ammonia, nitrite and nitrate never exceeded 0.05 mg of total ammonia-N/L ($NH_{3(aq)}$ and NH_4^+), 0.08 mg NO_2-N/L and 18 mg NO_3-N/L (Ward, 1991). The toxicity of ammonia is based primarily on unionized ammonia (NH_3) and the proportion of NH_3 species to NH_4^+ species is dependent on pH, ionic strength and temperature. It is strongly recommended that the concentrations of total ammonia, nitrite and nitrate do not exceed those reported here. The ammonia, nitrite, and nitrate levels can be checked by using color comparison test kits such as those made by LaMotte Chemical or equivalent methods.

4.5.4 Bacterial oxidation of excreted ammonia by two groups of autotrophic nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*), results in an increase of hydrogen ions, which causes a drop in pH and subsequent loss of buffering capacity. Typically, the culturist responds to the change in pH by adding Na_2CO_3 or $NaHCO_3$. However, such efforts to buffer against a drop in pH will result in an increase in alkalinity and the uncontrolled use of carbonates can affect reproduction, especially at higher alkalinity values (Ward; 1989, 1991). Therefore, when using carbonates to buffer against pH changes, alkalinity values should not exceed 120 mg/L, which is easily measured by using a titrator kit such as that available from LaMotte Chemical or equivalent methods.

4.5.5 Figure 4 (Ward, 1991) depicts juvenile production per aquarium, no buffer added, over a period of 24 weeks. A regression line was calculated for these data and the slope and correlation coefficient were analyzed by Student's t test. The data showed that even when the pH dropped as low of 7.5, there was a significant increase ($P < 0.001$) in juvenile production. However, the pH should be maintained above 7.8 by the controlled use of $NaHCO_3$ and frequent water exchanges.

4.6 FEEDING

4.6.1 Frequent feeding with live food is necessary to prevent cannibalism of the young by the adults. McKenny (1987) suggests feeding densities of 2-3 *Artemia* per mL of seawater and Lussier et al. (1988) suggest a feeding rate of 150 *Artemia* nauplii per mysid daily.

4.6.2 In the *M. bahia-Artemia* predator-prey relationship, it is also important to provide sufficient quantities of nutritionally viable free-swimming stage-I nauplii (Ward, 1987); final hatching from the membranous-sac (pre-nauplii) into stage-I nauplii does not always occur. *Artemia* cysts that have been incubated for 24 h should be periodically examined with a stereozoom microscope to enumerate free-swimming stage-I nauplii and prenauplii (membranous-sac stage).

4.6.3 It has also been found that heavy metals can affect the hatchability of *Artemia* (Rafiee et al., 1986; Liu and Chen, 1987), therefore, when using natural seawater the level of metals should always be checked.

4.6.4 Ward (1987; 1991) has tried different brands of *Artemia* from different geographic origins and lot numbers; many achieved stage I nauplii and still caused variability in production of mysids which suggests that they were nutritionally lacking. Leger et al. (1985; 1987a) have drawn attention to

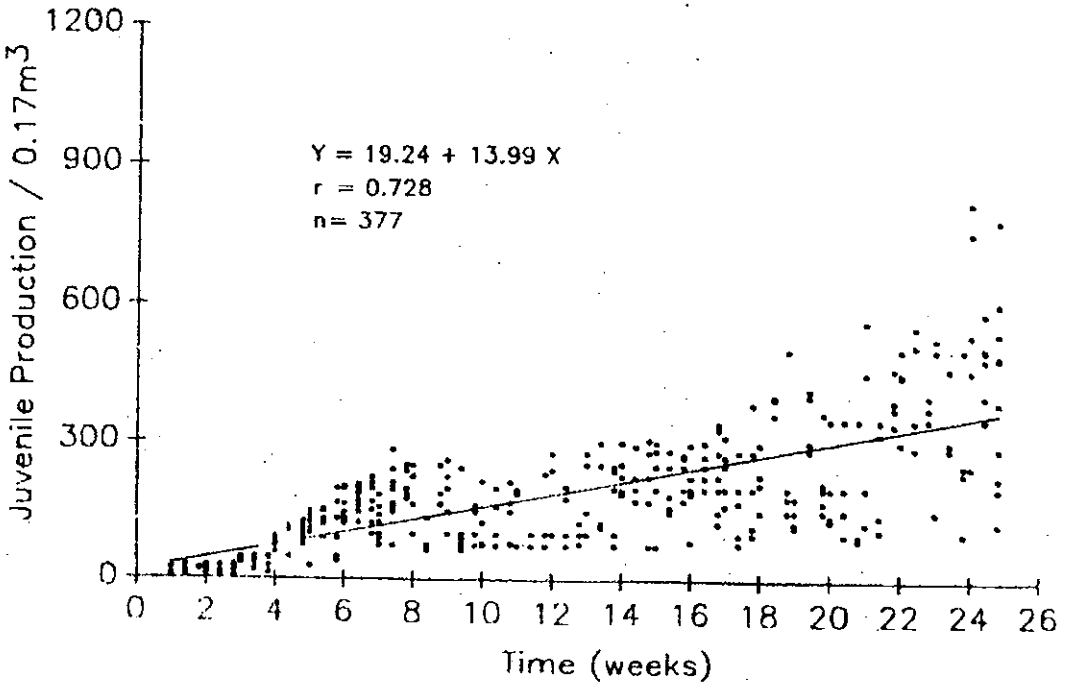

Figure 4. Juvenile production per aquarium over time (from Ward, 1991).

poor larval survival of *M. bahia* and low levels of certain polyunsaturated fatty acids found in the *Artemia* fed. The enhancement of *Artemia* has also been studied and there are numerous techniques that have been successful (Leger et al., 1986).

4.6.5 Ward (1987; 1991) has found that it is important to control the flow of seawater in recirculating systems (keep below 5 L/min) so that *Artemia* does not become limiting to the mysid. Newly hatched *Artemia* should be fed to mysids at least twice a day. To supply *Artemia* to the mysid population on the weekend and prevent cannibalism of newly released mysids, an automatic feeder such as described by Schimmel and Hansen (1975) or Ward (1984; 1991) could be used. Ward (1991) designed a system to hatch *Artemia* when personnel were not available to set up *Artemia* for the following morning and afternoon feeding, such as Monday. Cysts were placed in two 4-L Erlenmeyer flasks (dry), an airstone was placed in each flask, and two vessels overhead were filled with 3500 mL of 30‰ seawater each. The previously described timer (ChronTrol®, Model CD) was used to open the normally closed solenoids, allowing the seawater to gravity feed and hydrate the cysts.

4.6.6 It is possible that a surface dwelling diatom community acts as a secondary food that supplements deficient brands of *Artemia*, especially for newly released juveniles. Ward (1991) has observed that a strong fertilizing action is caused by the excretory products of the mysid population. As the concentration of nitrate increases (nitrification) to about 5 mg/L (in approximately 7-10 weeks in an aquarium), a bloom of surface dwelling diatoms, principally *Nitzschia*, but including *Amphora* and *Cocconeis*, occurs in natural or artificial seawater (Ward, 1993). It is interesting to note that, at the same time, there is a dramatic increase in the number of juveniles observed in the aquaria (Figure 4). The diatoms form layers on the walls of the aquarium and swarms of newly released juveniles have been found among them, possibly feeding upon them.

4.6.7 *Nitzschia* has been identified as a food source for the marine mud snail, *Ilyanassa obsoleta* (Collier, 1981), and the sea urchin, *Lytechinus pictus* (Hinegardner and Tuzzi, 1981). The diatom, *Skeletonema*, has also been used as a supplemental food for *M. bahia* (Venables, 1987). DeLisle and Roberts (1986) reported on the use of rotifers, *Brachionus plicatilis*, as a superior food for juvenile mysids. Rotifers are active swimmers, ranging in size from 100-175 μm as compared to 420-520 μm for *Artemia*, and would provide a good alternative food source if their fatty acid profile is adequate.

4.7 CULTURE MAINTENANCE

4.7.1 To avoid an excessive accumulation of algal growth on the internal surfaces of the aquaria, the walls and internal components should be scraped periodically and the shell substrate (coral or oyster) turned over weekly. Also, the filter plates must be completely covered so that the biological filter functions properly. After a culture tank has been in operation for approximately 2-3 months, detritus builds up on the bottom, which is removed with a fish net after first removing the mysids. The rate of water flow through the tanks should be maintained between 4-5 L/min, and 10-20% of the seawater in each aquarium should be exchanged weekly.

4.7.2 Some culturists have noted problems with hydrozoan pests in their cultures and there are procedures for their eradication, if necessary (Lawler and Shepard, 1978; Hutton et al., 1986).

4.8 PRODUCTION LEVEL

4.8.1 At least four aquaria should be maintained to insure a sufficient number of organisms on a continuing basis. If each 200-L aquarium is initially stocked with between 200 and 500 adults (do not exceed 500 adults), they will provide sufficient numbers of test organisms (Figure 4) each month. If the cultures are correctly maintained, at least 20 percent of the adult population should consist of gravid females (have a visible oostegite brood pouch with young). It is also advantageous to cull older mysids in the population every 4-6 weeks and to move mysids among the four aquaria to diversify the gene pool.

5. VIDEO TRAINING TAPE AVAILABLE FOR CULTURING METHODS

5.1 A video training tape and supplemental report (USEPA, 1990) on culturing *Mysidopsis bahia* are available from the National AudioVisual Center, Customer Services Section, 8700 Edgeworth Drive, Capitol Heights, MD 20743-3701, (Phone 301-763-1891), as part of a video package on culturing and short-term chronic toxicity test methods (Order No. A18657; cost $75.00).

6. TEST ORGANISMS

6.1 Juvenile *Mysidopsis bahia*, one to five days old, are used in the tests. To obtain the necessary number for a test, there are a number of techniques available. A mysid generator such as the one described by Reistsema and Neff (1980) has been successfully used. Another method to obtain juveniles is to take approximately 200 adult females (bearing embryos in their brood pouches) from the stock culture and place them in a large (10 cm X 15 cm) standard fish transfer net (2.0 to 3.0 mm openings) that is partially submerged in an 8-L aquarium containing 4 L of clean culture medium. As the juveniles are released from the brood pouches, they drop through the fish net into the aquarium. The adults and juveniles in the aquarium are fed twice daily 24-hour post hydrated *Artemia*. The adults are allowed to remain in the net for 48 h, and are then returned to the stock tanks. The juveniles that are produced in the small tank may be used in the toxicity tests over a five-day period. Another method for obtaining juveniles (Ward 1987; 1989) is simply to remove juveniles from the stock culture with a fine mesh net, place them in 2-L PYREX® crystalline dishes with media, positioned on a light table that has an attached viewing plate (2 mm squares), and remove juveniles less than 2 mm in length (approximately 24 h old).

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.4. BRINE SHRIMP (*ARTEMIA SALINA*)

1. SYSTEMATICS

1.1 MORPHOLOGY AND TAXONOMY

1.1.1 The taxonomic status of *Artemia* has long been controversial because there is considerable morphological variability over parts of its range. The present consensus is that there is a single cosmopolitan species, *Artemia salina*, which has numerous intergrading physiological and morphological varieties (Pennak, 1989). Brine shrimp belong to the subclass Branchiopoda which is characterized by many pairs of flattened appendages on the thorax (Figure 1), in contrast to other members of the Crustacea that have no more than six pairs. Probably the most distinctive feature of *Artemia salina* is the compressed, triangular, and blade-shaped distal segment of the second antenna of the male (Figure 2). The mature adult is 8 to 10 mm long with a stalked lateral eye, sensorial antennulae,a linear digestive tract and 11 pairs of thoracopods. In the male the antennae are transformed into muscular claspers used to secure the female during copulation.

2. DISTRIBUTION

2.1 *Artemia* are found nearly worldwide in saline lakes and pools. In North America, they have been reported throughout the western United States, in Nebraska and Connecticut and in Saskatchewan, Canada. They are probably more widely distributed than indicated because of limited effort in collecting from many areas of the country. They are absent from many suitable habitats, probably because of their limited dispersal methods.

3. ECOLOGY AND LIFE HISTORY

3.1 GENERAL ECOLOGY

3.1.1 The ecological conditions under which brine shrimp live are highly variable. The salinity can exceed 300‰, where most other life cannot survive. Favored by the absence of predators and food competitors in such places, *Artemia* develop very dense populations. Although not a marine species, they sometimes occur in bays and lagoons where brines are formed by evaporation of seawater (salt pans). They are more commonly found in highly saline lakes, such as the Great Salt Lake, where the shoreline may become ringed with brown layers of accumulated brine shrimp cysts. Brine shrimp are also common in evaporation basins used for the commercial production of salt.

3.1.2 The reproductive habits of different populations vary considerably. In parts of Europe parthenogenesis is the rule, males being rare or absent, but in North America most *Artemia* populations seem to be diploid with males common.

3.1.3 The principal mechanism of *Artemia* dispersion is transportation of the cysts by wind or waterfowl and by deliberate or accidental human inoculation.

3.1.4 Growth of brine shrimp is influenced by many factors and the tolerance of these factors is strain dependent. Optimum temperature for most strains ranges between 25 and 35°C but strains have been reported thriving at 40°C. Most geographical strains do not survive temperatures below 6°C except as cysts. These cysts are tolerant of temperatures from far below 0°C to near the boiling point of water. Although *Artemia* can survive and reproduce under a wide range of salinity, they are seldom found in nature in salinities below 45‰ or above 200‰. The pH tolerance of *Artemia* varies from neutral to highly alkaline but the cysts will hatch best at a pH of 8 or higher.

3.1.5 Many predators including many zooplankton that populate natural salt waters, many salt water fish, several insect groups (odonates, hemipterans and beetles), and birds feed on brine shrimp in situations where they can tolerate the conditions.

3.2 FOOD AND FEEDING

3.2.1 Brine shrimp are typically filter-feeders that consume organic detritus, microscopic algae and bacteria. Blooms of microscopic algae are favorite habitats of *Artemia*, and large populations develop in such areas where they feed on the algae and heterotrophic bacteria that are produced by these blooms. Brine shrimp populations have done well in cultures when fed algae, rice bran (Sorgeloos et al., 1979), soybean meal or whey powder (Bossuyt and Sorgeloos, 1979). The nauplii do not need food for four days after hatching.

3.3 LIFE HISTORY

3.3.1 Most strains of *Artemia* produce cysts that float (cysts from the Mono Lake, California strain sink). These cysts remain in diapause as long as they are kept dry or under anaerobic conditions. Upon hydration, the embryo in the cyst becomes activated. After several hours the outer membrane bursts and the embryo emerges still encased in the hatching membrane. Soon the hatching membrane is ruptured and the free-swimming nauplius is born. The first instar is brownish-orange colored and has three pairs of appendages (Figure 3). The larva grows through about 15 molts and becomes differentiated into male or female after the tenth molt. Copulation is initiated when the male grasps the female with its modified antennae (Figure 4). The fertilized eggs develop either into free-swimming nauplii, or they are surrounded by a thick shell and deposited as cysts which are in diapause.

Figure 1. Drawing of male (A) and female (B) brine shrimp (From Kuenen and Bass-Becking, 1938).

Figure 2. Head of adult male showing triangular distal segment of antennae modified as claspers (From Persoone et al., 1980a).

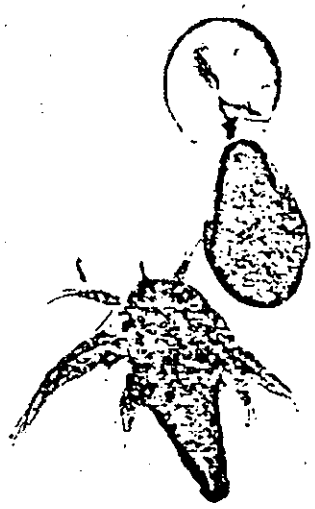

Figure 3. Pre-nauplius and freshly hatched first instar (From Persoone et al., 1980a).

Figure 4. Male and female brine shrimp nauplius preparing to copulate (From Persoone et al., 1980a).

4. METHODS FOR HATCHING ARTEMIA CYSTS

4.1 SOURCES OF CYSTS

4.1.1 Brine shrimp cysts are available from many commercial sources, representing several geographical strains. The cysts from any source can vary from batch to batch in terms of nutritional quality for the test organisms. Therefore, it is recommended that each new batch purchased should be analyzed chemically, and that a side-by-side feeding test be performed on their nutritional suitability by comparing the response of the test organisms with the new cysts and cysts of known quality (ASTM, 1993). A list of sources of cysts is provided at the end of this chapter.

4.2 STORAGE OF CYSTS

4.2.1 Sealed cans of *Artemia* cysts can be stored for years at room temperature, but once opened, should be used up within two months. After each use, the can should be tightly covered with a plastic lid and stored in the refrigerator. If the entire contents of a can will not be used up in two months, it is recommended that the portion that is expected to be unused be placed in a tightly closed container and frozen until needed.

4.3 HATCHING OF CYSTS

4.3.1 A 2-L separatory funnel makes a convenient brine shrimp hatching vessel, but nearly any transparent or translucent (preferably colorless) conical shaped container that will hold water may be used. A satisfactory apparatus can be prepared by removing the bottom of a 2-L plastic soft drink bottle and inserting a rubber stopper with a flexible tube and pinch cock. The hatching chambers must be clean and free from toxic material. All detergents should be completely removed by rinsing well with deionized water.

4.3.2 Salinity of the water used for hatching brine shrimp cysts should be between 25 and 35‰. Natural sea water or water made up from artificial sea salts may be used. The hatching medium can be prepared by placing 1800 mL of deionized water in the hatching chamber and adding 50-70 g non-iodized salt. After the salt is added, lower a 1 mL pipette or glass tube fitted to an air supply into the vessel, so that the tip rests on the bottom, and bubble air vigorously through it to dissolve the salt.

4.3.3 Add the desired quantity of cysts to the vessel. Approximately 15 mL of cysts in a 2-L hatching vessel will provide enough brine shrimp nauplii to feed three large stock cultures of mysids in 76-L aquaria, or 1000 to 1500 newly hatched fish in four to six 8-L tanks.

4.3.4 Continue the aeration to keep the cysts and newly hatched nauplii from settling to the bottom where the DO would quickly be depleted and the newly hatched animals would die.

4.3.5 The area in which the cysts are hatched should be provided with approximately 20 $\mu E/m^2/s$ (100 ft-c) of illumination.

4.3.6 The cysts will hatch in about 24 h at a temperature of 25°C. Hatching time varies with incubation temperature and the geographic strain of *Artemia* used.

4.4 HARVESTING THE NAUPLII

4.4.1 When the brine shrimp nauplii first emerge from the cyst, they are enclosed in a membranous sac (Figure 3). To be taken as food by the test organisms, the pre-nauplii must emerge from the sac and swim about (Stage-I or first instar nauplius).

4.4.2 The first instar (Stage-I) nauplii do not feed. Their value as food for the test organisms decreases from birth until they begin feeding. Because they do not feed in the hatching vessels, it is important to harvest and use the nauplii soon after hatching. The nauplii can be easily harvested in the following manner:

1. After approximately 24 h at 25°C, remove the pipet supplying air and allow the nauplii to settle to the bottom of the hatching chamber. The empty egg shells will float to the top and the newly hatched nauplii and unhatched eggs will settle to the bottom. A light trained on the bottom of the separatory funnel will hasten the settling process.
2. After approximately 5 min, using the stopcock, drain off the nauplii into a 250-mL beaker.
3. After another 5 min, again drain the nauplii into the beaker.
4. The nauplii are further concentrated by pouring the suspension into a small cylinder which has one end closed with #20 plankton netting or they may be washed through a 150-μm net or screen.
5. The concentrate is resuspended in 50 mL of appropriate culture water, mixed well, and dispensed with a pipette. (Mysids require approximately 100 to 150 nauplii/mysid/day).
6. Discard the remaining contents of the hatching vessel and wash the vessel with hot soap and water.
7. Prepare fresh salt water for each new hatch.

4.4.3 To have a fresh supply of *Artemia* nauplii daily, at least two hatching vessels should be used, so that the newly-hatched can be harvested daily.

4.5 FEEDING ASSAY

4.5.1 Before using brine shrimp nauplii from a new batch of cysts for routine feeding of cultures and test organisms, they should be tested for their ability to support life, growth, and reproduction of the test animals. Two treatments with four replicates each are required for this test. In Treatment (A), the test organisms are fed the nauplii from the new batch of *Artemia* cysts, and in Treatment (B), the test organisms are fed nauplii of known, good quality, such as from the reference *Artemia* cysts or from a batch of *Artemia* cysts that have been successfully used in culturing and testing.

4.5.2 If there is no significant difference in the survival, growth, and/or reproduction of the organisms in the two treatments at the end of a 7-day period, it is assumed that the new batch of *Artemia* cysts is satisfactory. If

the survival, growth, and/or reproduction in treatment A is significantly less than the response in treatment B over a 7-day test period it is assumed that the new batch of brine shrimp cysts are unsuitable for use as a food source for the organisms tested.

4.5.3 Test chambers and all test conditions during the feeding assay should be similar to those planned for use in the subsequent toxicity tests.

4.6 LIST OF COMMERCIAL SOURCES OF *ARTEMIA* CYSTS

Aquafauna Biomarine
P.O. Box 5
Hawthorne, CA 90250
Tel. (213) 973-5275
Fax (213) 676-9387
(Great Salt Lake, North Arm San Francisco Bay)

Aquarium Products
180L Penrod Court
Glen Burnie, MD 21061
Tel. (800) 368-2507
Tel. (301) 761-2100
(Colombia)

Argent Chemical
8702 152nd Ave. NE
Redmond, WA 98052
Tel. (800) 4266258
Tel. (206) 855-3777
Fax (206)885-2112
(Platinum Label - San Francisco Bay; Gold Lable - San Francisco Bay, Brazil, other; Silver Label - Great Salt Lake, Australia, other; Bronze label - China, Canada, other]

Artemia Systems
Wiedauwkaai 79
B-9000 Ghent, Belgium
Tel 011-32-91-534142
Fax 011-32-91-536893
(For marine species - AF grade [small nauplii], UL grade [large nauplii], for freshwater species - IH grade [small nauplii], EG grade [large nauplii]

Bonneville Artemia International, Inc.
P.O. Box 511113
Salt Lake City, UT 84151-1113
Tel. (801) 972-4704
Fax (801) 972-4795

Golden West Artemia
411 East 100 South
Salt Lake City, UT 84111
Tel. (801) 532-1400
Fax (801) 531-8160

Ocean Star International
P.O. Box 643
Snowville, UT
Tel. (801) 872-8217
Fax (801) 872-8272
(Great Salt Lake)

Pennsylvania Pet Products
Box 191
Spring City, PA
(Great Salt Lake)

Sanders Brine Shrimp Co.
3850 South 540 West
Ogden, UT 84405
Tel. (801) 393-5027
(Great Salt Lake)

San Francisco Bay Brand
8239 Enterprise Drive
Newark, CA 94560
Tel. (415) 792-7200
(Great Salt Lake, San Francisco Bay)

Sea Critters Inc.
P.O. Box 1508
Tavernier, FL 33070
Tel. (305) 367-2672

Western Brine Shrimp
957 West South Temple
Salt Lake City, UT 84104
Tel. (801) 364-3k642
Fax (801) 534-0211
(Great Salt Lake)

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Bengtson, D.A.S., A.D. Beck, S.M. Lussier, D. Migneault, and C.E. Olney. 1984. International study on *Artemia*. XXXI. Nutritional effects in toxicity tests: Use of different *Artemia* geographical strains. In: G. Persoone, E. Jaspers, and C. Claus, (eds.). Ecotoxicological testing for the marine environment, Vol. 2. State Univ. Ghent and Inst. Mar. Sci. Res., Bredene, Belgium. pp. 399-416.

Bossuyt, E., and P. Sorgeloos. 1979. Technological aspects of the batch hatching of *Artemia* in high densities. In: G. Persoone, P. Sorgeloos, O. Roels and E. Jaspers (eds.), The brine shrimp *Artemia*, Vol. 3. Ecology, culturing, use in aquaculture. Universa Press, Wetteren, Belgium. pp. 133-152.

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Johns, D.M., W.J. Berry, and W. Walton. 1981. International study on *Artemia*. XVI. Survival, growth and reproductive potential of the mysid, *Mysidopsis bahia* Molenock fed various geographical strains of the brine shrimp, *Artemia*. J. Exp. Mar. Biol. Ecol. 53:209-219.

Kuenen, D.J., and L.G.M. Baas-Becking. 1938. Historical notes on *Artemia salina* (L.). Zool. Med. 20:222-230.

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Persoone, G., P. Sorgeloos, O. Roels, and E. Jaspers, (eds.). 1980b. The brine shrimp *Artemia*. Vol. 2. Physiology, biochemistry, molecular biology. Universa Press, Wetteren, Belgium. 636 pp.

Persoone, G., P. Sorgeloos, O. Roels, and E. Jaspers, (eds.). 1980c. The brine shrimp *Artemia*. Vol. 3. Ecology, culturing, use in aquaculture. Universa Press, Wetteren, Belgium. 428 pp.

Sorgeloos, P. 1980. Life history of the brine shrimp *Artemia*. In: G. Persoone, P. Sorgeloos, D.A. Roels, and E. Jaspers (eds.), The brine shrimp, *Artemia*. Vol. 1. Morphology, genetics, radiobiology, toxicology. Universa Press, Wetteren, Belgium. pp. ixx-xxii.

