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SECTION 1

INTRODUCTION

1.1 This manual describes chronic toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in chronically toxic concentrations. The methods included in this manual are referenced in Table IA, 40 CFR Part 136 regulations and, therefore, constitute approved methods for chronic toxicity tests. They are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on the LC50, NOEC, IC50 or IC25 (see Section 9, Chronic Toxicity Endpoints and Data Analysis) and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from chronic toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988c; USEPA, 1989b; USEPA 1989c; USEPA, 1989d; USEPA, 1989e; USEPA, 1991a; USEPA, 1991b; and USEPA, 1992).

1.4 This methods manual serves as a companion to the acute toxicity test methods for freshwater and marine organisms (USEPA, 2002a), the short-term chronic toxicity test methods for marine and estuarine organisms (USEPA, 2002b), and the manual for evaluation of laboratories performing aquatic toxicity tests (USEPA, 1991c). In 2002, EPA revised previous editions of each of the three methods manuals (USEPA, 1993a; USEPA, 1994a; USEPA, 1994b).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1.6 These freshwater short-term toxicity tests are similar to those developed for marine and estuarine organisms to evaluate the toxicity of effluents discharged to marine and estuarine waters under the NPDES permit program. Methods are presented in this manual for three species of freshwater organisms from three phylogenetic groups. The methods are all static renewal type seven-day tests except the green alga, *Selenastrum capricornutum*, test which lasts four days.

1.7 The three species for which test methods are provided are the fathead minnow, *Pimephales promelas*; the daphnid, *Ceriodaphnia dubia*; and the green alga, *Selenastrum capricornutum*.

1.7.1 Two of the methods incorporate the chronic endpoint of growth in addition to lethality and one incorporates reproduction. The fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test incorporates teratogenic effects in addition to lethality. The green alga, *Selenastrum capricornutum*, growth test has the advantage of a relatively short exposure period (96 h).

1.8 The validity of the freshwater chronic methods in predicting adverse ecological impacts of toxic discharges was demonstrated in field studies (USEPA, 1984; USEPA, 1985b; USEPA, 1985c; USEPA, 1985d; USEPA, 1986a; USEPA, 1986b; USEPA, 1986c; USEPA, 1986d; Birge et al., 1989; and Eagleson et al., 1990).

1.9 The use of any test species or test conditions other than those described in the methods summary tables in this manual shall be subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.10 These methods are restricted to use by, or under the supervision of, analysts experienced in the use or conduct of aquatic toxicity tests and the interpretation of data from aquatic toxicity testing. Each analyst must demonstrate the ability to generate acceptable test results with these methods using the procedures described in this methods manual.

1.11 This manual was prepared in the established EMSL-Cincinnati format (USEPA, 1983).

SECTION 2

SHORT-TERM METHODS FOR ESTIMATING CHRONIC TOXICITY

2.1 INTRODUCTION

2.1.1 The objective of aquatic toxicity tests with effluents or pure compounds is to estimate the "safe" or "no effect" concentration of these substances, which is defined as the concentration which will permit normal propagation of fish and other aquatic life in the receiving waters. The endpoints that have been considered in tests to determine the adverse effects of toxicants include death and survival, decreased reproduction and growth, locomotor activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, olfactory function, and terata. Since it is not feasible to detect and/or measure all of these (and other possible) effects of toxic substances on a routine basis, observations in toxicity tests generally have been limited to only a few effects, such as mortality, growth, and reproduction.

2.1.2 Acute lethality is an obvious and easily observed effect which accounts for its wide use in the early period of evaluation of the toxicity of pure compounds and complex effluents. The results of these tests were usually expressed as the concentration lethal to 50% of the test organisms (LC50) over relatively short exposure periods (one-to-four days).

2.1.3 As exposure periods of acute tests were lengthened, the LC50 and lethal threshold concentration were observed to decline for many compounds. By lengthening the tests to include one or more complete life cycles and observing the more subtle effects of the toxicants, such as a reduction in growth and reproduction, more accurate, direct, estimates of the threshold or safe concentration of the toxicant could be obtained. However, laboratory life-cycle tests may not accurately estimate the "safe" concentration of toxicants because they are conducted with a limited number of species under highly controlled, steady-state conditions, and the results do not include the effects of the stresses to which the organisms would ordinarily be exposed in the natural environment.

2.1.4 An early published account of a full life-cycle, fish toxicity test was that of Mount and Stephan (1967). In this study, fathead minnows, *Pimephales promelas*, were exposed to a graded series of pesticide concentrations throughout their life cycle, and the effects of the toxicant on survival, growth, and reproduction were measured and evaluated. This work was soon followed by full life-cycle tests using other toxicants and fish species.

2.1.5 McKim (1977) evaluated the data from 56 full life-cycle tests, 32 of which used the fathead minnow, *Pimephales promelas*, and concluded that the embryo-larval and early juvenile life-stages were the most sensitive stages. He proposed the use of partial life-cycle toxicity tests with the early life-stages (ELS) of fish to establish water quality criteria.

2.1.6 Macek and Sleight (1977) found that exposure of critical life-stages of fish to toxicants provides estimates of chronically safe concentrations remarkably similar to those derived from full life-cycle toxicity tests. They reported that "for a great majority of toxicants, the concentration which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration for fish, and that the most sensitive life stages are the embryos and fry". Critical life-stage exposure was considered to be exposure of the embryos during most, preferably all, of the embryogenic (incubation) period, and exposure of the fry for 30 days post-hatch for warm water fish with embryogenic periods ranging from one-to-fourteen days, and for 60 days post-hatch for fish with longer embryogenic periods. They concluded that in the majority of cases, the maximum acceptable toxicant concentration (MATC) could be estimated from the results of exposure of the embryos during incubation, and the larvae for 30 days post-hatch.

