Designation: E1366 – 23

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

This standard is issued under the fixed designation E1366; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

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, independent of the location of the test.

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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1.5 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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*Specific hazard statements are given in Section 7.

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

D3978 Practice for Algal Growth Potential Testing with

[1] This practice is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

Pseudokirchneriella subcapitata
E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians
E943 Terminology Relating to Biological Effects and Environmental Fate
E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians
E1193 Guide for Conducting *Daphnia magna* Life-Cycle Toxicity Tests
E1733 Guide for Use of Lighting in Laboratory Testing
IEEE/SI 10 American National Standard for Use of the International System of Units (SI): The Modern Metric System

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 E729, Terminology E943, and Guide E1023. For an explanation of units and symbols, refer to IEEE/SI 10.

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**)[3]).

3.3.4 *community metabolism, n*—the oxygen or carbon dioxide 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. Because P and R often change in the same direction and magnitude, P/R maybe less sensitive than P or R considered separately.

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

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*—a culture which the exact composition of the organisms is known, down to the presence or absence of bacteria.

3.3.7.1 *Discussion*—Such cultures are developed from axenic cultures. The word implies know biota (**2**). The microcosms described here are not gnotobiotic because of the bacteria and other microbes are not known. 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 Cyanobacteria).

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

[3] Boldface numbers in parentheses refer to the list of references at the end of this practice.

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 nutrients 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. To the extent possible, all solutions are mixtures of distilled water and reagent grade chemicals (see Section 8) and all organisms are available in commercial culture collections.

5.2 The species used are easy to culture in the laboratory and some are routinely used for single species toxicity tests (Guide E729; Practice D3978, Guides E1192 and E1193). 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 published methods (**4-6**), interlaboratory testing (**7-10, 11**), intermediate studies (**12-23, 24**), statistical studies (**25-27**) and mathematical simulation results (**28**). Newer studies on jet fuels have been reported (**29**)(See 15.1 for multivariate statistical analyses) and on the implications of multispecies testing for pesticide registration (**30**). Environmental Protection Agency, (EPA) and Food and Drug Administration, (FDA) published similar microcosm tests (**31**). The methods described here were used to determine the criteria for Acceptable Tests (Section 16). Additional papers have been published using this method for measuring chemical stress on organisms (**32**).

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 ((**33**) 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 (**13**). 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.

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 °C 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 light-colored 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 (see Guide E1733). LED lights have been satisfactory for similar microcosms. For standard fluorescent tubes, 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 during the dark period.

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 0.1 N 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.*

