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Toxicity Identification Evaluation:

Characterization of Chronically Toxic Effluents, Phase I

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T.J. Norberg-King
Environmental Research Laboratory
Duluth, MN 55804

D.I. Mount,
J.R. Amato,
D.A. Jensen,
and
J.A. Thompson
ASci Corporation
Duluth, MN 55804

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Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Duluth, MN 55804

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Foreword

This guidance document has been prepared to assist dischargers and/or their consultant laboratories in conducting chronic aquatic toxicity identification evaluations (TIEs). TIEs may be required by the state or federal agencies as a result of enforcement actions or as a condition of the discharger's National Pollutant Discharge Elimination System (NPDES) permit or may be conducted voluntarily by permittees. This document will assist the state and federal agencies and permittees in overseeing and determining the adequacy of the TIE in toxicity reduction evaluations (TREs).

This document discusses methods to characterize the chemical/physical nature of the constituents in effluents which cause their chronic toxicity. The general approach for toxicity identification evaluations is described in the document *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures* (EPA, 1988A; EPA, 1991A), hereafter referred to as the *"acute Phase I manual."* The acute Phase I manual provides much of the basis for the statements and guidance provided in this chronic Phase I characterization document. This chronic TIE manual and the acute Phase I manual should be used as companion documents, because all the guidance of the acute Phase I manual is not repeated here.

The general approach for the chronic characterization is divided into Tier 1 and Tier 2. Tier 1 consists of the EDTA and sodium thiosulfate additions, the graduated pH test, aeration and filtration manipulations, and the use of the C_{18} solid phase extraction (SPE) resin. For Tier 1, the tests are all done using the effluent sample without any pH adjustments (i.e., at the initial pH (pH *i*) of the effluent). Tier 2 manipulations are added when Tier 1 tests are not definitive in characterizing the toxicity. Tier 2 includes the aeration, filtration, and C_{18} SPE steps of Tier 1 performed at pH 3 and pH 10 and returned to pH *i* prior to testing.

The chronic Phase I procedures should provide information on whether the toxicants are volatile, chelatable, filterable, reducible, non-polar, or pH sensitive. These characteristics are indicated by comparing the results of toxicity tests conducted using unaltered and manipulated effluent samples. As with the acute TIE, the characterization results from the chronic TIE can be used for the treatability approach in a TRE (EPA, 1991A).

These chronic TIE methods are not written as rigid, required protocols, but rather as general guidance for conducting TIEs with effluents. These acute and chronic methods should also be applicable to samples from ambient waters, sediment pore and elutriate waters, and leachates. The methods to identify (Phase II; EPA, 1989A) and confirm (Phase III; EPA, 1989B) the cause of toxicity in effluent samples evaluated with the acute Phase I procedure are also applicable to effluent samples evaluated with this chronic Phase I procedure. The identification and confirmation documents are being revised (EPA, 1992A; EPA, 1992B) to reflect additional information from this manual and the revised acute Phase I manual (EPA, 1991A) to discuss the aspects of TIEs for both acute and chronic toxicity.

In September of 1991, we solicited peer-review comments until January 31, 1992 from all persons who obtained the document from any of the following locations: EPA's Office of Water, Washington, D.C., each EPA Regional Water Division Office, EPA's Environmental Research Laboratory-Duluth, MN, or EPA's Center for Environmental Research Information (CERI), Cincinnati, Ohio. Appropriate technical comments were incorporated into this manual.

Abstract

This manual is intended to provide guidance to aid dischargers in characterizing the type of toxicants that are causing chronic toxicity in industrial and municipal effluents. In a regulatory context, a toxicity identification evaluation (TIE) may be required as part of the National Pollutant Discharge Elimination System (NPDES) permit or as an enforcement action. TIEs may also be conducted by permittees on a volunteer basis to characterize their discharge toxicity.

The Phase I chronic toxicity methods are modified from those described in the acute Phase I TIE manual (EPA, 1988A; EPA, 1991A) and additional techniques are incorporated. This chronic Phase I manual describes procedures for characterizing the physical/chemical nature of toxicants in effluents that exhibit chronic toxicity to freshwater species, although many of the principles and procedures are similar for TIEs on marine species. Aliquots of effluent samples are manipulated and the resulting effect on toxicity measured. The objective is to characterize the toxicants so that appropriate analytical methods can be chosen to identify the toxicants.

The general approach to the chronic toxicity characterization is a two tiered approach, where usually Tier 1 is applied before proceeding to Tier 2. Tier 1 consists of filtration, aeration, use of additives to chelate or reduce the toxicants, minor pH adjustments, and use of a separation technique with the C_{18} solid phase extraction (SPE) resin. Each effluent is characterized in Tier 1 by performing the manipulations at the initial pH (pH *i*) of the effluent. Tier 2 consists of the Tier 1 manipulations combined with pH adjustments of additional aliquots of the effluent sample, and the Tier 2 characterization steps include aeration, filtration, and the C_{18} solid phase extraction of effluent samples adjusted to pH 3 and pH 10.

The Phase I characterization methods were developed for the short-term *"chronic"* test methods using two species, *Ceriodaphnia dubia* and the fathead minnow (*Pimephales promelas*) (EPA, 1989C). Chronic threshold levels for the various additives (sodium thiosulfate, EDTA, methanol) used in some of the characterization tests are provided for these species. Although developed for these species, the characterization techniques should be applicable to other species as well, provided threshold levels are established.

The guidance provided in this manual is intended to be supplemental to that given in the acute Phase I manual (EPA, 1991A). Sections of this chronic Phase I TIE manual discuss quality assurance, effluent handling, facilities and equipment, health and safety, dilution water, principles of the chronic TIE testing, and the Phase I characterization tests as a two tiered approach. The use of the whole effluent test as a *baseline test* (in manner similar to the acute Phase I characterization procedure), the appropriate treatment of dilution water for blanks and the toxic levels of the additives for two species are described. Use of short-cuts, reduced test volumes, reduced test duration, and a small number of replicates are discussed. The importance of sample type, frequency of sample collection and renewal, and descriptions of all manipulations are discussed, along with a section on the application of combining several of the characterization tests.

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In the review comments, a suggestion was made to summarize all the effort on TIEs by government, state, academia, contract laboratories, and industries to date. While the TIEs at Duluth can be summarized, data from all the possible sources are difficult if not impossible to obtain. Contract laboratories and industrial data are protected for confidentiality and proprietary reasons, and information about the kinds of toxicants, the types of discharges, the time-frame for the TIE, and the costs are difficult to obtain. Numerous toxicity problems have been resolved as TIEs are initiated because of better plant operation. In fact, during a workshop (Aquatic Habitat Institute, 1992) held March 17 and 18, 1992 in Richmond, CA, these issues were discussed, and presenters of chronic TIE data indicated chronic TIEs have been much more successful than expected.

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Section 1
Introduction

The United States Federal Water Pollution Control Act Amendments (commonly referred to as the Clean Water Act (CWA); (Public Law 92-500 of 1972) states that the discharge of toxic pollutants in toxic amounts is prohibited. In the CWA, the National Pollutant Discharge Elimination System (NPDES) was established; this system provides a mechanism whereby point source wastewater discharges are permitted. NPDES permits contain effluent limits that require baseline use of treatment technologies (best available technology). The technology-based limits are independent of receiving water impact, and additional water quality-based limits may be needed in order to meet the goal of the CWA of "*no toxics in toxic amounts.*" State narrative and state numerical water quality standards are used in conjunction with EPA's water quality criteria and other toxicity databases to determine the adequacy of the technology-based permit limits and the need for any additional water quality-based controls.

When limits were first written into the permits, they were based primarily on physical factors such as biological oxygen demand (BOD), suspended solids (SS), and color. Additional components were added in subsequent amendments to the CWA; for example, the list of 126 *"priority pollutants"* of which many or most were required to be monitored by the permittees. Water quality criteria were used to develop the water quality-based limits for these pollutants. However, water quality criteria or discharge limits exist for only a few of the thousands of chemicals in use.

An important objective of the NPDES program is the control of toxicity of discharges and to accomplish this objective, EPA uses an integrated water quality-based approach. Published water quality criteria are converted to standards that consist of both chemical-specific numeric criteria for individual toxics and narrative criteria. The states' narrative water quality criterion generally requires that the waters be free from oil, scum, floating debris, materials that will cause odors, materials that are unsightly or deleterious, materials that will cause a nuisance, or substances in concentrations that are toxic to aquatic life, wildlife or human health. Use of toxicity testing and whole effluent toxicity limitations is based on a state's narrative water quality criterion and in some cases, a state numeric criterion for toxicity.

EPA, in 1984, issued a policy statement (Federal Register, 1984) that recommends an *"integrated approach"* for controlling toxic pollutants. This *integrated approach* is referred to as the water quality-based approach and is described in detail in the Technical Support Document (hereafter referred to as the TSD; EPA, 1985A; EPA, 1991B). The control regulations for EPA (Federal Register 23868, 1989) establish specific requirements that the *integrated approach* be used for water quality-based toxics control. This *integrated approach* results in NPDES permit limits to control toxic pollutants through the use of both chemical-specific and whole effluent toxicity limitations as a means to protect both aquatic life and human health. This combination of chemical specific and whole effluent toxicity limitations is essential to the control of toxic pollutants. Once the permit limits are set, compliance is established through routine monitoring of effluent quality. In this manner, water quality-based limits (when following EPA, 1991B) will protect water quality and prevent the state water quality standards from being violated.

The whole effluent toxicity limitation aspect involves using acute and chronic toxicity tests to measure the toxicity of wastewaters. *Acute toxicity* refers to toxicity that occurs in a short period of time, operationally defined as 96 h or less. *Chronic toxicity* occurs as the result of long exposures in which sublethal effects (fertilization, growth, reproduction) are measured in addition to lethality. The chronic test is used to measure the effects of long-term exposure to chemicals, wastewaters, and leachates to aquatic organisms. True chronic toxicity tests include the life-cycle of the organism. For fish, the life-cycle test is infrequently conducted (Norberg-King, 1989A), and abbreviated test methods have been used to estimate chronic toxicity. These tests are the 7-d growth and survival test (EPA, 1989C), or the 32-d embryo-larval early life stage test (Norberg-King, 1989A). These tests rely on the most sensitive life-cycle stages (i.e., embryos and larval fish) to estimate chronic toxicity (McKim, 1977; Woltering 1983; Norberg-King, 1989A). Hereafter, chronic tests refer to the short-term tests that are described in the EPA manuals (EPA, 1992C; EPA, 1992D; EPA, 1989C; EPA, 1985C).

Toxicity is a useful parameter to protect receiving waters from potential impacts on water quality and designated uses caused by the mixture of toxic pollutants in wastewaters. EPA has published manuals which provide test methods for use of freshwater and marine organisms to determine acute and chronic toxicity of effluents. These manuals have been available since 1978 and 1985, respectively (EPA, 1978; EPA,

1985B; EPA, 1985C; EPA, 1988B; EPA, 1989C) and have been recently revised (EPA, 1991C; EPA, 1992C; EPA, 1992D). These methods are used by federal, state and local governments to assess toxicity and determine compliance of permitted point source discharges. Since the late 1970's, toxicity has been measured in wastewaters; permit writers began using toxicity limits in the early 1980's. With the increased use of toxicity testing, substantial numbers of unacceptably toxic effluents have been identified. Now, some permittees are required to perform *toxicity reduction evaluations* (TREs) as a condition of the NPDES permit. The TSD defines a TRE as "*a site specific study conducted in a stepwise process designed to identify the causative agents of effluent toxicity, isolate the sources of toxicity, evaluate the effectiveness of toxicity control options, and then confirm the reduction in effluent toxicity.*" *Toxicity identification evaluations* (TIEs), which are a part of the TRE, consist of methods to characterize (Phase I; EPA, 1988A; EPA, 1991A; EPA, 1991D), identify (Phase II; EPA, 1989A; EPA, 1992A), and confirm (Phase III; EPA, 1989B; EPA, 1992B) the cause of acute and chronic toxicity in effluents.

The TIE approach (EPA, 1988A; EPA, 1991A) relies on the use of organisms to detect the presence of toxicants in the effluent. Information about the physical/chemical characteristics of the effluent's toxicity is gained (by the various manipulations) and if possible the number of constituents in the effluent is reduced before any analyses begin. Using this approach, analytical problems can be simplified and the costs reduced. Toxicity throughout the TIE must be tracked to determine if the toxicity is consistently being caused by the same substance. Once the physical/chemical characteristics of toxicants are known, a better choice of analytical methods can be made. Knowledge of physical/chemical characteristics of any effluent is used for the treatability approach to TRE's (EPA, 1989D; EPA, 1989E).

As with the acute Phase I TIE approach, the chronic Phase I TIE is based on manipulations designed to alter a group of toxicants (such as oxidants, cationic metals, volatiles, or non-polar organics) so that toxicity is changed. Chronic toxicity tests are conducted after each manipulation to indicate the effect on the toxicity of the effluent. Based upon the manipulations that change toxicity, inferences about the chemical/physical characteristics of the toxicants can be made. Using several samples of the effluent for these characterization steps provides information on whether the nature of compounds causing the chronic toxicity remains consistent. The tests do not provide information on the variability of toxicants within a characterization group. From these data the toxicant characteristics can be identified as pH sensitive, filterable, volatile, soluble, degradable, reducible, or EDTA chelatable. Such information indicates how samples must be handled for analyses and which analytical methods should be used.

The recommended procedure is to concentrate on the characterization steps that are most clean-cut and have the major effect of reducing the toxicity in the effluent. If toxicity in every effluent sample is not caused by the same toxicant(s), the characterization tests should indicate if the type of toxicant(s) is the same or different. Once identification is initiated, and suspects identified, the varying causes of toxicity can be evaluated because the concentration of toxicants should be tracking with the toxicity. In the earlier version of this document (EPA, 1991D) we suggested that samples be subjected to Phase I techniques until no additional responses are found (which was suggested to be at least three samples). After conducting several Phase I evaluations for chronic toxicity, we have determined that if the effluents' toxicity is readily characterized after Phase I even with one sample it may be prudent to proceed with Phase II (EPA, 1992C) to measure the toxicant(s). Use of toxicity patterns as the TIE progresses can be helpful if patterns are tracked, beginning with the first samples. Following characterization, a decision is made to proceed with identification (Phase II; EPA, 1989A; EPA, 1992A) and confirmation (Phase III, EPA, 1989B; EPA, 1992B) or to conduct treatability studies where the identification of the specific toxicants (cf., acute treatability procedures (EPA, 1989D; EPA, 1989E)) is not made.

Chronic toxicity must be present frequently enough so that an adequate number of toxic samples can be obtained. Enough routine toxicity testing should be done on each effluent before a TIE is initiated (EPA, 1991B), to ensure that toxicity is consistently present. It is not important that the same amount of toxicity is present in each sample; in fact, variable levels of toxicity can assist in determining the cause of toxicity. If toxicity is not consistently present, when it occurs the toxicity can be pursued and if a toxicant(s) is suspected, the non-toxic samples may be used to eliminate suspects. One cannot assume that if the effluent showed acute toxicity and a TIE was completed, identifying the cause(s) of acute toxicity and action taken to remove the acute toxicant from the effluent, that the sublethal toxicity exhibited is due to the same compound.