2.1.7 Because of the high cost of full life-cycle fish toxicity tests and the emerging consensus that the ELS test data usually would be adequate for estimating chronically safe concentrations, there was a rapid shift by aquatic toxicologists to 30 - 90-day ELS toxicity tests for estimating chronically safe concentrations in the late 1970s. In

1980, USEPA adopted the policy that ELS test data could be used in establishing water quality criteria if data from full life-cycle tests were not available (USEPA, 1980a).

2.1.8 Published reports of the results of ELS tests indicate that the relative sensitivity of growth and survival as endpoints may be species dependent, toxicant dependent, or both. Ward and Parrish (1980) examined the literature on ELS tests that used embryos and juveniles of the sheepshead minnow, *Cyprinodon variegatus*, and found that growth was not a statistically sensitive indicator of toxicity in 16 of 18 tests. They suggested that the ELS tests be shortened to 14 days posthatch and that growth be eliminated as an indicator of toxic effects.

2.1.9 In a review of the literature on 173 fish full life-cycle and ELS tests performed to determine the chronically safe concentrations of a wide variety of toxicants, such as metals, pesticides, organics, inorganics, detergents, and complex effluents, Woltering (1984) found that at the lowest effect concentration, significant reductions were observed in fry survival in 57%, fry growth in 36%, and egg hatchability in 19% of the tests. He also found that fry survival and growth were very often equally sensitive, and concluded that the growth response could be deleted from routine application of the ELS tests. The net result would be a significant reduction in the duration and cost of screening tests with no appreciable impact on estimating MATCs for chemical hazard assessments. Benoit et al. (1982), however, found larval growth to be the most significant measure of effect, and survival to be equally or less sensitive than growth in early life-stage tests with four organic chemicals.

2.1.10 Efforts to further reduce the length of partial life-cycle toxicity tests for fish without compromising their predictive value have resulted in the development of an eight-day, embryo-larval survival and teratogenicity test for fish and other aquatic vertebrates (USEPA, 1981; Birge et al., 1985), and a seven-day larval survival and growth test (Norberg and Mount, 1985).

2.1.11 The similarity of estimates of chronically safe concentrations of toxicants derived from short-term, embryo-larval survival and teratogenicity tests to those derived from full life-cycle tests has been demonstrated by Birge et al. (1981), Birge and Cassidy (1983), and Birge et al. (1985).

2.1.12 Use of a seven-day, fathead minnow, *Pimephales promelas*, larval survival and growth test was first proposed by Norberg and Mount at the 1983 annual meeting of the Society for Environmental Toxicology and Chemistry (Norberg and Mount, 1983). This test was subsequently used by Mount and associates in field demonstrations at Lima, OH (USEPA, 1984), and at many other locations. Growth was frequently found to be more sensitive than survival in determining the effects of complex effluents.

2.1.13 Norberg and Mount (1985) performed three single toxicant fathead minnow larval growth tests with zinc, copper, and DURSIBAN[®], using dilution water from Lake Superior. The results were comparable to, and had confidence intervals that overlapped with, chronic values reported in the literature for both ELS and full life-cycle tests.

2.1.14 Mount and Norberg (1984) developed a seven-day cladoceran partial life-cycle test and experimented with a number of diets for use in culturing and testing the daphnid, *Ceriodaphnia reticulata* (Norberg and Mount, 1985). As different laboratories began to use this cladoceran test, it was discovered that apparently more than one species was involved in the tests conducted by the same laboratory. Berner (1986) studied the problem and determined that perhaps as many as three variant forms were involved and it was decided to recommend the use of the more common *Ceriodaphnia dubia* rather than the originally reported *Ceriodaphnia reticulata*. The method was adopted for use in the first edition of the freshwater short-term chronic methods (USEPA, 1985e).

2.1.15 The green alga, *Selenastrum capricornutum*, bottle test was developed, after extensive design, evaluation, and application, for the National Eutrophication Research Program (USEPA, 1971). The test was later modified for use in the assessment of receiving waters and the effects of wastes originating from industrial, municipal, and agricultural point and non-point sources (USEPA, 1978a).

2.1.16 The use of short-term toxicity tests including subchronic and chronic tests in the NPDES Program is especially attractive because they provide a more direct estimate of the safe concentrations of effluents in receiving waters than was provided by acute toxicity tests, at an only slightly increased level of effort, compared to the fish full life-cycle chronic and 28-day ELS tests and the 21-day daphnid, *Daphnia magna*, life-cycle test.

2.2 TYPES OF TESTS

2.2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2.2 Effluent chronic toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that affects the hatchability, gross morphological abnormalities, survival, growth, and/or reproduction within the prescribed period of time (four to seven days). The results of the tests are expressed in terms of the highest concentration that has no statistically significant observed effect on those responses when compared to the controls or the estimated concentration that causes a specified percent reduction in responses versus the controls.

2.2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control **is not recommended**. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) $(RWC + 100)/2$, (3) RWC, (4) $RWC/2$, and (5) $RWC/4$. More specifically, if the $RWC = 50\%$, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

2.2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.2.5 A negative result from a chronic toxicity test does not preclude the presence of toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit chronic toxicity.

2.2.6 The frequency with which chronic toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.2.7 Tests recommended for use in this methods manual may be static non-renewal or static renewal. Individual methods specify which static type of test is to be conducted.

2.3 STATIC TESTS

2.3.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.3.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

2.4 ADVANTAGES AND DISADVANTAGES OF TOXICITY TEST TYPES

2.4.1 STATIC NON-RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Simple and inexpensive.
2. Very cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform many more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than static renewal, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.4.2 STATIC RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Reduced possibility of DO depletion from high COD and/or BOD, or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

Disadvantages:

1. Require greater volume of effluent than non-renewal tests.
2. Generally less chance of temporal variations in waste properties.