Sorgeloos, P., M. Baesa-Mesa, E. Bossuyt, E. Bruggeman, J. Dobbeler, D. Versichele, E. Lavina, and A. Bernardine. 1979. Culture of *Artemia* on rice bran: The conversion of waste-products into highly nutritive animal protein. Aquaculture 21:393-396.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.5. FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

1. MORPHOLOGICAL AND ANATOMICAL CHARACTERISTICS

1.1 Fathead minnows vary greatly in many characteristics throughout their wide geographic range. The morphology and characters for identification are taken from Clay (1962), Hubbs and Lagler (1964), Eddy and Hodson (1961), Scott and Crossman (1973), and Trautman (1981). Adults (Figure 1) are small fish, typically 43 mm to 102 mm, and averaging about 50 mm, in total length. The standard lengths are usually less than four and one-half times the body depth. The first rudimentary ray of the dorsal fin is more or less thickened and distinctly separated from the first well-developed ray by a membrane. The lateral line is usually incomplete, but may be complete in specimens from some geographic areas. The scales are cycloid and moderate in size. Andrews (1970), reporting on fish collected in Colorado, noted that no scales were found on fish smaller than 14 mm, and the average length for first scale formation was 16.3 mm. The scales in the lateral series number 41 to 54.

1.2 The mouth is terminal. The snout does not extend beyond the upper lip and is decidedly oblique. Nuptial tubercles occur on mature males only, are large and well-developed on the snout, and rarely extend beyond the nostrils.

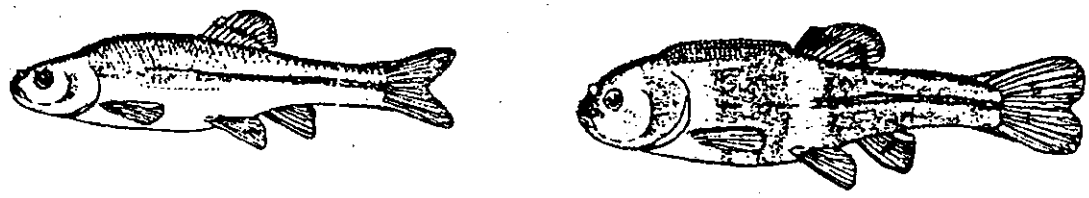

Figure 1. Fathead minnow: adult female (left) and breeding male (right). (From Eddy and Hodson, 1961).

They occur in three main rows, with a few on the lower jaw. In addition to nuptial tubercles, the males have an elongate, fleshy, or spongy pad extending in a narrow band from the nape to the dorsal fin. The pad is wide anteriorly, and narrows to engulf the first dorsal ray. In addition, the sides of the body become almost black except for two wide vertical bars which are light in color. In contrast to the males, the mature females remain quite drab.

1.3 The peritoneum is brownish-black, and the intestine is long and coiled one or more times.

1.4 Some external markings occur infrequently. Young occasionally have a dusky band on the snout and opercules. Other young and adults, from clear and weedy waters, have a distinct, lateral band across the body. The band may be absent in breeding males or, if present, becomes very diffuse anteriorly. This band is usually most apparent on preserved specimens. Dymond (1926), Trautman (1981), and others described the saddle-like pattern often associated with breeding males in which a light area develops just behind the head and another beneath the dorsal fin, the areas between producing a saddle affect. A dark spot is usually present in front of the dorsal fin in mature males, and a narrow, dark, vertical bar or spot is present at the base of the caudal fin, but often is not very distinct.

2. TAXONOMY

2.1 The specific name (*Pimephales promelas*) appears to be incorrectly applied to this fish because the fathead minnow does not fit the description originally given by Rafinesque (1820) (Lee et al., 1980). Common names include "northern fathead minnow", and "blackhead minnow," in addition to fathead minnow. The holotype was collected near Lexington, Kentucky.

2.2 Some geographic variations have been noted in the morphology of the fathead minnow. Vandermeer (1966) indicated that the introduction of this species outside its native range may have resulted in some local deviations from the broad patterns of geographic variation in taxonomic characters. Some populations have been designated as subspecifically distinct: *Pimephales promelas promelas*, the northern form; *P. p. harveyensis*, the Harvey Lake form, from Isle Royal in Lake Superior and *P. p. confertus*, the southern form (Hubbs and Lagler, 1949, 1964). However, Taylor (1954), Vandermeer (1966), and others expressed doubt concerning the validity of assigning subspecific status to the variants and recommended against their recognition. Vandermeer (1966), in a statistical analysis of the geographic variations in taxonomic characters, stated that two of the three described subspecies intergrade clinally.

2.3 Of the eight characters measured, two showed a north-south trend; (1) eye diameter, with the northern fish having smaller eyes, and (2) completeness of the lateral line, with the northern fish having the least complete lateral line. However, Scott and Crossman (1973), indicated that some Canadian populations exhibit a nearly complete lateral line. The American Fisheries Society (1980) does not recognize any of the fathead minnow subspecies.

3. DISTRIBUTION

3.1 The fathead minnow is widely distributed in North America (Figure 2). It is a popular bait fish, and the ease with which it is propagated has led to its widespread introduction both within and outside the native range of the species. It has been so widely distributed in the eastern and southwestern United States by bait transportation that it is difficult to determine its original range. The presumed native distribution (Vandermeer, 1966; Scott and Crossman, 1973; Lee, et al., 1980) extended from the Great Slave Lake in the northwest to New Brunswick, in eastern Canada, southward throughout the Mississippi valley in the United States, to southern Chihuahua in Mexico. Distribution records for this species also now include Oregon (Andreasen, 1975), and the Central Valley (Kimsey and Fisk, 1964) and other locations in California (Andreasen, 1975), but there are no records for British Columbia.

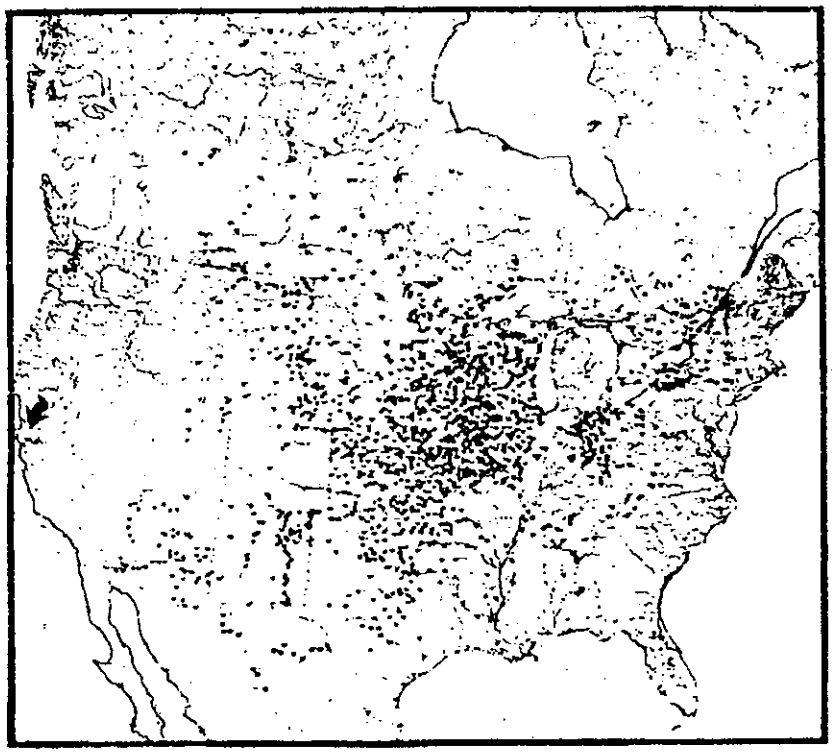

Figure 2. Map showing the distribution of the fathead minnow in North America. Open circles represent transplanted populations. Most Atlantic slope records are probably transplanted populations. (From Lee et al., 1980).

3.2 This species is found in a wide range of habitats. It is most abundant in muddy brooks, streams, creeks, ponds, and small lakes, is uncommon or absent in streams of moderate and high gradients and in most of the larger and

deeper impoundments, and is tolerant of high temperature and turbidity, and low oxygen concentrations.

3.3 Species associated with the fathead minnow seem to vary greatly throughout its range (Scott and Crossman, 1973; Trautman 1981). Trautman (1981) reported that fathead minnows and bluntnose minnows, *Pimephales notatus* (Rafinesque), were competitors, and that fathead minnows occurred in greatest numbers only where bluntnose minnows were absent or comparatively few in number. He also stated that the fathead minnow may hybridize with the bluntnose minnow

3.4 The fathead minnow is primarily omnivorous, although Coyle (1930) reported algae to be one of its main foods in Ohio. Elsewhere in the United States, young fish have been reported to feed on organic detritus from bottom deposits, and unicellular and filamentous algae and planktonic organisms. Adults feed on aquatic insects, worms, small crustaceans, and other animals. Scott and Crossman (1973) and others regard the fathead minnow as a highly desirable forage fish, providing food for other fishes and birds.

4. GENERAL LIFE HISTORY

4.1 The natural history and spawning behavior (Markus, 1934; Flickinger, 1973; Andrews and Flickinger, 1974; and others) of the fathead minnow are well known because of the early interest in raising the fish for bait and for feeding other pond fish, such as bass. Sexual dimorphism occurs at maturity. Breeding males develop a conspicuous, narrow, elongated, gray, fleshy pad of spongy tubercles on the back, anterior to the dorsal fin, and two or three rows of strong nuptial tubercles across the snout. The sides of the body become almost black except for two wide vertical bars which are light in color. In contrast, the females remain quite drab.

4.2 The initiation of spawning varies with temperature throughout its geographic range. Isaak (1961), Carlander (1969), and others reported that, in the wild, fathead minnows begin spawning in the spring, when the water temperature reaches 16-18°C, and continue to spawn throughout most of the summer. The minimum spawning temperature, however, may vary with population and latitude.

4.3 Markus (1934) reported that spawning always occurred at night, whereas Isaak (1961) observed spawning during the day, as well as at night. Gale and Buynak (1982) and others reported that spawning often began before dawn and usually was completed before noon. Observations of the fathead minnow cultures at EPA's Newtown Facility also indicate the majority of fathead minnows spawn in early morning.

4.4 Breeding males are very territorial and select sites for spawning, such as the underside of a log or branch, rock, board, tin can, or almost any other solid inanimate object, usually in water from 7 cm to 1 m in depth. A receptive female is sought out and brought into position below the nest site. After circling below the nesting site, the female is nudged and lifted on the male's back until she lies on her side immediately below the undersurface of the spawning substrate, where she releases a small number of eggs (usually 100

to 150) at a time. The eggs are adhesive and attach to the underside of the spawning substrate. The females have a urogenital structure (ovipositor) to help deposit the eggs on the underside of objects. Flickinger (1966) indicated that the ovipositor is noticeable at least a month prior to spawning. The reported size of the eggs varies from 1.15 mm (Markus, 1934) to 1.3 mm in diameter (Wynne-Edwards, 1932).

4.5 Immediately after the eggs are laid, they are fertilized by the male, and the female is driven off. Once eggs are deposited in the nest, the male becomes very aggressive and will use the large tubercles on his snout to help drive off all intruding small fishes. In addition to fertilizing and guarding the eggs, the male agitates the water around the eggs, which ventilates them and keeps them free of detritus. Some males will spawn with several females on the same substrate, so that the nest may contain eggs in various stages of development. The number of eggs per nest may vary from as few as nine or 10 to as many as 12,000.

4.6 The ovaries of the females contain eggs in all stages of development, and they spawn repeatedly as the eggs mature. A female may deposit eggs in more than one nest. Although the average number of eggs per spawn is generally 100 to 150, large females may lay 400 to 500 eggs per spawn.

4.7 Gale and Buynak (1982), in a study using five captive pairs of fathead minnows in separate outdoor pools, observed that each pair produced 16 to 26 clutches of eggs between May and August. The time between spawns, which ranged from two to 16 days, was affected by water temperature. As the temperature increased, the intervals between spawning sessions become shorter and more uniform. In their study, from nine to 1,136 (mean of 414) eggs were deposited per spawn. The average number of eggs deposited per spawn ranged from 371 to 480, and the total number of eggs spawned per female ranged from 6,803 to 10,164 (mean of 8,604). The length of the spawning period during a given season also varied greatly between females. The authors suggested that the fecundity of fathead minnows is much higher than has generally been recognized, but they noted that fecundity of fish in the natural environment, where conditions might be more or less favorable, might differ from that of captive fish.

4.8 The incubation time depends on temperature, and is 4.5 to 6 days at 25°C. The newly-hatched young (larvae) are about 5 mm long, white in color, and have large black eyes. The general appearance and typical pigmentation of the various larval stages are illustrated in Figures 3A-3M. In a warm, food-rich environment, growth is rapid. Markus (1934) stated that fish hatched in May in Iowa reached adult size and were spawning by late July. Hubbs and Cooper (1935) and others noted that such rapid growth is unlikely in more northerly waters, and that the young do not spawn the first year. In cooler water the adult size is probably not reached until the second year. The males generally grow faster than the females, a characteristic of minnow species.

4.9 The fathead minnow is short lived, and rarely survives to the third year. However, Scott and Crossman (1973) stated that longevity varies throughout the geographic range of the species. Post-spawning mortality was reported to be great by several authors, but was not observed by Gale and Buynak (1982).

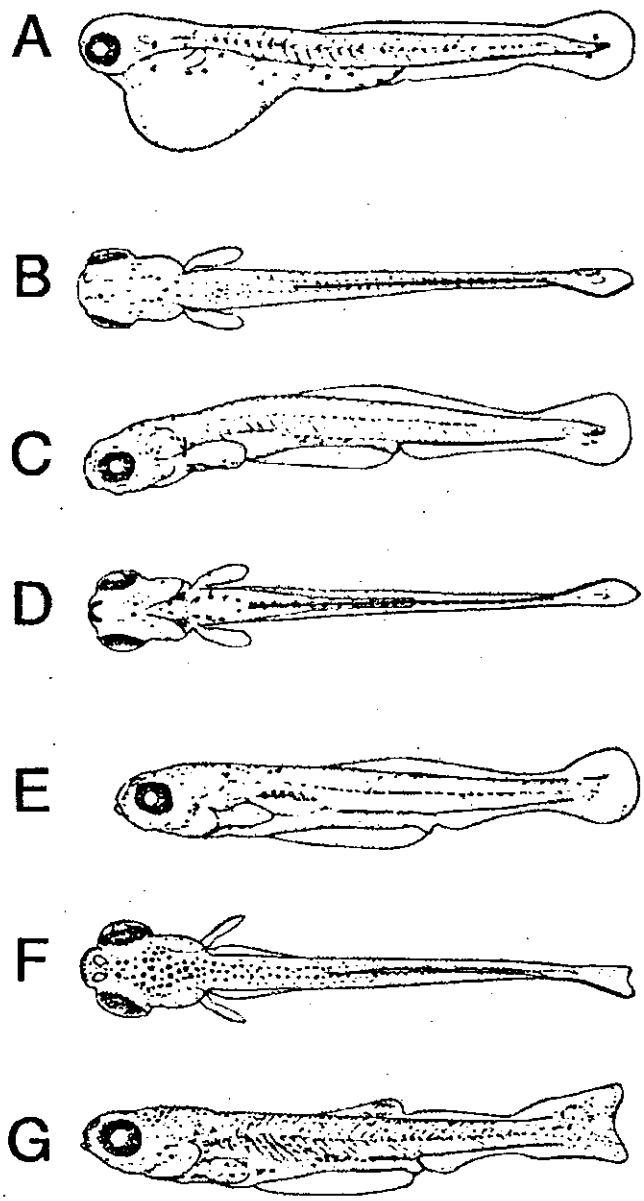

Figure 3. Fathead minnow (*Pimephales promelas*) larvae: A. protolarva, lateral view, 4.3 mm TL; B. protolarva, dorsal view, 5.6 mm TL; C. protolarva, lateral view, 5.6 mm TL; D. protolarva, ventral view, 5.6 mm TL; E. mesolarva, lateral view, 6.9 mm TL; F. mesolarva, dorsal view, 7.9 mm TL; G. mesolarva, lateral view, 7.9 mm TL; (From Snyder et al., 1977).

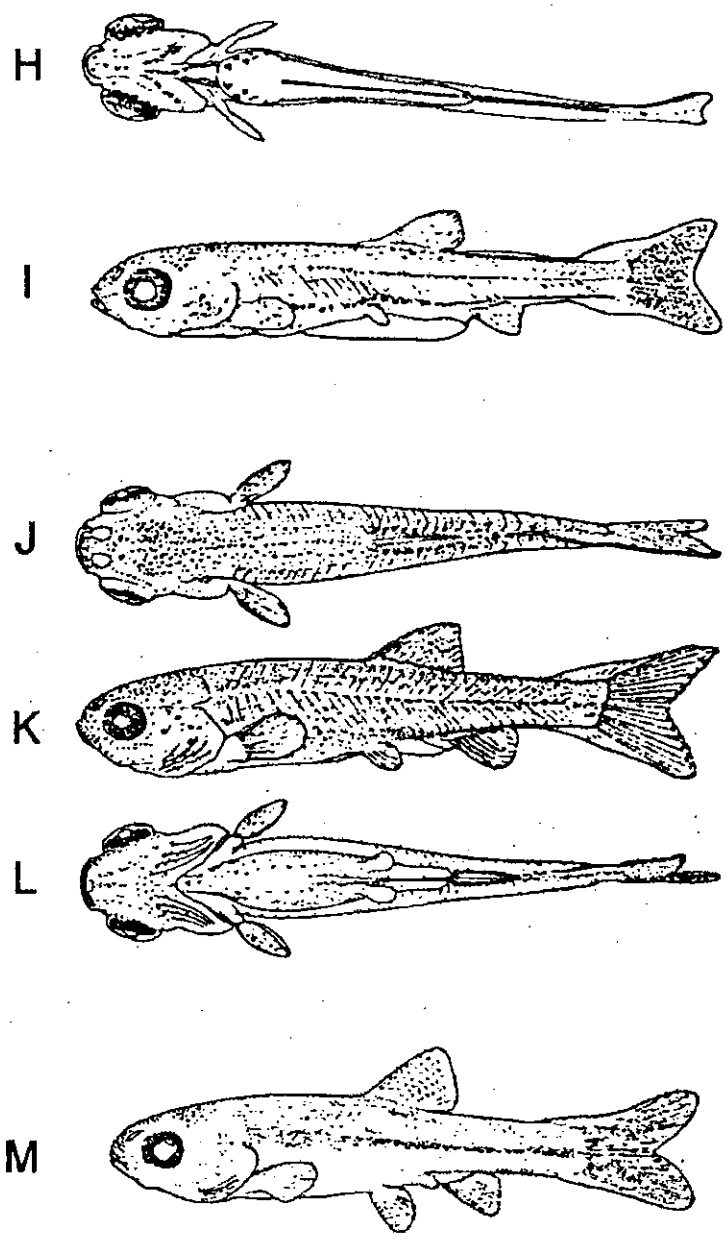

Figure 3. Fathead minnow (*Pimephales promelas*) larvae. H. mesolarva, ventral view, 7.9 mm TL; I. metalarva, lateral view, 9.3 mm TL; J. metalarva, dorsal view, 14.3 mm TL; K. metalarva, lateral view, 14.3 mm TL; L. metalarva, ventral view, 14.3 mm TL; M. late metalarva, lateral view, 19.6 mm TL (CONTINUED) (from Snyder et al., 1977).

However, in defending their territory, male fish, may become weakened by a lack of food over a prolonged period and their resistance to disease may be lowered. Also, at spawning time, many waters are warm and somewhat stagnate, favoring the spread of fish parasites and disease.

5. CULTURE METHODS

5.1 OUTSIDE SOURCES OF FATHEAD MINNOWS

5.1.1 Fathead minnows are available from commercial biological supply houses. Fish obtained from outside sources for use as brood stock or in toxicity tests may not always be of suitable age and quality. Fish provided by supply houses should be guaranteed to be of (1) the correct species, (2) disease free, (3) in the requested age range, (4) and in good condition. The latter can be done by providing the record of the date on which the eggs were laid and hatched, and information on LC50 of contemporary fish using reference toxicants.

5.2 INHOUSE SOURCES OF FATHEAD MINNOWS

5.2.1 Problems in obtaining suitable fish from outside laboratories can be avoided by developing an inhouse laboratory culture facility. Fathead minnows can be easily cultured in static, recirculating, or flow-through systems.

5.2.2 Flow-through systems require large volumes of water and may not be feasible in some laboratories. The culture tanks should be shielded from extraneous disturbances using opaque curtains, and should be isolated from toxicity testing activities to prevent contamination.

5.2.3 To avoid the possibility of inbreeding of the inhouse brood stock, fish from an outside source should be introduced yearly into the culture unit.

5.2.4 The inhouse culture facility consists of the following components:

5.2.4.1 Water Supply

5.2.4.1.1 Water Quality

5.2.4.1.1.1 Reconstituted (synthetic) water or dechlorinated tap water can be used, but natural water may be preferred. To determine water quality, it is desirable to analyze the water for toxic metals and organics quarterly (see Section 4, Quality Assurance). Temperature, dissolved oxygen, pH, hardness, and alkalinity should also be measured periodically.

5.2.4.1.1.2 If a static or recirculating system is used, it is necessary to equip each tank with an outside activated carbon filter system, similar to those sold for tropical fish hobbyists (or one large activated carbon filter system for a series of tanks) to prevent the accumulation of toxic metabolic wastes (principally nitrite and ammonia) in the water.

5.2.4.1.2 Dissolved oxygen

5.2.4.1.2.1 The DO concentration in the culture tanks should be maintained near saturation, using gentle aeration with 15 cm air stones if necessary. Brungs (1971), in a carefully controlled long-term study, found that the growth of fathead minnows was reduced significantly at all DO concentrations below 7.9 mg/L. Soderberg (1982) presented an analytical approach to the re-aeration of flowing water for culture systems.

5.2.4.2 Maintenance

5.2.4.2.1 Adequate procedures for culture maintenance must be followed to avoid poor water quality in the culture system. The spawning and brood stock culture tanks should be kept free of debris (excess food, detritus, waste, etc.) by siphoning the accumulated materials (such as dead brine shrimp nauplii or cysts) from the bottom of the tanks daily with a glass siphon tube attached to a plastic hose leading to the floor drain. The tanks are more thoroughly cleaned as required. Algae, mostly diatoms and green algae, growing on the glass of the spawning tanks are left in place, except for the front of the tank, which is kept clean for observation. To avoid excessive build-up of algal growth, the walls of the tanks are periodically scraped. The larval culture tanks are cleaned once or twice a week to reduce the mass of fungus growing on the bottom of the tank.

5.2.4.2.2 Activated charcoal and floss in the tank filtration systems should be changed weekly, or more often if needed. Culture water may be maintained by preparation of reconstituted water or use of dechlorinated tap water. Distilled or deionized water is added as needed to compensate for evaporation.

5.2.4.2.3 Before new fish are placed in tanks, salt deposits are removed by scraping or with 5% acid solution, the tanks are washed with detergent, sterilized with a hypochlorite solution, and rinsed well with hot tap water and then with laboratory water.

5.2.5 SPAWNING TANKS AND CULTURE CONDITIONS

5.2.5.1 For breeding tanks, it is convenient to use 60 L (15 gal) or 76 L (20 gal) aquaria. The spawning unit is designed to simulate conditions in nature conductive to spawning, such as water temperature and photoperiod. Spawning tanks must be held at a temperature of 25 ± 2°C. Each aquarium is equipped with a heater, if necessary, a continuous filtering unit, and spawning substrates. The photoperiod for the culture system should be maintained at 16 h light and 8 h darkness. For the spawning tanks, this photoperiod must be rigidly controlled. A convenient photoperiod is 5:00 AM to 9:00 PM. Fluorescent lights should be suspended about 60 cm above the surface of the water in the brood and larval tanks. Both DURATEST® and cool-white fluorescent lamps have been used, and product similar results. An illumination level of 10-20 $\mu E/m^2/s$ (50 to 100 ft-c) is adequate.

5.2.6 SPAWNING BEHAVIOR AND CONDITIONS

5.2.6.1 To simulate the natural spawning environment, it is necessary to provide substrates (nesting territories) upon which the eggs can be deposited and fertilized, and which are defended and cared for by the males. The recommended spawning substrates consist of inverted half-cylinders, such as 7.6 cm X 7.6 cm (3 in. X 3 in.) sections of schedule 40, PVC pipe. The substrates should be placed equi-distant from each other on the bottom of the tanks.

5.2.6.2 To establish a breeding unit, 15-20 pre-spawning adults six to eight months old are taken from a "holding" or culture tank and placed in a 76-L spawning tank. At this point, it is not possible to distinguish the sexes. However, after less than a week in the spawning tank, the breeding males will develop their distinct coloration and territorial behavior, and spawning will begin. As the breeding males are identified, all but two are removed, providing a final ratio of 5-6 females per male. The excess spawning substrates are used as shelter by the females.

5.2.6.3 Sexing of the fish to ensure a correct female/male ratio in each tank can be a problem. However, the task usually becomes easier as experience is gained (Flickinger, 1966). Sexually mature females usually have large bellies and a tapered snout. The sexually mature males are usually distinguished by their larger overall size, dark vertical color bands, and the spongy nuptial tubercles on the snout. Unless the males exhibit these secondary breeding characteristics, no reliable method has been found to distinguish them from females. However, using the coloration of the males and the presence of an enlarged urogenital structures and other characteristics of the females, the correct selection of the sexes can usually be achieved by trial and error.