Section 2
Quality Assurance, Health, and Safety, and Facilities and Equipment

2.1 Quality Assurance

The quality assurance plan (QAP), as described in *Standard Methods for the Examination of Water and Wastewater* (APHA, 1989) (describes standards to conduct performance evaluations) is primarily for analytical analyses. A QAP for toxicity testing can be developed, but determining the recovery of known additions for toxicity testing is not possible. For TIEs the combination of chemistry and biology requires a level of checks and balances not typically used under other situations. A step-by-step QAP for all steps of a TIE is not always possible due to the unknown toxicant(s) requiring various follow-up testing and analytical procedures; however as a TIE progresses, additional or different tests may be needed and many aspects of the TIE QAP can be addressed as the TIE proceeds. Adhering to the general guidelines of a strong QAP is important however, and should increase the probability of the TIE succeeding. As additional steps are recognized, the details should be added to the QAP.

Specific quality control (QC) procedures for aquatic toxicity tests are different than the specific QC procedures for chemical analytical methods. Both procedures have common goals that are to know that reliable data are generated, to recognize and eliminate unreliable data, and to have methods which assist investigations in resolving problems for future work. The quality assurance (QA/QC) guidance given by EPA (1989C) for the short-term tests lists numerous items of concern for toxicity testing. These are: (a) effluent sampling/handling, (b) test organisms, (c) facilities, equipment and test chambers, (d) analytical methods, (e) calibration and standardization, (f) dilution water, (g) test conditions, (h) test acceptability, (i) test precision, (j) replication and test sensitivity, (k) quality of organisms, (l) quality of food, (m) control charts, and (n) record keeping and data evaluation. Many of these should be closely followed, and the reader is encouraged to review the guidance in relation to QA/QC in both the short-term effluent test manual (EPA, 1989C; EPA, 1992C) and the acute Phase I manual (EPA, 1991A).

2.2 QA/QC Cost Considerations and Testing Requirements

For the chronic TIE, cost considerations are important and concessions in the requirements of the QC may have to be made. In some instances, the data will demand stringent control while in others, the QC can be lessened without impact to the overall endpoint of the TIE.

TIEs can require a great number of toxicity tests. The use of all aspects of the standard test protocols (EPA, 1989C; EPA, 1991C) is not necessary in Phase I. The factors of time requirements, number of tests and the test design (i.e., five replicates versus ten, four dilutions versus five) must be considered and weighed against the type of questions that are posed. For example, the need for water chemistry data are specific for each Phase I test. The testing requirement (EPA, 1989C) according to the permit requirement most likely included pH, daily measurements of DO, temperature, conductivity, alkalinity, and hardness measurements in the low, middle, and high concentrations for the five test dilutions of the effluent. However, hardness measurements are not pertinent for the methanol eluate collected from a solid phase extraction column. The post C_{18} SPE column effluent samples are more similar to the effluent and a concern for low dissolved oxygen (DO) exists, while the test solutions of the methanol eluate are more similar to the dilution water and the possibility of low DOs is not as great a concern. In contrast, frequent pH measurements on all test concentrations are needed to determine the impact of pH sensitive compounds.

As TIEs are reliant on a strong QAP, there are several aspects of a QA/QC program for chronic TIEs that should be delineated. In regard to test organism quality, there are steps for culturing organisms that should help provide the necessary QC verification that is needed to ensure the animals are representative in their sensitivity. These steps are simply routine items such as monitoring and recording the young production (for cladocerans) of the culture brood animals once a month, conducting monthly reference toxicant tests (including maintaining control charts), monitoring the preparation dates for the reconstituted waters used, and monitoring the types and age of the foods fed (Norberg-King, 1989B). For fathead minnows, it is useful to monitor the survival of the breeding stock, and the percent hatchability of the embryos, to verify that new genetic stock is introduced on a regular basis, and to conduct monthly reference toxicant tests (Norberg-King and Denny, 1989; Denny, 1988). Similar parameters for other species that are used are also desirable.

Since toxicity tests in the early part of the chronic Phase I do not generally follow all the effluent testing

requirements (EPA, 1989C), the QC measures are not as strict because the data are primarily informative rather than definitive. When Phases II (identification) and III (confirmation) are initiated, then QC aspects should be reconsidered and the tests modified. Phase I procedures frequently use one species and later stages of the TIE (Phase III) use more than one species to determine whether the cause of toxicity is the same for other species of the aquatic community.

Reference toxicant tests are not conducted with each set of Phase I manipulations because of the amount of labor and large numbers of animals required for testing. In general, the utility of the reference toxicant test is to know that the organisms are responding as expected. Since only relative differences are needed at this stage (Phase I), reference toxicant data are much less useful for the characterization interpretation but are important for the knowledge of the quality of the test organisms and general test procedures. For various manipulations of the TIE, organism responses are compared to either the *baseline test* (see Section 6) or the response of organisms in the dilution water treatments. Monthly reference toxicant tests should provide the necessary information about the quality of the organisms for the laboratory conducting the TIE. When a toxicant has been identified (Phase II) and tests for Phase III confirmation indicate it is the toxicant(s), that chemical should become the reference toxicant with the species used in the TIE.

Using receiving water as the dilution water in Phase III confirmation will help ensure that receiving water effects are properly considered (see Section 3, Dilution Water). The variability of the effluent, by nature of the TIE, is defined during the TIE, and this information will aid in determining the appropriate control option in order that the final effluent is safe upon discharge.

2.3 QA/QC and Chronic Testing Considerations

An inherent problem with effluents is that no effluent test can be repeated to assure that the toxicity is the same and that the toxicants are the same. However, repeated *baseline tests* (Section 6) can be done with the same effluent sample to determine how long that effluent sample can be used. The chemical and toxicological nature of the effluent shifts as an effluent is discharged or as an effluent sample is stored. Effluent constituents degrade (at unknown rates) and each constituent has its own rate of change. Analysis of each sample should be initiated as soon as the sample is received in the testing laboratory (generally <24 h). Until an effluent sample has been tested several times, there is no way to predict how long a sample can be stored before the toxicity changes. Testing of each sample can be done provided the toxicity remains and/or stabilizes; however this cannot be determined at the beginning of the Phase I battery of tests and will be known only through testing several samples a few times. Even though the toxicity remains, it is possible that the toxicant may change with time. The number of samples to evaluate and the number of tests to conduct must be weighed against the cost of the effort and how representative each effluent sample is of the effluent. Effluents that have low and non-persistent toxicity may need to be approached with the Tier 1 and Tier 2 characterization steps applied simultaneously (see Section 6).

In a chronic TIE, information obtained from a test should be maximized. This may mean paying particularly close attention to details such as small differences in the number of neonates the cladocerans are producing or the lack of food in the stomach of the larval fish. These parameters and any other observed characteristics during a test may be subtle indicators and quite informative about small changes in toxicity. For example, if all the animals exposed to the whole effluent die on day 4, and in some characterization test the animals don't reproduce or grow but are alive at day 7 of the exposure, that characterization manipulation reduced the toxicity, but did not remove it completely. Observations such as these may be just as useful as reductions in young production or growth.

While some abbreviations in the test design are made, the general principles for toxicity testing still apply. For example, all animals must be added to test solutions randomly. Animals must be placed in a test chamber one at a time. For the fathead minnows, use of an intermediate vessel to hold all 10 animals is preferable to ensure that animals are assigned randomly and that the volume of water added with the fish is minimized (1-2 ml). Also, transferring animals may require separate pipettes for each concentration or cleaning of the pipettes between concentrations to prevent cross contamination. However, we have observed that *C. dubia* do not have to be placed under the water; they can be added or transferred by dropping the water droplet containing the animal into the test solution. The problem frequently observed with *D. pulex* where animals are caught at the surface of the test solution (called "floaters") does not occur with *C. dubia*. Randomization, careful exposure time readings, use of animals of uniform narrow-age groups (i.e., *Ceriodaphnia* neonates 0-6 h old rather than 0-12 h old) should assist in quality data generation.

Standard operating procedures (SOPs) should be developed for each Phase I test, for preparing the reconstituted waters, preparing the foods for the test organisms, calibration and standardization for all measurements (temperature, DO, pH, conductivity, alkalinity, hardness, ammonia, chlorine), and other general routine practices.

An important aspect of TIEs is accurate and thorough data recording. All observations should be documented. Items that were not thought to be important at first may be useful in later stages of analysis and actually assist in the confirmation of the toxicant(s). These observations can be as simple as large bubbles produced during the aeration and filtration manipulations, large particles present in whole effluent, and low

pH upon arrival. It is best to record data so that any preconceived ideas of the toxicants are avoided. Data records should include records of test organisms (species, source, age, date of receipt, history and health), calibration records, test conditions, results of tests, and summaries of data. Once a control chart is developed using point estimates for reference toxicant tests, 1 out of 20 reference toxicity test results will be predicted to fall outside the acceptable limits if the 95% confidence intervals are used to develop the control chart (EPA, 1991C). If TIEs are conducted during such a period, the TIE data generated must be used with caution, and the investigator must carefully examine the TIE data to determine if the results are usable. The decision may be based on consistency of the concentration response data, *control blank* performance, and the consistency of the TIE results with those obtained with the same effluent sample.

2.4 QA/QC Blanks and Artifactual Toxicity

Throughout the TIE, dilution water samples are subjected to most of the procedures and analyses performed on the effluent sample (see Section 5.6). This is done to detect toxic artifacts (i.e., toxicity due to anything other than the effluent constituents causing toxicity) that are created during the effluent characterization manipulations (see Section 6). These manipulations can make QC/QA verifications difficult, as the use of such *blanks* for interpreting toxicity results is not standard toxicology. For example, typically organism responses from any toxicity test in standard aquatic toxicology are compared to the performance of *control* organisms which were in dilution water only. In the TIE, *controls* are used to judge organism performance (Section 5), and *toxicity controls* and *blanks* are used to evaluate whether a manipulation affected the toxicant(s), therefore the results of all characterization tests are not necessarily compared to the *baseline test.* For instance, post-column effluent samples that are collected and tested following concentration on a resin column have been filtered first. Therefore it is only logical to compare the post-column effluent toxicity (*post C_{18} SPE column test*; Section 6.6) to the toxicity observed in the filtered effluent sample (*filtration test*; Section 6.4) rather than to the unfiltered whole effluent (*baseline test*; Section 6.1) (see Section 5).

Artifactual toxicity can occur in several of the manipulations, particularly from the major pH adjustment manipulation (Tier 2). Toxicity results from tests relying on the addition of the reagents (EDTA, sodium thiosulfate, acids/bases) must be interpretable. Addition of both the acid (HCl) and the base (NaOH) can form a toxic product (e.g., NaCl). The addition of the acid and base may interfere with the growth and reproduction of the test organisms for the short-term chronic test, at lower levels than cause mortality in the acute test. Whether additives act in an additive, synergistic, or independent manner with the compounds in the effluent must be determined during the TIE but this is not likely to be clear during Phase I. Artifactual toxicity can occur in the aeration process, where contaminated air can be introduced. Also, contaminants can be leached from solid phase extraction (SPE) columns, and methanol leaching off the column can cause bacterial growth that will confound the results in the post-column *blank* and *post C_{18} SPE column tests.* Originality and judgement are needed to devise tests that will reveal artifactual toxicity (see Section 6) and some of these methods to deal with artifactual toxicity will be effluent specific.

2.5 Health and Safety Issues

For the toxicity identification work, hazards present in any effluent may not be known until Phase II identification steps have been started. Therefore, safety requirements for working with effluents (or other samples) of unknown composition must follow safety procedures for a wide spectrum of chemical and biological agents. Because all of the hazards in an effluent sample may not be known when a toxicant is identified, effluent samples should be treated as hazards of unknown composition throughout the TIE. Knowledge of the types of wastewater treatment applied to each effluent can provide some insight for the possible hazards. For example, unchlorinated primary treatment plant effluents containing domestic waste may contain pathogens. Chlorinated secondary effluents are less likely to contain such agents. Effluents from activated sludge treatment plants are less likely to contain volatile toxicants.

Because effluent characteristics are unknown, personnel should follow the guidelines for hazardous materials (EPA, 1991A; 1991C). Also, if any sample contains human waste, personnel should be immunized for diseases such as hepatitis B, tetanus, polio, and typhoid fever.

Each laboratory should provide a safe and healthy work place. All laboratories should develop and maintain effective health and safety programs (APHA, 1989; EPA, 1991C). Each program should consist of: (a) designated health and safety officers, (b) formal written health and safety plans, (c) on-going training programs, and (d) periodic inspections of emergency equipment and safety violations. Further guidance on safety practices is provided in other documents (APHA, 1989; EPA, 1991A; 1991C).

2.6 Facilities and Equipment

The laboratory facilities and equipment needed to conduct TIEs are discussed in the acute Phase I manual (EPA, 1988A; EPA, 1991A). Most of the equipment for conducting the short-term tests are delineated elsewhere (EPA, 1989C; EPA, 1992C). The reagents used for the chronic Phase I characterization are identical to those described in the acute Phase I manual (EPA, 1991A). Compressed air systems with oil-free compressors and air filters to provide high purity air are very important (EPA, 1991A). All glassware should be rigorously cleaned, and the glassware used for filtering must be rigorously cleaned to remove residual contaminants from the glass frit(s). Filtering equipment may

need to be made of plastic to avoid leaching of metals or other toxicants from glass when acid washes are used (see Section 6). Use of stainless steel frits can be used provided pH adjustments are not made since metals will rinse off the stainless steel at extreme pH's and cause toxicity. Ultra pure acids and bases (e.g., Suprapur®, E. Merck, Darmstadt, Germany) should be used to prevent impurities in the acids/bases from interfering in the toxicity results.

Section 3
Dilution Water

Dilution water used for chronic TIE's must meet several requirements. Obviously it must support adequate performance of the test animals in regard to growth, survival, and reproduction since these are the effects measured in the tests. Secondly, it must not substantially change the animals' response to the sample toxicants. Because the characteristics of the toxicants are not known, there is no way to be sure which dilution water characteristics are important. Hardness and alkalinity are most often used to select the dilution water but these parameters are generally of little importance for non-polar organics. Rarely is the organic matter content considered and yet for both non-polar organics and metals, organic matter has more effect on toxicity than hardness. Experience in the acute TIE work has shown pH to be the single most important water quality characteristic for characterizing the cause of toxicity.

The most important consideration, in addition to those mentioned above, is that the water be consistent in quality and not contain contaminants that could produce artificial toxicity. For example, if there was a nontoxic concentration of a non-polar organic present in the dilution water, when samples are concentrated, it might be toxic and this can confound the identification of the components causing toxicity in the effluent. The best policy is to use a high purity reconstituted water or a well water of known suitability. Receiving water should not be used until Phase III, when it is the water of choice to evaluate the toxicant in the receiving water system (see Section 2.2).

A reconstituted water of similar pH, hardness and alkalinity to that of the effluent is a first approximation of an appropriate water; however, organic matter is hard to duplicate. Experience has shown that for the *Ceriodaphnia* test, the addition of food[1] to the water has been helpful to provide some organic material. With food added, traces of contaminants can be less toxic. If higher concentrations of effluent are to be used, the choice of the dilution water is less important because the characteristics of the effluent dilution mixture will resemble those of the effluent. As information is gained about the toxicant characteristics, the choice of dilution water can be improved.