SECTION 3
HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Each laboratory should develop and maintain an effective health and safety program, requiring an ongoing commitment by the laboratory management. This program should include (1) a safety officer with the responsibility and authority to develop and maintain a safety program, (2) the preparation of a formal, written, health and safety plan, which is provided to each of the laboratory staff, (3) an ongoing training program on laboratory safety, and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel will determine that all necessary safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel should use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes. Plastic netting on glass beakers, flasks, and other glassware minimizes breakage and subsequent shattering of the glass.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, chemical spill clean up kits, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone or other means to enable personnel to summon help in case of emergency.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1 Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals listed in Section 3, Health and Safety, Subsection 3.5). It is recommended that personnel collecting samples and performing toxicity tests not work alone.

3.3.2 Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the toxicity test areas must be used whenever possible.

3.3.3 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.4 All containers are to be adequately labeled to indicate their contents.

3.3.5 Staff should be familiar with safety guidelines on Material Safety Data Sheets for reagents and other chemicals purchased from suppliers. Incompatible materials should not be stored together. Good housekeeping contributes to safety and reliable results.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.8 Mobile laboratories should be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, polio, and hepatitis B.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general safety manuals, including USEPA (1986e) and Walters and Jameson (1984).

3.6 WASTE DISPOSAL

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state, and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in performing the toxicity testing activities. Local fire officials should be notified of any potentially hazardous conditions.

SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

4.1.1 Development and maintenance of a toxicity test laboratory quality assurance (QA) program (USEPA, 1991a) requires an ongoing commitment by laboratory management. Each toxicity test laboratory should (1) appoint a quality assurance officer with the responsibility and authority to develop and maintain a QA program; (2) prepare a quality assurance plan with stated data quality objectives (DQOs); (3) prepare a written description of laboratory standard operating procedures (SOPs) for culturing, toxicity testing, instrument calibration, sample chain-of-custody procedures, laboratory sample tracking system, glassware cleaning, etc.; and (4) provide an adequate, qualified technical staff for culturing and testing the organisms, and suitable space and equipment to assure reliable data.

4.1.2 QA practices for toxicity testing laboratories must address all activities that affect the quality of the final effluent toxicity test data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

4.1.3 Quality control practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance and general guidance on good laboratory practices and laboratory evaluation related to toxicity testing, see FDA, (1978); USEPA, (1979d), USEPA (1980b), USEPA (1980c), and USEPA (1991c); DeWoskin (1984); and Taylor (1987).

4.1.4 Guidance for the evaluation of laboratories performing toxicity tests and laboratory evaluation criteria may be found in USEPA (1991c).

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.2.1 Separate test organism culturing and toxicity testing areas should be provided to avoid possible loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into organism culturing or testing areas, and from testing and sample preparation areas into culture rooms.

4.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities, Equipment and Supplies; and specific toxicity test method).

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are the fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia*, and the green alga, *Selenastrum capricornutum*. The fish and invertebrates should appear healthy, behave normally, feed well, and have low mortality in the cultures, during holding, and in test controls. Test organisms should be positively identified to species (see Section 6, Test Organisms).

4.4 LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER

4.4.1 The quality of water used for test organism culturing and for dilution water used in toxicity tests is extremely important. Water for these two uses should come from the same source. The dilution water used in effluent toxicity tests will depend in part on the objectives of the study and logistical constraints, as discussed in detail in Section 7, Dilution Water. For tests performed to meet NPDES objectives, synthetic, moderately hard water should be used.

The dilution water used for internal quality assurance tests with organisms, food, and reference toxicants should be the water routinely used with success in the laboratory. Types of water are discussed in Section 5, Facilities, Equipment and Supplies. Water used for culturing and test dilution should be analyzed for toxic metals and organics at least annually or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. The concentration of the metals Al, As, Cr, Co, Cu, Fe, Pb, Ni, and Zn, expressed as total metal, should not exceed 1 mg/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA, 1992). Pesticide concentrations should not exceed USEPA's Ambient Water Quality chronic criteria values where available.

4.5 EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING

4.5.1 Sample holding times and temperatures of effluent samples collected for on-site and off-site testing must conform to conditions described in Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

4.6 TEST CONDITIONS

4.6.1 Water temperature should be maintained within the limits specified for each test. The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. Temperature should be recorded continuously in at least one test vessel for the duration of each test. Test solution temperatures should be maintained within the limits specified for each test. DO concentration and pH should be checked at the beginning of each test and daily throughout the test period.

4.7 QUALITY OF TEST ORGANISMS

4.7.1 The health of test organisms is primarily assessed by the performance (survival, growth, and/or reproduction) of organisms in control treatments of individual tests. The health and sensitivity of test organisms is also assessed by reference toxicant testing. In addition to documenting the sensitivity and health of test organisms, reference toxicant testing is used to initially demonstrate acceptable laboratory performance (Subsection 4.15) and to document ongoing laboratory performance (Subsection 4.16).

4.7.2 Regardless of the source of test organisms (in-house cultures or purchased from external suppliers), the testing laboratory must perform at least one acceptable reference toxicant test per month for each toxicity test method conducted in that month (Subsection 4.16). If a test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test.

4.7.3 When acute or short-term chronic toxicity tests are performed with effluents or receiving waters using test organisms obtained from outside the test laboratory, concurrent toxicity tests of the same type must be performed with a reference toxicant, unless the test organism supplier provides control chart data from at least the last five monthly short-term chronic toxicity tests using the same reference toxicant and control conditions (see Section 6, Test Organisms).

4.7.4 The supplier should certify the species identification of the test organisms, and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

4.7.5 If routine reference toxicant tests fail to meet test acceptability criteria, then the reference toxicant test must be immediately repeated.