5.2.6.4 Sexually immature males are usually recognized by their aggressive behavior and partial banding. These undeveloped males must be removed from the spawning tanks because they will eat the eggs and constantly harass the mature males, tiring them and reducing the fecundity of the breeding unit. Therefore, the fish in the spawning tanks must be carefully checked periodically for extra males.

5.2.6.5 A breeding unit will remain in their spawning tank about four months. Thus, each brood tank or unit is stocked with new spawners about three times a year. However, the restocking process is rotated so that at any one time the spawning tanks contain different age groups of brood fish.

5.2.7 EMBRYO COLLECTION

5.2.7.1 Fathead minnows spawn mostly in the early morning hours. They should not be disturbed except for a morning feeding (approximately 8:00 AM) and daily examination of substrates for eggs in late morning or early afternoon. In nature, the male protects, cleans, and aerates the eggs until they hatch. In the laboratory, however, it is necessary to remove the eggs from the tanks to prevent them from being eaten by the adults, and for ease of handling for purposes of recording embryo count and hatchability, and for the use of the newly hatched for young fish for toxicity tests.

5.2.7.2 Daily, beginning six to eight hours after the lights are turned on (i.e., 11:00 AM - 1:00 PM), the substrates in the spawning tanks are each lifted carefully and inspected for embryos. Substrates without embryos are immediately returned to the spawning tank. Those with embryos are immersed in clean water in a collecting tray, and replaced with a clean substrate. A daily record is maintained of each spawning site and estimated number of embryos on the substrate.

5.2.8 EMBRYO INCUBATION

5.2.8.1 Three different methods are described for embryo incubation.

5.2.8.1.1 Incubation of Embryos on the Substrates: Several (2-4) substrates are placed on end in a circular pattern (with the embryos on the inner side) in 10 cm of water in a tray. The tray is then placed in a constant temperature water bath, and the embryos are aerated with a 2.5 cm airstone placed in the center of the circle. The embryos are examined daily, and the dead and fungused embryos are counted, recorded, and removed with forceps. At an incubation temperature of 25°C, 75-100% hatch occurs in five days. At 22°C, embryos incubated on aerated tiles require seven days for 50% hatch.

5.2.8.1.2 Incubation of Embryos in a Separatory Funnel: The embryos are removed from the substrates with a rolling action of the index finger ("rolled off")(Gast and Brungs, 1973), their total volume is measured, and the number of embryos is calculated using a conversion factor of approximately 430 embryos/mL. The embryos are incubated in about 1.5 L of water in a 2 L separatory funnel maintained in a water bath. The embryos are stirred in the separatory funnel by bubbling air from the tip of a plastic micro-pipette placed at the bottom, inside the separatory funnel. During the first two days, the embryos are taken from the funnel daily, those that are dead and fungused are removed, and those that are alive are returned to the separatory funnel in clean water. The embryos hatch in four days at a temperature of 25°C. However, usually on day three the eyed embryos are removed from the separatory funnel and placed in water in a plastic tray and gently aerated with an air stone. Using this method, the embryos hatch in five days.

5.2.8.1.2.1 Hatching time is greatly influenced by the amount of agitation of the embryos and the incubation temperature. If on day three the embryos are transferred from the separatory funnel to a static, unaerated container, a 50% hatch will occur in six days (instead of five) and a 100% hatch will occur in seven days.

5.2.8.1.3 Incubation in Embryo Incubation Cups: The embryos are "rolled off" the substrates, and the total number is estimated by determining the volume. The embryos are then placed in incubation cups attached to a rocker arm assembly (Mount, 1968). Both flow-through and static renewal incubation have been used. On day one, the embryos are removed from the cups and those that are dead and fungused are removed. After day one, only dead embryos are removed from the cups. Most of the embryos will hatch in five days if incubated at 25°C.

5.2.8.1.4 During the incubation period, the eggs are examined daily for viability and fungal growth, until they hatch. Unfertilized eggs, and eggs that have become infected by fungus, should be removed with forceps using a table top magnifier-illuminator. Non-viable eggs become milky and opaque, and are easily recognized. The non-viable eggs are very susceptible to fungal infection, which may then spread throughout the egg mass. Removal of fungused eggs should be done quickly, and the spawning substrates should be returned to the incubation tanks as quickly as possible so that the good eggs are not damaged by desiccation.

5.2.9 LARVAE REARING TANKS

5.2.9.1 Newly-hatched larvae are transferred daily from the egg incubation apparatus to small rearing tanks, using a large bore pipette, until the hatch is complete. New rearing tanks are set up on a daily basis to separate fish by age group. Approximately 1500 newly hatched larvae are placed in a 60-L (15-gal) or 76-L (20-gal) all-glass aquarium for 30 days. A density of 150 fry per liter is suitable for the first four weeks. The water temperature in the rearing tanks is allowed to follow ambient laboratory temperatures of 20-25°C, but sudden, extreme, variations in temperature must be avoided.

5.2.10 HOLDING OR CULTURE TANKS FOR REPLACEMENT SPAWNERS

5.2.10.1 Replacement spawners (brood stock) are cultured from larvae produced in the spawning tanks. After 30 days in a larval rearing tank, a number of juveniles, equivalent to 2-4 days hatch are transferred to brood stock tanks for a 30- to 60-day growth period. The sub-adults then are transferred to 500-L brood stock tanks to provide about 500 sub-adult fish per month for the brood tank rotation. The surplus fish are transferred to 2000-L fiber glass, or equivalent, holding tanks.

5.2.10.2 Surplus young males removed from spawning tanks, and other surplus mature males, are placed in all-male holding tanks for future use as spawners. Similarly, young and surplus mature females are held in all-female holding tanks until needed as spawners. Tanks holding replacement spawners need not be temperature-controlled, but for ease of transfer to the spawning tanks, it is preferable to hold the water temperature close to that of the spawning tanks (25 ± 2°C).

5.2.11 FOOD AND FEEDING

5.2.11.1 Newly hatched brine shrimp nauplii or frozen adult brine shrimp and commercial fish starter are fed to the fish cultures in volumes based on age, size, and number of fish in the tanks. The amount of food and feeding schedule affects both growth and egg production.

5.2.11.2 Fish from hatch to 30 days old are fed starter food at the beginning and end of the work day, and newly hatched brine shrimp nauplii (from the brine shrimp culture unit) twice a day, usually mid-morning and mid-afternoon. Utilization of older (larger) brine shrimp nauplii may result in starvation of the young fish because they are unable to ingest the larger food organisms (see Appendix A.4 for instructions on the preparation of brine shrimp

nauplii). Avoid introducing *Artemia* cysts and empty shells when the brine shrimp nauplii are fed to the fish larvae. Some of the mortality of the larval fish observed in cultures could be caused from the ingestion of these materials.

5.2.11.3 Fish older than four weeks are fed frozen brine shrimp and commercial fish starter (#1 and #2), which is ground fish meal enriched with vitamins. As the fish grow, larger pellet sizes are used, as appropriate. (Starter, No. 1 and N. 2 granules, U.S. Fish and Wildlife Service Formulation Specification Diet SD9-30, can be obtained from Zeigler Bros., Inc., P.O. Box 90, Gardners, PA 17324 (717-780-9009); Glencoe Mills, 1011 Elliott, Glencoe, MN 55336 (612-864-3181); and Murray Elevators, 118 West 4800 South, Murray, UT 84107 (800-521-9092).

5.2.11.4 The spawning fish and pre-spawners in holding tanks usually are fed all the adult frozen brine shrimp and tropical fish flake food or dry commercial fish food (No. 1 or No. 2 granules) that they can eat (ad libitum) at the beginning of the work day and in the late afternoon (i.e., 8:00 AM and 4:00 PM). The fish are feed twice a day, twice with dry food and once with adult shrimp, during the week, and once a day on weekends.

5.2.12 DISEASE CONTROL

5.2.12.1 Fish are observed daily for abnormal appearance or behavior. Bacterial or fungal infections are the most common diseases encountered. However, if normal precautions are taken, disease outbreaks will rarely, if ever, occur. Hoffman and Mitchell (1980) have put together a list of some chemicals that have been used commonly for fish diseases and pests.

5.2.12.2 Treatment of individual lots of infected fish should be carried out separate from the main culture. Use of treated fish should be avoided, if possible, and diseased cultures should be replaced.

5.2.12.3 In aquatic culture systems where filtration is utilized, the application of certain antibacterial agents should be used with caution. A treatment with a single dose of antibacterial drugs can interrupt nitrate reduction and stop nitrification for various periods of time, resulting in changes in pH, and in ammonia, nitrite and nitrate concentrations (Collins et al., 1976). These changes could cause the death of the culture organisms.

5.2.12.4 To prevent possible rapid spread of disease, do not transfer equipment from one tank to another without first disinfecting tanks and nets. If an outbreak of disease occurs, any equipment, such as nets, airlines, tanks, etc., which has been exposed to diseased fish should be disinfected with sodium hypochlorite. Also to avoid the contamination of cultures or spread of disease, each time nets used to remove live or dead fish from tanks, they are first sterilized with sodium hypochlorite or formalin, and rinsed in hot tap water. Before a new lot of fish is transferred to culture tanks, the tanks are cleaned and sterilized as described above.

5.2.13 RECORD KEEPING

5.2.13.1 Records are kept in a bound notebook, include: (1) type of food and time of feeding for all fish tanks; (2) time of examination of the tiles for embryos, the estimated number of embryos on the tile, and the tile position number; (3) estimated number of dead embryos and embryos with fungus observed during the embryonic development stages; (4) source of all fish; and (5) daily observation of the condition and behavior of the fish.

5.2.14 REFERENCE TOXICANTS

5.2.14.1 It is recommended that static acute toxicity tests be performed monthly with a reference toxicant. Fathead minnow larvae one to 14 days old are used to monitor the acute toxicity of the reference toxicant to the test fish produced by the culture unit.

6. VIDEO TRAINING TAPE AVAILABLE FOR CULTURING METHODS

6.1 A video training tape and supplemental report (USEPA, 1989) on culturing fathead minnows are available from the National AudioVisual Center, Customer Services Section, 8700 Edgeworth Drive, Capitol Heights, MD 20743-3701, (Phone 301-763-1891), as part of a video package on short-term chronic toxicity tests for freshwater organisms (Order No. EPA18036), which costs $45.00.

7. TEST ORGANISMS

7.1 Fish one to 14 days old are used in acute toxicity tests.

7.2 If the fish are kept in a holding tank or container, most of the water should be siphoned off to concentrate the fish. The fish are then transferred one at a time randomly to the test chambers until each chamber contain 10 fish. Alternately, fish may be placed one to two at a time into small beakers or plastic containers until they each contain five fish. Two of these beakers/plastic containers (total of 10 fish) are then assigned to each randomly-arranged control and exposure chamber.

7.3 The fish are transferred directly to the test vessels or intermediate chambers using a large-bore, fire-polished glass tube (6 mm to 9 mm I.D. X 30 cm long) equipped with a rubber bulb, or a large volumetric pipet with tip removed and fitted with a safety type bulb filler. The glass or plastic containers should only contain a small volume of dilution water.

7.4 It is important to note that larvae should not be handled with a dip net. Dipping small fish with a net may result in damage to the fish and cause mortality.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.6. RAINBOW TROUT, *ONCORHYNCHUS MYKISS* AND BROOK TROUT, *SALVELINIUS FONTINALIS*

1. RAINBOW TROUT

1.1 SYSTEMATICS AND TAXONOMY

1.1.1 Rainbow trout are native to the streams of the Pacific coast where several varieties or strains have developed. The seagoing form is known as the steelhead trout and is thought to be identical to the strictly freshwater rainbow form. Many other strains, for example, the inland lake form (Kamloops trout) are found in other watersheds. Because of the ease with which the eggs can be transported, different strains have been distributed all over the world.

1.1.2 Rainbow trout are a variable species that differ considerably over the whole of their range. Populations in different regions and watersheds of North America have been referred to over the years by different scientific names (e.g. species, distinct subspecies, or variants of a single species and different regional common names). In recent years the validity of the generic name, *Salmo*, for some western North American trout species has been questioned. Fish taxonomists agree that native "*Salmo*" trouts of the northern Pacific Ocean drainage are closely related with Pacific salmon *Oncorhynchus* spp. The American Society of Ichthyologists and Herpetologists and the American Fisheries Society have accepted *Oncorhynchus* as the appropriate generic name for all native Pacific drainage trouts that are presently called *Salmo*, based on new data and evidence by Smith and Stearly (1989). Furthermore, the Names of Fishes Committee of the American Fisheries Society has adopted the specific name, *Oncorhynchus mykiss*, for the rainbow trout and its anadromous form, steelhead trout. The new names for the other North American species affected are the following: Apache trout (*O. apache*), cutthroat trout (*O. clarki*), Gila trout (*O. gilae*), golden trout (*O. aguabonita*), and Mexican golden trout (*O. chrysogaster*).

1.2 DISTRIBUTION

1.2.1 The native range of the rainbow trout group (all varieties) in North America is west of the Rocky Mountains and along the eastern Pacific Ocean, but the species (*Oncorhynchus mykiss*) has now been introduced into many parts of the continent (Figure 1). Except for the northern and southern extremes of the rainbow trout range, anadromous populations occur in all coastal rivers. This species, under all its common names (rainbow trout, Kamloops trout, steelhead trout, steelhead, coast rainbow trout, and silver trout), has been so widely introduced in North America outside its natural range as to suggest it may occur throughout the United States in all suitable habitats. Rainbow trout are widely introduced and established in appropriate cold water habitats all over the world.

Figure 1. Map showing the distribution of the rainbow trout in North America. (Modified from Lee et al., 1980).

1.3 GENERAL LIFE HISTORY

1.3.1 In its natural environment of flowing streams of the western mountains, the rainbow trout (Figure 2) thrives best at temperatures ranging from 3°C in the winter to 21°C in the summer, but the optimum temperature is between 10-16°C. The rainbow trout can withstand higher and lower temperature if it is acclimated gradually. However, the rainbow trout's growth is impeded by extremes of temperature, for example, above 27°C which it can tolerate only for short periods of time.

1.3.2 Rainbow trout are basically spring spawners, but they can spawn at the beginning of summer or early winter, depending on climate, elevation, and genetic strain. If the spawning occurs in late fall or in winter, the eggs do not hatch until spring. Prior to the spawning season adult males develop a kype (elongated hooked snout) on the lower jaw and their colors intensify. Males and females usually migrate upstream and select spawning sites in beds of fine, clean gravel in riffles or runs above pools in streams. Long journeys may be made by lake-dwelling rainbow (or Kamloops) and steelhead trouts or anadromous, ocean-run rainbow steelheads. If the rainbow trout are confined to land-locked lakes, they move into shallow shoals or reefs of gravel and sand for spawning. Females dig out pits or sweep out depressions (redds) in the gravel or sand and later spawn with males. Males are capable

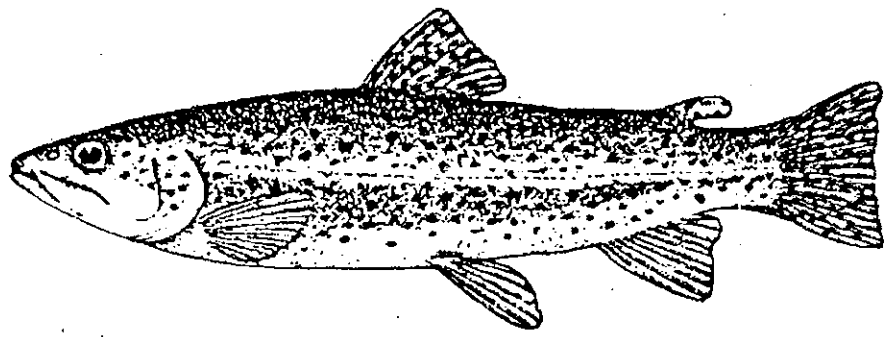

Figure 2. Rainbow trout (Modified from Eddy and Underhill, 1974).

of displaying aggressive behavior on the spawning grounds and can drive other males away from a redd occupied by a female. In general, one or more males court the digging female by sliding along side and crossing over her body and rubbing their snout against her caudal peduncle with body pressing and body vibrations. The female deposits her eggs, which are 3-5 mm in diameter, demersal, and pink to orange in color. The eggs are immediately fertilized by one or more males, fall into spaces between the gravel, and are covered with loose gravel or sand to depths of 20 cm or more by the female. Females are capable of digging and spawning in several redds with the same male or different males. The number of eggs released can range from 400-3000, depending on the size of the female.

1.3.3 Eggs usually hatch in approximately four to seven weeks. The time of hatching, however, varies greatly with region and habitat. If the stream temperature averages 7°C, eggs will hatch in about 48 days. The newly hatched fish, called alevins, have a yolk sac, which is absorbed in three to seven days. After the yolk sac is absorbed, the young are called fry, and begin feeding in 10-15 days. In general, rainbow trout feed on a variety of invertebrates. Also, depending on their size and the habitat in which they live, other fishes and fish eggs, especially salmon, can be important food. The fry of lake-resident spawners move up or down the spawning river to the lake, or they may spend as much as one to three years in the streams. The stream-resident spawners remain in the streams, whereas the steelhead trout, which are stream-spawners, migrate to the sea, usually after 1-4 years in freshwater.

1.3.4 The growth of rainbow trout is highly variable with the area, habitat, type of life history, and quantity and type of food. Some males may be good breeders at two years of age, but few females produce eggs until their third year of life. Rainbow trout young attain fingerling size of about 76 mm by the end of their first summer. The length may range between 178-204 mm at the end of the second year, 279-382 mm after the third year, 356-406 mm after the fourth year, and 406 mm or more after the fifth year. Lake- and ocean-run rainbows may grow over twice as fast as this. However, the average length of

rainbow trout (or Kamloops trout) is 305-458 mm and that of steelhead trout is 508-762 mm. Under favorable conditions of artificial propagation, yearlings average about 28 g, 2-year-olds about 255 g, 3-year-olds between .45-9 kg, and 4-year-olds between 1.4-1.8 kg. Returning sea-run individuals weigh up to 18 kg, or even more, but usually between 1.4-9 kg with the majority weighing less than 5.4 kg. Some western varieties weight up to 23 kg, but the midwest rainbows are much smaller. Those in streams are rarely over 1.4 kg, but in some large lakes (e.g., Lake Superior) and in some western lakes they may reach 7 kg or much larger. The life expectancy of rainbow trout can be as low as three or four years in many streams and Lake populations, but that of seagoing steelhead rainbow trout and Great lakes populations would appear to be 6 to 8 years (Scott and Crossman, 1973).

1.4 GENERAL DESCRIPTION

1.4.1 Adult rainbow trout are bluish or olive green above and silvery on the sides, with a broad pink lateral stripe that is enhanced during the spawning season. The back, the sides, and the dorsal and caudal fins are profusely dotted with small dark spots. Their color is variable with habitat, size, and sexual condition. Stream forms and spawners are generally darker with color more intense, lake forms lighter, brighter, and more silvery. Different color types are often called by different names, e.g., darker stream fish often called rainbows; larger, brighter, silvery fish in western lakes often called Kamloops trout, and large silvery specimens returning from the sea and in the Great Lakes or tributaries called steelhead trout. The scales are large, numbering 120 to 150 in the lateral line. The caudal fin is very slightly forked. The dorsal fin has 11 rays, and the anal fin has from 10 to 12 rays.

1.4.2 Young rainbow trout are typically blue to green on the dorsal surface, silver to white on the sides and white ventrally. There are 5-10 dark marks on the back between the head and dorsal fin. Also, there are 5-10 short, dark, oval parr marks widely spaced on the sides, straddling the lateral line with some small dark spots above but not below the lateral line. The dorsal fin has a white to orange tip and a dark leading edge, or a series of bars or spots. The adipose fin is edged with black, and the anal fin has an orange to white tip.

2. BROOK TROUT

2.1 SYSTEMATICS AND TAXONOMY

2.1.1 Brook trout can be found exhibiting some variation in growth rate and color throughout its range, but is considered a stable and well-defined species (American Fishery Society, 1980). Male brook trout may be crossed with female lake trout (*Salvelinus namaycush*) to produce fertile hybrids that are known as splake. Trautman (1981) and other papers cited in this section indicate that brook trout can naturally and artificially hybridize with brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*). For additional information and discussion on freshwater and anadromous brook trout stocks and systematic notes of brook trout, see Scott and Crossman (1973) and other papers cited in this section.

2.3 GENERAL LIFE HISTORY

2.3.1 Brook trout (Figure 4) are generally found in clear brooks, streams, and rivers in which the mean temperature rarely exceeds 10°C. The optimum temperature is reported as ranging from 7 to 13°C, but they may be found living in waters with temperatures ranging from 1 to 22°C (Piper et al. 1982). The brook trout usually inhabits waters which flow less swiftly than those inhabited by the rainbow. Brook trout also thrive in the small cold-water lakes of the Great Lakes region, provided that suitable spawning conditions exist.

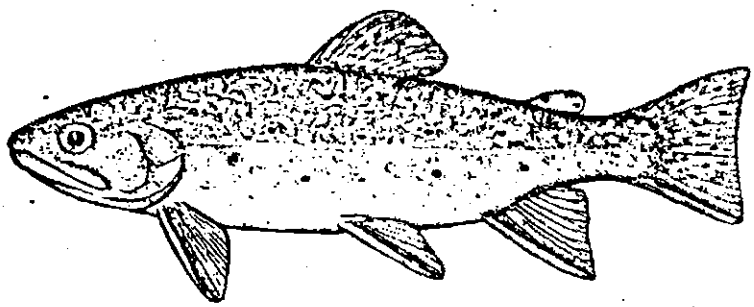

Figure 4. Brook trout. (Modified from Eddy and Underhill, 1974).

2.3.2 Brook trout spawn in late summer or autumn, the date varying with latitude and temperature, usually from late October to December when the water temperature is suitable although some may start spawning in September in certain streams flowing into large lakes. Some females are capable of spawning when they are a year old, while others do not mature until the second year. When the spawning season occurs, brook trout move upstream into small head waters or brooks where they select gravel and sand substrates usually in shallow riffle areas or the tail-ends of pools for the spawning beds. Spawning usually occurs during the day.

2.3.3 The female prepares a nest (redd), similar to those of the rainbow trout, by sweeping out a depression in the gravel and sand substrate. During preparation of the redd, the male starts courtship by quivering around the female and driving off all intruders. When the female is ready to spawn, she takes a position above and close to the redd. The male gets close to her side and arches his body over hers, discharging milt as the female deposits her eggs. Occasionally second male may join them in the spawning. After spawning, the male leaves.

2.3.4 The eggs are 3.5 to 5.0 mm in diameter, are adhesive, and adhere to the gravel at the bottom of the redd. The female pushes loose gravel and sand to

the center, covering the entire redd, and then desert the nest. A female may spawn several times, and the number of eggs can vary from 100 to 5000, depending on the size of the female.

2.3.5 The eggs remain in the redd until the water temperature rises during the following spring. If the level of DO is adequate, the eggs will hatch in approximately 75 days at an average water temperature of 6.1°C, and in approximately 50 days at an average temperature of 10°C. The upper lethal temperature limit for developing eggs is about 11.7°C (Scott and Crossman, 1973).

2.3.6 After the eggs hatch, the larvae (sac fry) remain in the gravel of the redd until the yolk is absorbed. Depending on the water temperature, it may take from one to three months for the yolk sac to be absorbed (Lagler, 1956). While the yolk sac is absorbed, the fry work themselves free from the gravel and start feeding. They become free swimming at about 38 mm long. Under natural conditions, newly hatched brook trout establish small feeding territories in the stream and feed on small aquatic insects, insect larvae, and other organisms.

2.3.7 Growth of brook trout is extremely variable, depending on the suitability of the environment. The average length attained at various ages may approximate 8.9 cm the first year; 15.2 cm the second year, 22.9 cm the third year, 30.5 cm the fourth year, and 33 cm the fifth year. Brook trout generally do not exceed a length of 54 cm and a weight of 1.5 kg (Trautman, 1981). However, Scott and Crossman (1973) reported a brook trout as large as 6.6 kg. Rumors of larger brook trout have been circulated, but none have been verified. Brook trout may overpopulate small streams, resulting in large numbers of small trout less than 25.4 cm long. Wild brook trout seldom live longer than five years, and rarely live more than eight years.

2.4 GENERAL DESCRIPTION

2.4.1 The sides of large young and adult brook trout are dark olive, sprinkled with light spots and red spots outlined with purplish or blue hue. Some forms have red spots with light brown margins. The scales are cycloid, small, in about 215 to 250 rows at the lateral line. The top of the head and back is dark olive and heavily vermiculated. There are no black or brown spots on the head, back, adipose, or caudal fin. The anterior rays of the pectoral, pelvic, and anal fins are milk-white, bordered posteriorly with a dusky hue and the remainder of the fins yellowish or reddish.

2.4.2 The back of young or immature brook trout is olive, the sides are lighter and more silvery, and the belly is whitish. There are between 8-12 rectangular parr marks on the sides, also a few yellow and blue spots, but no black spots.

2.4.3 The dorsal fin has 10 rays, and the anal fin has 9 rays. The belly of breeding males is red, and some males may develop a hook (or kype) at the front of the lower jaw. The tail or caudal fin is slightly notched in the young but is generally square in older brook trout.