The impact of dilution water choice depends on the IC25 (see Section 5.8) of the effluent. If toxicity changes substantially from sample to sample, but the dilution water selected does not match the effluent in water characteristics yet is kept the same throughout several samples for Phase I, then the effect of the effluent in the dilution water can also vary across samples. As the TIE progresses into Phase II, attributing relative toxicity to various constituents must be more refined. For instance, suppose the suspect toxicant is a cationic metal whose toxicity is hardness dependent. Also, suppose that the whole effluent has a hardness of 300 mg/l as $CaCO_3$ (very hard water) but the dilution water has a hardness of 40 mg/l as $CaCO_3$. In this case, the hardness in each of the test dilutions will be different from that of either the whole effluent or the dilution water. Provided the cationic metal concentrations vary over the course of the TIE period, the amount of toxicity (as toxic units[2], TUs) due to a particular metal concentration will also vary depending upon the effect concentration in the effluent. If the first whole effluent sample contains 160 µg/l of zinc (for this example, 160 µg/l is 1.0 TU_c in very hard water) and the test is conducted using a dilution water of 40 mg/l as $CaCO_3$ (soft water), the no effect concentration would be 100% where hardness is 300 mg/l and the effluent would have <1 TU_c. The second whole effluent sample contains 480 µg/l of zinc. One would expect this sample to possess 3 TUs (480 µg/l ÷ 160 µg/l). The toxicity due to the second effluent sample would likely contain more than 3 TUs because the hardness at the effect level (<100%) would be much lower than at 100% effluent (where hardness is 300 mg/l as $CaCO_3$). The effect

[1] Food added for the *C. dubia* tests are the yeast-cerophyll-trout food (YCT) and the algae (*Selenastrum capricornutum*) at a rate of 0.1 ml/15 ml (EPA, 1989C). Although at ERL-Duluth the algae has been added at the rate of 0.05 ml/15ml until May of 1991 when we increased the level (EPA, 1989C).

[2] TUs is a means of normalizing the concentration term (i.e., LC50, NOEC, IC25 as percent effluent; see Section 5.8) to a unit of toxicity. The use of the TUs approach allows effluent toxicity to be compared (provided test species and test duration are the same) to a suspect toxicant's toxicity. The toxicity of an effluent and a chemical are different and different concentrations of each equal one LC50 (1 TU). TUs of an effluent can be calculated for either acute or chronic toxicity endpoints. The number of acute TUs in the effluent is 100% ÷ LC50 = TU_a and the chronic TUs in the effluent is 100% ÷ NOEC = TU_c or 100% ÷ IC25 = TU_c (EPA, 1991B). For specific chemicals the TU_c is equal to the concentration of the compound present in the effluent divided by the acute test LC50 for TU_a or the chronic test NOEC or IC25 for the TU_c. The assignment of TU_c is necessary for the correlation step (Phase III) when effluent toxicity TUs are compared to suspect toxicant(s) TUs.

level would be near 20-25% effluent where hardness would be <100 mg/l as $CaCO_3$ and 1 TU of zinc would be <160 µg/l. In addition, if one were to use receiving water for the diluent, the hardness might change dramatically and confound calculation of TU's in a like manner if the effect concentration was <100% effluent.

Section 4
Effluent Samples

To determine whether an effluent sample is typical of the wastewater discharge may require a number of samples to be tested. Experience has shown that the use of several samples spanning two to three months has been successful in characterizing many effluents. TIE work on atypical samples may be problematic and these TIE procedures were not developed for one-time episodic events. However, the very nature of atypical samples may provide valuable assistance in the TIE effort by identifying the type of toxicant(s) that previously was not suspect. This is probably more likely when an atypical sample has greater toxicity than the other samples. In addition, the atypical toxic sample may aid a discharger in recognizing wastewater treatment plant upsets and assist the discharger in implementing prevention procedures or generally improve and maintain better wastewater plant housekeeping efforts, which in turn may eliminate the episodic toxicity problems.

The acute Phase I manual discusses the quantitative and qualitative changes in effluents (EPA, 1988A; EPA, 1991A) that may affect toxicity. Varying concentrations of toxicants, different toxicants, water quality characteristics, and analytical and toxicological error are all factors in determining the toxicity of an effluent. Although the toxicity of an effluent over time appears unchanged, there may be more than one toxicant involved in each sample, and not necessarily the same ones.

At the same time a sample is collected, information on the facilities treatment system (normal operation; aberrant processes) may be useful. When dealing with industrial discharges, details of the process being used may be helpful. These details and others should be recorded and provided to the laboratory conducting the TIE at the time of sample shipment. When samples are received, temperature, pH, chronic toxicity, hardness, conductivity, total residual chlorine (TRC), total ammonia, alkalinity and DO should be measured. Figure 4-1 provides a typical format to record such information.

Since most TIEs are not performed on-site, the effluent samples must be shipped on ice to the testing location. The samples should be cooled to 4°C or less prior to shipment and they should be shipped in sturdy ice chests to prevent either temperature increases or container breakage during shipment. Primary requirements of the TIE are that toxicity occurs frequently in the effluent samples and that the toxicity of each sample (held at 4°C) remains in the efiluent sample for a sufficient period of time. If samples repeatedly lose their toxicity after shipment, steps should be taken to preserve toxic fractions (Section 6.7) for later testing and analysis. For example, if the initial characterization tests indicate the presence of non-polar organics, one tool to use is to concentrate large volumes (5-10 L) of effluent when the sample arrives (see Section 6). Use of the Phase II (EPA, 1992A) non-polar fractionation procedure is the preferred way to concentrate the non-polar toxicants for subsequent analysis and testing. While efforts must be expended on this procedure, it can be a crucial step to aid in identifying potential toxicants (in instances where toxicity is present and lost in the effluent). The information on when toxicity degrades or is lost may become useful as the toxicant(s) is identified (see Section 9; EPA, 1991A). Filterable toxicants which degrade quickly in the effluent may be recovered from the filters with solvent and stored for future use (cf., *filtration test*; Section 6.4).

For one chronic Phase I TIE, a typical volume of effluent needed to ship is 19 L (5 gal) but of course this will depend on the options chosen for the TIE (Section 6) and 38 L (10 gal) may be more helpful once identification and confirmation begin on any sample. The second edition of the acute Phase I TIE manual (EPA, 1991A) recommends that samples be initially collected and stored in both glass and plastic to determine whether effluent stored in either container affects the toxicity. Some compounds (such as surfactants) are less toxic if water samples containing them are stored in plastic containers. Prior to initiating the characterization it may be useful to collect and test several preliminary samples to determine which containers to use during the TIE to provide samples that are the most representative of the effluent (see Phase I, Section 6 (EPA, 1991A) for more details). Less volume (≤2 L) is needed for these tests.

Composite samples should be used for Phase I. Later, in Phases II and III, where variability is desired, grab samples should be used. Samples that are consistent (i.e., composite samples) give results that are easier to interpret and lead more rapidly to identification (Phase II) and confirmation (Phase III) of the cause of toxicity. Grab samples can provide the maximum effluent toxicity; however, it is more difficult to catch

Figure 4-1. Example data sheet for logging in samples.

Sample Log No.: _____

Date of Arrival: _____

Date and Time
of Sample Collection: _____

Facility: _____

Location: _____

NPDES No.: _____

Contact: _____

Phone No. _____

Sampler: _____

Sample Type: ☐ Grab ☐ Composite
☐ Glass ☐ Plastic
☐ Prechlorinated
☐ Chlorinated
☐ Dechlorinated

Sample Conditions Upon Arrival:

Temperature _____

pH _____

Total Alkalinity _____

Total Hardness _____

Conductivity/Salinity _____

Total Residual Chlorine _____

Total Ammonia _____

Condition of treatment system at time of sampling:

Status of process operations/production (if applicable):

Comments:

intermittent peaks of toxicity (such episodic events may not be caused by the same toxicant that causes routine toxicity).

Multiple effluent samples in each test should *not* be used in Phase I as is done for permit testing (EPA, 1989C). We have found that using only *one* composite sample for each set of Phase I characterization tests is adequate. If several effluent samples are used for renewals during the chronic Phase I TIE and the toxicants are different or change in their ratios one to another, the interpretation of Phase I will be nearly impossible. Indeed such variability must be identified but it should be done after at least one or preferably most of the toxicants are known. The use of *one* sample is more important in Phase III, (EPA, 1992B) where toxicity data are correlated to the measured concentrations in the effluent. If multiple samples are used, this correlation can not be readily done because the same toxicant may not be present in each sample, or it is present in varying concentrations and other toxicants may appear.

Existing routine toxicity test data should be examined. If one notes a sudden response such as death in the middle to the end of the test period and especially if it is associated with a new sample, the effect being measured may actually be acute rather than chronic and if so the approach may be switched to an acute TIE approach. The investigative approach should be adjusted to respond to such situations. When the permit test is conducted and the test fails, it may be desirable to try to identify the toxicants in those permit compliance samples. This can be done by collecting the appropriate volume needed for a chronic TIE of either the daily samples or the three samples used for the short-term toxicity test (EPA, 1992C). Additional short-term toxicity tests can be conducted on each

sample prior to any TIE tests on each sample or preferably additional short-term tests would be initiated on each new sample during the 7-d test to evaluate whether it is the cumulative toxicity from all samples or whether one or two samples are driving the toxicity. We have observed in several effluent tests that the toxicity during the short-term chronic test can be caused by one or two samples and these samples cause the chronic test to demonstrate that the effluent is toxic in less than the full 7-d of the *C. dubia* or fathead minnow tests. When the toxicity that occurs in ≤48 h (*C. dubia*) or ≤96 h (fathead minnow) with any one of the samples from the permit compliance samples or any sample collected for the TIE, is observed as >50% mortality, acute TIE procedures can be applied to more quickly characterize the toxicant(s).

Section 5
Toxicity Testing

5.1 Principles

The test organism is used as the detector of chemicals causing chronic toxicity in effluents and other aqueous media. The response to toxic levels of chemicals is a general one; however the organism is the only tool that can be used specifically to measure toxicity. Only when the cause of toxicity is characterized can chemical analytical methods be applied to identify and quantify the toxicants.

Chronic TIE's will usually be triggered by the use of the toxicity test methods as found in the short-term chronic toxicity test manuals (EPA, 1989C; EPA, 1992C). These methods rely on sublethal endpoints as the indicator of chronic toxicity for the Phase I manipulations, therefore conducting the tests strictly as detailed in those manuals is not always necessary and sometimes not possible. Modifications have been developed and these include: (a) reduced test volumes, (b) shorter test duration, (c) smaller number of replicates, (d) reduced number of test concentrations, and (e) reduction in the frequency of the test solution renewal. In addition, the frequency of preparation of manipulated samples for test solution renewal must be established and this issue is discussed in the following section. Any loss of test precision due to these modifications is not as critical during Phase I characterization as it is in Phase II and Phase III (EPA, 1992A; EPA, 1992B). During Phase I the analyst is searching for an obvious alteration in effluent toxicity, which may be obtained using modified chronic test methods. Confirmation testing (Phase III) conducted according to the standard methodologies will confirm whether the toxicant(s) detected in the characterization and identification steps (Phases I and II) is the true toxicant.

5.2 Test Species

In most cases, freshwater effluents will be subjected to this evaluation because they have been found to be chronically toxic to the cladoceran, *C. dubia*, or to the fish, fathead minnow (*P. promelas*), or possibly to the cladocerans, *D. magna* or *D. pulex*. Freshwater effluents discharged into marine environments are evaluated for toxicity using marine species or may be assessed with freshwater species (EPA, 1991D). TIE guidance for the marine species will be forthcoming in the fall of 1992 (George Morrison, personal communication, ERL-Narragansett, RI).

The species which detected the toxicity which in turn triggered the TIE, is the first choice for the TIE species. When an alternative species is chosen one must prove that it is being impacted by the same toxicant(s) as the species which initially detected the toxicity. The species need not have the same sensitivity to the toxicant(s), but each species' threshold must be at or below the toxicant concentration(s) present in the effluent. One method of proving that the species are being affected by the same compound(s) is to test several samples of the effluent over time to both species. If the effluent possesses sufficient variability, and the two species IC25s (see Section 5.8 below for a description of the IC25) change in proportion to one another, the analyst may assume that the organisms are reacting to changing concentrations of the same compound. Further proof that the two species are responding to the same toxicant should surface during Phase III. If the toxicant is the same for both species, then characterization manipulations which alter toxicity to one species should also alter toxicity to the second species. The extent to which toxicity is altered for each will depend upon the efficiency of the manipulation and the organism's sensitivity to the toxicant. Steps applied in Phase III will confirm whether the two species are indeed sensitive to the same toxicant in the effluent. Extensive time and resources may be wasted if one discovers during Phase III that the organism of choice is not responding to the same toxicant as the species which triggered the TIE.

For the above mentioned reasons, we recommend when at all possible to use the organism which prompted the TIE. Our chronic TIE experience has been based on tests with *C. dubia* and/or larval fathead minnows. Obvious constraints on the use of other species are availability, size, age, and adaptability to test conditions. Also, the threshold levels for additives and reagents must be determined for other species.

5.3 Toxicity Test Procedures

Measures to conserve time and resources required to conduct a chronic Phase I must be used in order to make the procedures cost-effective. The application of all aspects of the standard short-term chronic tests to Phase I in terms of replicates, routine water chemistries, test duration, and volume is not practical due to time constraints and expense. Variations of the procedures need to be implemented whenever possible.

As mentioned above, smaller test volumes can be used in all tests with *C. dubia* and in most instances with fathead minnows. For example, 10 ml in a 1 oz

plastic cup (or 30 ml glass beaker) has been adequate for *C. dubia* and 50 ml in a 4 oz plastic cup (10 fish per cup) has been used successfully to test the fathead minnows (or 100 ml in a 400 ml glass beaker). There are two precautions to watch for in the chronic TIE tests—1) evaporation of test solutions and 2) transfer of toxicants while moving the animals. If evaporation reduces test volumes, efforts to reduce the evaporation must be made or larger volumes must be used. The volume of water added with each transfer should be minimized, because the volume used in the test is small, and the resultant test concentration could be diluted, thereby reducing toxicity. Using the same size test chambers and consistent volumes should be maintained in Phase I; when Phases II and III are initiated, tests should be conducted following the test protocol that was used to trigger the TIE. This may be important in Phase I to be as sure that the oxygen requirements for the test species are met and that toxicity is not due to physical restrictions of the test procedure.

If a reduction in the number of replicates per test concentration is used, one must assume that precision is sufficient enough to decipher changes in toxicity that must be measured. For the *C. dubia* test, five animals per concentration (one per cup) and for the fathead minnow test, two replicates per concentration and 10 fish per replicate have been found to be adequate for interpreting the changes in toxicity. However this smaller data set is not amenable to all statistical requirements as described for the short-term tests (EPA, 1989C; see Section 5.8). Use of more organisms and more replicates may be preferable if Phase I data are likely to be used in Phase III confirmation (see Sections 2.2 and 2.3).

A shortened version of the 7-d *C. dubia* test, referred to as the 4-d test, may be useful in the TIE. The 4-d test does not have to be as sensitive as the 7-d test, just sensitive enough that the toxicity changes occurring in Phases I and II of the TIE (using 4-d tests) would be the same as the 7-d tests. The 4-d day test was found to produce similar results for single chemicals (Oris et al., 1991), but in tests in our laboratory with effluents, the 4-d test has not been as sensitive for all effluents tested as the 7-d test in determining the effects on young production and survival. Masters et al. (1991) tested *C. dubia* to one effluent (three times), three surfactants, three metals, and three organic compounds with the 4-d and 7-d exposures. They found that for the most part the effluent toxicity was similar for the 4-d and 7-d test results but for the surfactants the 7-d test was more sensitive. For the metals (cadmium, lead, and zinc), ethylene glycol, and pentachlorophenol, the chronic toxicity values for both tests were very similar while the 4-d test was more sensitive for phenol.

In the 4-d test, when animals are initially exposed at 72 h they are ready to produce their first brood. Therefore, toxicity can be underestimated because these animals are predisposed to produce their first brood, unlike the animals exposed as neonates (24 h old). The exposure during a 4-d test may miss their most sensitive life stage. However for the Phase I where the purpose is to detect differences following various manipulations, this issue is not as important as the ability to rapidly conduct the characterization. Use of the shorter term test will decrease the cost of Phase I TIE's. In the confirmation of toxicity (Phase III), the 7-d test is required because the toxicity as measured in the 7-d test (with more replicates, more dilutions, more volume) was used to detect toxicity for the permit, and should be used to confirm the cause of toxicity.