4.8 FOOD QUALITY

4.8.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. *Artemia* cysts, and other foods must be obtained as described in Section 5, Facilities, Equipment, and Supplies.

4.8.2 Problems with the nutritional suitability of food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the affect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in chronic toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration chronic tests, using the reference toxicant regularly employed in the laboratory QA program.

4.8.3 New batches of food used in culturing and testing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. If the concentration of total organochlorine pesticides exceeds 0.15 mg/g wet weight, or the concentration of total organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight, or toxic metals (Al, As, Cr, Cd, Cu, Pb, Ni, Zn, expressed as total metal) exceed 20 µg/g wet weight, the food should not be used (for analytical methods see AOAC, 1990 and USDA, 1989). For foods (e.g., such as YCT) which are used to culture and test organisms, the quality of the food should meet the requirements for the laboratory water used for culturing and test dilution water as described in Section 4.4 above.

4.9 ACCEPTABILITY OF SHORT-TERM CHRONIC TOXICITY TESTS

4.9.1 For the tests to be acceptable, control survival in fathead minnow, *Pimephales promelas*, and the daphnid, *Ceriodaphnia dubia*, tests must be 80% or greater. At the end of the test, the average dry weight of surviving seven-day-old fathead minnows in control chambers must equal or exceed 0.25 mg. In *Ceriodaphnia dubia* controls, 60% or more of the surviving females must have produced their third brood in 7 ± 1 days, and the number of young per surviving female must be 15 or greater. In algal toxicity tests, the mean cell density in the controls after 96 h must equal or exceed 1×10^6 cells/mL and not vary more than 20% among replicates. If these criteria are not met, the test must be repeated.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of the test would depend on the experience and professional judgment of the laboratory investigator and the reviewing staff of the regulatory authority. Any deviation from test specifications must be noted when reporting data from the test.

4.10 ANALYTICAL METHODS

4.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions must include established quality assurance practices outlined in USEPA methods manuals (USEPA, 1979a and USEPA, 1979b).

4.10.2 Reagent containers should be dated and catalogued when received from the supplier, and the shelf life should not be exceeded. Also, working solutions should be dated when prepared, and the recommended shelf life should be observed.

4.11 CALIBRATION AND STANDARDIZATION

4.11.1 Instruments used for routine measurements of chemical and physical parameters such as pH, DO, temperature, and conductivity, must be calibrated and standardized according to instrument manufacturer's procedures as indicated in the general section on quality assurance (see USEPA Methods 150.1, 360.1, 170.1, and 120.1 in USEPA, 1979b). Calibration data are recorded in a permanent log book.

4.11.2 Wet chemical methods used to measure hardness, alkalinity and total residual chlorine must be standardized prior to use each day according to the procedures for those specific USEPA methods (see USEPA Methods 130.2 and 310.1 in USEPA, 1979b).

4.12 REPLICATION AND TEST SENSITIVITY

4.12.1 The sensitivity of the tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

4.13 VARIABILITY IN TOXICITY TEST RESULTS

4.13.1 Factors which can affect test success and precision include (1) the experience and skill of the laboratory analyst; (2) test organism age, condition, and sensitivity; (3) dilution water quality; (4) temperature control; and (5) the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions, such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between sample size (number of fish) and the standard error of the test, and considered 20 fish per concentration as optimum for Probit Analysis.

4.14 TEST PRECISION

4.14.1 The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with reference toxicants before they attempt to measure effluent toxicity. The single-laboratory precision of each type of test to be used in a laboratory should be determined by performing at least five tests with a reference toxicant.

4.14.2 Test precision can be estimated by using the same strain of organisms under the same test conditions and employing a known toxicant, such as a reference toxicant.

4.14.3 Interlaboratory precision data from a 1991 study of chronic toxicity tests with two species using the reference toxicants potassium chloride and copper sulfate are shown in Table 1. Table 2 shows interlaboratory precision data from a study of three chronic toxicity test methods using effluent, receiving water, and reference toxicant sample types (USEPA, 2001a; USEPA, 2001b). The effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Additional precision data for each of the tests described in this manual are presented in the sections describing the individual test methods.

TABLE 1. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 1991: SUMMARY OF RESPONSES USING A REFERENCE TOXICANT¹

Organism	Endpoint	No. Labs	% Effluent ²	SD	CV(%)
<i>Pimephales promelas</i>	Survival, NOEC	146	NA	NA	NA
	Growth, IC25	124	4.67	1.87	40.0
	Growth, IC50	117	6.36	2.04	32.1
	Growth, NOEC	142	NA	NA	NA
<i>Ceriodaphnia dubia</i>	Survival, NOEC	162	NA	NA	NA
	Reproduction, IC25	155	2.69	1.96	72.9
	Reproduction, IC50	150	3.99	2.35	58.9
	Reproduction, NOEC	156	NA	NA	NA

¹ From a national study of interlaboratory precision of toxicity test data performed in 1991 by the Environmental Monitoring Systems Laboratory- Cincinnati, U.S. Environmental Protection Agency, Cincinnati, OH 45268. Participants included Federal, state, and private laboratories engaged in NPDES permit compliance monitoring.

² Expressed as % effluent; in reality it was a reference toxicant (KCl) but was not known by the persons conducting the tests.

TABLE 2. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 2000: PRECISION OF RESPONSES USING EFFLUENT, RECEIVING WATER, AND REFERENCE TOXICANT SAMPLE TYPES¹.

Organism	Endpoint	Number of Tests ²	CV (%) ³
<i>Pimephales promelas</i>	Growth, IC25	73	20.9
<i>Ceriodaphnia dubia</i>	Reproduction, IC25	34	35.0
<i>Selenastrum capricornutum</i> (with EDTA)	Growth, IC25	21	34.3
	Growth, IC50	22	32.2
<i>Selenastrum capricornutum</i> (without EDTA)	Growth, IC25	21	58.5
	Growth, IC50	22	58.5

¹ From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

² Represents the number of valid tests (i.e., those that met test acceptability criteria) that were used in the analysis of precision. Invalid tests were not used.