3. HOLDING AND ACCLIMATION PROCEDURES FOR TROUT STOCKS

3.1 SOURCES OF ORGANISMS

3.1.1 Trout fry are obtained from commercial hatcheries during March through July. However, if trout are needed for toxicity testing, it is advisable to contact the hatchery for its trout hatching and rearing schedule. If trout must be ordered from out-of-state, the State Fish and Game Agency should be contacted concerning regulations on fish importation. The recommended age for test organisms is approximately 15-30 days (after yolk sac absorption to 30 days) for rainbow trout and 30-60 days for brook trout. Trout are purchased 36 to 48 h prior to their use as testing organisms, but they must have time to stabilize over the acclimation period. Trout should appear disease-free and unstressed, with fewer than 5% of the animals dying during the 24-48 hours preceding use in a toxicity test.

3.1.2 Trout fry are usually transported in plastic bags of at least 4-mil plastic or thicker in shipping containers. The bags are partially filled with water saturated with oxygen. During warm weather the shipping containers are cooled with ice or cold packs to prevent temperature increases which will result in the loss of fish. Trout should be acclimated gradually from the temperature of the transportation unit to that of holding environment. Upon arrival at the destination the plastic bags should be allowed to float unopened in the holding tank for about 30 minutes to acclimate the fish.

3.2 HOLDING CONDITIONS

3.2.1 Trout are held in 200-L (50-gal) or larger tanks supplied with a flow-through water system, or with recirculated water and a biological filtration system. The holding water should be moderately hard and free of chlorine, have low concentrations of metals, and should have a pH between 6 and 9. Provide a daily photoperiod of 16 hours light, 8 hours darkness with an illumination at 10-20 $\mu E/m^2/s$ (50-100 ft-c, or ambient laboratory levels). A 15-min dimmer timer should be used to gradually increase or decrease the illumination when lights are turned on or off. The gradual increase and decrease of illumination at the beginning and ending of the photoperiod is important because trout tend to jump when startled by a sudden change in light intensity. Holding water temperature is maintained at 12°C ± 2°C and is aerated as close as possible to saturation. Measurements of temperature, DO, pH, conductivity, and ammonia are made on holding water daily.

3.3 FEEDING

3.3.1 Trout are fed fine texture trout chow which can be obtained from Zeigler Bros., Inc., P.O. Box 95, Gardners, PA 17324 (717-780-9009); Glencoe Mills, 1011 Elliott, Glencoe, MN 55336 (612-864-3181); and Murray Elevators, 118 West 4800 South, Murray, UT 84107 (800-521-9092). The fry in the holding tank are fed (ad libitum) up to 24 hours before the start of the acute toxicity test. Dead or moribund fish should be removed from the holding tanks every day. Excess food and feces are vacuum-siphoned off the bottom of the tank daily.

3.3.2 Daily records should be maintained for organism survival, health, and acclimation conditions.

4. TEST ORGANISMS

4.1 Rainbow trout fry 15 to 30 days old, and brook trout 30-60 days old, are used in acute tests (see summary tables of test conditions in Section 9, Acute Toxicity Test Procedures). The fry in the holding tank are not fed for 24 hours prior to the start of the test. The fry are caught carefully with a fine mesh net and placed gently in the 5 L (4 L test solution volume) test chambers, until 10 fish are reached per test chamber. Larger test chambers or 5 fish/chambers may be necessary if DO or pH problems are encountered. Placement of the test chambers is random.

4.2 After the fish are introduced, the behavior should be noted and recorded throughout the test period. At the beginning and ending of the photoperiod, during the test, the light intensity should be raised and lowered gradually over a 15-min period using a dimmer switch or suitable device. Between observations the test vessels are covered to act as a dust barrier and to prevent fish from jumping out.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.7. SHEEPSHEAD MINNOW (*CYPRINODON VARIEGATUS*)

1. MORPHOLOGY AND TAXONOMY

1.1 The sheepshead minnow (*Cyprinodon variegatus*) belongs to the family Cyprinodonitidae (killifishes), which includes 45 genera and 300 species worldwide, occurring on all continents except Australia. Most species are freshwater, but some occur in brackish and coastal marine waters. There are thirteen species in the Genus *Cyprinodon* in the United States (American Fisheries Society, 1980). The sheepshead minnow is the only marine species, and is widely distributed in the coastal waters of the Atlantic and Gulf of Mexico.

1.2 Adult sheepshead minnows (see Hardy, 1978, for a complete description), can attain a total length of 93 mm, but the average standard length report for adults is 35-50 mm. The males are usually somewhat longer than females. The fish have the following morphological characteristics: lack a lateral line; have 24-29 lateral scale rows; have a large elongate humeral scale just above the pectoral base; the dorsal fin has nine to 13 rays; the anal fin has nine to 12 rays; the caudal fin has 14-16 principal rays and a total of 28-29 rays; the pectoral fin has 14-17 rays, and the ventral fin has five to seven rays.

1.3 The body of males is short, compressed, and deep. The depth increases with age. The upper profile is evenly elevated. The males are olivaceous above with a lustrous steel blue or bluish green area on the back from nape to dorsal or beyond, and have a series of poorly defined dark bars on the sides and a belly that is yellowish white to deep orange. The dorsal fin ocellus on posterior rays is lacking or developed as faint dusky spot.

1.4 The females are light olive, brown, brassy, or light orange above with 14 dark crossbars on the lower sides alternating with seven to eight crossbars on the back. The lower sides and belly are yellowish or white. The dorsal fin is olive or dusky and has one or two prominent ocelli on the posterior rays.

2. LIFE HISTORY

2.1 DISTRIBUTION AND GENERAL ECOLOGY

2.1.1 Sheepshead minnows occur in estuaries along the Atlantic and Gulf coasts (Figure 1). They are a schooling, euryhaline species that inhabit a variety of shallow water habitats, such as coves, bays, ponds, inlets, harbors, bayous, salt marshes, and along open beaches. In some cases, they may be very abundant where the bottom is partially sandy, emergent vegetation lacking, and little current or wave action are present. This species may establish populations in inland lakes containing relatively high concentrations of

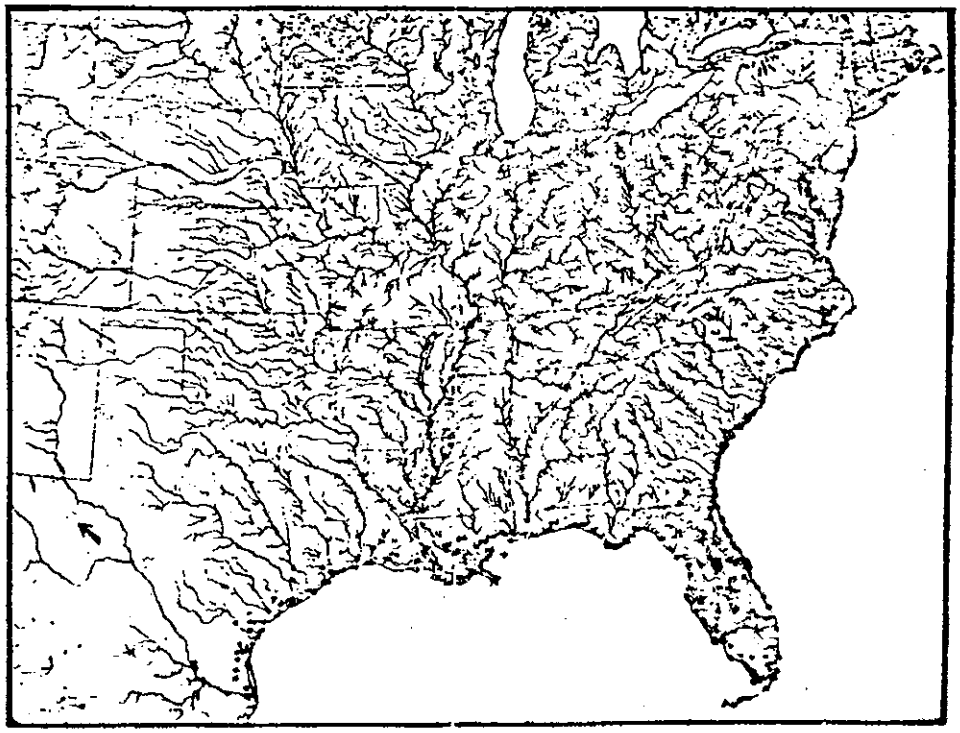

Figure 1. Map showing the distribution of the sheepshead minnow (*Cyprinodon variegatus*) in North America. Open circles represent transplanted populations (From Lee et al., 1980).

dissolved salts. They are tolerant of extreme changes in water temperatures, ranging from 0-40°C, and in salinities, ranging from 0.1 to 149‰ (Simpson and Grunter, 1956; Nordlie, 1987).

2.1.2 This omnivorous fish is an important component of the estuarine ecosystem serving as a link in transferring energy from lower trophic levels, detritus and benthic plants and animals, to carnivores in higher trophic levels (Hansen and Parrish, 1977). Sheepshead minnows serve as forage fish for commercially and recreationally valued fish species, such as the black drum (*Pogonias cromis*), red drum (*Sciaenops ocellata*), bluefish (*Pomatomus saltatrix*), spotted seatrout (*Cynoscion nebulosus*), striped bass (*Morone saxatilis*), and snook (*Centropomus undecimalis*) (Gunter, 1945; Darnell, 1958; Grant, 1962; Sekavec, 1974, and Carter et al., 1973).

2.2 GENERAL SPAWNING BEHAVIOR

2.2.1 Sheepshead minnows (Figures 2, 3, 4) spawn at depths of 2.5 to 60 cm in shallow bays, tide pools, mangrove lagoons, and pools in shallow, gently flowing streams, and other similar habitats over bottoms of sand, black silt, or mud. Males occupy territories up to 0.3-0.6 m in diameter and may or may not construct nest pits. Spawning may take place out of both pit and

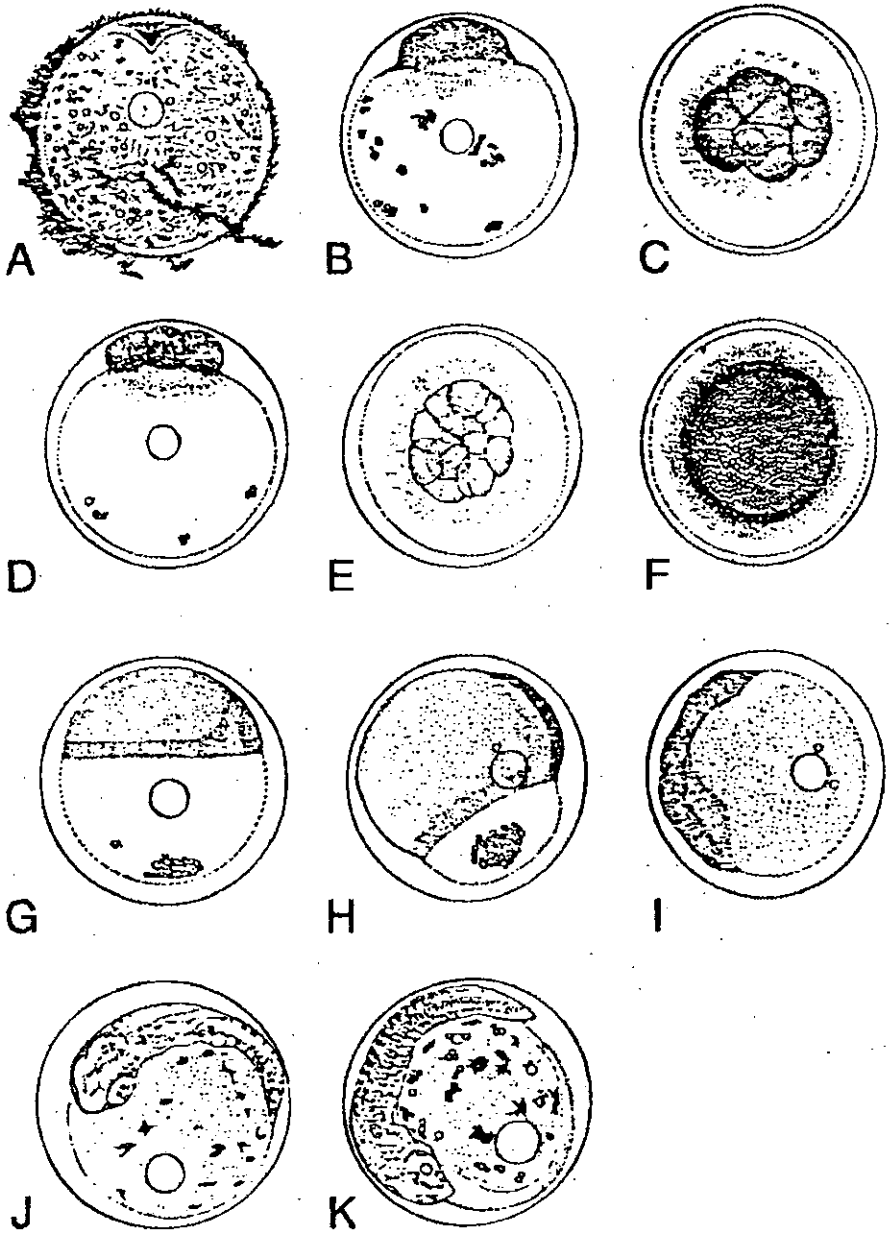

Figure 2. Sheepshead minnow (*Cyprinodon variegatus*). A. unfertilized egg; B. blastodisc stage; C-D. 8-cell stage; E. 16-cell stage; F. late cleavage; G. germ ring formed; H. blastoderm over 1/4 of yolk; I. early embryo; J. embryo 48-hours old; K. tail-free embryo. (From Kuntz, 1916).

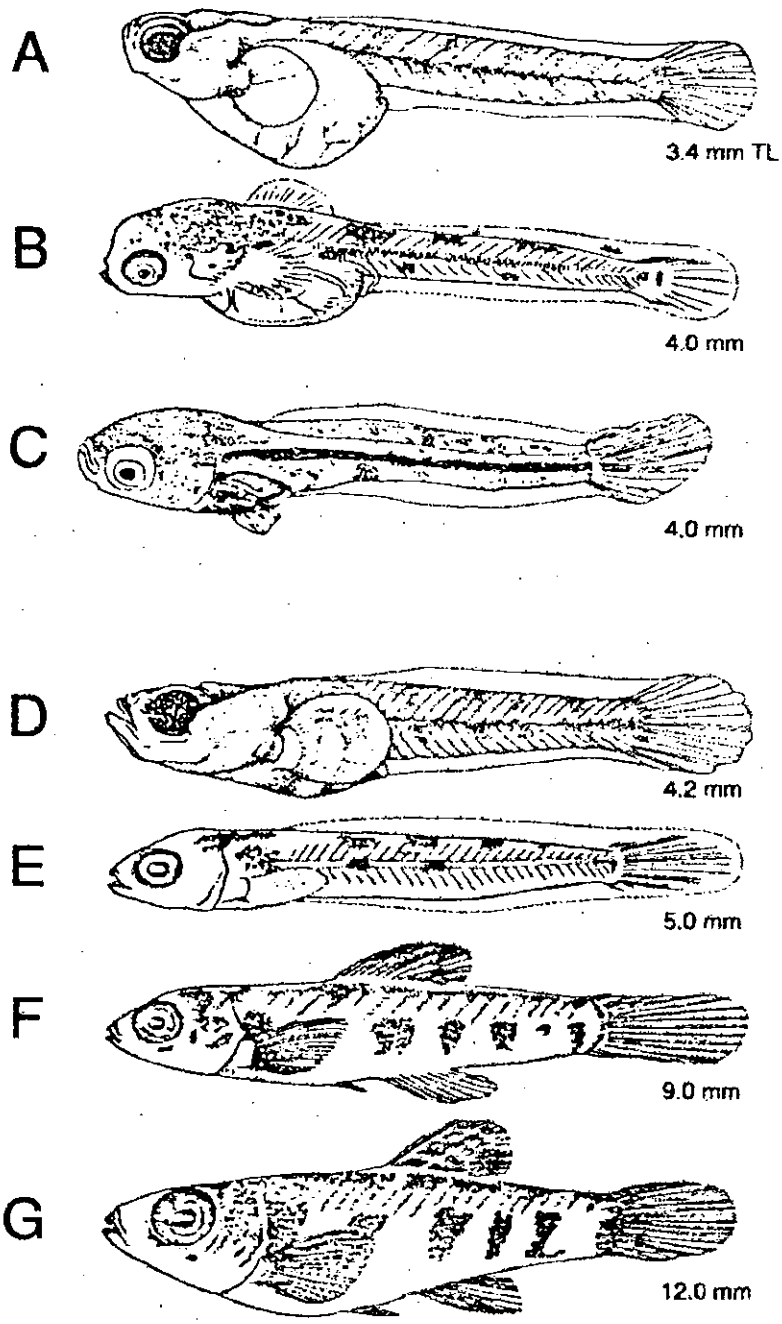

Figure 3. Sheepshead minnow (*Cyprinodon variegatus*). A-E. yolk-sac larvae; F. larvae; G. juvenile; (B-C, E-G, from Kuntz, 1916; A, D, from Foster, 1974).

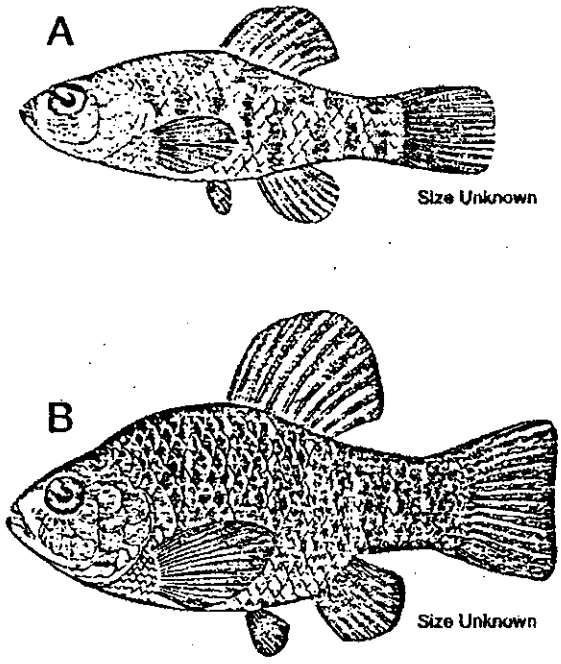

Figure 4. Sheepshead minnow (*Cyprinodon variegatus*). A. juvenile; B. adult (From Jordan and Evermann, 1896-1900).

territory. Besides temperature, Martin (1972) reported that sudden changes in salinity can initiates spawning activities. Eggs (Figure 2) are demersal, adhesive or semi-adhesive with very minute attachment filaments (threads) more or less evenly distributed over the chorion. They stick to a variety of substrates, such as plants, sand, rocks, logs, and to each other. Sometimes they stick to plants near the surface, and at other times become partially buried in the bottom. The yolk contains one very large and many minute oil globules. Adults spawn possibly throughout the year on the Gulf coast of the United States. Hansen and Parrish (1977) reported that in an estuary near Pensacola, Florida, spawning may occur during any month of the year. Ripe females are found April to October in North Carolina, throughout the summer in the Chesapeake Bay, May to August in Delaware Bay, May to September in New Jersey and New York, and June to mid-July in Massachusetts.

3. CULTURE METHODS AND FACILITIES

3.1 SOURCES OF ORGANISMS

3.1.1 Juvenile and adult sheepshead minnows (Figure 4) for use as brood stock spawners may be obtained from commercial biological supply houses or taken by seine in coastal estuaries of the Atlantic coast and Gulf of Mexico. They may also be obtained from young fish raised to maturity in the laboratory. Feral brood stock and first generation laboratory fish may be preferred, to minimize inbreeding. A continuous supply of wild stock, however, may be more cost effective. Neither fish nor eggs of feral stock should contain excessive contaminants nor exhibit excessive mortality, and the fish should demonstrate normal behavior. Before being used as a source of gametes, field-caught adults should be maintained and observed in the laboratory for at least one week to permit detection of disease and to allow time for acute mortality resulting from stress of capture. Injured or diseased fish should be discarded.

3.2 LABORATORY CULTURE FACILITIES

3.2.1 Sheepshead minnows can be cultured in a static, recirculated, or flow-through systems. Flow-through systems require large volumes of water and may not be feasible in some laboratories.

3.3 LABORATORY YEAR-ROUND SPAWNING

3.3.1 In the laboratory, adults may be kept in breeding condition year round. Females may spawn a number of times at intervals of one to seven days, and will generally produce an average of 10 to 30 eggs per spawning (USEPA, 1978a). To obtain large number of eggs at one particular time, adult fish of 27 mm standard length or greater should be used. If fish are taken in the field, they should be acclimated for at least one to two weeks in 20-30‰ salinity, a water temperature of 25-28°C, and a photoperiod of 16 h light and 8 h dark.

3.3.2 Sheepshead minnows can be continuously cultured in the laboratory from eggs to adults. The eggs (embryos), larvae, juveniles, and adults (Figures 2, 3, 4) should be kept in rearing and holding tanks of appropriate size and

maintained at ambient laboratory temperature. The larvae should be fed sufficient newly-hatched *Artemia* nauplii daily to assure that live nauplii are always present. At the juvenile stage, they are fed frozen adult brine shrimp and a commercial flake food, such as TETRA SM-80® (available from Tetra Sales, (U.S.A.), 201 Tabor Rd, Morris Plains, NJ 07950, phone: 800-526-0650), MARDEL AQUARIAM® Tropical Fish Flakes (available from Mardell Laboratories, Inc., 1958 Brandon Court, Glendale Heights, IL 60139, phone: 312-351-0606), or equivalent. Adult fish are fed flake food two or three times daily, supplemented with frozen adult brine shrimp.

3.3.3 Sheepshead minnows normally reach sexual maturity three to five months after hatching, and have an average standard length of approximately 27 mm for females and 34 mm for males, if held at a temperature of 25-30°C in rearing tanks of adequate size, and fed adequately. At this time, the males begin to exhibit sexual dimorphism and initiate territorial behavior. When the fish reach sexual maturity, and are to be used to obtain large number of embryos by natural spawning, the brood stock should be kept in a temperature controlled system at 18-20°C. To initiate spawning, the spawners are moved to spawning tanks with a temperature of 25°C. Adults can be maintained in natural or artificial seawater in a flow-through, static, or recirculating, aerated system consisting of an all-glass aquarium, or a LIVING STREAM® fiberglass, circular or rectangular tank (Figid Unit, Inc., 3214 Sylvania Ave., Toledo, OH 43613, phone 419-474-6971), or equivalent (see USEPA, 1985 and USEPA, 1987).

3.3.4 Static systems are equipped with an undergravel filter. Recirculating systems are equipped with an outside biological filter constructed in the laboratory using a reservoir system of crushed coral, crushed oyster shells, or dolomite and gravel, charcoal, floss, (see Spotte, 1973; 1979, Bower, 1983 for information on filters and conditioning the biological filter), or a commercially available cartridge filter, such as a MAGNUM® Filter, available from Carolina Biological Supply Co., Burlington, NC 27215, phone 800-334-5551, an EHEIM® Filter, available from Hawaiian Marine Imports Inc., P.O. Box 218687, Houston, TX 77218, phone 713-492-7864, or an equivalent system. The culture conditions should include seawater at 20-30‰, and a photoperiod of 16 h light and 8 h dark. Water temperature may be controlled or maintained at ambient laboratory levels.

3.4 OBTAINING EGGS (EMBRYOS) FOR TOXICITY TESTS

3.4.1 Embryos can be shipped to the laboratory from an outside source or obtained from adults held in the laboratory. Ripe eggs can be obtained either by natural spawning or by intraperitoneal injection of the females with human chorionic gonadotrophin (HCG) hormone, available from United States Biochemical Corp., Cleveland, OH 44128, phone, 216-765-5000. If the culturing system for adults is temperature controlled, natural spawning can be induced to obtain large number of embryos by raising the temperature to 25°C. Natural spawning is preferred because repeated spawning can be obtained from the same brood stock, whereas with hormone injection, the brood stock is sacrificed in obtaining gametes. It should be emphasized that the injection and hatching schedules given below are to be used only as guidelines. Response to the hormone varies with brood stock and temperature. Time-to-hatch and percent

hatch also vary among stocks and among batches of embryos obtained from the same stock, and are dependent on temperature, DO, and salinity.

3.5 NATURAL SPAWNING

3.5.1 Adult fish should be maintained at 18-20°C in a temperature controlled system. The number of spawning chambers and fish to be spawned should be based on the requirements for providing sufficient numbers of viable embryos. As indicated above, an adult female in spawning condition will generally produce an average 10 to 30 eggs per spawn. To obtain embryos for a test, adult fish (generally, at least eight-to-ten females and three males) are transferred to a spawning chamber in a 57 L (15 gal) aquarium with the correct photoperiod and temperature (16 h light/8 h dark, and a temperature of 25°C), seven to eight days before the larval fish are needed. The spawning tank is fitted with a spawning chamber and an embryo collection tray. The spawning chamber consists of a basket of 3-5 mm NITEX® mesh, approximately 20 X 35 X 22 cm high (USEPA, 1978a), designed to fit into the aquarium. Spawning generally will begin within 24 h or less. The embryos will fall through the bottom of the spawning chamber and lightly adhere onto a collecting screen or tray placed on the bottom of the tank. The collecting tray should be checked for embryos the next morning. The number of eggs produced is highly variable. The number of spawning units required to provide the fish needed to perform a toxicity test (generally two to four) as determined by experience. If the collecting trays do not contain sufficient embryos after the first 24 h, discard the embryos, replace the tray, and collect the embryos for another 24 h. To help keep the embryos clean, the adults are fed while the screens are removed. Spawning fish should be shielded from excessive outside disturbance, e.g. an opaque curtain should surround the entire culture system. Care should also be taken so that outside light sources do not interfere with the photoperiod.