To conduct a 4-d test with *C. dubia*, neonates (0-12 h old) are placed in the dilution water that will be used to conduct the TIE. At present these animals are held in groups of three, two or individually in test containers (with 15 ml of culture water) and fed daily until they are 72 h (±6 h) old in a similar test fashion (Oris et al., 1991). The animals are then transferred to the *baseline test* solutions or the various characterization test solutions. The test is then continued for 4-d using the endpoint of three broods.

The use of known parentage (EPA, 1989C) for the *C. dubia* test is important when the number of replicates is reduced, and helpful for Phase I, II or III tests and in routine tests as well (EPA, 1992C). For Phase I, this known parentage approach allows the young of one female to be used across one replicate of all dilutions and the control (i.e., 5 animals), the young from another female for the next replicate set of dilutions and control, and so on until all test cups contain one young animal. By this technique, animals from a given female that later appear atypical in appearance or movement or produce no young when others in the same test concentration are producing normally can legitimately be dropped from the data set without statistical bias (Norberg-King et al., 1989). The ability to discard such data without bias improves precision. Precision will be better when $n \geq 7$ per treatment for *C. dubia* or $n \geq 4$ for the fathead minnow test.

5.4 Concentrations to Test

The level of toxicity for any given discharger most likely will have been established with some degree of certainty from previous tests that were conducted on the effluent that triggered the TIE.

Therefore during Phase I of the TIE, we have found that four effluent dilutions and a control are adequate to define the toxicity of the sample while reducing the cost of the tests. Now for the TIE, the key to choosing the concentrations to test is to select those that will assist in the detection of small changes in toxicity, which is essential in the chronic TIE. For example, if the NOEC (from a previous data set) is 12% (or IC25 is 10%), then a concentration series such as 6.3%, 12.5%, 25%, and 50% would be logical; or perhaps closer concentration intervals may be desired. Using 20% as the high concentration and a dilution factor of 0.7, would mean the concentrations to test would be 7%, 10%, 14%, and 20%. If the NOEC (from historical data) is 40-50% (or above 50%), then the concentrations to test should be, for example, 25%, 50%, 75%, and 100% or

40%, 60%, 80%, and 100%. Choice of dilution factor and test concentration range is a matter of judgement and depends on needed precision and practicality.

In nearly all examples in this document, the concentrations of 12.5%, 25%, 50%, and 100% are used. We are assuming that if effluents have ICp (or NOEC) values below 10%, the effluent is likely to show acute toxicity and if so, an acute TIE approach should be used. If chronic work is to be done on a highly toxic effluent, the same recommendations given in the acute manual should be used; that is, use concentrations of 4x, 2x, 1x and 0.5x the IC25 or IC50 value (see Section 5.8 for which value to select). For example, if the IC25 is 5% effluent, we would suggest using a range such as 20%, 10%, 5% and 2.5% for the various tests. It is best to use the same dilution sequence within a series of tests (Tier 1) when tests are to be compared to each other for differences in toxicity.

5.5 Renewals

For *C. dubia*, daily renewals of the test media (as required in the chronic manual, EPA, 1989C) are not necessary in Phase I as long as the toxicity of the effluent can be measured with one or two renewals. Because available sample volume is limiting in some manipulations, fewer renewals are desirable. As with the test duration (4-d vs. 7-d) the acceptability of less frequent renewals must be established by comparison with whichever test duration is selected. However in Phase III, tests must be conducted similarly to the routine biomonitoring test. For the fathead minnow test the frequency of sample replacement must be daily to maintain adequate water quality because the live food organisms (brine shrimp, *Artemia salina*) die 2-8 h after being added to the freshwater test solutions. A *baseline test* (see Section 6) is always conducted when the sample is received. The suitability of reduced renewal frequency can efficiently be evaluated at this time by conducting comparative *baseline tests* simultaneously with different renewal frequencies.

The number and types of chemical measurements taken initially and at the renewal intervals (referred to as finals) should be based on the need for these measurements and their usefulness (see Section 2). Initially, little judgement about the value of these can be made, but as toxicant characteristics are identified, the usefulness of various measurements can be judged. Initially, the usual measurements (hardness, alkalinity, conductivity; EPA, 1989C) should be made but some of these can be dropped as the TIE progresses. For example, if non-polar toxicity is found, then hardness and alkalinity need not be closely monitored. However if a metal is suspected, then these measurements are important. Low levels of dissolved oxygen in the fathead minnow test are a greater concern than in the *C. dubia* test, and the pH between the two tests will be dissimilar after 24 h of exposure. The pH measurement is frequently needed and for toxicants such as ammonia it is extremely important (EPA, 1992A). If an effluent contains greater than 5.0 mg/l of ammonia, the pH should be carefully measured at least daily (or more often) in *all* test concentrations. Since ammonia is a highly pH dependent toxicant, one must be aware of variable pH drift in the Phase I treatments which may lead to erroneous conclusions. One generalization, however, can be made. For characteristics that are unlikely to change, such as conductivity and hardness, both initial and final measurements need not be made-once is enough.

5.6 Toxicity Blanks

A risk of the reliance on a toxicity response in the characterization step of TIEs is the probability that artifactual toxicity is created during sample manipulations (see Section 2.4). While a particular manipulation may cause some degree of artifactual toxicity, if the toxicity is predictable the test may still retain its validity. Since chronic tests are more sensitive to artifactual toxicity, lower concentrations of additives or less severe conditions must be used as compared to the acute test.

The presence of artifactual toxicity caused by contaminated acids, bases, air, filters and columns and by intentional additives are detected by treatment *blanks* and *toxicity controls*. A *blank* is dilution water manipulated the same as the effluent, and then it is toxicity tested to determine if the manipulation added any toxicity. The *toxicity control* is the reference used to judge the impact of a manipulation. Sometimes the *toxicity control* is the *baseline test*, at other times it will be a characterization test. For example, the *toxicity control* for the *EDTA addition test* is the *baseline test* while the *toxicity control* for the *post C_{18} SPE column test* is the *filtration test* (filtered whole effluent). Treatment *blanks* for either the *EDTA addition test* or the *sodium thiosulfate addition test* are not appropriate as the testing of these additives in clean dilution water is not representative of the effluents' characteristics. The *toxicity control* must be distinguished from the *control* treatment (animals in standard culture or dilution water; also described as "performance controls") which is always used. *Controls* provide information on the health of the test organism and the test conditions while the *blanks* provide information on the cleanliness of the acids and bases, the aeration system, the filter apparatus, the C_{18} SPE column, and other apparatus used.

Although artifactual toxicity may appear in the dilution water *blanks*, artifactual toxicity in the effluent matrix may not be observed. One must decide whether the test results from that manipulated sample are meaningful. For example, if the aeration manipulation caused toxicity in the dilution water *blank* but aeration removed the effluents' toxicity then the conclusion that aeration was an effective treatment is valid. However, if the dilution water *blank* was toxic and it appeared aeration did not remove the effluent's toxicity then one cannot conclude that aeration was not effective without further investigation.

5.7 Renewal of Manipulated Samples

One must decide whether a manipulated sample to be used for renewal during the test should be prepared (e.g., aerated or passed over a C_{18} SPE column) as a batch sample for the entire test or prepared separately for each renewal. This choice may be dependent on the persistence of the effluent toxicity, but whether daily samples are prepared or batch samples are prepared and used for renewals of the tests should be decided by the investigator, and the same methods should be performed consistently throughout the TIE. As a general guideline, we have chosen to discuss these Phase I steps as though one aliquot of effluent samples prepared for the characterization tests is used for all renewals. However for either daily or batch samples, the same techniques should be used for all the manipulations. For example, a sample for the *filtration test* (Section 6) may be batch prepared on day 1. Then on day 2, a batch sample for the *aeration test* should be prepared. Yet for the *EDTA and sodium thiosulfate addition tests*, these additives should be added to the effluent dilutions on the day of each renewal as batch solutions for each dilution (e.g., add EDTA to 50 ml of 100% effluent, let sample sit and dispense to test cups). This is true for the methanol addition and the graduated pH manipulations as well. To test the post C_{18} SPE column samples for some effluents, daily samples may need to be prepared because of bacterial growth problems in samples stored for several days.

Since Phase I TIE work is often concerned with the qualitative evaluation of toxicity, rather than quantitative, there is no reason why a test could not be terminated sooner than 7 d, if the answer to the particular question posed has been found. For example, if the *baseline test* with a sample indicates a complete inhibition of *C.dubia* reproduction by day 5 of a 7-d test, and one of the manipulated samples (e.g., aeration) shows normal reproduction, there may be little point in continuing that test, because toxicity was altered. This type of judgmental decision is harder to make in a chronic fathead minnow test based on growth; however, by careful observation of factors such as survival or behavior, the trend of the toxicity response may be discerned earlier than 7 d. Sufficient measurable growth of the fathead minnows may have been achieved by 5-d. Experiments with fish exposed to zinc and selenium for 5-d and 7-d indicated that sufficient growth differences could distinguish the toxic effect even at 5-d (Norberg-King, 1989). However, if this information is needed in Phase III, it is important to correlate the same type of data and terminating the test early may require additional tests later on.

Because the chronic test is longer and requires more laboratory work than the acute test, loss of toxicity of any effluent sample is more troublesome when it occurs. If the presence of toxicity is not measured in the whole effluent before Phase I tests begin, much work will be wasted if the sample is non-toxic initially. On the other hand, to delay by waiting for the test may also result in the loss of toxicity. The best approach is to examine existing data sets for evidence of toxicity loss due to storage of samples. If there are none then start a *baseline test*, and upon the onset of chronic toxicity (e.g., 60% mortality, no reproduction by day 5 in high test concentrations of a 7-d test, absence of food in the gut of the fishes), additional follow-up manipulations of Phase I tests should be started. Toxicity degradation can be a useful tool in identification and confirmation (cf., Section 2). Once it has been determined that the sample toxicity degrades quickly, Tier 1 and Tier 2 steps should be started on the day of arrival. Removal of headspace in effluent storage containers may help minimize the loss of toxicity.

5.8 Test Endpoints and Data Analysis

For evaluating whether any manipulation changed toxicity, the investigator should not rely on statistical evaluations only. Some treatments may have a significant biological effect that was not detected by the statistical analysis. Judgement and experience in toxicology should guide the interpretation.

Endpoints for the most commonly used freshwater short-term chronic tests are growth, reproduction, and survival. Historically, the effect and no effect concentrations have been determined using the statistical approach of hypothesis testing to determine a statistically significant response difference between a control group and a treatment group. The no effect level, called the no observed effect concentration (NOEC), and the effect concentration, called the lowest observed effect concentration (LOEC), are then statistically defined endpoints. The NOEC/LOEC are heavily affected by choice of test concentrations and test design. For example, these effect levels are dependent not only on the concentration intervals (dilution sequence) chosen, but the number of organisms, the number of replicates used, and the choice of the statistical analysis for the data (i.e., parametric or non-parametric). The minimum significant difference detected in hypothesis tests can be quite variable (e.g., 10% or 50%; Stephan and Rogers, 1985) and yet this difference is used to determine the NOEC. In the chronic testing manual (EPA, 1989C), the minimum number of replicates (a relatively large number), organisms, and dilutions for the *C. dubia* and fathead minnow short-term tests are needed to meet the hypothesis testing requirements. When less replicates, fewer numbers of dilutions and fewer test organisms are used (as in the chronic TIE) the hypothesis tests will not be able to detect smaller differences that are needed for chronic TIEs. Therefore, hypothesis testing is not suitable for Phase I purposes and a point estimation method must be used.

The linear interpolation method described in the supplement to the freshwater chronic manual (EPA, 1989C) calculates a point estimate of the effluent concentration that causes a given percent reduction based

on the organisms response. The inhibition concentration (ICp[3]) program (Norberg-King, 1989; DeGraeve et al., 1988; EPA, 1989C) was developed for the purpose of analyzing data from the short-term tests. This method of analysis is not as dependent on the test design as hypothesis analysis and is particularly useful for analyzing the type of data obtained from Phase I testing. When analyzing data for the ICp estimates, only one test endpoint is determined. For *C. dubia* all the data are used. If all animals have died, the data are entered as zeros and if some animals have some young but the adult dies, the partial brood values are used. We have found with some effluents that when the 4-d test is routinely applied during a chronic TIE, often the first brood is produced and then the adult dies. In other cases we have observed no adult mortality in the 4-d or 7-d test, but at the same effluent exposure concentrations the 7-d test animals will not produce any young while the 4-d test animals produce their first brood. The dose response from this 4-d test is not typical in the 7-d test, and the production of young can be problematic in data interpretation and analysis since mortality also occurred. For example, when analyzing the data using the ICp program, the effects of survival and young production are incorporated into one estimate for the IC50 and IC25. Yet there is no doubt that 0-40% survival is a significant reduction in survival that indicates toxicity, and would cause a routine test to fail (EPA, 1989C). Therefore when this occurs, to track toxicity in the TIE, it may require calculating the IC25/IC50 for young production and survival and then recalculating the IC25/IC50 for survival alone. For the fathead minnow test in the routine monitoring test and the TIE tests, the weights are calculated as mean weight per original fish rather than mean weight per surviving fish (EPA, 1992C). Also the program allows direct comparison of results from tests conducted using different concentration intervals. The level of inhibition (p) used as an endpoint (e.g., 25 or 50%) is not critical, although the IC25 is generally suggested as an equivalent for the NOEC (EPA, 1991B). Confidence intervals are calculated using a bootstrap technique, and these confidence intervals can be used to determine the significance of toxicity alterations observed in Phase I. A *"significant reduction"* in toxicity must be determined by each laboratory for each effluent and in combination with the precision of reference toxicant tests that the performing laboratory achieves. The use of the IC50 for Phase I TIEs may be more useful when trying to correlate the characterization test results to the effluent toxicity. However, an IC50 may not be able to be estimated while the IC25 can; use of a consistent endpoint effect level is important for subsequent TIE work (EPA, 1992A: EPA, 1992B). We have observed substantial toxicity reductions in characterization tests, yet it does not always appear to be a significant reduction when only the IC25s are compared. When this happens the sample size should be increased with subsequent testing in order to more clearly differentiate the toxicity and the dose response curve should be studied. Once the toxicant is identified, the number of replicates is increased and more dilutions are used (Phase III; EPA, 1992B), which increases the confidence in the IC25.

[3] The ICp program (Release 1.1) calculates confidence intervals which are limiting when the sample size is ≤5 and these confidence intervals are less than 95% in version 1.1 (R. Regal, personal communication, University of Minnesota, Duluth, MN). This is being corrected in the revision of the program now underway (for more information, contact Teresa Norberg-King). The ICp program is available by sending a formatted disk to Teresa Norberg-King, EPA, 6201 Congdon Boulevard, Duluth, MN 55804.

Section 6
Characterization Tests

The chronic Phase I manipulations follow the same approach and employ the same type of manipulations used in the acute TIE (EPA, 1991A). These include aeration, filtration, C_{18} SPE extraction and chromatography, chelation with EDTA, oxidant reduction and/or complexation with sodium thiosulfate, and toxicity testing at different pH values (Figure 6-1). The main differences between the acute and chronic techniques are that the concentrations of additives must be lower and the test conditions must be less severe in a chronic TIE because the chronic test organisms are more sensitive to these conditions. The pH adjustment procedures in Tier 2 are changed from the acute Phase I because we found that consistent, representative blanks with reconstituted water could not be obtained at higher pH's.