³ CVs based on total interlaboratory variability (including both within-laboratory and between-laboratory components of variability) and averaged across sample types. IC25s or IC50s were pooled for all laboratories to calculate the CV for each sample type. The resulting CVs were then averaged across sample types.

4.14.4 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-based Control (see pp. 2-4, and 11-15 in USEPA, 1991a).

4.14.5 In cases where the test data are used in Probit Analysis or other point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis), precision can be described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated endpoints from the replicated tests. In cases where the test data are used in the Linear Interpolation Method, precision can be estimated by empirical confidence intervals derived by using the ICPIN Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). However, in cases where the results are reported in terms of the No-Observed-Effect Concentration (NOEC) and Lowest-Observed-Effect Concentration (LOEC) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis) precision can only be described by listing the NOEC-LOEC interval for each test. It is not possible to express precision in terms of a commonly used statistic. However, when all tests of the same toxicant yield the same NOEC-LOEC interval, maximum precision has been attained. The "true" no effect concentration could fall anywhere within the interval, $NOEC \pm (NOEC \text{ minus } LOEC)$.

4.14.6 It should be noted here that the dilution factor selected for a test determines the width of the NOEC-LOEC interval and the inherent maximum precision of the test. As the absolute value of the dilution factor decreases, the width of the NOEC-LOEC interval increases, and the inherent maximum precision of the test decreases. When a dilution factor of 0.3 is used, the NOEC could be considered to have a relative variability as high as $\pm 300\%$. With a dilution factor of 0.5, the NOEC could be considered to have a relative variability of $\pm 100\%$. As a result of the variability of different dilution factors, **USEPA recommends the use of the dilution factor of 0.5 or greater.** Other factors which can affect test precision include: test organism age, condition, and sensitivity; temperature

control; and feeding.

4.15 DEMONSTRATING ACCEPTABLE LABORATORY PERFORMANCE

4.15.1 It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. To meet this requirement, the intralaboratory precision, expressed as percent coefficient of variation (CV%), of each type of test to be used in the laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and the same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations.

4.16 DOCUMENTING ONGOING LABORATORY PERFORMANCE

4.16.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method conducted in the laboratory during that month. For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations, in the same dilution water, using the same data analysis methods. Precision may vary with the test species, reference toxicant, and type of test. Each laboratory's reference toxicity data will reflect conditions unique to that facility, including dilution water, culturing, and other variables; however, each laboratory's reference toxicity results should reflect good repeatability.

4.16.2 A control chart should be prepared for each combination of reference toxicant, test species, test conditions, and endpoints. Toxicity endpoints from five or six tests are adequate for establishing the control charts. Successive toxicity endpoints (NOECs, IC25s, LC50s, etc.) should be plotted and examined to determine if the results (X_1) are within prescribed limits (Figure 1). The chart should plot logarithm of concentration on the vertical axis against the date of the test or test number on the horizontal axis. The types of control charts illustrated (see USEPA, 1979a) are used to evaluate the cumulative trend of results from a series of samples, thus reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. For endpoints that are point estimates (LC50s and IC25s), the cumulative mean (\bar{X}) and upper and lower control limits ($\pm 2S$) are recalculated with each successive test result. Endpoints from hypothesis tests (NOEC, NOAEC) from each test are plotted directly on the control chart. The control limits would consist of one concentration interval above and below the concentration representing the central tendency. After two years of data collection, or a minimum of 20 data points, the control chart should be maintained using only the 20 most recent data points.

4.16.3 Laboratories should compare the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). If the calculated CV exceeds the 75th percentile of CVs reported nationally, the laboratory should use the 75th and 90th percentiles to calculate warning and control limits, respectively, and the laboratory should investigate options for reducing variability. Note: Because NOECs can only be a fixed number of discrete values, the mean, standard deviation, and CV cannot be interpreted and applied in the same way that these descriptive statistics are interpreted and applied for continuous variables such as the IC25 or LC50.

4.16.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified. In the case of endpoints that are point estimates (LC50s and IC25s), at the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone. If more than one out of 20 reference toxicant tests fall outside the control limits, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Control limits for the NOECs will also be exceeded occasionally, regardless of how well a laboratory performs. In those instances when the laboratory can document the cause for the outlier (e.g., operator error, culture health or test system failure), the outlier should be excluded from the future calculations of the control limits. If two or more consecutive tests do not fall within the control limits, the results

must be explained and the reference toxicant test must be immediately repeated. Actions taken to correct the problem must be reported.

4.16.5 If the toxicity value from a given test with a reference toxicant falls well outside the expected range for the other test organisms when using the standard dilution water and other test conditions, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Performance should improve with experience, and the control limits for endpoints that are point estimates should gradually narrow. However, control limits of $\pm 2S$ will be exceeded 5% of the time by chance alone, regardless of how well a laboratory performs. Highly proficient laboratories which develop very narrow control limits may be unfairly penalized if a test result which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not a reference toxicant test result falls “well” outside the expected range. The width of the control limits may be evaluated by comparing the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). In determining whether or not a reference toxicant test result falls “well” outside the expected range, the result also may be compared with upper and lower bounds for $\pm 3S$, as any result outside these control limits would be expected to occur by chance only 1 out of 100 tests (Environment Canada, 1990). When a result from a reference toxicant test is outside the 99% confidence intervals, the laboratory must conduct an immediate investigation to assess the possible causes for the outlier.