3.5.2 The embryos are collected in a tray placed on the bottom of the tank. The collecting trays are fabricated from plastic fluorescent light fixture diffusors (grids), with cells approximately 14 mm deep X 14 mm square. A screen consisting of 250-500 μm mesh is attached to one side (bottom) of the grid with silicone adhesive. The depth and small size of the grid protects the embryos from predation by the adult fish. The collecting trays with newly-spawned embryos are removed from the spawning tank, and the embryos are collected from the screens by washing them with a wash bottle or removing them gently with a fine brush. The embryos from several spawning units are generally pooled in a single container to provide a sufficient number to conduct the test(s). The embryos are transferred to a petri dish, or equivalent, filled with fresh culture water, and are examined using a dissecting microscope or other suitable magnifying device. Damaged and infertile eggs are discarded (see Figure 2). The embryos are then placed in incubation dishes (e.g. KIMAX® or PYREX® crystallizing dishes, Carolina culture dishes, or equivalent; see 3.8, Embryo Incubation and Hatching Facility). It is recommended that the embryos be obtained from fish cultured inhouse, rather than from outside sources, to eliminate the uncertainty of damage caused by shipping and handling that may not be observable, but which might affect the results of the test. After sufficient number embryos are

collected for the test, the adult fish are returned to the (18-20°C) culture holding tanks.

3.6 SUSTAINED NATURAL EMBRYO PRODUCTION

3.6.1 Sustained (long-term), daily, embryo production can be achieved by maintaining mature fish (ratio of approximately 12-15 males to 50-60 females) in tanks, such as a 285-L LIVING STREAM® tank, or equivalent, at a temperature of 23-25°C. Embryos are collected seven or eight days prior to starting the acute or chronic toxicity tests for less than 24 hour or older larvae. Embryos are produced daily, and when needed, collecting trays are placed on the bottom of the tank. The next morning, the embryo collectors are removed, and the embryos are washed into a shallow glass culture dish using artificial seawater. Four collecting trays, each approximately 20 cm X 45 cm, will cover the bottom of a 285-L tank.

3.7 FORCED SPAWNING

3.7.1 Human chorionic gonadotrophin (HCG) is reconstituted with sterile saline or Ringer's solution immediately before use. The standard HCG vial contains 1,000 IU, which is reconstituted in 10 mL of saline. Freeze dried HCG, which comes with premeasured and sterilized saline, is the easiest to use. The reconstituted HCG may be used for several weeks if kept in the refrigerator.

3.7.2 Each female is injected with HCG on two consecutive days. The HCG is injected into the peritoneal cavity, just below the skin, using the smallest needle possible. A 50 IU dose (0.5 mL of reconstituted hormone solution) is recommended for females approximately 27 mm in standard length. A larger or smaller dose may be used for fish which are significantly larger or smaller than 27 mm. It may be helpful if fish that are to be injected are maintained at 20°C before injection, and the temperature raised to 25°C on the day of the first injection. Injected females should be isolated from males.

3.7.3 With injections made on days one and two, females which are held at 25°C should be ready for stripping on Days 4, 5, or 6. Ripe females should show pronounced abdominal swelling, and release at least a few eggs in response to a gentle squeeze. Eggs are stripped from the ripe females and mixed with sperm derived from excised, macerated testes. At least ten females and five males are used per test to ensure that there is a sufficient number of viable embryos.

3.7.4 Prepare the testes immediately before stripping the eggs from the females. Remove the testes from three-to-five males. The testes are paired, dark-grey organs along the dorsal midline of the abdominal cavity. If the head of the male is cut off and pulled away from the rest of the fish, most of the internal organs can be pulled out of the body cavity, leaving the testes behind. The testes are placed in a few mL of seawater until the eggs are ready.

3.7.5 Strip the eggs from the females into a dish containing 50-100 mL of seawater, by firmly squeezing the abdomen. Sacrifice the females and remove

the ovaries if all the ripe eggs do not flow out freely. Break up any clumps of ripe eggs and remove clumps of ovarian tissue and under-ripe eggs. Ripe eggs are spherical, approximately 1.0-1.7 mm in diameter, and almost clear. Place the testes in a fold of NITEX® screen (250-500 μm mesh), dampen with seawater, and macerate while holding over the dish containing the eggs. Rinse the testes with seawater to remove the sperm from the tissue, and wash the remaining sperm and testes into the dish with the eggs. Let the eggs and sperm stand together for 10-15 minutes, swirling occasionally.

3.7.6 Pour the contents of the dish into a crystallizing dish or equivalent and insert an airstone. Aerate gently, so that the water moves slowly over the eggs, and incubate at 25°C for 60-90 min. After this period of time, wash the fertilized eggs on a NITEX® screen, place them in clean seawater in an incubation chamber.

3.8 EMBRYO INCUBATION AND HATCHING FACILITY

3.8.1 Embryos are incubated in KIMAX® or PYREX® crystallizing dishes, Carolina culture dishes, or equivalent, at a temperature of 25°C and 14-h light/10-h dark photoperiod. An air stone is placed in each dish, and the contents are gently aerated for the duration of the incubation. The water in the incubation chambers is replaced daily. Approximately 24 h prior to hatching, the salinity of the seawater in the incubation chambers is changed to that of the test salinity, if different. The salinity must remain within the 20 to 30‰ range. The embryos should hatch in 6-7 days at 25°C, and in 4 to 5 days at 30°C.

3.9 FEEDING AND STOCKING DENSITY

3.9.1 The sheepshead minnow cultures should be provided a sufficient amount of high quality nutrition without over-feeding. The adult and juvenile sheepshead minnows are fed, frozen adult brine shrimp and flake food, ad libitum, daily. The larvae are fed newly hatched *Artemia* nauplii and crushed flake food, ad libitum, daily. Methods for culturing brine shrimp are discussed in Appendix A.4. The stocking of adult fish in the holding tanks depends on the biological filter system (see Biological Filters and Substrate Conditioning). A circular, 1.3 m (48 in.) diameter, 880 L (235 gal), fiberglass tank will hold approximately 30-50 adult fish with a varied sex ratio. A stocking density of about 300 larvae is suitable in a 76 L aquarium. Brood stock should be replaced with feral fish annually, or whenever the fecundity of the females diminishes, and they appear spent with age and from frequent breeding.

3.10 CULTURE TANKS

3.10.1 Larvae, juvenile, and adult fish should be kept in holding and rearing tanks of appropriate size. The tanks can be all-glass aquaria, fiberglass tanks, or equivalent. All tanks should have appropriate biological filtration systems, and the culture filtration system should be conditioned properly before adding the fish (see Spotte, 1973, 1979; Bower, 1983).

3.11 BIOLOGICAL FILTERS AND SUBSTRATE CONDITIONING

3.11.1 Holding and rearing aquaria and tanks can accommodate as many fish as its biological filter will permit. The substrate conditioning for the undergravel or outside filters is also important to the life and health of the fish. Substrate conditioning is the process to develop nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) that can convert ammonia and nitrite to nitrate. A conditioned filter bed is defined as one in which the capacity for ammonia and nitrite oxidation is sufficient to keep pace with the production of ammonia by the fish. Consult Spotte (1973; 1979) or Bower (1983) for a thorough understanding of the biological filter and conditioning process.

3.12 CULTURE WATER

3.12.1 Artificial seawater is prepared by dissolving FORTY-FATHOMS® or equivalent artificial sea salts in deionized water to a salinity of 20-30‰. Synthetic sea salts are packaged in plastic bags and mixed with deionized (MILLI-Q® or equivalent) water. The instructions on the package of sea salts should be followed carefully, and the salts should be mixed in a separate container, and not in the culture tank. The deionized water used in hydration should be in the temperature range of 21-26°C. Seawater made from artificial sea salts is conditioned (see Spotte, 1973, 1979; Bower, 1983) before it is used for culturing by aerating mildly for at least 24 h.

3.12.2 Adequate aeration will bring the Ph and concentration of dissolved oxygen and other gases into equilibrium. The concentration of dissolved oxygen in the water supply should be 90-100% saturation before it is used. If a residue or precipitate is present, the solution should be filtered before use. The seawater should be monitored periodically to insure a constant salinity.

3.13 CULTURE CONDITIONS

3.13.1 Holding and rearing tanks and any area used for manipulating live sheepshead minnows should be located in a room or space separated from that in which toxicity test(s) are to be conducted. The salinity of the culture systems should be between 20 and 30‰. Water temperature for the brood stock should be maintained at 18-20°C. A photoperiod of 14 h illumination (10-20 $\mu E/m^2/s$, or 50-100 ft-c) and 10 h dark, should be provided. The holding and rearing tanks should be aerated so that the DO is not less than 1.0 ppm below saturation at any given temperature, with 5.0 ppm (60% saturation) being the absolute lowest limit.

3.14 CULTURE MAINTENANCE

3.14.1 Replace approximately 10% of the culture water every two weeks, or 25% monthly. The culture water should be clear. If the water appears cloudy or discolored, replace at least 50% of it. Replacement water should be well oxygenated and at the same temperature and salinity as the culture water. Salinity is maintained at the proper level by adding deionized water to compensate for evaporation. A replenisher, made of the trace elements, iodine

(KI) and bromine (KBr), is added (1 mL/400 L) to the culture water each week, or commercial trace elements replenisher should be used as directed by the artificial sea salt manufacturer.

3.14.2 To avoid excessive build up of algal growth, periodically scrape the walls of culture system. Some of the algae will serve as a supplement to the diet of the fish. A partial activated carbon "charcoal" change in the filtration systems should be done monthly or as needed. The detritus (dead brine shrimp nauplii and cysts, adult brine shrimp, other organic material accumulation) should be siphoned from the bottom of rearing and holding aquaria or tanks each week or as needed.

3.15 WATER QUALITY MONITORING

3.15.1 Checking the chemistry of the sea water is critical to the success of the marine culture system. The water quality will determine whether the life support processes in the filter bed work at reasonable and steady rates. The culture water is checked routinely for temperature, alkalinity, pH, DO, total ammonia, nitrite, and nitrate. More frequent monitoring of these parameters is recommended during periods of organism procurement and starting new culture systems with inside underground filters and outside-of-tank biological filtration. The DO should be maintained at greater than 60% saturation. The pH should not go below 7.5 with an acceptable range between 7.5 to 8.3. Low pH levels can result from overcrowding, overfeeding, or waste accumulation, especially in static or recirculating culture systems.

3.15.2 Acceptable pH levels can be re-established by siphoning off 50-75% of the water and replacing it with conditioned artificial seawater of the same temperature. Also, sodium bicarbonate or commercially available liquid buffers can be added to the tanks whenever the pH falls below 7.5. Un-ionized ammonia, total (NH_3 + NH_4), and nitrite ion (NO_2) levels should not exceed 0.1 ppm in the holding tanks. It is recommended that the ammonia and nitrite concentrations be determined prior to starting new culture systems. It is recommended that nitrate (NO_3) concentrations be determined prior to starting new culture systems, and the nitrate ion concentrations should not exceed 20 mg/L.

3.15.3 A specific schedule for water quality monitoring should be established for each culture system. All water quality measurements and data are recorded in the culture and environmental conditions log books.

3.16 DISEASE CONTROL AND TREATMENT

3.16.1 Discussions of identification and treatment of common parasites of marine fish culturing can be found in Spotte (1973), Sindermann (1970), and Bower (1983). Several commercial companies, e.g. Aquatronics, P.O. Box 12107, La Costa Station, Malibu, CA 09265; Marine Enterprises, Inc., Baltimore, MD (301) 321-1189; and Hawaiian Marine Imports, Inc., Houston, TX (713) 492-7864, sell various kinds of medication to treat common parasites of marine fish.

3.16.2 A colorless medication, FORMALITE II®, available from Aquatronics, has been used successfully for the treatment of the protozoan parasites, *Chilodonella*, *Costia*, *Trichoina*, *Scyphidia*, *Trichophrya*, and *Ichyophirius*.

4. VIDEO TRAINING TAPE AVAILABLE FOR CULTURING METHODS

4.1 A video training tape and supplemental report (USEPA, 1990) on culturing sheepshead minnows are available from the National AudioVisual Center, Customer Services Section, 8700 Edgeworth Drive, Capitol Heights, MD 20743-3701, (Phone 301-763-1891), as part of a video package on short-term chronic toxicity tests for marine organisms (Order No. AI8545). The package includes methods for sheepshead minnows, inland silversides, sea urchins, and *Champia*, and costs $85.00.

5. TEST ORGANISMS

5.1 Sheepshead minnows 1-14 days old are used in the acute toxicity test. If the larvae are used one or two days after hatching, they can be held in the crystallizing or culture dishes. If they are to be used later, they should be placed in larger holding aquarium or tanks. Prior to beginning the test, the larvae can be transferred to small beakers or plastic cups, using a large-bore, fire-polished glass tube (6 mm to 9 mm I.D. X 30 cm long) equipped with a rubber bulb.

5.2 If the larvae are to be moved to holding aquaria, a large-bore, fire-polished glass tube should also be used to move them. It is important to note that larvae and fry should not be handled with a dip net. Dipping larvae and fry with a net can result in very high mortality. Some of the water in the holding aquarium or tank containing the larvae should be siphoned off before they are transferred using the large-bore tube. This should make them easier to catch. The same large-bore, fire-polished glass tube discussed above should be used to gently transfer the fish from the holding vessels to the test vessels. As the fish are counted, they can be transferred to small plastic cups before they are added to the test vessels. It is more convenient to first transfer five fish to each of several small beakers or plastic containers with a few mL of 20-30‰ saline dilution water. The appropriate number of fish (multiples of five) can then be added to the test vessels.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.8. SILVERSIDES: INLAND SILVERSIDE (*MENIDIA BERYLLINA*), ATLANTIC SILVERSIDE, (*M. MENIDIA*), AND TIDEWATER SILVERSIDE (*M. PENINSULAE*)

1. MORPHOLOGY AND TAXONOMY

1.1 Adult Atlantic silversides attain a total length of up to 117 mm (Figure 1A and 1B). Females in general are slightly larger than males. The first dorsal fin has three to seven, usually four or five spines. The second dorsal fin has one spine and eight or nine rays; the anal fin has one spine and 19 to 29, usually 21 to 26, rays; and the pectoral fin has 12 to 16, usually 14 or 15, rays (Robbins, 1969). Atlantic silverside embryos are easily distinguished from those of the closely related inland silverside, *Menidia beryllina*. The former have a bundle of elastic filaments attached to the chorion at one small area of insertion (Figure 1C and 1D). These filaments, typically longer than the diameter of the egg, are all the same diameter. In contrast, inland silverside eggs posses one or two thick, elongated filaments, up to 50 mm long and four to nine shorter, thinner filaments (Figure 1E and 1F).

2. GENERAL LIFE HISTORY

2.1 DISTRIBUTION

2.1.1 Silversides occur in estuaries along the Atlantic, Gulf, and Pacific coasts (Figures 2-4). The Atlantic silverside, *Menidia menidia*, is a resident of estuaries from Maine to northern Florida. It occurs at intermediate to high salinities, typically of 12 to 30 parts per thousand (ppt), and remains in Atlantic estuaries throughout most of the year (DeSylva et al., 1962; Dahlberg, 1972). Recent evidence indicates an offshore migration at northern latitudes in the fall and reappearance of adults in estuaries in late spring (Conover and Kynard, 1984). This species is an important component in estuarine ecosystems, serving as forage fish for commercially and recreationally valued species such as striped bass, bluefish and spotted seatrout (Merriman, 1941; Bayliff, 1950; Middaugh, 1981).

2.1.2 Although the culturing methods described in this section were written primarily for *Menidia menidia*, they are also suitable for the inland silverside, *M. beryllina*, and the tidewater silverside, *M. peninsulae* (USEPA, 1987). The staff of the Environmental Research Laboratory, Gulf Breeze, Florida, have developed procedures for spawning, culturing, and testing of other fishes, including the California grunion, *Leuresthes tenuis*, and the topsmelt, *Atherinops affinis*. The availability of these fishes as test organisms will permit the use of indigenous fish in toxicity tests of wastes discharged along the entire coast line of the contiguous United States and Alaska.

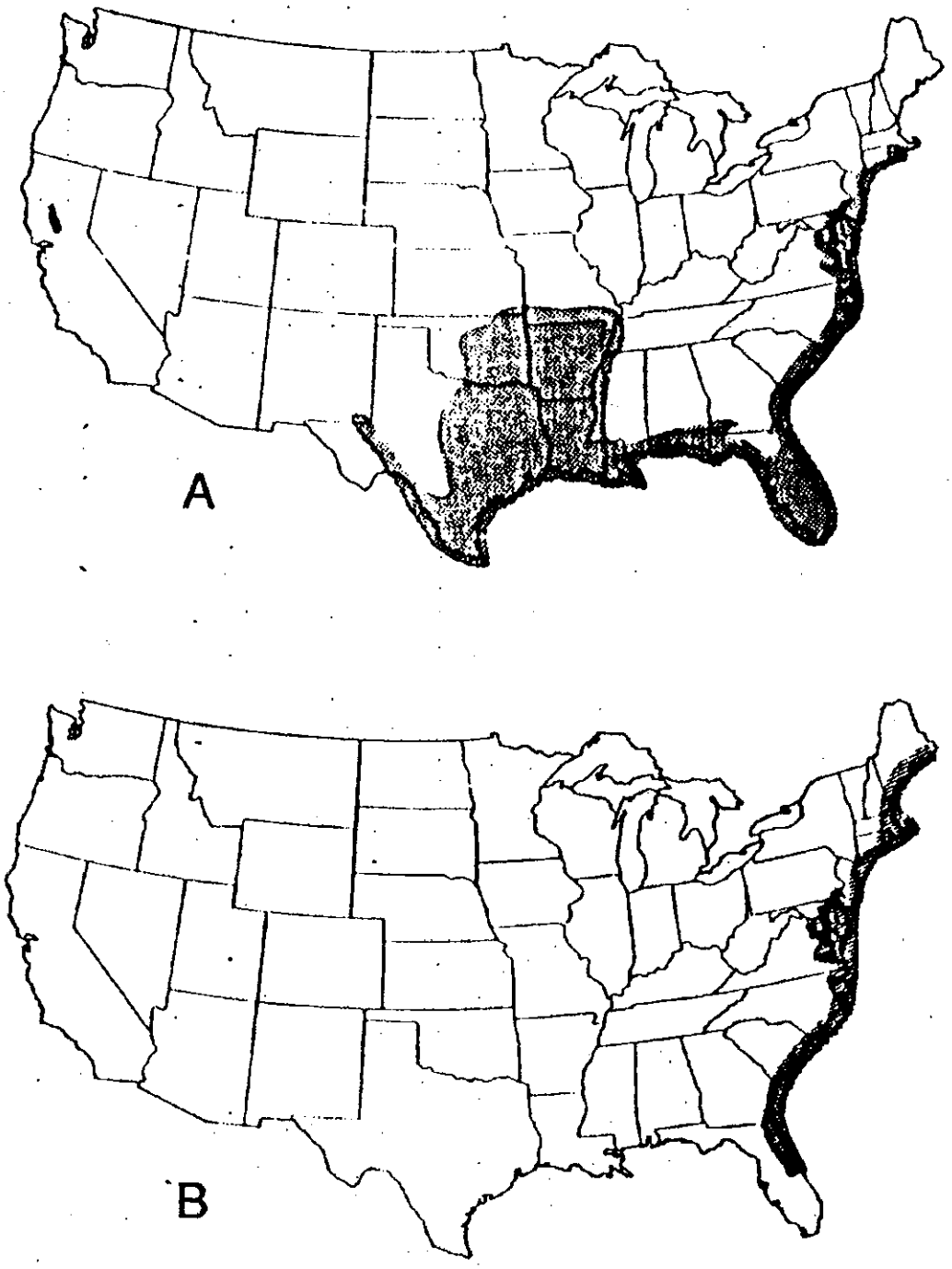

Figure 2. Biographical Distribution: A, inland silverside, *Menidia beryllina*; B. Atlantic silverside, *M. menidia*. (From USEPA, 1987).

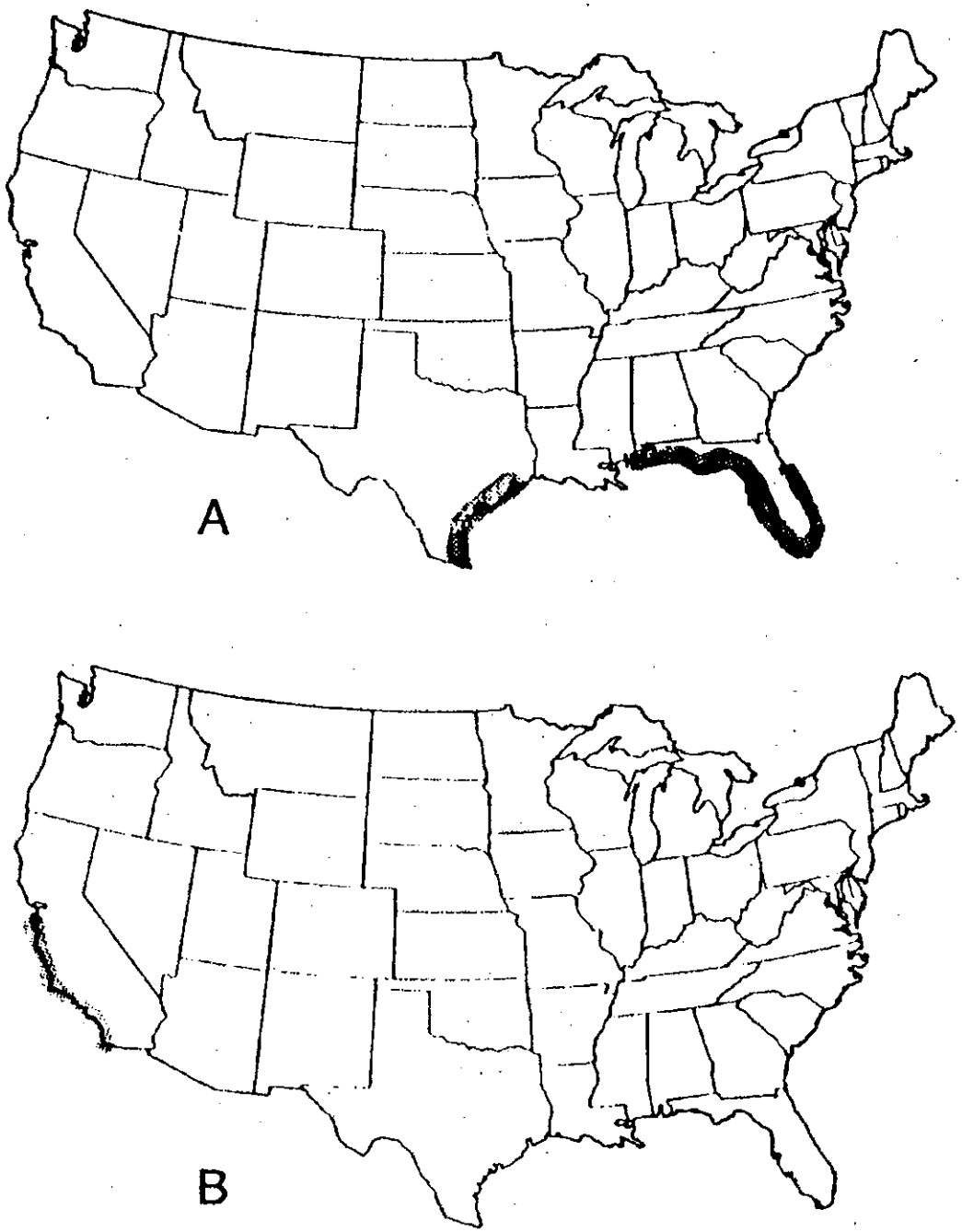

Figure 3. Biogeographical Distribution: A, tidewater silverside, *Menidia peninsulae*; B, California grunion, *Leuresthes tenuis*. (From USEPA, 1987).

Figure 4. Biogeographical Distribution: Topsmelt, *Atherinops affinis*. (From USEPA, 1987).

2.2 SPAWNING BEHAVIOR

2.2.1 The Atlantic silverside spawns during spring and summer. Spawning runs generally occur during April - June or July at northern latitudes, and March through July or August at southern latitudes (Bayliff, 1950; Hildebrand and Schroeder, 1928; Middaugh and Lempesis, 1976). Spawning occurs in the upper intertidal zone during daytime high tides (Middaugh, 1981). Eggs are deposited on a variety of substrates which provide protection from thermal stress and desiccation (Middaugh et al., 1981; Conover and Kynard, 1981). Females typically release 200 to 800 eggs, 1.0-1.2 mm diameter, as they spawn. Individuals may spawn up to five or six times, at two week intervals, during the reproductive season. The life span is generally 12-15 months, although year class-2 fish are occasionally found (Beck, 1979).

3. **CULTURING METHODS**

3.1 SOURCES OF ORGANISMS

3.1.1 *Menidia* may be obtained from commercial biological supply houses or collected in the field.