The following characterization steps are all based on the use of *Ceriodaphnia* or fathead minnows. Obviously, use of other species will require consideration of appropriate test volumes and additive concentrations. As discussed in the acute manual, if the TIE is done with species different from the species used in the permit, one must demonstrate that both species are sensitive to the same toxicant(s) (see Section 5).

More than one effect is measured in chronic tests (reproduction or growth and survival) and because partial effects are more frequent in short-term chronic tests than in acute tests, a graded response with concentration is often seen. A graded response allows one to better judge small changes in toxicity—an advantage not often available in acute tests. Also, effects (initial mortality, delayed mortality, aborted young, reduced young, poor growth) can be observed and used in interpreting the results as can the time to onset of effect be used. Such effects can be useful in distinguishing the response to different toxicants.

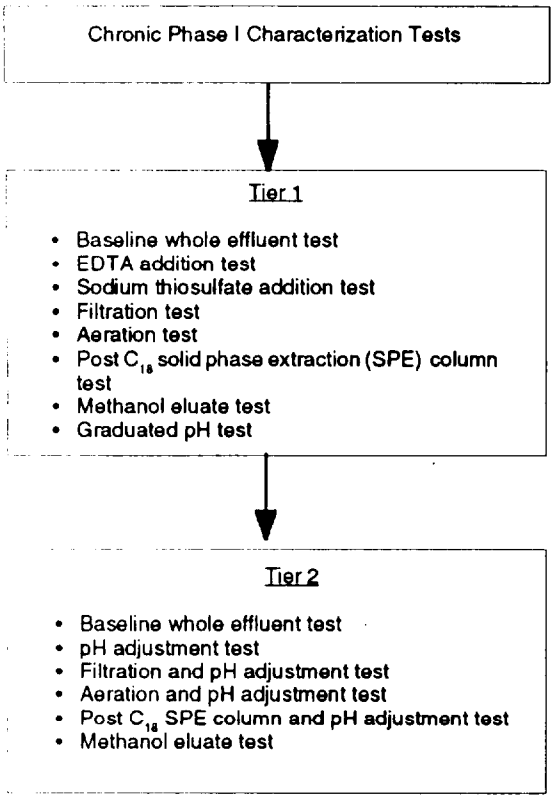
For acute TIEs, tests are quick and relatively inexpensive, so the need to maximize their usefulness is lessened. The chronic test is more work not only because the test is longer and more complex, but also because more sample volume is needed. For example, for tests such as the sublation test (a subsequent step in the *aeration test* (Section 6.4)) sample size can be very restricting. In addition, if an effluent is not always toxic, a decision has to be made as to whether to test for the presence of toxicity first, before manipulations are started. If the effluent is not toxic and all the manipulations are set up, the results may be of no value. On the other hand, if the presence of toxicity is first established, often a week will have passed and by the time manipulations are tested, the toxicity may have degraded. Unfortunately, there is no clear answer to which way to proceed. When there are data for effluent toxicity for preceding months, examination of these data may assist in the decision.

In the acute TIE, the *initial test* (EPA, 1991C) is used to set the range of concentrations to test. However in the chronic TIE, an equivalent of the *initial test* is not practical, therefore historical data must be used to make such judgements. Lacking historical data, a judgement will have to be made to set the test range and guidance for this is given in Section 5.4.

For chronic Phase I characterization, the use of two tiers of characterization tests is suggested (Figure 6-1). Tier 1 is done without major pH adjustments. Experience with acute TIEs has shown that major pH adjustments are usually not needed. Tier 2 is performed only when Tier 1 does not provide sufficient information, and consists of filtration, aeration and the C_{18} separation technique of Tier 1 with an effluent sample adjusted to both pH 3 and pH 10. Therefore when the characterization tests indicate Tier 2 is not required, resources needed to conduct the TIE are significantly reduced.[4] Each characterization test used in the Tier 1 or Tier 2 has as its foundation the information in the acute Phase I manual (EPA, 1988A; EPA, 1991A). The principles, methods, and interpretation of results are based on the acute manual, and the tests for Tier 1 (Figure 6-2) are discussed in Sections 6.1-6.8. All tests within a Tier (1 or 2) should be started on the same day. Starting chronic tests involves more effort than acute tests, and logistics must be planned (for instance, available animals of the appropriate age for the chronic test, sufficient food supply for more chronic tests, adequate supply of dilution water for all test renewals). Tests need to be started on the same day in order to compare results of each manipulation test to others and to the *baseline test* (Section 6.3) results (Table 6-1). Once the Tier 1 data are generated, they are compared, and interpretations are made to see which inferences can be drawn concerning the nature of the toxicants. Usually, multiple manipulations and a retest of selected manipulations will be effective in

[4] A recent estimate of the cost of the Tier 1, Phase I for chronic toxicity was equivalent to the full Phase I acute TIE (Aquatic Habitat Institute, 1992).

Figure 6-1. Overview of characterization tests.



yielding information concerning the nature of toxicants before additional effluent samples are tested (see Sections 6.15, 6.16 and acute Phase I manual, EPA 1991A).

Sample Preparation for the Characterization Tests

As for acute TIE tests, we suggest doing certain chemical measurements and the manipulations on one day and then starting the tests the next day (Table 6-1). This schedule balances the work load more evenly. When the sample is received (day 1), various measurements (Section 4) are taken and some preparatory manipulations for the Tier 1, Phase I are done.

First, the routine chemical measurements are taken as discussed in Section 4. DO, conductivity, and pH should be measured on the 100% effluent to ensure that the values are in the physiologically tolerable range for the test species. If these are at levels that could be toxic (EPA, 1989C), there is little point to test the effluent sample without some sample manipulation. In addition, the water hardness and alkalinity should be measured so that the appropriate dilution water can be selected (see Section 3, Dilution Water). As the TIEs have progressed, we have begun to match both the hardness and the alkalinity of the dilution water to similar values for the effluent.

Figure 6-2. Tier 1 sample preparation and testing overview.

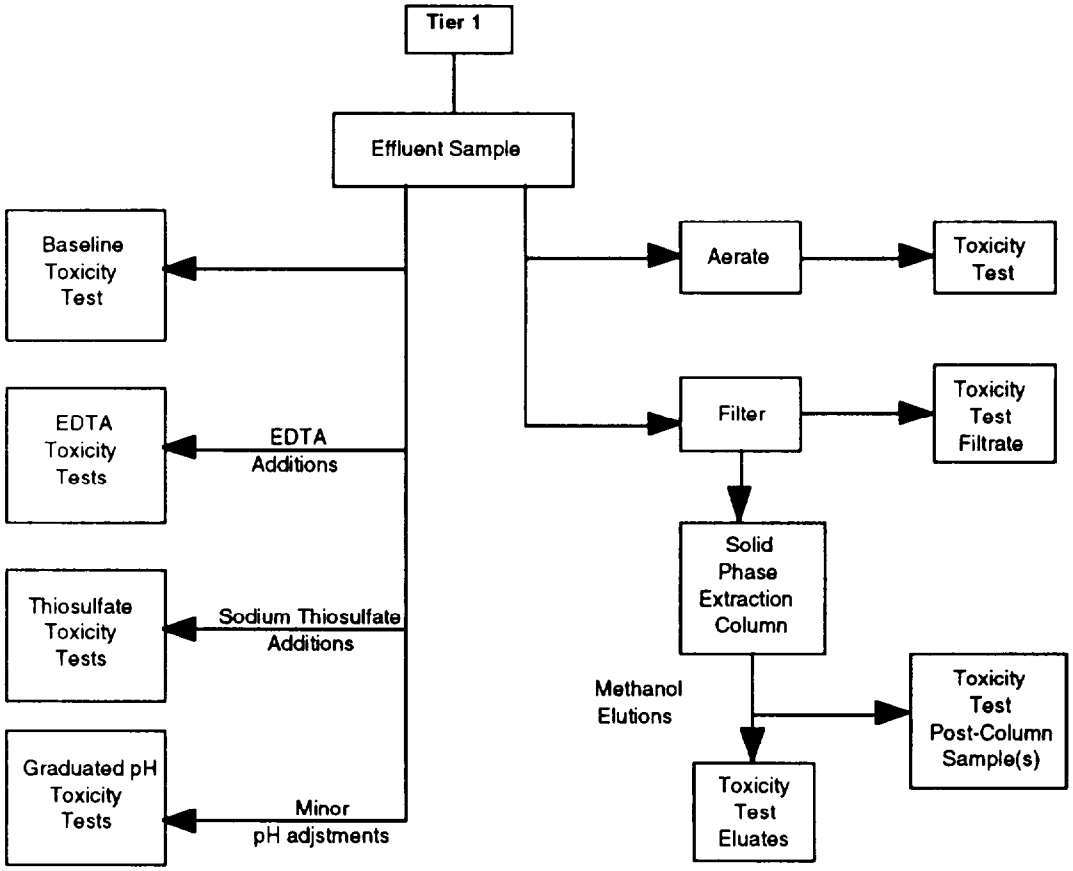


Table 6-1. Outline of Phase I effluent manipulations Tier 1 and Tier 2.

Description	Section
DAY 1 SAMPLE ARRIVAL:	
Measure	4.0
• temperature	
• conductivity	
• pH	
• DO	
• alkalinity	
• hardness	
• total ammonia	
• total residual chlorine	
Perform Sample Manipulations	6.0
• filter effluent	6.4
• perform solid phase extraction (SPE)	
• collect effluent	6.6
• collect methanol eluate	6.7
DAY 2 TOXICITY TESTING:	
Warm aliquot of whole effluent and aliquots of filtered effluent, post C_{18} SPE column effluent, and methanol eluates.	
Initiate Tier 1 Tests	
• *baseline toxicity test*	6.1
• *EDTA addition test*	6.2
• *sodium thiosulfate addition test*	6.3
• *aeration test*	6.4
• *filtration test*	6.5
• *post C_{18} SPE column test*	6.6
• *methanol eluate test*	6.7
• *graduated pH test*[1]	6.8
ADDITIONAL TESTING ON SUBSEQUENT DAYS[2]*:*	
Tier 2 Tests	
• *pH adjustment test*	6.10
• *aeration and pH adjustment test*	6.11
• *filtration and pH adjustment test*	6.12
• *post C_{18} SPE column and pH adjustment test*	6.13
• *methanol eluate test for pH adjusted samples*	6.14

[1] Experimentation may be needed for this test (see text for details).

[2] Tier 2 is primarily for those effluents where the results from Tier 1 did not indicate any clear pattern of toxicity change following manipulation (see text for details).

The initial pH of effluent upon arrival at the testing laboratory is referred to as pH *i*, which is not necessarily the pH of the effluent at air equilibrium[5]. The pH of the sample after being warmed, may be selected as pH *i* rather than the pH upon arrival. The important point is to use the same pH *i* for all subsequent tests. As an effluent warms to 25°C in an open container, CO_2 escapes and the pH may rise from 7.2-7.6 to 8-8.5. In some tests, once the food is added the pH may rise faster or in some cases (e.g., the fathead minnow growth test), once the food has been in the test solution for a period of time, the pH may be lower (e.g., 7.5-7.6). These changes may be important for interpreting the data in a chronic TIE, and pH should be measured in the test dilutions that determine the test endpoint. Of course, since the endpoint may be unknown, pH is typically measured in all test concentrations.

Since samples are cooled for shipping and storage, upon warming to 25°C, some of the samples are apt to be supersaturated. Supersaturation can usually be monitored by measuring DO. If DO is too high, it should be reduced to acceptable levels as described by EPA (1989C) for the routine monitoring test or by maximizing surface-to-volume ratio of the container to facilitate more rapid exchange of equilibrium of the sample and atmospheric oxygen. *Ceriodaphnia* are less sensitive to supersaturation than newly hatched fathead minnows. For chronic Phase I tests, routine water chemistry measurements (such as DO, pH, temperature) are more important than in acute Phase I tests.

The manipulations performed the day the sample arrives are filtering, extraction on the C_{18} SPE column, and collection of the methanol eluates (see Sections 6.5 and 6.7 below). The aliquots of filtered effluent and post-column effluent will be held until the next day (day 2) to start the tests. Of course these samples should be stored in the refrigerator at 4 (± 2°C). This sample preparation schedule is particularly convenient for laboratories who rely on courier services to deliver samples, which typically occurs late in the morning.

On day 2, the EDTA addition test should be prepared first so that compounds that are EDTA chelatable, yet may require an equilibration time for complexation, can be chelated (see Section 6.4). Then the rest of the manipulations (aeration, sodium thiosulfate additions, graduated pH adjustments) should be started. For the laboratory that is experienced in chronic toxicity testing, the amount of time required to conduct the Tier 1 sample manipulations and set up the toxicity tests is about 6-10 h.

6.1 Baseline Test

General Approach: To determine the effects of Phase I manipulations on the toxicity of the effluent, its inherent toxicity must be determined. The toxicity measured in this test is used to gauge toxicity changes caused by some manipulations and to detect changes in the sample's toxicity during storage. *Baseline tests* must be repeated each time additional manipulation tests are started.

Methods: The *baseline test* will be initiated using concentrations based on the historical data for each particular discharger. For the TIE, use of four (and

[5] EPA suggests that toxicity must be prevented under worst case scenarios (EPA, 1991B) which may mean the routine monitoring tests were conducted at high pH's.

three) dilutions have been sufficient for defining toxicity (Section 5.4). If the toxicity is low, in order to draw distinctions between the concentrations used in the test for the various characterization tests, the dilutions may need to be set closer, for example, 40%, 60%, 80%, 100%. In this test, and all subsequent characterization tests, the test concentrations, test volumes and number of replicates should be kept the same as described in Section 5, Toxicity Testing.

On day 2, an aliquot of the effluent is warmed slowly in a warm water bath to test temperature (25°C). The various test concentrations are prepared using the appropriate hardness reconstituted water. Next, routine chemistries are measured (initial pH, temperature, DO). The use of *dilution water controls* is not required for every manipulation but at least two sets of *controls* should be included to estimate reproducibility. In addition, the tests are conducted using one *C. dubia* per one 10 ml test volume in a 1 oz plastic cup (or glass beaker) and five animals per treatment. For the fathead minnow tests, two replicates per treatment, 10 fish in 50 ml in a 4 oz plastic cup, or 100 ml in a 400 ml beaker, are assumed.

Interpretation of Results/Subsequent Tests: The *baseline tests* serve as the basis for determining the effects produced by various characterization tests. This test serves as the *toxicity control* for some of the other tests. If *baseline tests* done on subsequent days with additional manipulations indicate that the toxicity of the effluent is decreasing, either every effort should be expended to characterize the toxicity more quickly (i.e., Phase II identification or Tier 2 tests) or another sample should be obtained. The *"shelf life"* of the toxicity can be determined after a few samples have been evaluated.

Special Considerations/Cautions: The *controls* in this test will provide information on the health of the test organisms, the dilution water, the test glassware and equipment used to prepare the test solutions and the cleanliness of the test chambers. This *baseline test* serves as the *toxicity control* for some subsequent Tier 1 or Tier 2 tests.

6.2 EDTA Addition Test

General Approach: This test is designed to detect effluent toxicity caused by certain cationic metals. The addition of EDTA to water and effluent solutions can produce non-toxic complexes with many cationic metals. Loss of toxicity with EDTA addition(s) suggests that cationic metals are causing toxicity.

EDTA is a strong chelating agent and because of its complexing strength, it will often displace other soluble forms (such as chlorides and oxides) of many metals. The ability of EDTA to chelate any metal is a function of pH, the type and speciation of the metal, other ligands in the solution, and the binding affinity of EDTA for the metal. And the complexation of metals by EDTA may vary according to the sample matrix. The specific form of metal that causes toxicity in the water matrix may be more important than the total concentration of the metal.