4.16.6 Reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. Reference toxicant testing is used for evaluating the health and sensitivity of organisms over time and for documenting initial and ongoing laboratory performance. While reference toxicant test results should not be used as a *de facto* criterion for test rejection, effluent and receiving water test results should be reviewed and interpreted in the light of reference toxicant test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increased test organism sensitivity or toward decreased test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test.

4.17 REFERENCE TOXICANTS

4.17.1 Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride (CdCl₂), copper sulfate (CuSO₄), sodium dodecyl sulfate (SDS), and potassium dichromate (K₂Cr₂O₇), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests. EMSL-Cincinnati hopes to release USEPA-certified solutions of cadmium and copper for use as reference toxicants through cooperative research and development agreements with commercial suppliers, and will continue to develop additional reference toxicants for future release. Standard reference materials can be obtained from commercial supply houses, or can be prepared inhouse using reagent grade chemicals. The regulatory agency should be consulted before reference toxicant(s) are selected and used.

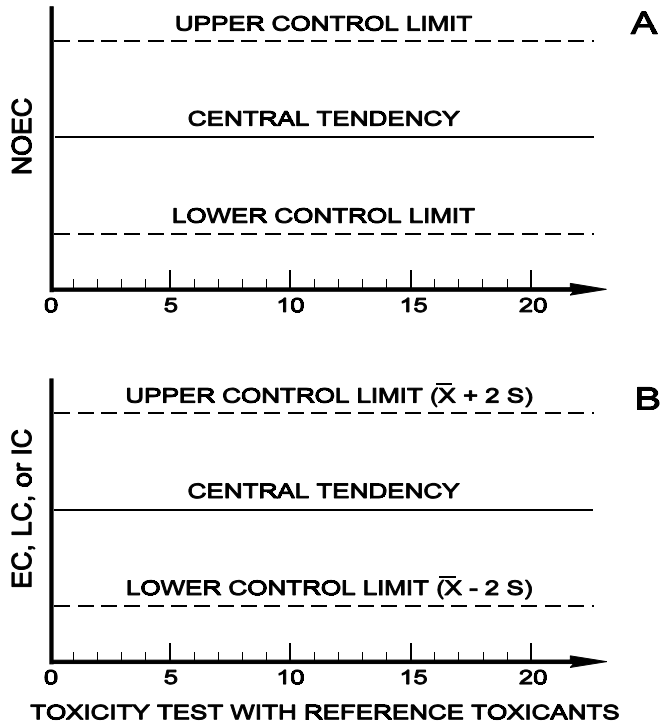


Figure 1. Control charts. (A) hypothesis testing results; (B) point estimates (LC, EC, or IC).

$$\bar{X} = \frac{\sum_{i=1}^n X_i}{n}$$

$$S = \sqrt{\frac{\sum_{i=1}^n X_i^2 - \frac{(\sum_{i=1}^n X_i)^2}{n}}{n-1}}$$

Where: X_i = Successive toxicity values from toxicity tests.

n = Number of tests.

\bar{X} = Mean toxicity value.

S = Standard deviation.

4.18 RECORD KEEPING

4.18.1 Proper record keeping is important. A complete file should be maintained for each individual toxicity test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

4.18.2 The regulatory authority should retain records pertaining to discharge permits. Permittees are required to retain records pertaining to permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

SECTION 5

FACILITIES, EQUIPMENT, AND SUPPLIES

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities must include equipment for rearing and/or holding organisms. Culturing facilities for test organisms may be desirable in fixed laboratories which perform large numbers of tests. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be ground water, receiving water, dechlorinated tap water, or reconstituted synthetic water. Dechlorination can be accomplished by carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg (anhydrous) sodium thiosulfate/L will reduce 1.0 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable. Air used for aeration must be free of oil and toxic vapors. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters, and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

5.1.2 The facilities must be well ventilated and free from fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories and/or sample holding areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Sample preparation, culturing, and toxicity test areas should be separated to avoid cross contamination of cultures or toxicity test solutions with toxic fumes. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely-fitting doors. Organisms should be shielded from external disturbances.

5.1.3 Materials used for exposure chambers, tubing, etc., that come in contact with the effluent and dilution water should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used as test chambers or to ship, store and transfer effluents and receiving waters, but they should not be reused unless absolutely necessary, because they could carry over adsorbed toxicants from one test to another, if reused. However, these containers may be repeatedly reused for storing uncontaminated waters, such as deionized or laboratory-prepared dilution waters and receiving waters. Glass or disposable polystyrene containers can be used for test chambers. The use of large (≥ 20 L) glass carboys is discouraged for safety reasons.

5.1.4 New plastic products of a type not previously used should be tested for toxicity before initial use by exposing the test organisms in the test system where the material is used. Equipment (pumps, valves, etc.) which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below (see Section 5, Facilities, Equipment and Supplies, Subsection 5.3.2). Fiberglass and stainless steel, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system, but once contaminated with pollutants the fiberglass should not be reused. All material should be flushed or rinsed thoroughly with the test media before using in the test.

5.1.5 Copper, galvanized material, rubber, brass, and lead must not come in contact with culturing, holding, acclimation, or dilution water, or with effluent samples and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers), may be toxic and should be tested before use.

5.1.6 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 TEST CHAMBERS

5.2.1 Test chamber size and shape are varied according to size of the test organism. Requirements are specified in each toxicity test method.

5.3 CLEANING TEST CHAMBERS AND LABORATORY APPARATUS

5.3.1 New plasticware used for sample collection or organism exposure vessels does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and rinsed well in deionized water and dilution water.

5.3.2 All non-disposable sample containers, test vessels, tanks, and other equipment that have come in contact with effluent must be washed after use to remove contaminants as described below.