3.1.1.1 The optimal time for collecting ripe *M. menidia* in the field is just prior to daytime high tides between 8:00 AM and noon (usually one to four days after the occurrence of a new or full moon), when prespawning schools move into the upper intertidal zone (Middaugh, 1981; Middaugh et al., 1981). Since the Atlantic silverside prefers relatively high salinities, it is recommended that collections be made in areas with salinities of 20‰ or greater. Sandy

beaches, bordering open but protected estuarine bays, are suitable for collecting adults. A 1 X 10-m bag seine with knotless 5-mm mesh is ideal for collecting. Since Atlantic silversides typically reside in shallow water, 1.5 m deep, they are easily captured by seining close to shore. It is important to avoid total beaching of the bag seine when collecting *M. menidia*. These fragile fish will quickly die if removed from water and, more importantly, ripe females often abort their eggs if stranded. Ideally, the bag portion of the seine, containing captured adults, should remain in water 5-15 cm deep (Middaugh and Lempesis, 1976).

3.1.1.2 It is possible to transport the spawn (fertilized eggs) or adults to the laboratory. The following procedure is recommended for stripping, fertilizing and transporting eggs from the field to the laboratory:

1. Immediately after seining (while still on the beach) three to five ripe females should be dipped into a bucket of seawater to remove sand and detritus.
2. Eggs are stripped into a glass culture dish containing seawater or onto a nylon screen (0.45 to 1.0 mm mesh) (Figure 5), which is then gently lowered into a culture dish of seawater with the eggs on the upper surface of the screen (Barkman and Beck, 1976). If excessive pressure is required to strip the eggs, the female should be discarded. Mature eggs, 1.0-1.2 mm in diameter, are clear, and have an amber hue.
3. Milt from several males can then be stripped into the culture dish and mixed with the eggs by gently tilting the dish from side to side. Upon contact with seawater, adhesive threads on mature eggs uncoil, making enumeration and separation difficult. If eggs are stripped directly into the culture dish, one end of a nylon string may be dipped into the dish and gently rolled so the embryos adhere (Middaugh and Lempesis, 1976). The Barkman and Beck (1976) technique for attaching the eggs to nylon screening minimizes the natural clumping tendency due to entanglement of the filaments on M. menidia eggs.
4. Strings of embryos or embryos on screens may be transported to the laboratory by placing them in an insulated glass container filled with seawater at the approximate temperature and salinity of fertilization. If gravid fish are transported to the laboratory for subsequent spawning, care must be taken to avoid overcrowding of fish in transport containers. Continuous, vigorous aeration is required and any increase in container water temperature should be minimized (Beck, 1979). A mass culture system for incubating the screen-adhered eggs and collecting the hatched larvae in a flowing seawater system (Figure 5) was described in detail by Beck (1979). A similar procedure utilizing a recirculating system was described by Middaugh and Lempesis (1976).

3.2 Laboratory Year-round Spawning

3.2.1 Atlantic, inland, and tidewater silversides may be spawned in the laboratory on a year-round basis. Procedures described by Middaugh and Takita (1983), and Middaugh and Hemmer (1984), provide for maintenance of a brood stock of 30 to 50 fish, sex ratio 1:1, in 1.3 m diameter, circular holding tanks which are part of a recirculating seawater system (Figure 6). The photoperiod should be adjusted to 14 L:10 D (lights on at 5:00 AM and off at

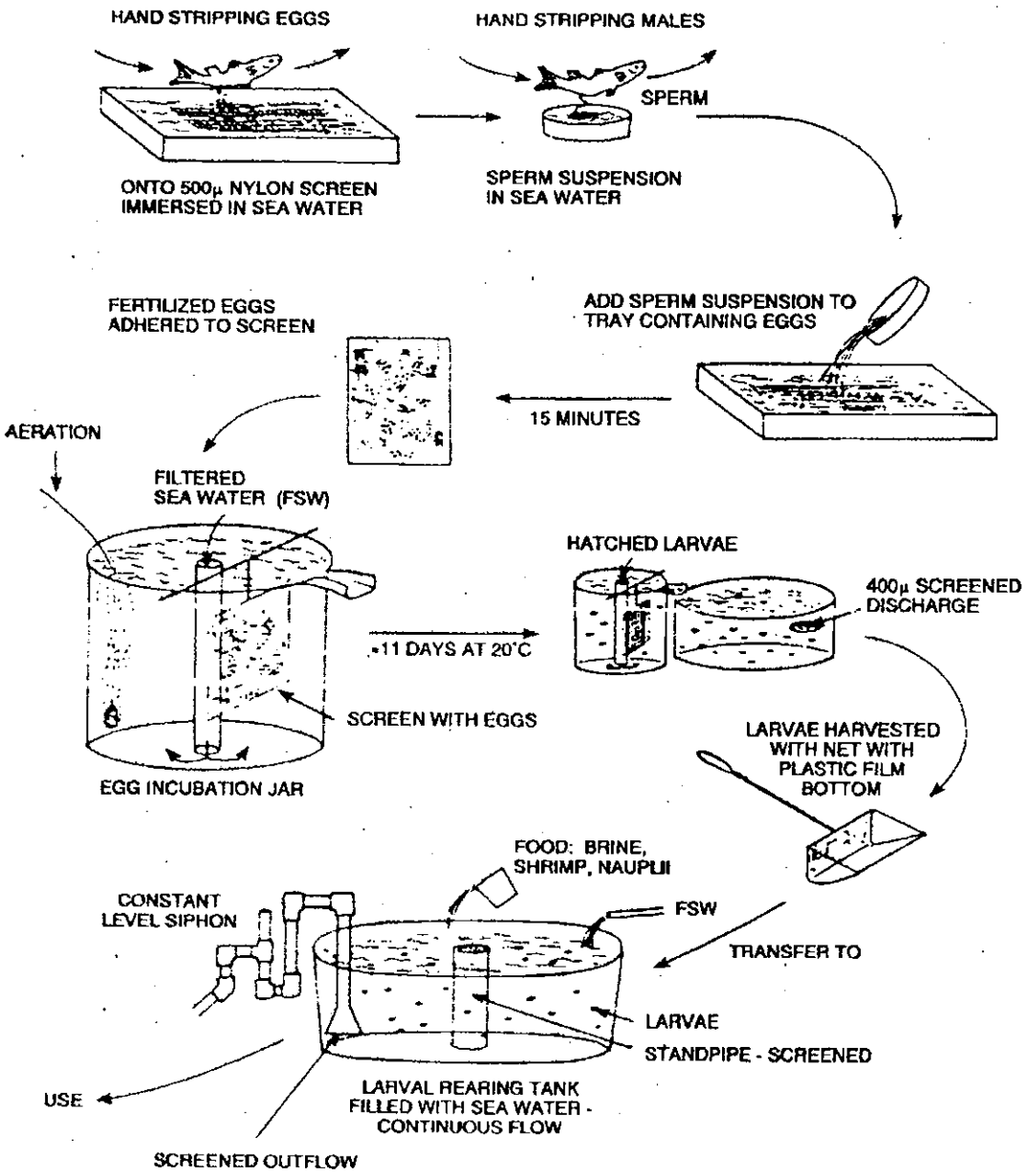

Figure 5. Techniques for collection of silverside eggs in the field, and production of larvae in the laboratory (From Beck, 1979).

7:00 PM, intensity 10-20 $\mu E/m^2/s$, or 50-100 ft c), with the water temperature maintained at 18-20°C for fish from northern latitudes, and 20-25°C for southern latitudes. Suitable salinities for the culture units would be 25-30 ppt for the Atlantic and tidewater silversides, and 7‰ for the inland silverside. Fish are fed 8 g TETRAMIN® each morning and afternoon, and concentrated *Artemia* nauplii (hatch obtained from approximately 15 mL of eggs after 48 h of incubation at 25°C) in mid-afternoon (see section on *Artemia* culture). Excess food should be siphoned from the holding tanks weekly. Filter media (activated charcoal) located in a reservoir tray should be changed weekly, immediately after cleaning the holding tanks. To induce spawning by the Atlantic silverside, the circulation current velocity in the holding tanks should be reduced to zero (from 8 to 0 cm/sec) twice daily by turning off the seawater circulation pump from midnight to 1:00 AM, and from noon to 1:00 PM. Atlantic silversides will spawn in response to interrupted current velocities during daytime (noon to 1:00 PM). Spawning of the tidewater silverside also is enhanced by reducing the current velocity twice daily, but spawns primarily during nighttime. No interruption in current is necessary to enhance spawning by the inland silverside.

3.2.2 A suitable spawning substrate can be made by cutting enough 25 cm lengths of No. 18 nylon string to form a small bundle, and tying a string around the middle of the bundle to form a "mop." The mop is suspended just below the surface of the water, in contact with the side of the holding tanks. Spawning fish will deposit eggs on this substrate. The mops are removed from the holding tanks daily and suspended in incubation vessels. Typical egg production ranges from 300 to 1200 per spawn. Fish generally can be expected to spawn three to four days each week.

3.2.3 It is essential that light-tight curtains surround the holding tanks. These curtains should remain closed except during periodic feedings, tanks cleaning, and during removal and replacement of spawning substrates.

3.2.4 Embryos attached to nylon screening or nylon string may be suspended in a culture system such as shown in Figure 6. The culture chambers for embryos should be constructed of glass. Upon hatching, larvae may be transferred from the collection container to a 90-cm diameter glass or fiberglass tank with a volume of 350 L. Tanks receive a continuous flow of seawater at 2 L/min. Water is introduced at the tank periphery causing a gentle current sufficient to induce orientation to water movement and normal schooling behavior. Water is discharged from the tank by two automatic siphons. Siphon openings are protected by a 400 μm nylon screen to prevent escape of larvae. An inverted funnel is used at the siphon to decrease the velocity of discharge water, thus preventing impingement of larvae.

3.2.5 Embryos can also be incubated in small (4-10 L) glass aquaria, by placing the nylon screening or strings just below the surface of the water. Gentle aeration should be provided by an airstone positioned near the bottom of the holding aquaria.

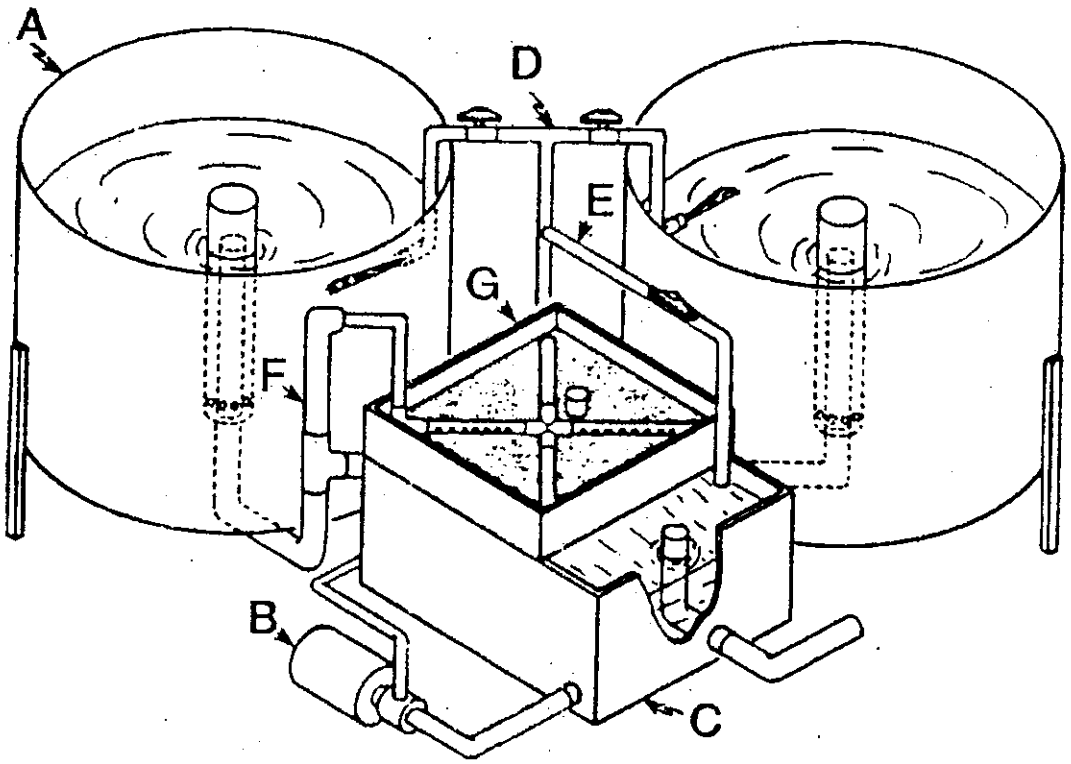

Figure 6. Holding and spawning system utilized in the culture of silversides (*Menidia*). A, 1.3 m diameter tanks; B, circulation pump; C, reservoir; D, seawater distribution system; E, by-pass line; F, seawater return line; and G, reservoir filter system. (From Middaugh and Hemmer, 1984).

3.3 CULTURE MEDIA

3.3.1 Use natural seawater if it is available and unpolluted. Otherwise use synthetic seawater prepared by adding artificial marine salts, such as FORTY FATHOMS®, to deionized water. If synthetic seawater is used, it should be aged for a least one week before being utilized in culture aquaria.

3.4 CULTURE CONDITIONS

3.4.1 The salinity maintained during incubation should be similar to that of the water from which the adults were taken, if collected in the field, or at which the adults are being maintained in the laboratory, if the embryos originate from laboratory brood stock. Water temperature should be maintained at 20 to 25°C depending upon the latitude where fish are collected. Provide a photoperiod of 12-14 h of illumination daily at 10-20 $\mu E/m^2/s$, or 50-100 ft-c (12 h minimum light/24 h). Embryos will hatch in seven to 14 days, depending upon the incubation temperature and salinity (Middaugh and Lempesis, 1976).

3.5 FEEDING AND STOCKING DENSITY

3.5.1 Upon hatching, *Menidia* larvae should be fed immediately. Newly hatched brine shrimp (*Artemia*) nauplii (less than eight hours old) are fed to the larvae twice daily. It is essential to feed *M. menidia* and *M. peninsulae* larvae newly-hatched brine shrimp nauplii (USEPA, 1987). Utilization of older, larger, brine shrimp nauplii will result in starvation of the larvae since they are unable to ingest the larger food organisms. Three to four days after hatching, the fish are able to consume older (larger) brine shrimp nauplii. Because of their small size *M. beryllina* larvae must be fed a mixohaline rotifer, *Branchionus plicatilus* from day of hatch through day five. Thereafter, they are able to consume newly-hatched and older *Artemia* nauplii (USEPA, 1987; USEPA 1988). Methods for culturing brine shrimp are discussed in the Appendix. A stocking density of about 300 larvae is suitable in an 76-L aquarium.

3.6 CULTURE MAINTENANCE

3.6.1 To avoid excessive build up of algal growths, periodically scrape the walls of aquaria. Activated charcoal in the aquarium filtration systems should be changed weekly and detritus (dead brine shrimp nauplii or cysts) siphoned from the bottom of holding aquaria each week. Salinity may be maintained at the proper level by addition of distilled or deionized water to compensate for evaporation.

4. VIDEO TRAINING TAPE AVAILABLE FOR CULTURING METHODS

4.1 A video training tape and supplemental report (USEPA, 1990) on culturing inland silversides are available from the National AudioVisual Center, Customer Services Section, 8700 Edgeworth Drive, Capitol Heights, MD 20743-3701 (Phone 301-763-1891), as part of a video package on short-term chronic toxicity tests for marine organisms (Order No. A18545). The package includes methods for sheepshead minnows, inland silversides, sea urchins, and *Champia*, and costs $85.00.

5. TEST ORGANISMS

5.1 Fish one to 14 days old are used in acute toxicity tests. Most of the water in the holding aquarium should be siphoned off before removal of larvae. Larvae can then be siphoned from the holding tanks into a holding vessel. It is essential that larvae not be handled with a dip net, because it will result in very high mortality. A large-bore, fire-polished glass tube, 6 mm I.D. x 500 mm long (1/4 in. ID X 18 in. long), equipped with a rubber squeeze bulb should be used to transfer the larvae from the holding vessel to the test vessels. It is more convenient to first transfer five fish to each of several small beakers containing 20 mL of saline dilution water. The appropriate number of fish (multiples of five) can then be added to test vessels.

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APPENDIX B

SUPPLEMENTAL LIST OF ACUTE TOXICITY TEST SPECIES

TEST ORGANISM		TEST TEMP (°C)	LIFE STAGE
FRESHWATER SPECIES: VERTEBRATES - WARMWATER			
Cyprinella leedsi[1]	Bannerfin shiner	25	1-14 days
Lepomis macrochirus	Bluegill sunfish	20,25	" "
Ictalurus punctatus	Channel catfish	"	" "
FRESHWATER SPECIES: INVERTEBRATES - COLDWATER			
Pteronarcys spp.	Stoneflies*	12	larvae
Pacifastacus leniusculus	Crayfish*	"	juveniles
Baetis spp.	Mayflies*	"	nymphs
Ephemerella spp.	"	"	"
FRESHWATER SPECIES: INVERTEBRATES - WARMWATER			
Hyalella spp.	Amphipods	20,25	juveniles
Gammarus lacustris	"	"	"
G. fasciatus	"	"	"
G. pseudolimnaeus	"	"	"
Hexagenia limbata	Mayflies	"	nymphs
H. bilineata	"	"	"
Chironomus spp.	Midges	"	larvae

*Stoneflies, crayfish, and mayflies may have to be field collected and acclimated for a period of time to ensure the health of the organisms and that stress from collection is past. Species identification must be verified.

[1]Test conditions for *Cyprinella leedsi* are found in Table 13.

SUPPLEMENTAL LIST OF ACUTE TOXICITY TEST SPECIES (CONTINUED)

TEST ORGANISM		TEST TEMP (°C)	SALINITY (‰)	LIFE STAGE
MARINE AND ESTUARINE SPECIES: VERTEBRATES - COLDWATER				
Parophrys vetulus	English sole	12	32-34	1-90 days
Citharichys sitigmaeus	Sanddab	"	"	" "
Pseudopleuronectes americanus	Winter flounder	"	"	post metamorphosis
MARINE AND ESTUARINE SPECIES: VERTEBRATES - WARMWATER				
Paralichthys dentatus	Flounder	20,25	32-34	1-90 days
P. lethostigma	"	"	"	" "
Fundulus similis	Killifish	"	20-32	1-30 days
Fundulus heteroclitus	Mummichog	"	25-32	" "
Lagodon rhomboides	Pinfish	"	20-32	1-90 days
Orthipristis chrysoptera	Pigfish	"	15-30	" "
Leostomus xanthurus	Spot	"	10-30	" "
Gasterosteus aculeatus	Threespine stickleback	"	20-32	1-30 days
Atherinops affinis	Topsmelt	21	10-30	7-15 days

SUPPLEMENTAL LIST OF ACUTE TOXICITY TEST SPECIES (CONTINUED)

TEST ORGANISM		TEST TEMP (°C)	SALINITY (‰)	LIFE STAGE
MARINE AND ESTUARINE SPECIES: INVERTEBRATES - COLDWATER				
Pandalus jordani	Oceanic shrimp	12	25-32	juvenile
Strongylocentrotus droebachiensis	Green sea urchin	"	32-34	gametes/embryo
Strongylocentrotus purpuratus	Purple sea urchin	"	"	" "
Dendraster excentricus	Sand dollar	"	"	" "
Cancer magister	Dungeness crab	"	"	juvenile
Holmesimysis costata[2]	Mysid	"	"	1-5 days
MARINE AND ESTUARINE SPECIES: INVERTEBRATES - WARMWATER				
Callinectes sapidus	Blue crab	20,25	10-30	juvenile
Palaemonetes pugio,	Grass shrimp	"	10-32	1-10 days
P. vulgaris,	" "	"	"	" "
P. intermedius	" "	"	"	" "
Penaeus setiferus	White shrimp	"	20-32	post-larval
Penaeus duorarum	Pink shrimp	"	"	" "
Penaeus aztecus	Brown shrimp	"	"	" "
Crangon septemspinosa	Sand shrimp	"	25-32	" "
Mysidopsis almyra	Mysid	"	10-32	1-5 days
Neomysis americana	"	"	"	" "
Metamysidopsis elongata	"	"	"	" "
Crassostrea virginica	American oyster	"	20-32	embryo
Crassostrea gigas	Pacific oyster	"	25-32	"
Arbacia punctulata	Purple sea urchin	"	32-34	gametes/embryo

[2]Test conditions for *Homesimysis costata* are found in Table 15.

APPENDIX C

DILUTOR SYSTEMS

Two proportional dilutor systems are illustrated: the solenoid valve system. and the vacuum siphon system.

1. Solenoid and Vacuum Siphon Dilutor Systems

The designs of the solenoid and vacuum siphon dilutor systems incorporate features from devices developed by many other Federal and state programs, and have been shown to be very versatile for on-site bioassays in mobile laboratories, as well as in fixed (central) laboratories. The Solenoid Valve system is fully controlled by solenoids (Figures 1, 2, and 3), and is preferred over the vacuum siphon system. The Vacuum Siphon system (Figures 1, 4, and 5), however, is acceptable. The dilution water, effluent, and pre-mixing chambers for both systems are illustrated in Figures 6, 7, and 8. Both systems employ the same control panel (Figure 9).

If in the range-finding test, the LC50 of the effluent falls in the concentration range, 6.25% to 100%, pre-mixing is not required. The pre-mixing chamber is bypassed by running a TYGON® tube directly from the effluent in-flow pipe to chamber E-2 (see Figures 3 and 5), and Chambers E-1 and D-1 and the pre-mixing chamber are deactivated.

The dilutor systems described here can also be used to conduct tests of the toxicity of pure compounds by equipping the control panel with an auxiliary power receptacle to operate a metering pump to deliver an aliquot of the stock solution of the pure compound directly to the mixing chamber during each cycle. In this case, chamber E-1 is de-activated and chamber D-1 is calibrated to deliver a volume of 2000 mL, which is used to dilute the aliquot to the highest concentration used in the toxicity test.

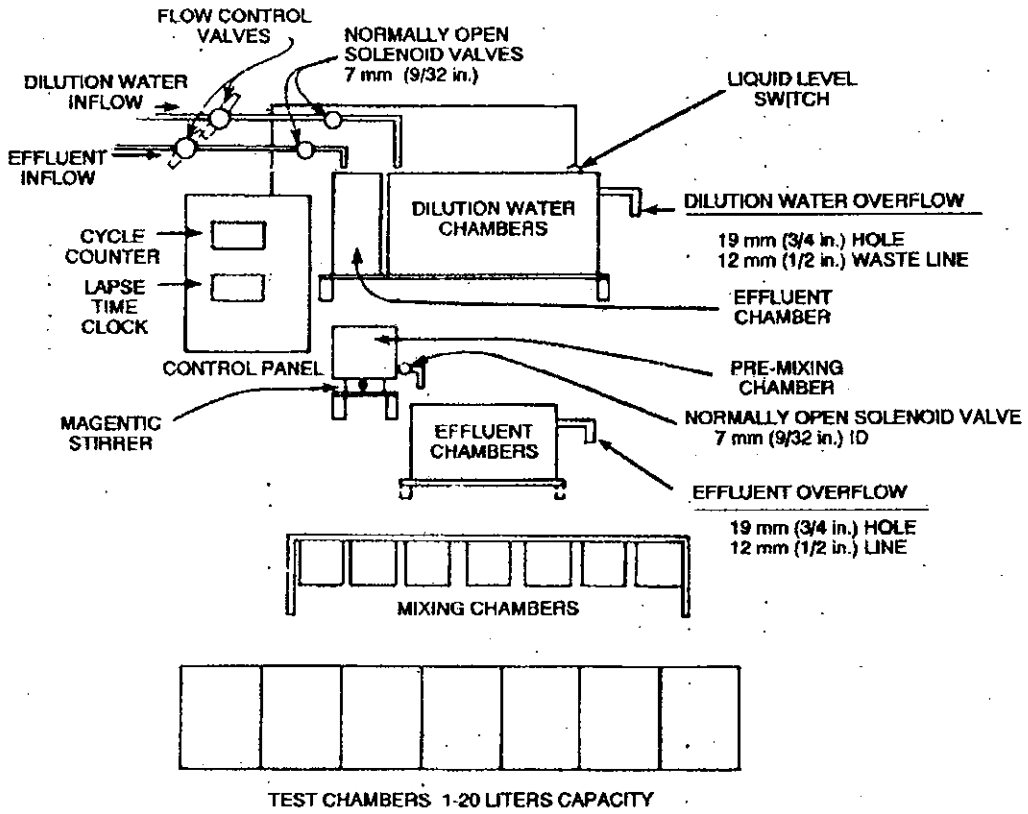
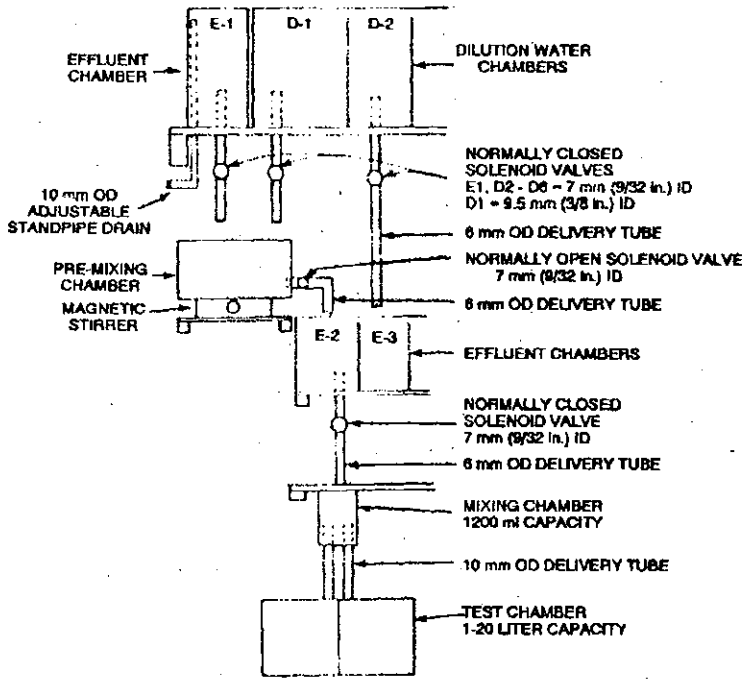


Figure 2. Solenoid valve dilutor system, general diagram (not to scale).