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

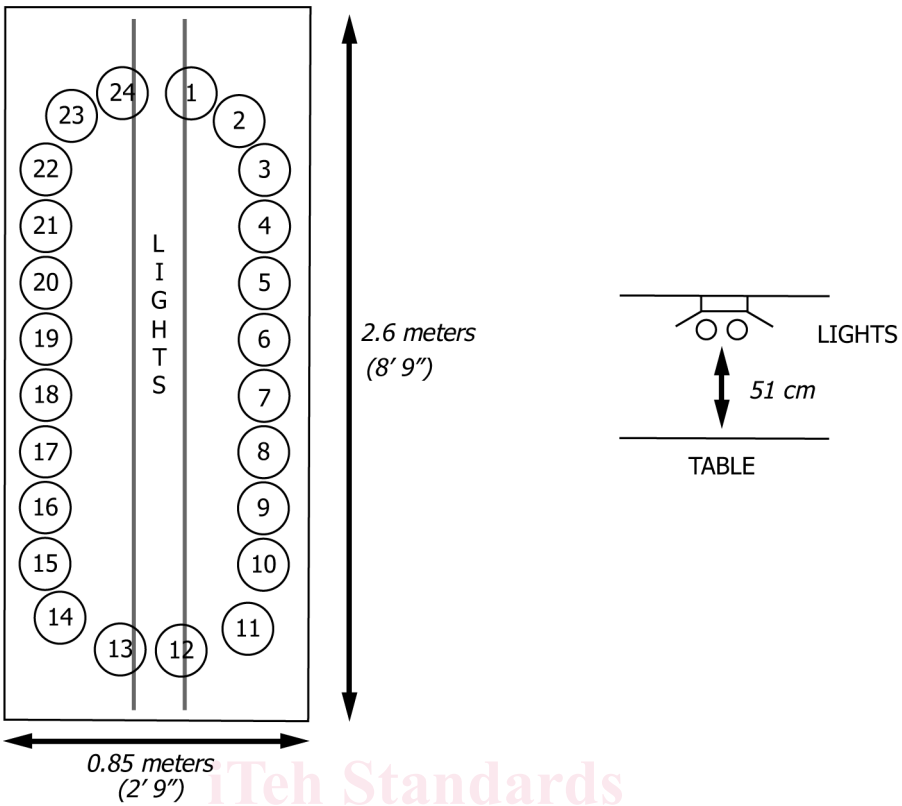

FIG. 1 Position of Microcosms under Lights (6.2.3 and 12.3.1)

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 (**34**), recommended handling procedures (**35**) 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 (**36**) and with materials that are, or are suspected of being carcinogenic (**37**).

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 (**13, 38**). 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*—T82MV (Table 1) is recommended and was used in the interlaboratory testing experiments (**7-10**). Related media used for organism culture maintenance (see Section 10) are described in Appendix X1. Alternative microcosm medium (T86MVK) with additional trace metals is also described (Appendix X1) but has not been as extensively tested. All of these media are designed to have low pH buffer and low metal chelation capacity and some might be suitable for complete microcosm studies. 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 D1193).

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 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).

Master Solution	Salt	mL/L	mL/18 L	Concentration m*M* (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	$Al_2(SO_4)_3 \cdot 18H_2O$	1	18	0.0048
I	$Na_2SiO_3 \cdot 9H_2O$	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 liquid medium and 200.1 g of sediment (see 8.2 – 8.4 for direction).

NOTE 2—pH adjusted to 7.0 with 0.1 N HCl.

Medium T82MV Composition				
Compound	Molecular Weight	Concentration		
		m*M*	Element	mg/L
$NaNO_3$	85.0	0.5	N	7.0
$MgSO_4 \cdot 7H_2O$	246.5	0.1	Mg	2.43
KH_2PO_4	136.0	0.04	P	1.23
NaOH[A]	40.0	0.032	Na	0.74
$CaCl_2 \cdot 2H_2O$	147.0	1.0	Ca	40.0
NaCl	58.5	1.5	Na	34.5
$Al_2(SO_4)_3 \cdot 18H_2O$	666.5	0.0048	Al	0.26
$Na_2SiO_3 \cdot 9H_2O$[B]	284.0	0.80	Na	36.8
			Si	22.4
Trace Metals		µ*M*		mg/L
$FeSO_4 \cdot 7H_2O$	278.0	1.12	Fe	0.0625
EDTA	292.0	1.42	EDTA	0.4146
H_3BO_3	61.8	0.75	B	0.008
$ZnSO_4 \cdot 7H_2O$	287.5	0.025	Zn	0.0015
$MnCl_2 \cdot 4H_2O$	197.9	0.25	Mn	0.0135
$Na_2MoO_4 \cdot 2H_2O$	242.0	0.025	Mo	0.0024
$CuSO_4 \cdot 5H_2O$	249.7	0.005	Cu	0.00032
$Co(NO_3)_2 \cdot 6H_2O$	291.0	0.0025	Co	0.00015
Murphy's Vitamins[C]		µ*M*		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	

[A] NaOH is added with the KH_2PO_4 master solution. There are additional minor sources of Na^+ (trace metals and Murphy's vitamins); NaCl and Na_2SiO_3-$9H_20$ are the major sources of Na^+. It is important that Na^+ and not K^+ be the major monovalent cation.

[B] If diatoms are not used, the Na_2SiO_3-$9H_20$ concentration can be reduced to 0.08 mM (3.6 mg/L) see Table 2. The NaCl solution will assure that Na^+ is the major monovalent cation in the final medium.