Cations strongly chelated by EDTA include aluminum ($^{3+}$), cadmium, copper, iron, lead, manganese ($^{2+}$), nickel, and zinc (Stumm and Morgan, 1981). EDTA weakly chelates barium, calcium, cobalt, magnesium, strontium, and thallium (Flaschka and Barnard, 1967). EDTA can form relatively weak chelates with arsenic and mercury and anionic forms of metals (selenides, chromates and hydrochromates) will not be chelated.

For some cationic metals for which EDTA forms relatively strong complexes, the acute toxicity to *C. dubia* is reduced (Mount, 1991; Hockett and Mount, In Preparation). EDTA was shown to chelate the metal causing the acute toxicity (at 4x the LC50) for copper, cadmium, lead, manganese ($^{2+}$), nickel, and zinc to *C. dubia* in both dilution water and effluents. However, they also found that EDTA did not remove/reduce the acute toxicity of silver, selenium (either as sodium selenite or sodium selenate), aluminum ($Al(OH)_4^-$), chromium (either as chromium chloride or potassium dichromate), or arsenic (either sodium m-arsenite or sodium arsenate) when tested using moderately hard water and *C. dubia* (Hockett and Mount, In Preparation).

In the acute Phase I manual (EPA, 1988A), the recommended amount of EDTA to be added was high because the authors thought calcium and magnesium had to be complexed in order to complex toxic metals (D. Mount, personal communication, NETAC, Duluth, MN). The mass of EDTA required was approximated by the amount needed for the titration of hardness or the measurement of calcium and magnesium when titration was not possible due to interferences. A third choice was to use 0.5x the EDTA LC50 for the test species (EPA, 1991A). Ideally the amount of EDTA to add would be just enough to chelate the toxicant(s) without causing toxicity or otherwise changing the matrix of the effluent. Without knowing how much toxicant must be chelated, the amount of EDTA to add must be estimated. Recently, the role of calcium and magnesium was tested in our laboratory. Acute toxicity tests with *C.dubia* were conducted in moderately hard and very hard reconstituted water using copper, cadmium, and zinc at 4x, 2x, and 1x the LC50 of each. When one metal and EDTA were present at approximately a 1:1 molar basis, all the toxicity was removed regardless of water hardness (J. Thompson, personal communication, NETAC, Duluth, MN). These results indicate that calcium and magnesium concentrations do not affect the levels of EDTA needed to remove the acute cationic metal toxicity. Whether toxicity reduction using the 1:1 molar ratio is true for chronic toxicity has not yet been evaluated in a likewise manner (cf., *Interpretation of Results/Subsequent Tests* below). However, EDTA and nitrotriacetic acid (NTA) were effective in chelating the toxicity of one concentration of either cadmium or copper to *C. dubia* at molar ratios of less than 1:1 (Zuiderveen and Birge, 1991). However, NTA pos-

sesses the characteristic of increasing the toxicity of some metals therefore NTA is limited in its usefulness for the TIE.

The threshold levels for *C. dubia* and fathead minnows to EDTA were determined using 7-d tests in different hardness waters and the results are given in Table 6-2. For *C. dubia*, the chronic toxicity of EDTA is not water hardness dependent, but for fathead minnows the sublethal toxicity appears to be greater in softer waters. This is in contrast to the acute toxicity of EDTA to *Ceriodaphnia* which indicated that EDTA toxicity decreased with increased water hardness (Phase I; EPA, 1991A). Natural waters and effluents have many constituents in addition to those added to reconstituted waters, and the behavior of EDTA in effluents (or receiving waters) could be different than in simple reconstituted water.

Methods: The goal is to add enough EDTA to reduce metal toxicity, without causing EDTA toxicity or substantially changing the water quality. The toxicity of EDTA as determined in clean reconstituted water is likely to be higher than the toxicity of EDTA added to an effluent. Therefore, the EDTA toxicity values contained in Table 6-2 represent maximum toxicity in any effluent. The toxic concentration of EDTA in one effluent will probably not be the same as the concentration causing toxicity in a different effluent or even a different sample of the same effluent. To be safe, the concentrations of EDTA added to any effluent should be less than the expected effect concentration of EDTA in clean water. For either species, two EDTA concentrations are added to two sets of two effluent dilutions. EDTA stock solution is added after the effluent dilutions are prepared so that the EDTA concentrations for each addition are constant across each set of effluent dilutions. A stock solution of EDTA (ethylene–diaminetetraacetic acid, disodium salt dihydrate) is prepared in distilled water. This EDTA stock solution should be prepared so that only microliter amounts of the stock are needed to minimize effluent dilution. No more than 5% dilution of the effluent aliquot by EDTA stock should occur.

To perform the effluent dilution test, two sets of effluent dilution concentrations are prepared (e.g, 100%, 50%, 25%,) and each set receives one of two addition levels of EDTA (Table 6-3). By using non-toxic concentrations of EDTA, there is less chance for artifactual toxicity; since the total amount of metal to be chelated is probably low for most chronically toxic effluents, there is no reason to add high levels of EDTA. The additive levels are based on the assumption that the calcium and magnesium need not be chelated in order to chelate the toxic metals, although the amount of EDTA added is most likely still an excess.

An EDTA stock solution of 2500 mg/l can be prepared. For the *C. dubia* tests, 0.06 ml is added to three separate 50 ml aliquots in the first effluent dilution set (i.e., 25%, 50%, 100%) to obtain a 3.0 mg/l final EDTA concentration. In the second dilution set, 0.16 ml is added to the other set of 50 ml effluent aliquots for a final concentration of 8.0 mg/l. For the fathead minnow tests, the same concentration of an EDTA stock solution can be used but the volume of stock additions must be doubled for the 100 ml test volume/concentration.

Table 6-2. Chronic toxicity of EDTA (mg/l) to *C. dubia* and *P. promelas* in various hardness waters using the 7-d tests.

Species	Water Type	IC50 95% C.I.	IC25 95% C.I.	NOEC	LOEC
C. dubia	VSRW	4.5 3.6-6.0	3.0 2.1-3.9	2.5	5.0
	SRW	7.5 6.2-8.3	4.9 3.7-5.7	3.1	6.3
	MHRW	8.8 4.7-13	5.9 3.4-10	5.0	10
	HRW	7.5 6.2-9.8	5.5 0.98-6.9	5.0	10
	VHRW	7.8 6.7-8.6	6.1 4.0-6.8	5.0	10
	VHRW	12 10-14	8.3 4.2-10	7.5	15
P. promelas	SRW	136 130-139	103 94-110	100	200
	MHRW	163 150-188	132 123-144	100	200
	HRW	236 227-248	—[1]	200	400
	VHRW	287 269-300	230 203-247	200	400

[1] Value could not be determined, value would be less than lowest test concentration.

Note: C.I. = confidence interval; VSRW = very soft reconstituted water; SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water.

Table 6-3. Concentrations of EDTA to add for chronic TIEs. Values given are the final exposure concentration in mg/l.

Species	Water Type[1]	Concentrations (mg/l)	
C. dubia and *P. promelas*	SRW, MHRW, HRW, VHRW	3.0	8.0

[1] In very soft water, the final concentrations of EDTA must be lower in order to not have EDTA induced toxicity, for example 1.0 mg/l and 5.0 mg/l.

Note: SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water.

To allow the EDTA time to complex the metals, solutions should be set up on day 2 and all solutions containing EDTA are allowed to equilibrate while other manipulations are being prepared before test organisms are introduced. A minimum of 2 h equilibration time should elapse before organisms are added.

Since EDTA is an acid, the pH of the effluent after addition of EDTA should be checked, although additions at these low levels should not lower the pH of the effluent. The amount of change in solution pH will depend upon the buffering capacity of the effluent and the amount of reagent added. If the pH of the effluent has changed, readjustment of the test solution pH to pH *i* should be performed.

The EDTA is not added to one batch of effluent on day 2; rather at each renewal EDTA is added to the renewal test solutions prior to dispensing into the test chambers in the identical way that the test solution was first made (allowing equilibration time).

Interpretation of Results/Subsequent Tests: Toxicity may be removed at all exposures provided the addition of EDTA does not cause toxicity. If the effluent is less toxic (i.e., EDTA addition IC50 (or IC25) shows less toxicity than *baseline test* IC50 (or IC25)) in either of the EDTA addition dilution tests, then EDTA removed or reduced the toxicity and cationic metal toxicity is probably present. If, in either test, the effluent is more toxic than in the *baseline test*, EDTA itself may be causing toxicity and the test should be repeated using lower EDTA concentrations. If toxicity is not reduced below the *baseline test*, the probability of cationic metals causing toxicity in the effluent is low and higher concentrations of EDTA can be tried, although this may or may not be useful.

Table 6-4 shows the results of a chronic zinc test and the reduction of the toxicity by the addition of EDTA. When *C. dubia* were tested in very hard reconstituted water, zinc was chronically toxic at 55 µg/l and EDTA was chronically toxic at 15 mg/l. When EDTA was added to solutions of 55 µg/l zinc at 2.5, 5.0, and 7.5 mg/l EDTA respectively, the toxicity of the zinc was removed but at 15 mg/l EDTA, EDTA itself was toxic. Such trends may be similar to the toxicity reduction observed in effluents. If toxicity is reduced in a systematic manner, such as in the example, proceed to Phase II methods for identification of those metal(s) which are chelated by EDTA. Additions of EDTA at 3 mg/l and 8 mg/l removed the toxicity of copper to *C. dubia* in a 7-d two-renewal test with hard reconstituted water at levels of 210 µg/l and 105 µg/l of copper. In addition to removing toxicity due to metals, EDTA reduces the acute toxicity of some cationic surfactants. This reduction of toxicity may also occur in chronically toxic effluents, and the toxicity reduced by EDTA should not be assumed to be due only to cationic metals. See Section 6.4 *Aeration Test* for subsequent tests to conduct if cationic metals are not present in the effluent at chronically toxic levels but EDTA reduced toxicity.

Table 6-4. The chronic toxicity of zinc (µg/l) to *C. dubia* in very hard reconstituted water and the toxicity of zinc when EDTA is added.

Zinc[1] Conc.	Mean Young per Female				
	EDTA Additions (mg/l)				
µg/l	0	2.5	5.0	7.5	15
0	19.2	18.6	17.5	17.6	6.8
3.4	19.4	—[2]	—	—	—
14	17.8	22.0	23.2	20.8	1.8
55	8.2	20.8	19.0	16.6	5.3

[1] Measured values.
[2] EDTA not added to this zinc concentration.

Special Considerations/Cautions: If pH in the EDTA tests is greatly different from that in the *baseline test*, the test might need to be redone. There is no way to distinguish the effect of pH change on the toxicity of a pH sensitive toxicant (e.g., ammonia) from toxicity changes caused by EDTA. A change of 0.1 pH unit can cause substantial errors if ammonia is involved. Before the test is reinitiated, data from the *graduated pH test* should be examined to evaluate whether the toxicity is pH dependent. This test data may be useful in deciding whether the *EDTA addition test* should be redone. EDTA additions to dilution water are not relevant *controls* for the EDTA additions to effluent; therefore, the *toxicity control* is the *baseline test*. The *control* of the *baseline test* serves as the QC for the health of the test organisms, the quality of the dilution water, and general test conditions.

If all dilutions where EDTA is added should cause mortality, one possibility is that the stock solution of EDTA is contaminated and the stock solution should be checked by conducting another test with a new EDTA stock.

6.3 Sodium Thiosulfate Addition Test

General Approach: Oxidative compounds (such as chlorine) and other compounds (such as copper and manganese) can be made less toxic or non-toxic by additions of sodium thiosulfate ($Na_2S_2O_3$). Toxicity from bromine, iodine, ozone, and chlorine dioxide is also reduced. Sodium thiosulfate has been routinely used to reduce the toxicity of substances such as chlorine (EPA, 1989C).

Reductions in effluent toxicity observed with sodium thiosulfate additions may also be due to the formation of metal complexes with the thiosulfate anion (Giles and Danell, 1983). The ability of sodium thiosulfate to form a metal complex is rate dependent and metal dependent (Smith and Martell, 1981) and sodium thiosulfate is not a particularly strong ligand for metal complexation. Cationic metals that appear to have this potential for complexation, based upon their equilibrium

stability constants, include cadmium, copper, silver, and mercury ($^{2+}$) (Smith and Martell, 1981). The rate of complexation is specific for various metals and some cationic metals may remain toxic in the 24-h or 48-h renewal period of the chronic toxicity test due to the slow rate of complexation or the stability of the complex. The thiosulfate anion is not very stable, and the ability of sodium thiosulfate to complex the compound(s) causing chronic toxicity without daily renewals has not been tested completely.

Recent findings have shown that the acute toxicity of certain cationic metals may be reduced by levels of sodium thiosulfate added in the acute Phase I tests (EPA, 1988A; EPA, 1991A). The acute toxicity of several cationic metals was shown to be removed by sodium thiosulfate in standard laboratory water. The acute toxicity at 4x the LC50s of copper, cadmium, mercury, silver, and selenium (as selenate) to *C.dubia* was removed by sodium thiosulfate additions at levels suggested in the acute Phase I manual. However, for zinc, manganese, lead, and nickel, the acute toxicity was not removed by the sodium thiosulfate additions (Mount, 1991; Hockett and Mount, In Preparation). The toxicity of mercury with the addition of sodium thiosulfate was reduced for 24 h but not 48 h which indicates it may not have been completely complexed by the thiosulfate. If the acute toxicity of metals can be reduced or complexed by sodium thiosulfate, the same may be true for chronic toxicity. However, for *C. dubia* 7-d tests with hard reconstituted water, sodium thiosulfate levels of 5 mg/l and 10 mg/l did not remove or reduce the chronic toxicity of copper at the same concentrations where EDTA complexed the toxicity (cf., Section 6.2).

The test animals will probably tolerate more sodium thiosulfate than would ever be needed to render oxidants or metals non-toxic in effluent samples, especially the fathead minnows in comparison to the *C. dubia* (Table 6-5). The presence of oxidants or complexable metals will reduce the concentrations of sodium thiosulfate below the nominal concentrations added.

Table 6-5 gives the toxicity values in various reconstituted waters. The effect concentrations for *C. dubia* and fathead minnows were measured in waters of different hardnesses (soft, moderately hard, hard, and very hard water (EPA, 1989C)). For *Ceriodaphnia*, the results indicate that the sublethal toxicity is unchanged regardless of the water type (Table 6-5). The toxicity tests with sodium thiosulfate and fathead minnows (7-d growth test) indicate that the toxicity due to sodium thiosulfate is greater in softer waters.

Methods: Two sets of effluent dilutions (such as 25%, 50%, 100%) each set with a different level of thiosulfate concentration (Table 6-6) are prepared regardless of whether *C. dubia* or fathead minnows are used as the TIE test organism. The concentration of thiosulfate remains constant across one set of effluent concentrations within a series (identical to *EDTA addition test*). Small volumes (microliter) of the sodium thiosulfate stock solution should be added to minimize the dilution (5% of total volume). Non-toxic concentrations of sodium thiosulfate are used to reduce the pro–

Table 6-5. Chronic toxicity of sodium thiosulfate (mg/l) to *C. dubia* and *P. promelas* in various hardness waters using the 7-d tests.

Species	Water Type	IC50 95% C.I.	IC25 95% C.I.	NOEC	LOEC
C. dubia	SRW	39 30-42	26 15-33	30	60
	HRW	38 26-44	27 20-36	30	60
	VHRW	43 37-44	34 21-37	30	60
P. promelas	SRW	1,070 1,041-1,1005	820 785-859	750	1,500
	MHRW	2,001 1,891-2,161	720 550-1,528	750	1,500
	HRW	4,871 4,633-5,051	3,590 3,226-3,800	3,000	6,000
	VHRW	8,522 8,053-8,704	6,780 6,065-7,073	6,000	12,000

Note: C.I. = confidence interval; SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water.