NOTE: WHEN 100% EFFLUENT IS USED AS THE HIGHEST EFFLUENT CONCENTRATION, E-1, D-1, AND THE PRE-MIXING CHAMBER ARE BYPASSED BY CONNECTING A TYGON TUBE TO THE EFFLUENT INFLOW, AND RUNNING IT DIRECTLY TO E-2. IN THIS CASE, SOLENOIDS FOR E-1 AND D-1, AND THE PRE-MIXING CHAMBER ARE DISCONNECTED. D-2 + E-3 = 50% EFFLUENT; D-3 + E-4 = 25% EFFLUENT, ETC.

Figure 3. Solenoid valve dilutor system, detailed diagram (not to scale).

SOLENOID SYSTEM EQUIPMENT LIST

1. Dilator Glass.
2. Stainless Steel Solenoid Valves
   a. 3, normally open, two-way, 55 psi, water, 1/4" pipe size, 9/32" orifice size, ASCO 8262152, for incoming effluent and dilution water pipes and mixing chamber pipe.
   b. 1, normally closed, two-way, 15 psi, water, 3/8" pipe size, 3/8" orifice size, ASCO 8030B65, for D-1 chamber evacuation pipe.
   c. 12, normally closed, two-way, 36 psi, water, 1/4" pipe size, 9/32" orifice size. ASCO 8262C38, for remaining dilution chambers (D2-D6) and effluent chamber (E1-E6) evacuation pipes.
3. Stainless steel tubing, seamless, austenitic, 304 grade for freshwater and 316 grade for saline water.
   a. 10 ft of 3/8" OD, 0.035" wall thickness, for dilution water and effluent pipes.
   b. 60 ft of 1/4" OD, 0.035" wall thickness, for dilution water and effluent pipes.
   c. 1 ft of 3/4" OD, 0.035" wall thickness, for standpipe in D1 chamber.
4. Swagelok tube connectors, stainless steel.
   a. 4, male tube connectors, male pipe size 1/4", tube OD 3/8".
   b. 2, male tube connectors, male pipe size 1/2", tube OD 3/8".
   c. 26, male tube connectors, male pipe size 1/4", tube OD 1/4".
   d. 2, male tube connectors, male pipe size 3/8", tube OD 3/8".
   e. 2, male adaptor, tube to pipe, male size 1/2", tube OD 3/8".
5. 7, 1200 mL stainless steel beakers.
6. Several lbs each of Neoprene stoppers, sizes 00, 0, and 1; 1 lb of size 5.
7. 14 - aquarium (1-20 liters).
8. Magnetic stirrer.
9. 2 - PVC ball valves, 1/2" pipe size.
10. Dilutor control panel - see Fig. 32 and equipment list.
11. Plywood sheeting, exterior grade: one - 4' x 8' x 3/4", one - 4' x 8' x 1/2".
12. Pine or redwood board, 1" x 8", 20 ft.
13. Epoxy paint, 1 gal.
14. Assorted wood screws, nails, etc.
15. 25 ft - 14" ID, TEFLON® tubing, to connect the mixing chambers to the test chambers.

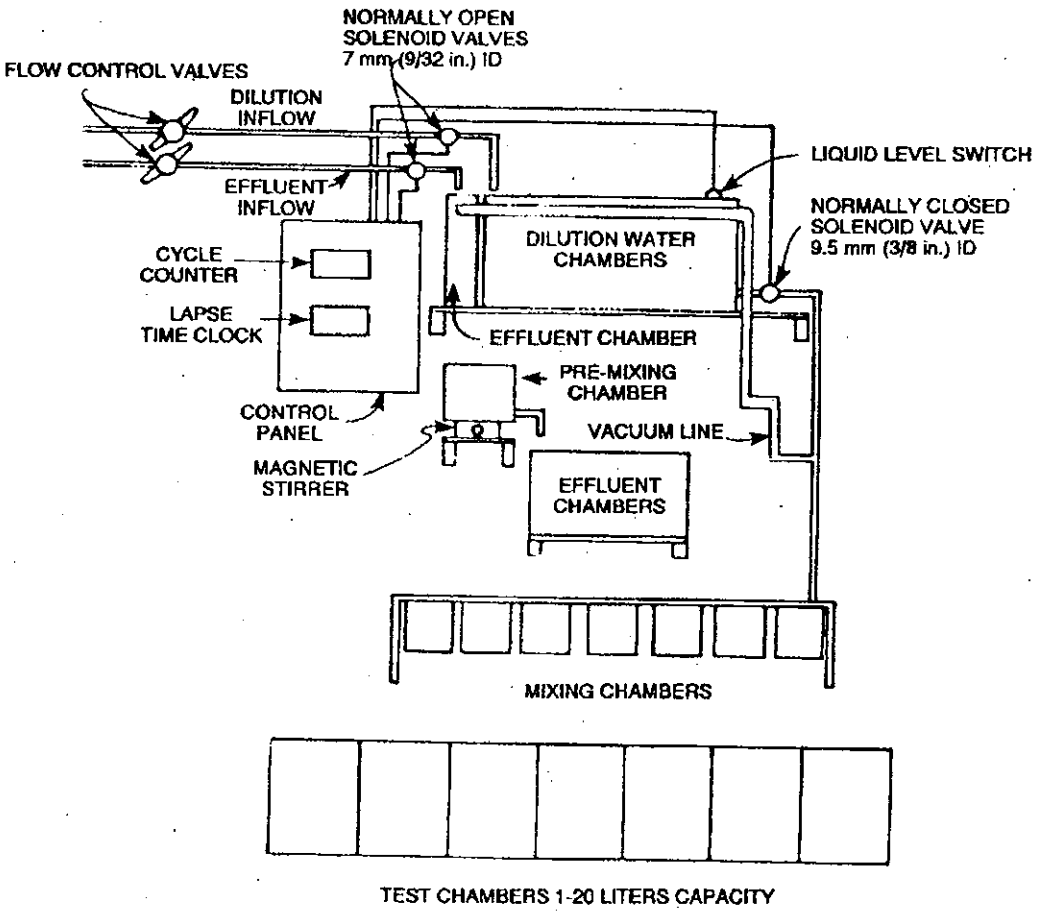


Figure 4. Vacuum siphon dilutor system, general diagram (not to scale).

VACUUM SIPHON SYSTEM EQUIPMENT LIST

1. Dilutor Glass.
2. Stainless steel solenoid valves.
   a. 2, normally open, two-way, 55 psi, water, 1/4" pipe size, 9/32" orifice size, ASCO 8262152, for incoming effluent and dilution water pipes.
   b. 2, normally closed, two-way, 15 psi, water, 3/8" pipe size, 3/8" orifice size, ASCO 8030B65, for dilution water chamber D-6 and effluent chamber E-2.
3. Stainless steel tubing, seamless, austenitic, 304 grade for freshwater and 316 grade for saline water.
   a. 60 ft of 3/8" OD, 0.035" wall thickness, for dilution water and effluent pipes.
   b. 20 ft of 5/16" OD, 0.035" wall thickness, for standpipes in mixing chambers.
   c. 1 ft of 3/4" OD, 0.035" wall thickness, for standpipe in D1 chamber.
4. Swagelok tube connectors, stainless steel.
   a. 4, male tube connectors, male pipe size 1/4", tube OD 3/8".
   b. 2, male tube connectors, male pipe size 3/8", tube OD 3/8".
   c. 2, male adaptor, tube to pipe, male pipe size 1/2", tube OD 3/8".
   d. 2, male tube connectors, male pipe size 1/2", tube OD 3/8".
5. 7, 1,200 mL stainless steel beakers.
6. Several lbs each of Neoprene stoppers, sizes 00, 0, and 1; 1 lb of size 5.
7. 14 - aquarium (1-20 liters).
8. Magnetic stirrer.
9. 2, PVC Ball valves, 1/2" pipe size.
10. Dilutor control panel equipment - see Fig. 32 and equipment list.
11. 7, 120 ml NALGENE® bottles.
12. 3 ft, 1-in-2 aluminum bar, for siphon support brackets.
13. Stainless steel set screws, box of 50, for securing SS tubing in siphon support brackets.
14. Stainless steel hose clamps, box of 10, size #4 or 5, (need 3 boxes).
15. 6, NALGENE® T's, 5/16" OD.
16. 12, TYGON® Y connectors, 3/8" I.D.
17. TYGON® tubing, 3/8" OD, 10 ft.
18. Plywood sheeting, exterior grade: one - 4' x 8' x 3/4", one - 4' x 8' x 1/2".
19. Pine or redwood board, 1" x 8", 20 ft.
20. Epoxy paint, 1 gal.
21. Assorted wood screws, nails, etc.
22. 25 ft of 5/16" ID, TEFLON® tubing, to connect the mixing chambers to the test chambers.

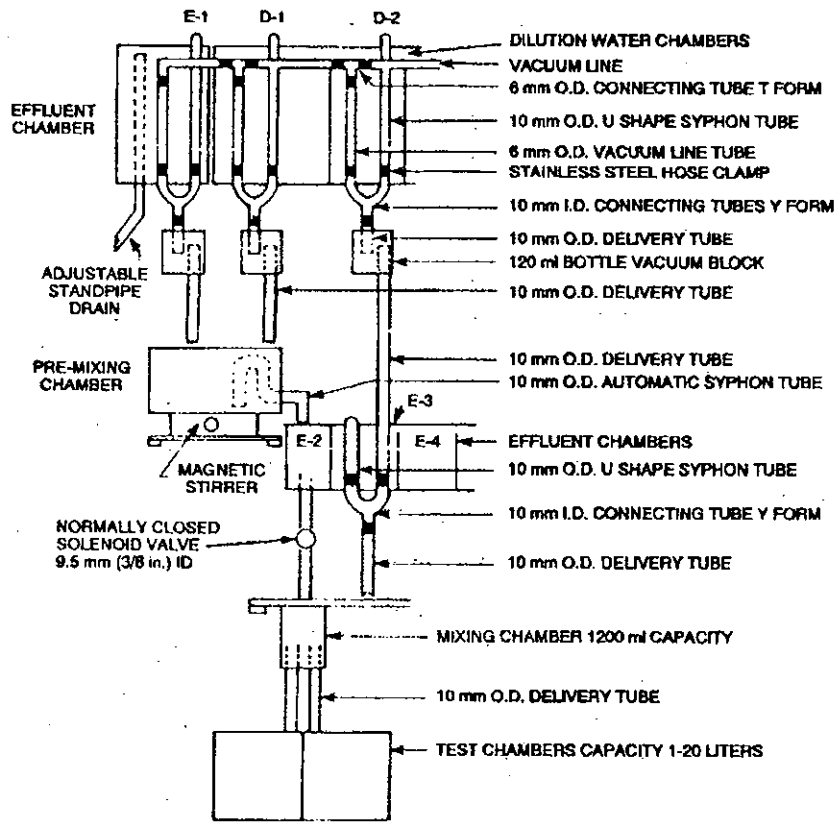
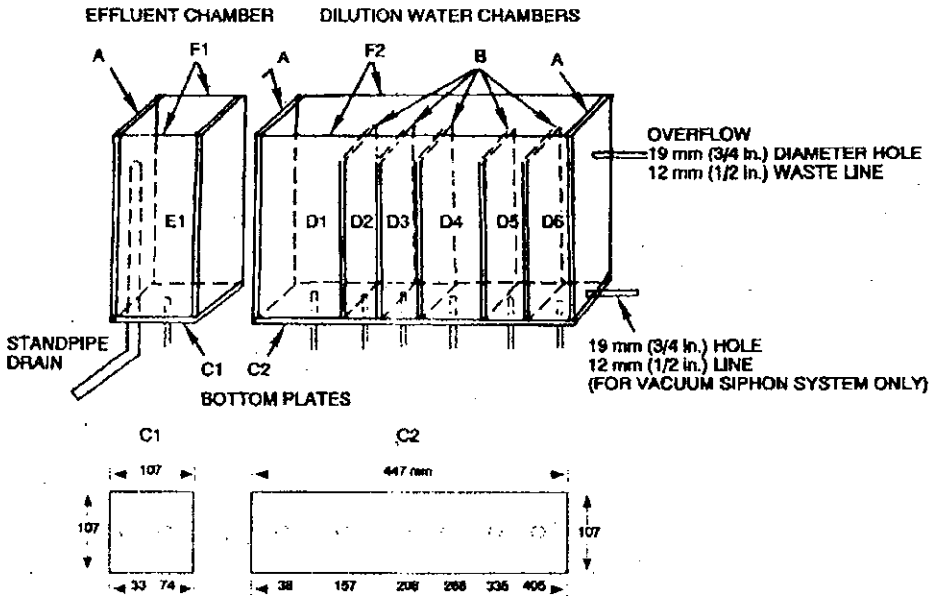


Figure 5. Vacuum siphon dilutor system, detailed diagram (not to scale).



DRAIN HOLES IN BOTTOM PLATE (C1 AND C2) SHOWN FOR SOLENOID VALVE DILUTOR SYSTEM. FOR VACUUM SIPHON DILUTOR SYSTEM, DRAIN HOLE IS REQUIRED ONLY FOR CHAMBER E1

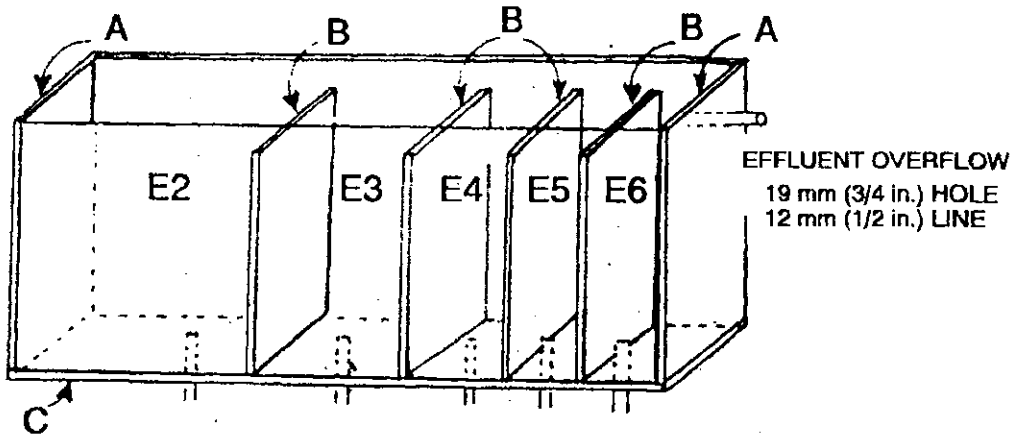
INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING 6 mm (1/4 in.) PLATE GLASS. NOTE: 1.6 mm (1/16 in.) No. 304 GRADE (FOR FRESH WATER) OR No. 316 GRADE (FOR SALINE WATER) STAINLESS STEEL MAY BE SUBSTITUTED FOR GLASS.

	LENGTH	WIDTH	NO. PIECES (14)	
A:	231 mm	x 96 mm	= 4	(END PLATES)
B:	200 mm	x 96 mm	= 6	(PARTITIONS)
C1:	107 mm	x 107 mm	= 1	(BOTTOM PLATE FOR E1)
C2:	447 mm	x 107 mm	= 1	(BOTTOM PLATE FOR D1-D6)
F1:	107 mm	x 231 mm	= 2	(FRONT AND BACK PANELS FOR E1)
F2:	447 mm	x 231 mm	= 2	(FRONT AND BACK PANELS FOR D1-D6)

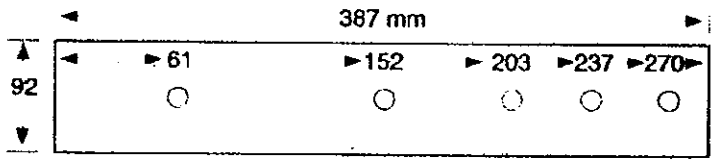
INSIDE CELL MEASUREMENTS AND APPROXIMATE VOLUMES

	WIDTH	LENGTH	HEIGHT	VOLUME
E1:	95 mm	x 95 mm	x 231 mm =	2085 mL
D1:	125 mm	x 95 mm	x 200 mm =	2375 mL
D2:	40 mm	x 95 mm	x 200 mm =	760 mL
D3:	50 mm	x 95 mm	x 200 mm =	950 mL
D4:	60 mm	x 95 mm	x 200 mm =	1140 mL
D5:	60 mm	x 95 mm	x 200 mm =	1140 mL
D6:	70 mm	x 95 mm	x 200 mm =	1330 mL

Figure 6. Effluent and dilution water chambers (not to scale).



BOTTOM PLATE (C)



DRAIN HOLES IN BOTTOM PLATE (C) SHOWN FOR SOLENOID VALVE DILUTOR SYSTEM ONLY. FOR VACUUM SIPHON DILUTOR SYSTEM, A DRAIN HOLE IS REQUIRED ONLY FOR CHAMBER E2.

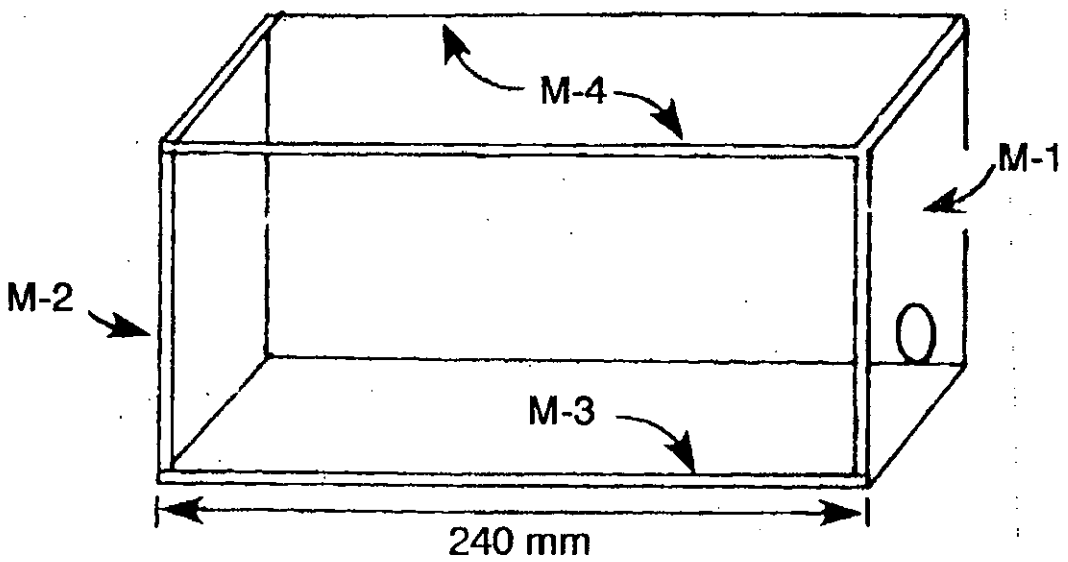
INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING A 6 mm (1/4 in.) PLATE GLASS ARE SHOWN BELOW. NOTE: 1/16 in. No. 304 (FOR FRESH WATER) OR No. 316 STAINLESS STEEL (FOR SALINE WATER) MAY BE SUBSTITUTED FOR GLASS.

	LENGTH	WIDTH	NO. PIECES (9)
A	180 mm x	80 mm	= 2 (END PLATES)
B	155 mm x	80 mm	= 4 (PARTITIONS)
C	296 mm x	92 mm	= 1 (BOTTOM PLATE)
D	296 mm x	180 mm	= 2 (FRONT AND BACK PLATES)

INSIDE CHAMBER MEASURMENTS AND APPROXIMATE VOLUMES.

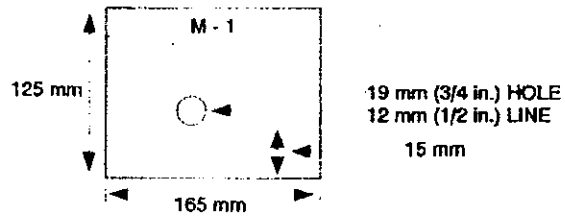
	WIDTH	LENGTH	HEIGHT	VOLUME
E2:	110 mm x	80 mm	x 155 mm	= 1364 mL
E3:	60 mm x	80 mm	x 155 mm	= 744 mL
E4:	30 mm x	80 mm	x 155 mm	= 372 mL
E5:	30 mm x	80 mm	x 155 mm	= 372 mL
E6:	30 mm x	80 mm	x 155 mm	= 372 mL

Figure 7. Effluent chambers (not to scale).



SIDE VIEW

END VIEW



INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING 6 mm (1/4 in.) PLATE GLASS. APPROXIMATE CAPACITY 4360 mL.

M-1	125	mm	x	153	mm	-	1	(END PLATE, WITH HOLE)
M-2	125	mm	x	153	mm	-	1	(END PLATE)
M-3	240	mm	x	165	mm	-	1	(BOTTOM PLATE)
M-4	240	mm	x	125	mm	-	2	(SIDE PLATES)

Figure 8. Pre-mixing chamber (not to scale).

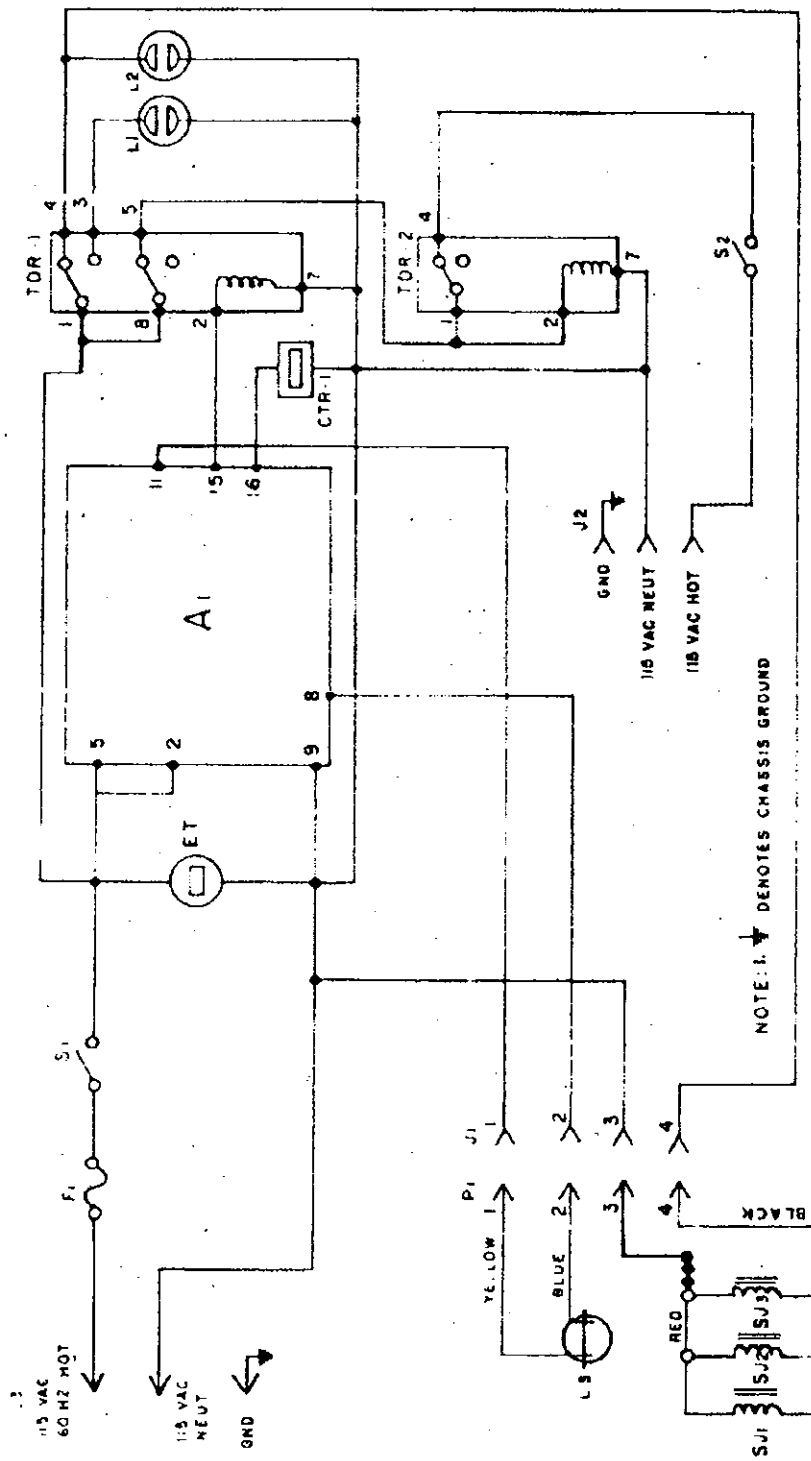


Figure 9. Dilutor control panel wiring diagram.