[C] Murphy's vitamins (Table 3) were used in the development and testing of the protocol **(1-28)**. More recent work **(39-44)** has indicated that not all of these organic compounds are needed, at least for algae-*Daphnia* magna microcosms, if Keating's Metal Solution of trace metals (Table 4) and 3 vitamins (B_{12}, Biotin, and Thiamine) are added. See the footnote B to Table 3 for preparation of the vitamin master mixture.

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	$MgSO_4·7H_2O$	246.5	24.65	0.1
C[A]	KH_2PO_4	136.0	13.6	0.1
	NaOH	40.0	3.2	0.08
D	$CaCl_2·2H_2O$	147.0	14.7	0.1
E	NaCl	58.5	5.84	0.1
F[B]	$FeSO_4·7H_2O$	278.0	24.9	0.0895
	EDTA[C]	292.0	26.1	0.0895
	NaOH	40.0	10.7	0.268
G[D]	H_3BO_3	61.8	1.85	0.03
	$ZnSO_4·7H_2O$	287.5	0.287	0.001
	$MnCl_2·4H_2O$	197.9	1.98	0.01
	$Na_2MoO_4·2H_2O$	242.0	0.242	0.001
	$CuSO_4·5H_2O$	249.7	0.0499	0.0002
	$Co(NO_3)_2·6H_2O$	291.0	0.0291	0.0001
H	$Al_2(SO_4)_3·18H_2O$	666.5	3.2	0.0048
I	$Na_2SiO_3·9H_2O$	284.0	4.55	0.016
I (10×)			45.5	0.16
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 $FeSO_4·7H_2O$ 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. With reduced $Na_2SiO_3·9H_2O$ concentrations pH adjustments are not likely to be needed.

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.

TABLE 3 Modified Murphy's Vitamin Solution[A,B]

Name	Molecular Weight	Concentration	
		mg/L	m*M*
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 **(45)**; 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).
[B] Murphy's vitamins (Table 3) were used in the development and testing of the protocol **(4-28)**. More recent work **(39-44)** has indicated that not all of these organic compounds are needed, at least for algae-Daphnia magna microcosms, if Keating's Metal Solution of trace metals (Table 4) and 3 vitamins (B_{12}, Biotin, and Thiamine) are added. The vitamin master solution is made by adding 5 mg of Biotin and 5 mg of B_{12} to distilled water in a 1 liter volumetric flask. In another 1 liter volumetric flask containing approximately 500 ml of distilled water, dissolve 100 mg of Thiamine; add 100 ml of the Biotin and B_{12} mixture, and bring the total volume to 1 liter. The final concentrations of this master solution are: Biotin 0.5 mg/L, Thiamine 100 mg/L, B_{12} 0.5 mg/L. Divide the master solution into approximately 100 ml aliquots in sterile plastic bags and store in the freezer. Discard the remaining biotin-B_{12} solution. Add 1 ml of the master vitamin solution per liter of final medium; lower concentrations may be adequate.

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 0.1N HCl, then foil covers are replaced with 150 by 15-mm plastic petri dishes. A laboratory worksheet, should document the media preparation.

9. Test Material

9.1 *General*—The test material should be reagent grade[4] or better, unless a test on an effluent, a formulation, commercial product, or technical-grade or use-grade material is specifically

[4] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Annual Standards for Laboratory Chemicals,* BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary,* U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

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	m*M*
NaBr	102.89	64.4	0.626
$SrCl_2 \cdot 6H_2O$	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_4VO_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 **(39).** 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 **(46).** 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 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 X2). 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 known 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).