1. Soak 15 min in tap water and scrub with detergent, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh, dilute (10%, V:V) hydrochloric or nitric acid to remove scale, metals and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

5.3.3 Special requirements for cleaning glassware used in the green alga, *Selenastrum capricornutum*, toxicity tests (Method 1003.0, Section 14). Prepare all graduated cylinders, test flasks, bottles, volumetric flasks, centrifuge tubes and vials used in algal assays as follows:

1. Wash with non-phosphate detergent solution, preferably heated to $\geq 50^{\circ}\text{C}$. Brush the inside of flasks with a stiff-bristle brush to loosen any attached material. The use of a commercial laboratory glassware washer or heavy-duty kitchen dishwasher (under-counter type) is highly recommended.
2. Rinse with tap water.
3. Test flasks should be thoroughly rinsed with acetone and a 10% solution (by volume) of reagent grade hydrochloric acid (HCl). It may be advantageous to soak the flasks in 10% HCl for several days. Fill vials and centrifuge tubes with the 10% HCl solution and allow to stand a few minutes; fill all larger containers to about one-tenth capacity with HCl solution and swirl so that the entire surface is bathed.
4. Rinse twice with MILLIPORE[®] MILLI-Q[®] OR QPAKTM₂, or equivalent, water.
5. New test flasks, and all flasks which through use may become contaminated with toxic organic substances, must be rinsed with pesticide-grade acetone or heat-treated before use. To thermally degrade organics, place glassware in a high temperature oven at 400°C for 30 min. After cooling, go to 7. If acetone is used, go to 6.
6. Rinse thoroughly with MILLIPORE[®] MILLI-Q[®] or QPAKTM₂, or equivalent water, and dry in an 105°C oven. All glassware should be autoclaved before use and between uses.
7. Cover the mouth of each chamber with aluminum foil or other closure, as appropriate, before storing.

5.3.4 The use of sterile, disposable pipets will eliminate the need for pipet washing and minimize the possibility of contaminating the cultures with toxic substances.

5.3.5 All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

5.4 APPARATUS AND EQUIPMENT FOR CULTURING AND TOXICITY TESTS

5.4.1 Apparatus and equipment requirements for culturing and testing are specified in each toxicity test method. Also, see USEPA, 2002a.

5.4.2 WATER PURIFICATION SYSTEM

5.4.2.1 A good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, must be available in the laboratory and in sufficient quantity for laboratory needs. Deionized water may be obtained from MILLIPORE® Milli-Q®, MILLIPORE® QPAK™₂ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade deionizer with preconditioned water from a Culligan®, Continental®, or equivalent mixed-bed water treatment system.

5.5 REAGENTS AND CONSUMABLE MATERIALS

5.5.1 SOURCES OF FOOD FOR CULTURE AND TOXICITY TESTS

1. Brine shrimp, *Artemia* sp., cysts -- Many commercial sources of brine shrimp cysts are available.
2. Frozen adult brine shrimp, *Artemia* -- Available from most pet supply shops or other commercial sources.
3. Flake fish food -- TETRAMIN® and BIORIL® are available from most pet shops.
4. Trout chow -- Available from commercial sources.
5. Cereal leaves, CEROPHYLL® or equivalent -- Available from commercial sources.
6. Yeast -- Packaged dry yeast, such as Fleischmann's, or equivalent, can be purchased at the local grocery store or commercial sources.
7. Alfalfa Rabbit Pellets -- Available from feed stores as Purina rabbit chow.
8. Algae - Available from commercial sources.

5.5.1.1 All food should be tested for nutritional suitability and chemically analyzed for organochlorine pesticides, PCBs, and toxic metals (see Section 4, Quality Assurance).

5.5.2 Reagents and consumable materials are specified in each toxicity test method section. Also, see Section 4, Quality Assurance.

5.6 TEST ORGANISMS

5.6.1 Test organisms should be obtained from inhouse cultures or from commercial suppliers (see specific test method; Section 4, Quality Assurance; and Section 6, Test Organisms).

5.7 SUPPLIES

5.7.1 See test methods (see Sections 11-14) for specific supplies.

SECTION 6

TEST ORGANISMS

6.1 TEST SPECIES

6.1.1 The species used in characterizing the chronic toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test. It is essential that good quality test organisms be readily available throughout the year from inhouse or commercial sources to meet NPDES monitoring requirements. The organisms used in the toxicity tests must be identified to species. If there is any doubt as to the identity of the test organism, representative specimens should be sent to a taxonomic expert to confirm the identification.

6.1.2 Toxicity test conditions and culture methods for the species listed in Subsection 6.1.3 are provided in this manual also, see USEPA, 2002a.

6.1.3 The organisms used in the short-term chronic toxicity tests described in this manual are the fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia* (Berner, 1986), and the green alga, *Selenastrum capricornutum*.

6.1.4 Some states have developed culturing and testing methods for indigenous species that may be as sensitive, or more sensitive, than the species recommended in Subsection 6.1.3. However, USEPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the recommended species in Subsection 6.1.3. Where state regulations prohibit importation of non-native fishes or the use of recommended test species, permission must be requested from the appropriate state agency prior to their use.

6.1.5 Where states have developed culturing and testing methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and the one or more recommended species must be obtained in side-by-side toxicity tests with reference toxicants and/or effluents, to ensure that the species selected are at least as sensitive as the recommended species. These data must be submitted to the permitting authority (State or Region) if required. USEPA acknowledges that reference toxicants prepared from pure chemicals may not always be representative of effluents. However, because of the observed and/or potential variability in the quality and toxicity of effluents, it is not possible to specify a representative effluent.

6.1.6 Guidance for the selection of test organisms where the salinity of the effluent and/or receiving water requires special consideration is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1. Where the salinity of the receiving water is < 1‰, freshwater organisms are used regardless of the salinity of the effluent.
2. Where the salinity of the receiving water is ≥ 1‰, the choice of organisms depends on state water quality standards and/or permit requirements.