Table 6-6. Concentrations of sodium thiosulfate to add for chronic TIEs. Values given are the final exposure concentration in mg/l.

Species	Water Type[1]	Concentrations (mg/l)	
C. dubia and *P. promelas*	SRW, MHRW, HRW, VHRW	10	25

[1] In very soft water, the final concentrations of sodium thiosulfate must be lower in order to not have sodium thiosulfate induced toxicity, for example 1.0 mg/l and 5.0 mg/l.

Note: SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water.

bability of artifactual toxicity, yet sufficient concentrations are needed to remove/reduce oxidants.

For a *C. dubia* test, to the first effluent dilution set (i.e., 25%, 50%, 100%), 0.20 ml of sodium thiosulfate stock (2500 mg/l) is added to each 50 ml dilution to obtain final concentrations of sodium thiosulfate of 10 mg/l. To the second effluent dilution set, 0.50 ml of the same stock solution is added to 50 ml of each test dilution to obtain final concentrations of 25 mg/l (Table 6-6).

The fathead minnow test is similar except that twice the volume of the same thiosulfate stock is needed (because of 100 ml test volumes) to achieve the same final concentrations (Table 6-6).

The sodium thiosulfate is not added to a batch of the effluent on day 2; rather, at each renewal, sodium thiosulfate is added to the renewal test solutions in a manner identical to the way they were first prepared.

Interpretation of Results/Subsequent Tests: The results of the *sodium thiosulfate addition tests* are compared to one another and to the *baseline test* results to determine whether or not toxicity reduction occurred. Toxicity may be completely reduced, partially reduced, or not reduced. If toxicity appears to be reduced and/or removed, then more tests to determine whether the toxicity is due to an oxidant or to some metal should be performed. When chlorine concentrations are ± 0.1 mg/l total residual chlorine (TRC), there may be a toxicity problem for *C. dubia*. A significant drop in the chlorine level in the whole effluent may occur in the first 24-h period after sample collection and testing. Therefore, tests repeated on an aged sample may give different results if an oxidant is involved but may give the *same* results if a metal is involved.

For cases where oxidants account for only part of the toxicity, sodium thiosulfate may only reduce, not eliminate, the toxicity. Yet the *sodium thiosulfate addition test* is useful even when chlorine appears to be absent in the effluent. Oxidants other than chlorine occur in effluents, and even if the effluent is not chlorinated this test should not be omitted. Both thiosulfate and EDTA reduce the toxicity of some metals and this information can be helpful in identifying the toxicant. (However, this effect of thiosulfate/metal complexation has not been demonstrated for chronic toxicity.) In cases where both the *sodium thiosulfate addition test* and the *EDTA addition test* reduce the toxicity in the effluent sample, there is a possibility that the toxicant(s) may be a cationic metal(s). Many oxidants are reduced by aeration but if aeration does not reduce toxicity, Phase II methods for identification of cationic metal(s) toxicants should be investigated. No change in toxicity suggests either no oxidants or certain metals.

Special Considerations/Cautions: The general test conditions, quality of the dilution water, and health of the test organisms are tracked by the *controls* in the *baseline test*. Additions of sodium thiosulfate to dilution water are not relevant *controls* for thiosulfate additions to effluent to determine if the thiosulfate was toxic. Therefore the *toxicity control* is the *baseline test*.

If all dilutions where sodium thiosulfate is added should exhibit mortality, one possibility is that the stock solution of sodium thiosulfate is contaminated and this phenomena should be checked by conducting another test.

6.4 Aeration Test

General Approach: Changes in toxicity due to aeration at pH *i* may be caused by substances that are oxidizable, spargeable, or sublatable. The chemical/physical conditions of the aeration process will also affect whether or not the toxicity is reduced or removed.

Sparging of samples is done using air which includes oxidation as a means of toxicity removal. In our experience, typically volatile compounds that are highly water soluble (such as ammonia) will not be air-stripped at pH *i* by this method. If aeration is one of the mechanisms that removes the toxicity, then additional tests must be performed to identify which mechanism is removing the toxicity. Subsequent tests with nitrogen can be used to determine if toxicity reduction was due to oxidation. Also, air or nitrogen sparging can cause surface active agents to sublate. As bubbles break at the surface, sublatable compounds will be deposited on the sides of the aeration vessel. Sublatable toxicity identification requires special sample removal and rinsing (see below). A visible deposit does not indicate the presence or absence of such toxicants.

Methods: For the aeration process, the volume of effluent and dilution water aerated is kept the same even though all of the dilution water volume is not needed for the *aeration blank*. The flow rate, bubble size, geometry of apparatus and time of aeration should be consistent among treatments. Taller water columns and smaller bubbles should ensure better stripping; therefore, the aeration vessel should be half-full or greater for this process. Each aliquot (effluent and dilution water) should be moderately aerated for a standard length of time (60 min). Use of gas washing bottles (Kontes Glass Co., Vineland, NJ) fitted with

glass frit diffusers located at the bottom of the vessel for aeration is suggested because they sparge the sample effectively. During aeration, the pH of the effluent is not maintained at "pH *i*."

The volume of effluent aerated should be the same for either a 4-d *C. dubia* test or a 7-d *C. dubia* two renewal test (four dilutions, five replicates for each dilution; see Section 5), although there is excess of solutions for the 4-d test. Use of 300 ml of effluent (or dilution water) in a 500 ml gas washing bottle or 500 ml in a 1 L bottle and a flow-rate of 500ml/min is suggested. Any loss of volume and any formation of precipitates should also be recorded.

Interpretation of Results/Subsequent Tests: If the aerated effluent has less toxicity than the *baseline test*, and the *aeration blank* is not toxic, aeration was effective in reducing toxicity. If the toxicity of the aerated effluent is less than the *baseline test*, even though the *aeration blank* is toxic, the results indicate that aeration is an effective removal technique. If the effluent toxicity is not reduced or it is more toxic after aeration than in the *baseline test* (and the *aeration blank* was non-toxic, then either toxicity was concentrated during the aeration process or toxicity was added or created during the aeration process (see *Special Considerations/Cautions* below).

Typically, using this aeration technique, ammonia is not air-stripped from the sample at pH *i*. However, if total ammonia is at least 10 mg/l or higher and the pH is above 8.0, ammonia measurements in the aerated sample may be useful if the aeration manipulation resulted in a toxicity reduction.

If a substantial reduction in toxicity is observed, then the mechanism for the toxicity removal must be determined. To determine if the reduction is due to oxidation, sparging, or sublation, the air should be replaced by nitrogen. The flow of nitrogen through the sample must be the same as for air. If nitrogen sparging as well as air sparging removes or reduces the toxicity, then oxidation as the removal process is eliminated. If aeration only succeeds in reducing toxicity, then oxidation may be involved. It is possible that a toxicant can be removed through sparging *and* oxidation in which case air should reduce toxicity more than nitrogen.

The presence of sublatable substances can be determined (whether air or nitrogen is used) by removing the aerated sample from the aeration vessel by siphoning or pipetting without contact with the sides of the aeration vessel. The geometry of the aeration vessel (i.e., at least a half-full cylinder) must remain the same as in the initial aeration experiment but the recovery of sublated compounds can be difficult. Dilution water added to the aeration vessel is used as a rinse to remove the sublate residue on the walls. To attempt this recovery, use of graduated cylinders with ground glass stoppers has been successful for acute testing (EPA, 1991A) because the water can be shaken vigorously to contact all surface areas to recover the sublatables. This sublation procedure is effective for dissolved surfactants, and while sewage particles adsorb surface active particles tightly, the actual sublation process may take some time (i.e., >1 h) (AHPA, 1989). If toxicity is not recovered from the vessel walls, the presence of such compounds cannot be ruled out. Specific procedures, for the larger volumes needed in the chronic tests, have not yet been developed.

In some instances, sublatable toxicants may not be removed by dilution water, and the use of solvents (e.g., methanol) may be needed for better recovery. However, the solvent will have to be reduced in volume (aired down) in order to have an adequate concentration factor in the test solution and a sufficiently low concentration of solvent for the subsequent toxicity tests (see Sections 6.7 and 6.8 for methanol toxicity information). Of course, dilution water *blanks* must also be subjected to all steps to check for artifactual toxicity.

Special Considerations/Cautions: Removal of compounds by precipitation can occur through oxidation. However, the *filtration test* should not change toxicity of the effluent if oxidation is involved but filtration might also remove the toxicity of some sublatable compounds absorbed to particles and therefore the results of the *aeration test* can be compared to the *filtration test*.

Use of nitrogen to sparge the sample is likely to drastically reduce the DO. For instance, 1 h of nitrogen sparging has caused the DO to drop below 4 mg/l. To increase the DO before initiating the test after a sample has been sparged with nitrogen, transfer the sample to a container with a large surface area to water volume ratio. The DO should rise to >5 mg/l without additional aeration.

The *baseline test* serves as the *toxicity control* and the aeration of the dilution water (*aeration blank*) provides information on the system apparatus. The general test conditions, quality of the dilution water, and health of the test organisms are tracked by the *controls* in the *baseline test*. No significant toxicity should occur in the *aeration blank*. Toxicity in the *aeration blank* implies toxic artifacts from the aeration process, the glassware, or a dilution water problem. If the *aeration blank* is toxic, check the results of the test of the *filtration blank*. If both *blanks* are toxic, then most likely there is a problem with the dilution water but if only the *aeration blank* is toxic, artifactual toxicity arose during that manipulation.

6.5 Filtration Test

General Approach: Filtration of the effluent sample provides information on whether the toxicity is filterable yet provides relatively little specific information about which class of toxicant may be causing the toxicity. Reductions in the toxicity caused by filtering alone may imply toxicity associated with suspended solids or removal of particle-bound toxicants. Whether compounds in the effluent are in solution or sorbed to particles is

dependent on particle surface charge, surface area, compound polarity and charge, solubility, and the matrix of the effluent. If particles are removed, other compounds may be bound to them and are not available to cause toxicity. The way the toxicant is bound to the particulates is probably more important when using filter feeders as the toxicity test organism in short-term chronic tests. This is primarily a route of exposure for filter feeders as compared to the fathead minnow. Toxicity can also be reduced by filtering if a toxicant(s) is not particle-associated; we have observed that some chemicals in a dilution water stock are removed by filtering (e.g., DDT).

The filtration step also serves an important purpose for another Phase I manipulation, the solid phase extraction (SPE) (Section 6.6), where aliquots of the effluent typically must be filtered before application to the SPE sorbent (see *Interpretation of Results/Subsequent Tests* below). If many particles are present in the sample, the sorbent may act as a filter itself or the column will become plugged.

Methods: The use of a positive pressure filtration system is superior to the use of a vacuum filter because volatile compounds may be removed by vacuum filtering and hence confuse the effect of filtering (see *Interpretation of Results/Subsequent Tests*).

As in the acute Phase I, prepare the filters (typically 1 µm glass fiber filters without organic binder) by passing an appropriate volume (approximately one-fourth of effluent volume to be filtered) of high purity water over the filter(s) in the filter housing. This water is discarded, a small aliquot of the dilution water is filtered (prepare excess, at least 500 ml for the *C. dubia* 7-d test and 800 ml for the fathead minnow 7-d test) and discarded (100 ml) and the rest collected. A portion of the filtered dilution water is collected and used for testing and a portion reserved for the *post C_{18} SPE column test blank* (Section 6.6). For example, the last 400 ml of the filtrate is collected for the *C. dubia* 7-d *filtration blank* and *post C_{18} SPE column blank* tests.

Next the effluent sample is filtered using the same filter, and a portion of the filtrate is collected for toxicity testing and a portion set aside that will be concentrated on the C_{18} column. When filtering the effluent, filter enough sample for this test and enough sample (>1 L) to use for the SPE step described below. For some effluents, one filter will not suffice. A technique we use is to prepare several filters at once by stacking 5-8 filters together followed by rinses of high purity water and dilution water using the same rinse volumes as above. Then the filters are separated, and set aside, using one at a time for the effluent sample. If the samples measure quite high in total suspended solids, pre-filtering using a larger pore size filter may help. Again, appropriate *blanks* must be obtained for any pre-filtering. Low levels of metals on the glassware or the filters could cause interferences in toxicity interpretation. Pre-rinsing the filters and glassware with high purity water adjusted to pH 3 may provide consistently clean *blanks* and possibly less contamination in effluent samples. If the sample cannot be effectively/easily filtered due to many fine particles, centrifuging may be better (again *blanks* must be prepared).

The filter housing should be thoroughly cleaned between effluent samples to prevent any particle build-up or toxicity carryover. We have found large filter apparatus (1 L), removable glass frits, or plastic filtering apparatus (Millipore®) to be useful. The glassware cleaning procedure that is described in the acute Phase I TIE manual should be sufficient for chronic TIE work (EPA, 1991A). The glass frits may require rigorous cleaning (i.e., soak in strong acid (10% v/v) for 20-40 min) to remove residuals that may remain after filtering, since the glass frit may itself act as a filter.

Interpretation of Results/Subsequent Tests: If toxicity in the whole effluent is reduced by filtration, a method for separating the toxicants from other constituents in the effluent has been achieved. This should advance the characterization considerably because any subsequent analysis will be less confused by non-toxic constituents. If appropriate, one should determine if toxicity loss was due to volatilization. Comparisons of pressure filtering and vacuum filtering should indicate if volatilization is involved. For further characterization, the mechanism of removal should be determined (precipitation, sorption, changes in equilibrium or volatilization).

Identification efforts should be focused on the residue on the filter after testing indicates that the toxicant(s) is not volatile. To recover the toxicity from the filter(s), use of acidic and basic water as well as various organic solvents can be tried. The recovery achieved by these various methods provides information about pK_a and water solubility of the toxicants. Filtration has reduced the quantity of total cationic metals present in some effluents. The recovery of the metal and acute toxicity was successful when dilution water adjusted to pH 3 was used to extract the filter (EPA, 1991A). Filter extraction into smaller volumes than that of the effluent sample filtered will give a higher concentration of toxicant, perhaps allowing the use of acute test endpoints. However, evidence then must be gathered to be sure the toxicants causing acute toxicity are the same as those causing chronic toxicity. Use of solvents will require solvent reduction or solvent removal (exchange) before testing (see Phase II; EPA, 1992A). Sonication of filters is another approach but the manipulation must be accompanied by proper *blanks* in similar fashion to those needed for the pH 3 extraction of the filter extraction step described above.

If large volumes of an effluent (~2 L over one 1 µm filter) can be readily filtered, the effluent should be filtered for the *filtration test* and unfiltered effluent can be passed over the C_{18} SPE column (see Section 6.6; *Post C_{18} SPE column test*). Once it has been demonstrated that filtration does not reduce toxicity in the effluent, and the toxicity is recovered in the *methanol eluate test* the routine filtering can be eliminated. By

this approach the amount of testing to be done is decreased, yet the tracking of toxicity is possible. We have infrequently experienced any effluents that have low amounts of filterable solids where the effluent could be concentrated without filtering. If any effluent sample has reduced toxicity in the *filtration test* and toxicity is not observed in the *methanol eluate test*, characteristics of the toxicant(s) will be described as filterable and not C_{18} recoverable.

If the toxicity cannot be recovered from the filter, was not volatile (see Section 6.4 *aeration test*) and no other manipulations changed toxicity, use of Tier 2 is a good subsequent step. Toxicity could have been removed by the glass frit, and use of a plastic filter apparatus or stainless steel frits may assist in identifying that the toxicant(s) removed is on the frit or filter. Filter-removable toxicity in Tier 2 is more difficult to identify (because of the radical pH adjustments) because of irreversible reactions and potential for artifactual toxicity (see Section 6.12 below).