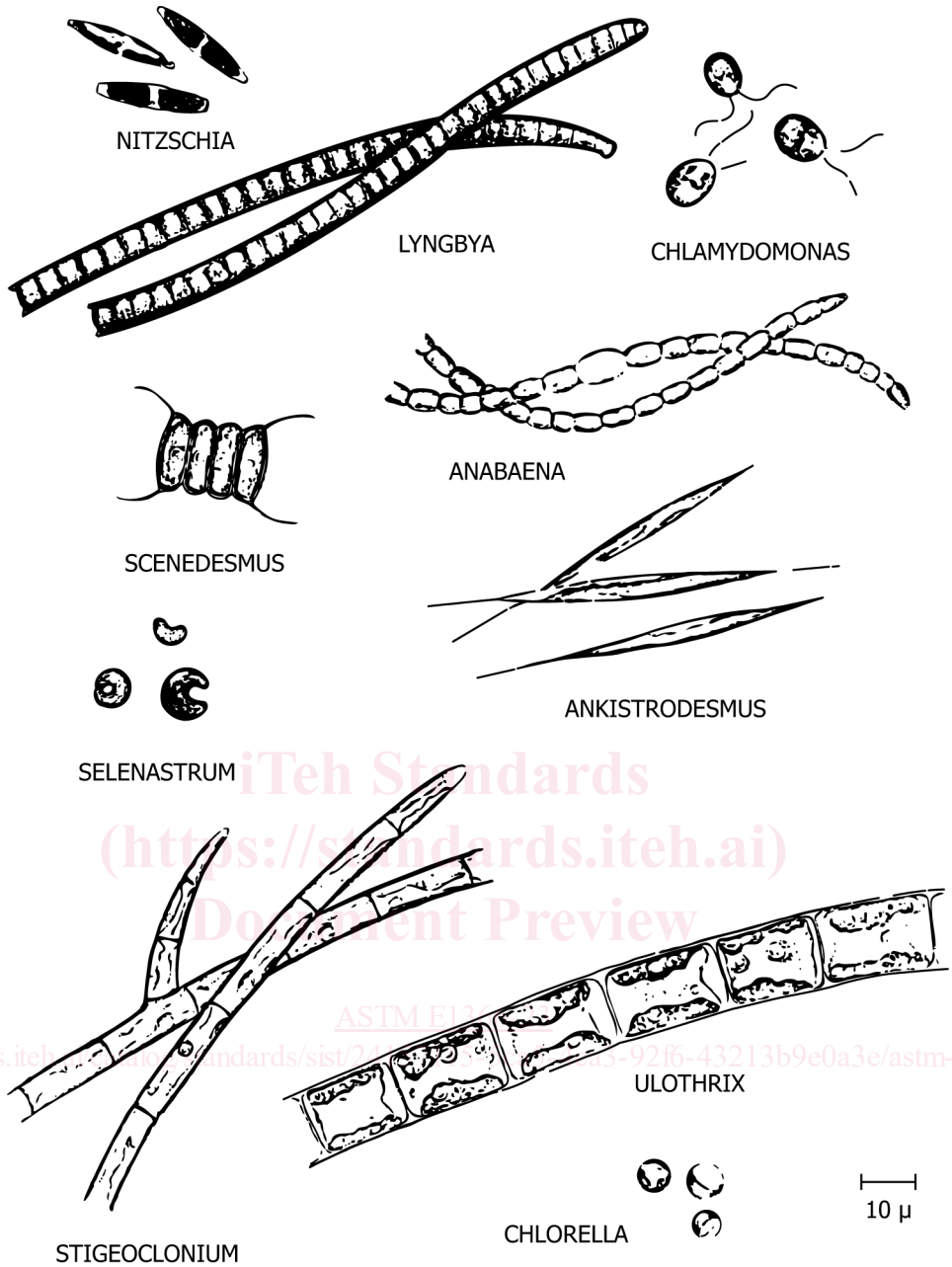

FIG. 2 Algae Used in the Standardized Aquatic Microcosm (10.1)

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.

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).

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. Tubes are then laid at a slant and allowed to solidify. The phosphate (Solution C) is likely to precipitate with 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 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.

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 E1193 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 lake water 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 parthenogenic 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 **(39).** Only differences from T82MV are noted (see Table 6). A simpler media was also satisfactory in other studies **(47)**.

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 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.

