire regression equation in its final form, along with the standard error of the residuals and 95 % confidence limits for estimated parameters.

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