6.2 SOURCES OF TEST ORGANISMS

6.2.1 The test organisms recommended in this manual can be cultured in the laboratory using culturing and handling methods for each organism described in the respective test method sections. The fathead minnow, *Pimephales promelas*, culture method is given in Section 11 and not repeated in Section 12. Also, see USEPA (2002a).

6.2.2 Inhouse cultures should be established wherever it is cost effective. If inhouse cultures cannot be maintained or it is not cost effective, test organisms or starter cultures should be purchased from experienced commercial suppliers (see USEPA, 2002a).

6.2.3 Starter cultures of the green algae, *Selenastrum capricornutum*, *S. minutum*, and *Chlamydomonas reinhardtii* are available from commercial suppliers.

6.2.4 Because the daphnid, *Ceriodaphnia dubia*, must be cultured individually in the laboratory for at least seven days before the test begins, it will be necessary to obtain a starter culture from a commercial source at least three weeks before the test is to begin if they are not being cultured inhouse.

6.2.5 If, because of their source, there is any uncertainty concerning the identity of the organisms, it is advisable to have them examined by a taxonomic specialist to confirm their identification. For detailed guidance on identification, see the individual test methods.

6.2.6 FERAL (NATURAL OCCURRING, WILD CAUGHT) ORGANISMS

6.2.6.1 The use of test organisms taken from the receiving water has strong appeal, and would seem to be a logical approach. However, it is generally impractical and not recommended for the following reasons:

1. Sensitive organisms may not be present in the receiving water because of previous exposure to the effluent or other pollutants.
2. It is often difficult to collect organisms of the required age and quality from the receiving water.
3. Most states require collecting permits, which may be difficult to obtain. Therefore, it is usually more cost effective to culture the organisms in the laboratory or obtain them from private, state, or Federal sources. The fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia*, and the green alga, *Selenastrum capricornutum*, are easily cultured in the laboratory or readily available commercially.
4. The required QA/QC records, such as the single laboratory precision data, would not be available.
5. Since it is mandatory that the identity of the test organism be known to species level, it would be necessary to examine each organism caught in the wild to confirm its identity. This would usually be impractical or, at the least, very stressful to the organisms.
6. Test organisms obtained from the wild must be observed in the laboratory for a minimum of one week prior to use, to assure that they are free of signs of parasitic or bacterial infections and other adverse effects. Fish captured by electroshocking must not be used in toxicity testing.

6.2.6.2 Guidelines for collecting natural occurring organisms are provided in USEPA (1973), USEPA (1990) and USEPA (1993b).

6.2.7 Regardless of their source, test organisms should be carefully observed to ensure that they are free of signs of stress and disease, and in good physical condition. Some species of test organisms can be obtained from commercial stock certified as "disease-free".

6.3 LIFE STAGE

6.3.1 Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as larval fish, is required for all tests. In a given test, all organisms should be approximately the same age and should be taken from the same source. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

6.4 LABORATORY CULTURING

6.4.1 Instructions for culturing and/or holding the recommended test organisms are included in the respective test methods (also, see USEPA, 2002a).

6.5 HOLDING AND HANDLING TEST ORGANISMS

6.5.1 Test organisms should not be subjected to changes of more than 3°C in water temperature in any 12 h period or 2 units of pH in any 24-h period.

6.5.2 Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch a dry surface or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from small-mesh nylon netting, silk batting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes (4 to 8 mm ID) with rubber bulbs or pipettors (such as PROPIPETTE®) should be used for transferring smaller organisms such as larval fish.

6.5.3 Holding tanks for fish are supplied with good quality water (see Section 5, Facilities, Equipment, and Supplies) with flow-through rate of at least two tank volumes per day. Otherwise use a recirculation system where water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photodegrade dissolved organics.

6.5.4 Crowding must be avoided because it will stress the organisms and lower the DO concentrations to unacceptable levels. The solution of oxygen depends on temperature and altitude. The DO must be maintained at a minimum of 4.0 mg/L. Aerate gently if necessary.

6.5.5 The organisms should be observed carefully each day for signs of disease, stress, physical damage, or mortality. Dead and abnormal organisms should be removed as soon as observed. It is not uncommon for some fish mortality (5-10%) to occur during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation. Organisms in the holding tanks should generally be fed as in the cultures (see culturing methods in the respective methods).

6.5.6 Fish should be fed as much as they will eat at least once a day with live brine shrimp nauplii, *Artemia*, or frozen adult brine shrimp, or dry food (frozen food should be completely thawed before use). Adult brine shrimp can be supplemented with commercially prepared food such as TETRAMIN® or BIORIL® flake food, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning.

6.5.7 A daily record of feeding, behavioral observations, and mortality should be maintained.

6.6 TRANSPORTATION TO THE TEST SITE

6.6.1 Organisms are transported from the base or supply laboratory to a remote test site in culture water or standard dilution water in plastic bags or large-mouth screw-cap (500 mL) plastic bottles in styrofoam coolers. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags or by use of an airstone supplied by a portable pump. The DO concentration must not fall below 4.0 mg/L.

6.6.2 Upon arrival at the test site, the organisms are transferred to receiving water if receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning and replaced slowly over a 10 to 15 minute period with dilution water. If receiving water is to be used as the dilution water, caution must be exercised in exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially

to the dilution water. If this group does not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms may be transferred to the dilution water.

6.6.3 A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed, or if mortality appears to exceed 10% preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The mortality may be due to the presence of toxicity, if the receiving water is used as dilution water, rather than a diseased condition of the test organisms. If the acclimation process is repeated with a new group of test organisms and excessive mortality occurs, it is recommended that an alternative source of dilution water be used.

6.7 TEST ORGANISM DISPOSAL

6.7.1 When the toxicity test(s) is concluded, all test organisms (including controls) should be humanely destroyed and disposed of in an appropriate manner.