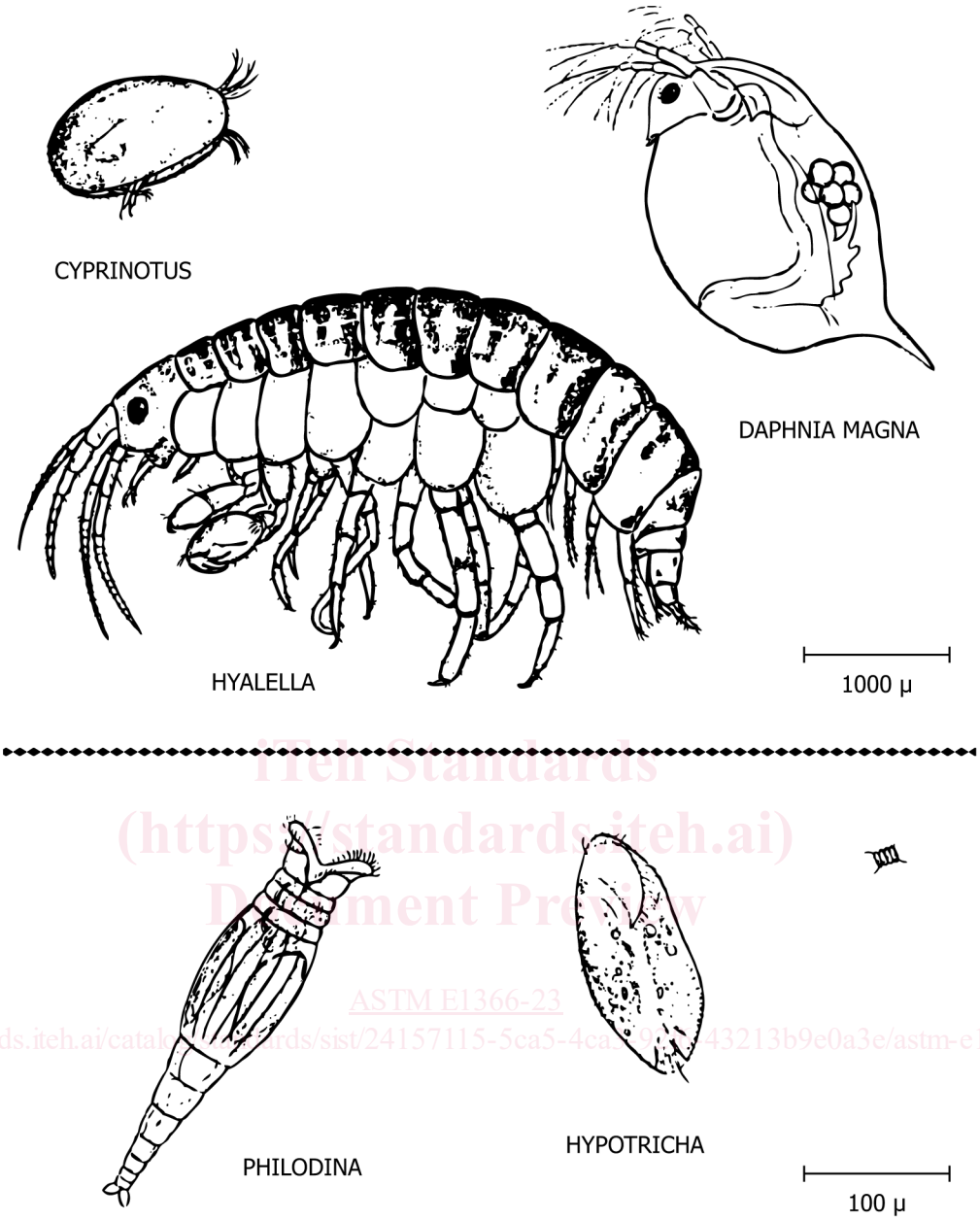

FIG. 3 Animals Used in the Standardized Aquatic Microcosm (10.2)

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	$MgSO_4 \cdot 7H_2O$	1	0.1 mM
C	KH2PO4	0.4	0.04mM
	NaOH		0.099 mM
D	$CaCl_2 \cdot 2H_2O$	10	1.0 mM
E	NaCl	15	1.5 mM
H	$Al_2(SO_4)_3 \cdot 18H_2O$	1	0.0048 mM
I	$Na_2SiO_3 \cdot 9H_2O$	5	0.080 mM
K	Trace metal mixture	0.05	same as T82
	Distilled Water		to 1000 mL
Bacto-Agar			15 g

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 (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