DILUTOR CONTROL PANEL EQUIPMENT LIST*

Designation	CKT Description	Manufacturer
A_1	Encapsulated amplifier	Cutler Hammer 13535H98C
CTR-1	Cycle counter	Redington #P2-1006
ET	Elapsed time indicator	Conrac #636W-AA H&T
F_1	Input power fuse	Little fuse 342038
J_1	Receptacle	Amphenol 91PC4F
J_2	Aux A.C. output jack	Stand. 3-prong AC Rcpt.
J_3	Main input power cord	Stand. 3-prong AC male plug
L_1	Fill indicator light	Dialco 95-0408-09-141
L_2	Emptying indicator light	Dialco 95-0408-09-141
L.S.	Liquid level sensor (Dual Sensing Probe)	Cutler Hammer 13653H2
P_1	Plug	Amphenol 91MC4M
S_1	On-off main power switch (spst)	Cutler Hammer 7580 K7
S_2	On-off aux power switch (spst)	Cutler Hammer 7580 K7
SJ_1	Solenoid	(See Solenoid and Vacuum System equipment lists)
SJ_2	"	" " "
SJ_3	"	" " "
SJ_4-SJ_6	Additional Solenoids for Solenoid Valve System	" " "
TDR-1	Time delay relay	Dayton 5x829
TDR-2	Aux time delay relay	Dayton 5x829

*Consult local electric supply house.

APPENDIX D

PLANS FOR MOBILE TOXICITY TEST LABORATORY

D.1. TANDEM-AXLE TRAILER

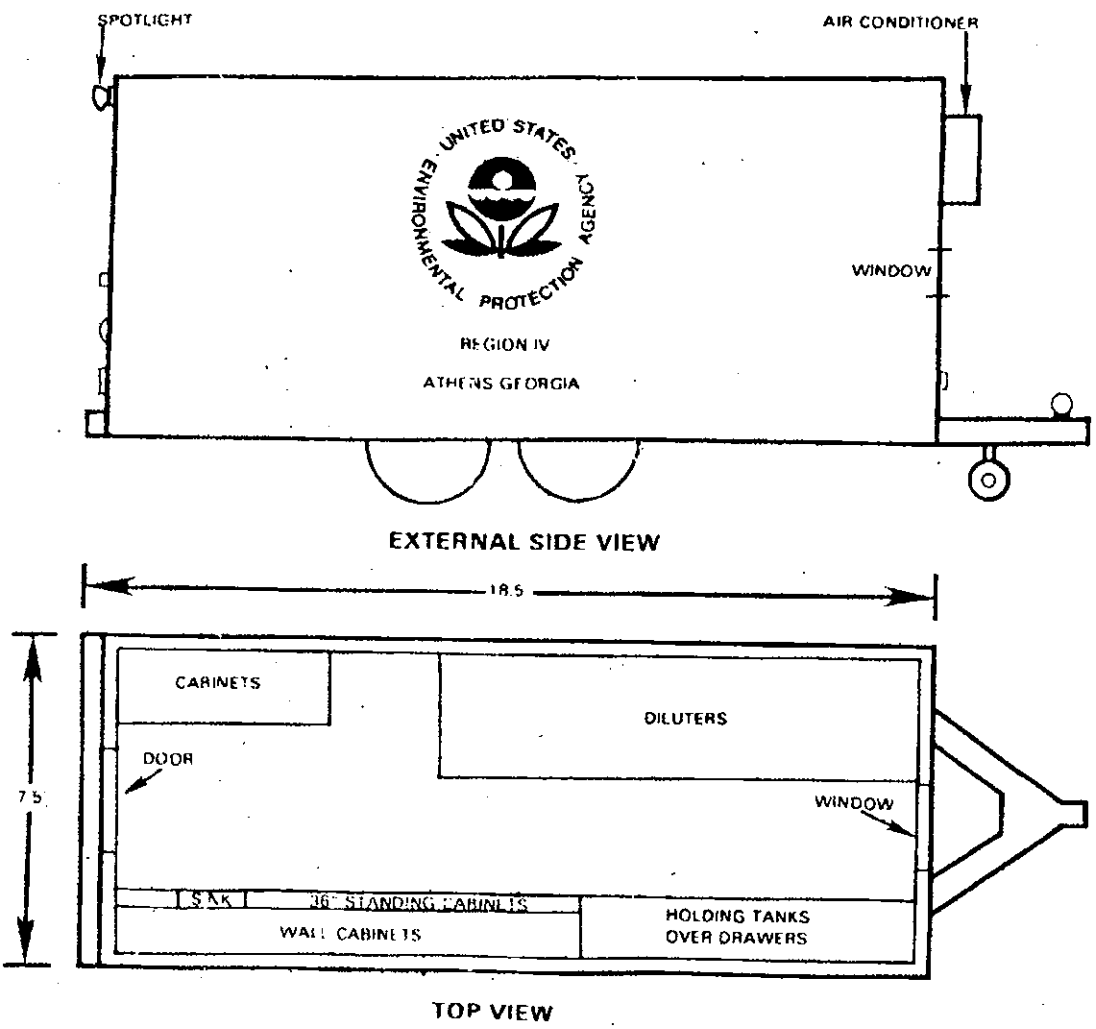


Figure 1. Mobile bioassay laboratory, tandem axle trailer. Above - external side view; below - internal view from above.

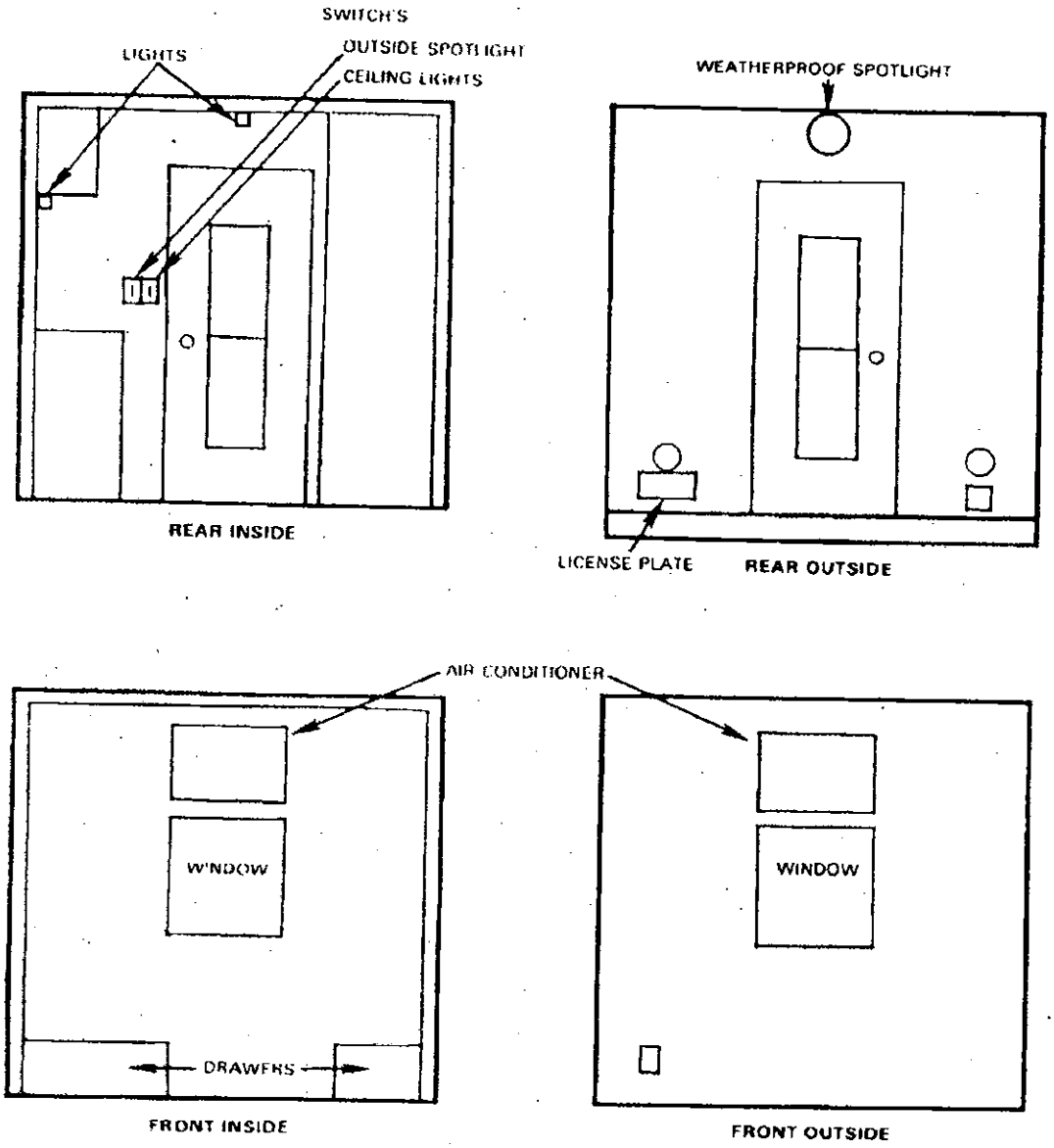


Figure 2. Mobile bioassay laboratory, tandem-axle trailer, external and internal end views.

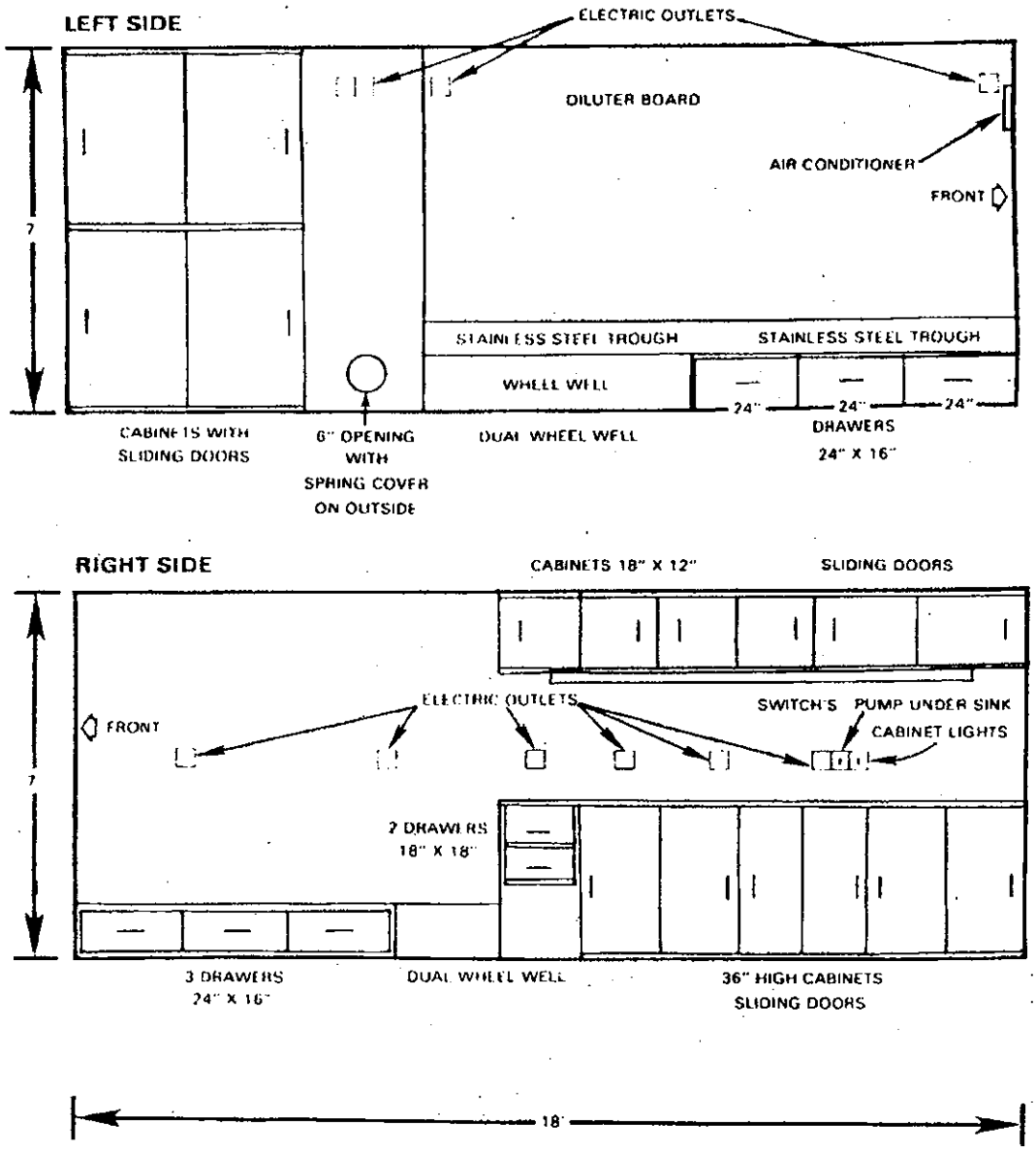


Figure 3. Mobile bioassay laboratory, tandem-axle trailer, internal views of side walls.

APPENDIX D

PLANS FOR MOBILE TOXICITY TEST LABORATORY

D.2. FIFTH WHEEL TRAILER

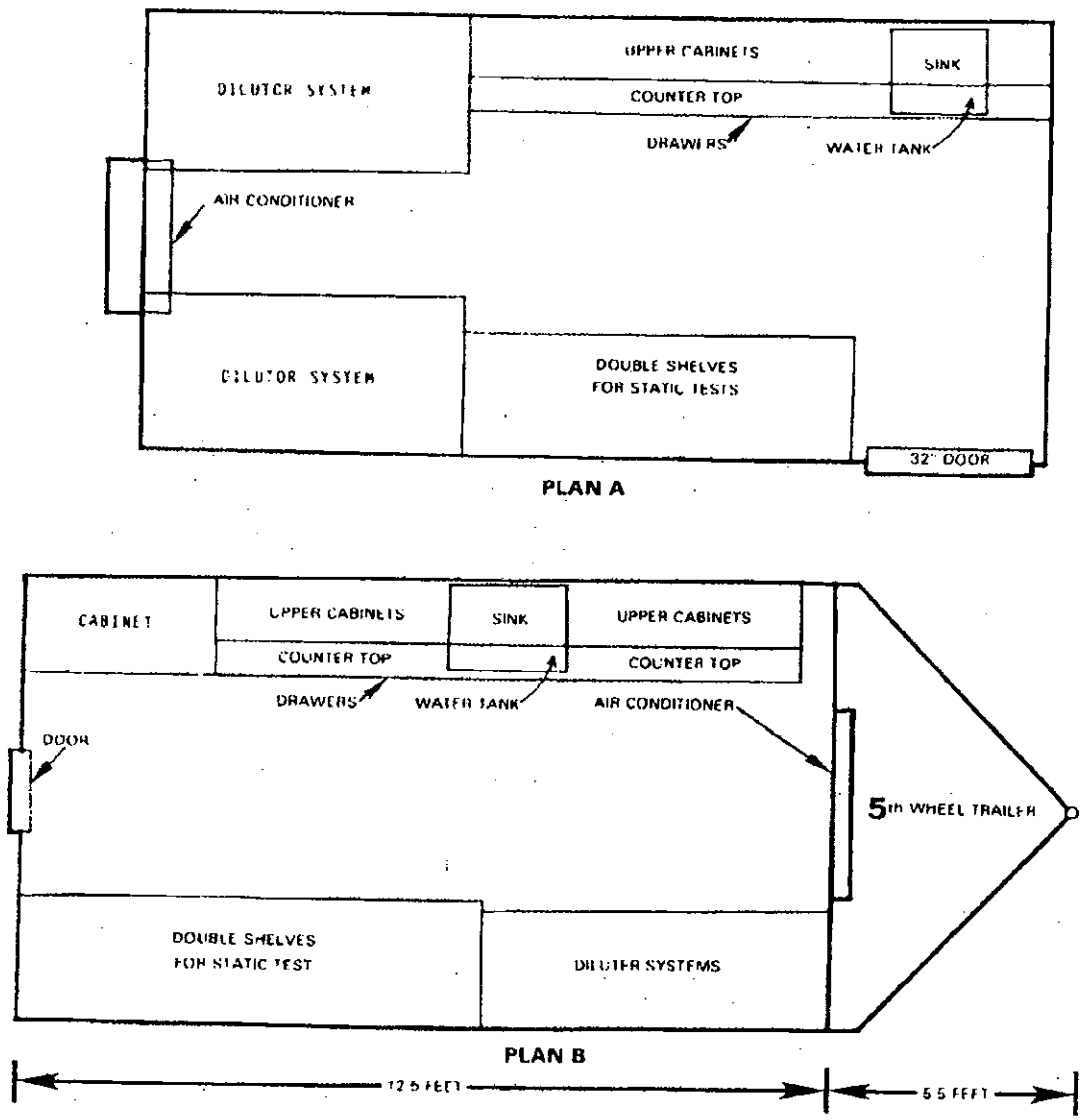


Figure 4. Mobile bioassay trailer, fifth-wheel trailer, internal view from above.

APPENDIX E

CHECK LISTS AND INFORMATION SHEETS

E.1. TOXICITY TEST FIELD EQUIPMENT LIST

Truck

____Boards
____Cinder blocks
____Drums: ____500 gal nalgene
____ 55 gal metal - diesel fuel
____ 22 gal
____Gas can ____ 5 gal
____Jacks
____Jumper cables
____Oil
____Pumps: ____ (2) Homelite
____ ____ Hoses & couplings
____Shovels
____Spare tires (trailer, generator)

Trailer

____Acetone
____Aerators (battery operated)
____Air line: ____Clamps
____Aerators (battery operated)
____Air line: ____Clamps
____Stones
____Tubing
____Valves
____Alcohol
____Aluminum foil
____Alkalinity analysis (0.02 N H_2SO_4)
____Boots: ____safety
____wading
____Batteries ____D cell
____Beakers: ____150 mL nalgene
____200 mL glass (3 boxes)
____Bottles: ____B.O.
____wash
____Sample
____VOA vials
____500 mL plastic
____Glass organic
____Qt. w/teflon liner
____Brine shrimp eggs
____Broom
____Brushes (wash)
____Buckets
____Camera
____Chlorine kit (w/chem)
____Cleanser
____Clip board (lg, sm)
____Cork borer set
____Culture dishes (200 mL, Daphnia)
____Daphnia food
____Data sheets: ____Bioassay (static)
____Bioassay (flow-thru)
____Dilutor volume delivery
____Calibrator delivery sheet
____Daily events log
____Dish pan
____Dish rack
____Dissolved oxygen:
____KCL membrane solution
____Membranes
____Meter (YSI)
____Probes
____Reagent: ____$MnSo_4$
____Alkaline azide
____H_2SO_4
____0.0375 Na thiosulfate
____Starch
____Distilled H_2O
____Emergency road kit
____Enamel pans (lg, sm)
____Erlenmeyer flasks: ____ 500 mL (2)
____1000 mL
____2000 mL
____Extension cords
____Fire extinguisher
____First aid kit
____Fish nets, (lg, sm)
____Flash light
____Generator: ____Oil
____Filter - fuel
____Funnel
____Grease gun (wheels)
____Credit card
____Lock/key
____Siphon hose

E.1. TOXICITY TEST FIELD EQUIPMENT LIST (CONTINUED)

____Glass cutter
____Gloves (plastic)
____Graduated cylinders:
25 mL, 50 mL, 100 mL
250 mL, 500 mL, 1000 mL, 2000 mL
____Ground wire & rod
____Hand soap
____Hard hats
____Hardness analysis: ____Buffer
____EDTA
____indicator
____HCl (20%)
____Heaters: ____Aquarium
____Space
____Hose: ____Clamps
____Connectors
____Ice chests

____Jars: ____750 mL (4 boxes)
____3 gal (glass) (1)
____5 gal (glass) (1)
____Sample jugs (2)
____Kimwipes (lg, sm)
____Lab coats (2)
____Level
____Light 110 V
____Log book
____Magnetic stirrers: ____Lighted
____Other

____Mop

____Paper towels
____Parachute cord
____Parafilm
____Pencils, pens
____pH: ____Meters, Orion
____ ____Meters, corning
____Buffers, 4,7,10
____Probes (extras)
____Pipets: ____Bulbs
____Eyedroppers
____Volumetric (1 mL, 5 mL, 10 mL)
____Plastic bags
____Quality assurance - SPCP
____Rain gear
____Reconstituted hard water
____Refractometer
____Respirators (cartridges)
____Rubber bands
____Ruler
____Safety glasses
____Safety manual
____Sample labels
____Scissors
____Screen bioassay cups
____Sea salts
____Separatory funnels & racks
____Silent giants
____Silicon sealant
____Solenoids (spare)
____Stainless steel tubing pieces
____Standard Methods Hand Book
____Stirring bars
____Stoppers (assorted)
____Submersible pumps: ____lg, sm.
____screens
____Super ice
____Tablets (paper)
____Tape: ____Cellophane
____Color coded
____Electrician
____Masking
____Nylon
____Thermometers: ____Dial
____Glass
____Tools (lock/key)
____Tygon tubing, 1/8", 1/4", 3/8"
____Volumetric flasks (1000 mL, 2000 mL)
____WD40
____Weigh boats
____Wire tags

APPENDIX E

CHECK LISTS AND INFORMATION SHEETS

E.2. INFORMATION CHECK LIST FOR ON-SITE INDUSTRIAL OR MUNICIPAL WASTE TOXICITY TEST

1. PRE-TRIP INFORMATION

Facility Name: _____

Address: _____

Phone number: _____

Plant Representative(s): _____

Names, Titles, Addresses of Company Personnel:

A. To Receive Correspondence: _____

_____

_____

B. To Receive Carbons: _____

_____

_____

Date of Notification Letter: _____

State Making Notification and Arrangements: _____

Special Plant Safety/Security Requirements for EPA Personnel to Observe:

_____

Local Accommodation Recommendations: _____

Directions to Plant: _____

_____

_____

Availability of Power Hookups (three 20-amp, 110-V Circuits): _____

_____

Distance from Power Source to Trailer: _____ (Feet)

Trailer Location: _____

Possible Source of Dilution Water: _____

_____

_____

Major Products: _____

Raw Materials: _____

_____

Name of Receiving Water: _____

Schedule of Plant Operation (continuous, weekdays only, etc.): _____

_____

Treatment Steps: _____

_____

_____

_____

Treatment Level (BPT, BAT, etc.): _____

Wastewater Retention Time by Lagoon or Treatment Step:

Lagoon Designation	Retention Time Hours	Time Days
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Total Wastewater Retention Time: _____ Hours; _____ Days

Retention Time Determination: _____ Calculated; _____ Actual

Calculation method: _____

Description of Wastewater Tap Point: _____

_____

_____

Description of Outfall (surface, submerged diffuser, etc.): _____

_____

_____

Description of Other Waste Disposal Alternatives in Use (spray irrigation, deepwell, municipal discharge, etc.): _____

_____

_____

2. ON-SITE INFORMATION

Wastewater General Characteristics:

Color: _____

Odor: _____

Solids: _____

Other: _____

Serial Number(s) of Discharge(s) to be Tested: _____

Description of Receiving Water: ____ Uniflow; ____ Tidal;
____ Approximate amplitude, feet

Color: _____

Odor: _____

Solids: _____

Salinity: High tide _____; Low tide _____

Other: _____

_____

7Q10: _____; Ave. flow _____

Description of Receiving Water Zone of Dilution: _____

_____

_____

Location and Description of Water Sampling Point(s): _____

_____

Fresh: _____

Salt: _____

Dilution Waste General Characteristics:

Color: _____

Odor: _____

Solids: _____

Other: _____

_____

Description of Toxicity Test Anomalies (plant production changes, power failure, rain events, etc.):

Duration		
Time & Date	Time & Date	Anomaly

Description of Plant maintenance: _____

_____

_____

_____

Attach: DIAGRAM OF WASTEWATER TREATMENT FACILITIES.

3. FOLLOW-UP INFORMATION

Date of follow-up letter: _____

Wastewater Flow (data supplied by discharger):

Week Prior to Testing		Week of Testing	
Date	Discharge (MGD)	Date	Discharge (MGD)

Average Discharge (MGD): _____

Organisms Tested On-site or In-Lab:

Species	Flow-thru test duration (h)	Static test duration (h)	Test Location	Dates	Results

Possible Recommended Action as a Result of These Findings:

APPENDIX E

CHECK LISTS AND INFORMATION SHEETS

E.3. DAILY EVENTS LOG

Date: _____ Page ____ of ____ Pages

Site: _____ Day #____ of Study

Initials:_____ Day #____ of Flow-through Test

Time: _____ Notes: _____

DILUTOR VOLUME DELIVERY INFORMATION

Industry: _____
Date: _____
Test Start Date: _____ Time: _____

Observation Number	Date	Time	Volume Delivery to Metering Boxes Each 10 Seconds (mL)		Measured Cycle Time (min)	Control Box		Comments
			Dilution Water	Wastewater		Hour	Cycles	
Final								Ave 4-day turnover = Min/turnover

USEPA
Athens, GA

STANDARD DILUTOR FLOW RATES

Cycle Time (min)	Flow rate (mL/10 seconds) Dilution Water	Wastewater	Turnover Time (min)
2.5	532	248	43
5.0	199	92	85
7.5	123	57	128
10.0	89	41	170
12.5	69	32	213
15.0*	67	22	300

* This turnover rate provides approximately five 90% turnovers per day