Semi-continous Culture Apparatus for Algae

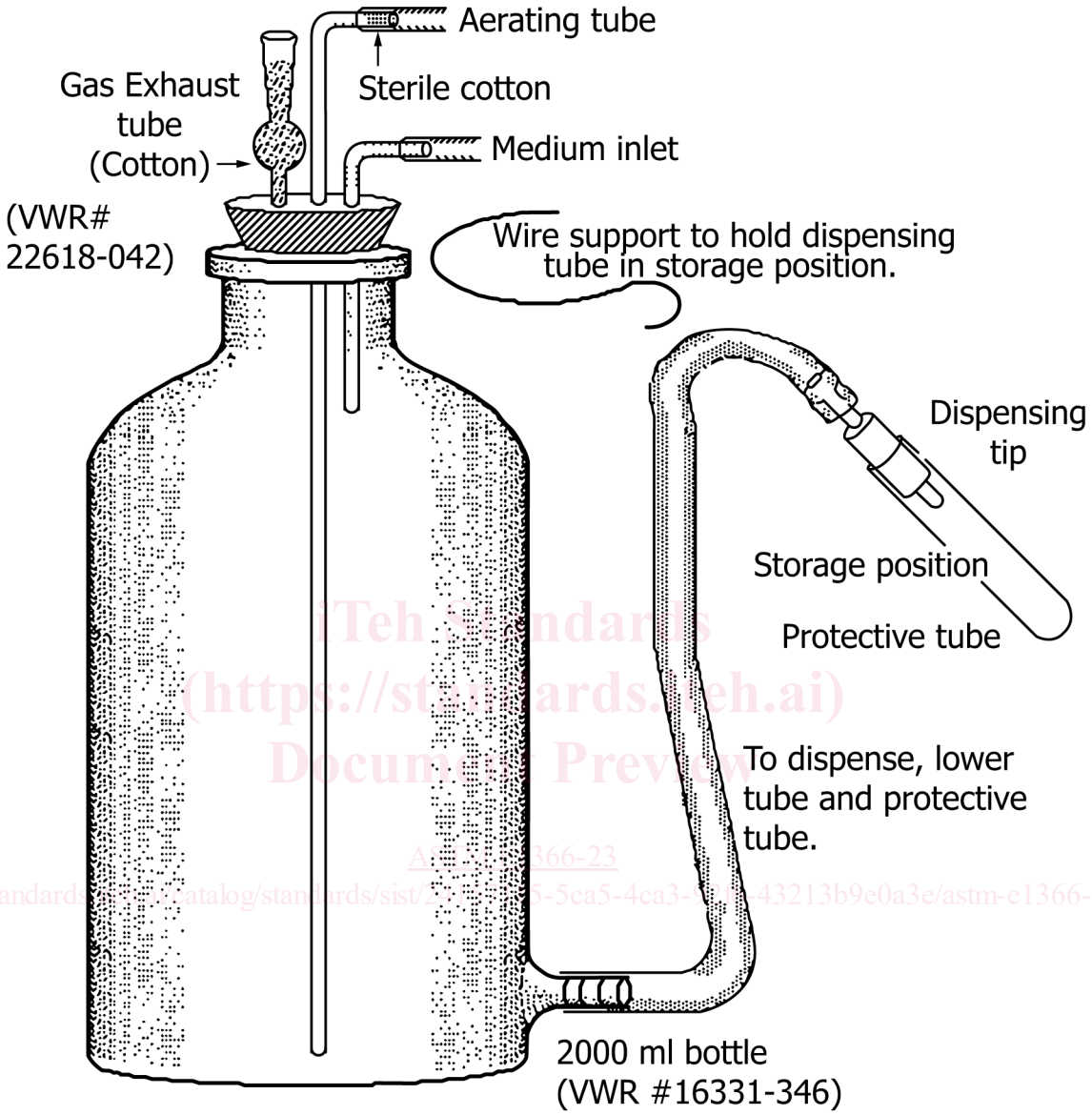

FIG. 4 Semicontinuous Algal Culture Unit (10.5.4.1)

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 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) or other satisfactory *Rotifer* media. 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 X2). The minimum desirable number of test chambers per treatment