Special Considerations/Cautions: The filtered dilution water and filtered effluent sample also serve as the *toxicity blank* and *toxicity control* respectively for the *post C_{18} SPE column test* (see Section 6.6). The results of the effluent *filtration test* should be compared with the *filtration blanks* and no major change in the trend of young production, growth or survival should occur in the *filtration blanks* in comparison to the *controls* in the *baseline test*. If the *filtration blanks* are acceptable, then the results of the *filtration test* and the *baseline test* should be compared.

As a *toxicity blank* for the SPE tests, if the *filtration blank* is either slightly or completely toxic, but the post C_{18} SPE column effluent is not toxic (and effluent toxicity was unchanged after filtration), the *filtration blank* toxicity can be ignored since the effluent toxicity was removed. However, as work proceeds to identification, the *blank* toxicity will have to be eliminated or else it could introduce an artifact and lead to a misidentification of the cause of toxicity.

6.6 Post C_{18} Solid Phase Extraction Column Test

General Approach: The C_{18} SPE column is used to determine the extent of the effluent's toxicity that is due to compounds that are removed or sorbed onto the column at pH *i* (cf., *post C_{18} SPE column and pH adjustment test*; Section 6.13 below). By passing effluent through a SPE column, non-polar organics, some metals, and some surfactants are removed from the sample. In addition, these columns may also behave as a filter (see *filtration test* above).

Compounds in effluent samples interact with the C_{18} and depending upon the polarity and solubility of the compounds, the sorbent may extract the chemicals from the water solution/effluent onto the column. Extraction occurs when the compounds have a higher affinity for sorbent than for the aqueous phase. Non-polar organic chemicals are extracted because the C_{18} sorbent is very non-polar in comparison to the polar water phase; this extraction process is referred to as reverse phase chromatography.

The effluent that passes over the column is collected and the post-column effluent is toxicity tested in order to determine if the column removed toxicity. If the toxicity of the post-column sample is decreased, removal of toxicant(s) by the column is probable but if it is not, artifactual toxicity may be obscuring the removal. Steps to deal with this are given below in *Interpretation of Results/Subsequent Tests*. If the post-column sample is highly toxic, the capacity of the column to extract the toxicants may be exceeded or the column may have been inadequately conditioned.

Because toxicity may be retained by the C_{18} column, efforts to recover the toxicity are necessary. After a sample is passed over the C_{18} column, many of the compounds extracted by the sorbent at a neutral pH should be soluble in less polar solvents than water (i.e., hexane, methylene chloride, methanol, chloroform). However, most of the non-polar solvents are highly toxic to aquatic organisms. Sorbed non-polar organics are eluted from the column because they have higher affinity for the non-polar solvent than the C_{18} sorbent. The *methanol eluate test* (Section 6.7) is designed to determine if toxicants are non-polar.

Methods: The toxicity of the effluent, the type of test to be conducted, and the frequency of the solution renewal affect how much effluent must be filtered and passed over the C_{18} SPE column. First, the concentrations and the volume of the eluate needed for the *methanol eluate test* (Section 6.7) to test at 2x or 4x the whole effluent concentrations should be determined (keeping in mind that the methanol test level must be below the chronic threshold level for the species used; Section 6.7). However, limiting factors of the maximum volume to apply to a column, the minimum elution volume required, and the concentration that can be obtained within these confines must be calculated (Tables 6-7 and 6-8).

For example, our procedure has been to pass 1000 ml of 100% effluent over a 1 g (6 ml) column and elute with 3 ml of methanol which results in a theoretical 333x concentrate. The 1000 ml is the limit of sample volume over a 1 g (6 ml) column and the 3 ml methanol elution is slightly more than the minimum elution volume required (Table 6-7). However to test *C. dubia* at 4x, and to have the methanol concentration at a non-toxic chronic level (Table 6-9), the 3 ml must be further concentrated to 1.5 ml (now 666x whole effluent concentration). At present 3 ml of the eluate is concentrated in graduated centrifuge tubes to 666x by using a gentle stream of nitrogen gas over the surface of the methanol eluate in a warm water bath (25-30°C) to concentrate the 333x eluate to a final volume of 1.5 ml. For five replicates of 10 ml each, 0.30 ml of the eluate can be added to 50 ml of dilution water and the resultant effluent concentration is 4x and the methanol concentration is 0.6%. However the 1.5 ml eluate from the

Table 6-7. Factors to consider for the size of available pre-packed C_{18} SPE columns. Appropriate volumes of sample to apply to each column with respect to maximum volumes of sample and minimum elution volumes, and elution volumes frequently used in the TIE process.

Columns Available[1]		Conditioning Volume (ml)	Maximum Volume (ml) of Effluent	Minimum Elution Volume[2]	Methanol Elution Used (ml)[3]	No. Methanol Fractions[4]	Eluate Concentration
Size (ml)	g of Sorbent						
6	1	10	1,000	2.0, 2.4	3[5], 2.4	3	333x[5], 417x
12	2	24	2,000	4.8	3	3	417x
20	5	40	5,000	12	6	3	417x
60	10	120	10,000	24	12	3	417x

[1] 1 g columns are available from J.T. Baker Chemical Company, Phillipsburg N.J. (1 g, 6 ml columns have been extensively used at ERL-Duluth). 1 g, 2 g, 5 g, and 10 g columns are available from Analytichem International, Mega Bond Elut™, Harbor City, CA. Pumping rates for each column are proportional to volume based on 1 L at 5 ml/min; therefore 2 L at 10 ml/min, 5 L at 25 ml/min, and 10 L at 50 ml/min. We are currently evaluating the minimum elution volumes to determine if less eluting solvent can be used. Pumping rates for 5 L and 10 L may need to be slower when eluting each column. Yet how much the pump should be slowed will be a function of the toxicants. The contact time of the elution solvent with C_{18} sorbent may need to be increased if toxicity is not recovered in the methanol eluates.

[2] Minimum elution volume as recommended by the manufacturers. For the 1 g column, J.T. Baker recommends 2.0 ml and Mega Bond Elut™ recommends 2.4 ml, but 2.0 ml is probably adequate.

[3] Elution of two one-half volume aliquots is better for optimizing the elution efficacy

[4] For each fractionation of any size column, collect three separate 100% methanol fractions to use in *methanol eluate test* to attempt recovery of the non-polar toxicants (see text for more details).

[5] This procedure has been routinely used for acute TIEs. To maximize concentration and minimize methanol levels in concentration and minimize methanol levels in toxicity tests it is best to use the minimum elution volumes recommended by the manufacturer.

1 L fractionation will allow testing of 4x, 2x, 1x only if two solution renewals are used (Table 6-8). Daily renewals for a 7-d *C. dubia* test require a total of 3.7 ml at a water concentration of 0.6% methanol (which means 3 L of effluent must be fractionated to obtain 9 ml of 333x eluate which is concentrated to 4.5 ml to test at 4x) (Table 6-8).

To test at 2x using a 417x eluate from a 2.4 ml elution, 0.048 ml in 10 ml will result in the 2x test concentration. For a 7-d, daily renewal test at 2x, 1x, 0.5x, 3.0 ml is needed (5 replicates of 10 ml each) which will require 1 L of effluent to be concentrated (Table 6-8). By this procedure the final methanol concentration is 0.48%. The 417x concentrate can also be concentrated to 834x and use 0.048 ml/10 ml to test the eluate at 4x.

For the 7-d fathead minnow test using 50 ml per replicate and two replicates, a total of 7.4 ml of a methanol eluate is needed for test initiation and six renewals, which requires fractionation of 3 L of effluent. This assumes the methanol test concentration between species are kept the same. Actually the fathead minnows could probably be tested at methanol concentrations of ~1%, and using 0.96 ml of the 417x eluate per 100 ml will result in 4x effluent test concentration and a 1% methanol concentration (Table 6-9).

The methods below assume one effluent volume (usually the 100%) is concentrated and the post column effluent sample collected and used for all solution renewals during the test (Table 6-8). The procedure described below is an overview of the steps needed to prepare the column, collect methanol *blanks*, recondition the column, collect post-column effluent, and collect methanol eluate (steps needed for this test and the next test—Section 6.7). All steps are detailed in the acute Phase I manual (EPA, 1991A), and the major difference for the chronic Phase I is that fewer post-column samples (one or two versus three) are collected.

The general technique for conditioning and using the prepackaged SPE columns is as follows. Using a pump system with a reservoir for the effluent sample and teflon tubing, first pump 10-120 ml of HPLC grade methanol over the column to condition the sorbent (Table 6-7). This methanol is discarded. Without letting the column go to dryness, 10-120 ml of high purity water is passed over the column and discarded. Next, before the methanol *blank* is collected, the column is allowed to go to dryness. For 1 L of sample and a 1 g (6 ml) column, two 1.5 ml aliquots of 100% methanol are collected, combined, and tested as the *blank*. The elution is more efficient when two aliquots of 1.5 ml are collected in contrast to one elution of 3 ml. The collection of three 100% methanol eluates (2.4 or 3 ml each) has been more helpful for tracking toxicity than only one 100% methanol eluate sample. The use of three 100% methanol elutions is replaced when the Phase II fractionation procedures are applied. These 100% methanol eluates may need to be concentrated prior to testing (see Section 6.7). The containers to collect the methanol should be acid leached, hexane and acetone rinsed, and allowed to dry before use. After the methanol *blank* is collected, the column must

Table 6-8. Test volume of eluate needed for *methanol eluate test* with *C. dubia* or *P. promelas*. Volumes described are based on minimum elution volumes recommended (Table 6-7) and the highest test concentration possible with the methanol level at an acceptable concentration.

Test Species	Test Duration	No. Renewals & Original Sample	High Test Conc.	No. Rep.	Volume of Eluate Needed for Testing at: 333x[1]	417x[2]	Test Concentrations	Minimum Volume (L) of Effluent[3]
C. dubia	4-d	2	2x	5	1.05	0.84	2x, 1x, 0.5x	1
C. dubia	4-d	4	2x	5	2.10	1.68	2x, 1x, 0.5x	1
C. dubia	7-d	3	2x	5	1.58	1.26	2x, 1x, 0.5x	1
C. dubia	7-d	7	2x	5	3.68	2.94	2x, 1x, 0.5x	2
C. dubia	4-d	2	2x	10	2.10	1.68	2x, 1x, 0.5x	1
C. dubia	4-d	4	2x	10	4.10	3.36	2x, 1x, 0.5x	2
C. dubia	7-d	3	2x	10	3.16	2.52	2x, 1x, 0.5x	2
C. dubia	7-d	7	2x	10	7.35	5.88	2x, 1x, 0.5x	3
P. promelas	7-d	7	2x	2	7.35	5.88	2x, 1x, 0.5x	3
P. promelas	7-d	7	2x	4	14.70	11.76	2x, 1x, 0.5x	5
P. promelas	7-d	7	4x	2	14.70	11.76	4x, 2x, 0.5x	5
P. promelas	7-d	7	4x	4	29.40	23.52	4x, 2x, 0.5x	10

[1] For the 333x eluate concentration, this volume is based on the assumption that the *C. dubia* test solutions are prepared as 300 µl of 333x into 50 ml for 2x, 150 µl into 50ml for 1x, and 75 µl into 50 ml for 0.5x. More volume will be needed if serial dilutions are prepared (600 µl vs 525 µl). For the fathead minnow tests this assumes test solutions are prepared as 600 µl into 100 mL for 2x, 300 µl into 100 mL for 1x, and 150 µl into 100 mL for 0.5x. More volume will be needed if serial dilutions are prepared (1200 µl vs 1050 µl).

[2] For the 417x eluate concentration, this volume is based on the assumption that the *C. dubia* test solutions are prepared as 240 µl of 333x into 50 ml for 2x, 120 µl into 50ml for 1x, and 60 µl into 50 ml for 0.5x. More volume will be needed if serial dilutions are prepared. For the fathead minnow tests this assumes test solutions are prepared as 480 µl into 100 ml for 2x, 240 µl into 100 mL for 1x, and 120 µl into 100 ml for 0.5x. More volume will be needed if serial dilutions are prepared. For the 4x fathead minnow test, 960 µl per 100 ml must be prepared for the 4x solution.

[3] Volume is based on high test concentration (2x or 4x) tested without concentration to obtain eluate twice as concentrated. If further concentration is needed, twice as much effluent will be needed.

Table 6-9. Chronic toxicity of methanol (%) to *C. dubia* and *P. promelas* using the 7-d tests.

Species	Water Type	Test Renewal	IC50 95% C.I.	IC25 95% C.I.	NOEC	LOEC
C. dubia	SRW	daily	1.2 1.1-1.2	0.45[1] 0.35-1.0	<0.5	—
	SRW[2]	twice	1.4 —	0.45[1] 0.36-0.70	<0.5	—
	SRW[2]	twice	1.2 0.69-1.7	0.59 0.29-0.95	0.75	1.5
	SRW[2]	twice	1.3 —	0.83 0.34-1.0	0.75	1.5
P. promelas	SRW	daily	2.1 2.0-2.2	1.34 0.27-1.5	1.3	2.5

[1] Value is extrapolated.
[2] Tests all conducted independently.

Note: C.I. = confidence interval; SRW = soft water

be reconditioned with 10-120 ml of methanol (which is discarded). Without allowing the column to go to dryness, follow the methanol with an aliquot (10-120 ml) of high purity water, immediately followed by an aliquot of filtered dilution water. The amount of filtered dilution water needed will be dependent on the species and type of test to be conducted. The initial aliquot of the post-column water should be discarded (~200 ml) and the remainder of the post column dilution water should be collected. This post-column dilution water sample will serve as the dilution water *blank* for the *post C_{18} SPE column test.*

In order to optimize concentration of an effluent sample and not exceed the specifications of the sorbent capacity, when the maximum volume (Table 6-7) of a sample is passed over a column, the sorbent must be reconditioned following the collection of the post column dilution water. For example if 1.2 L of dilution water is needed and 5 L of effluent is to be concentrated on a 5 g column, without reconditioning the column between the dilution water and the effluent, the sorbent's capacity is likely to be exceeded. Toxicity might be observed in the *post C_{18} SPE column test* because of the excessive volume of dilution water and 5 L of effluent. The procedures for conditioning the column are similar to those above. The appropriate amount of methanol (Table 6-7) is used to condition the sorbent and the methanol is discarded. Before the column goes to dryness, follow the methanol with an aliquot (10-120 ml) of high purity water, immediately followed by the volume of filtered effluent to be concentrated. Again, collect about 200 ml of the post-column effluent and discard it. This is discarded to reduce the possibility of higher background concentrations of methanol in the post-column sample which might contribute to artifactual toxicity. Collect remainder of post-column effluent as a batch or in aliquots. If small quantities (<500 ml) of post-column effluent are needed for toxicity testing, separate post-column effluent samples may help determine if toxicity breakthrough occurred, and concentration factors will be different for the lower volumes.

Interpretation of Results/Subsequent Tests: The extraction efficiency of the column is evaluated by comparing the toxicity in the *post C_{18} SPE column test* to the *filtration test* data. This *post C_{18} SPE column test* is most useful when there is no post-column toxicity, and filtration did not reduce toxicity.

When toxicity in the post-column effluent is reduced or removed, then the next step is to compare the results with the *methanol eluate test.* If toxicity was recovered in the methanol eluates (see Section 6.7 below), then efforts to identify the toxicants (Phase II) should be initiated immediately.

If the post-column effluent toxicity was removed or reduced, but toxicity was not recovered in the methanol eluates (see below), it is possible that the toxicant is not eluted into 100% methanol and the C_{18} SPE column contains the toxicant. Use of the gradient of methanol and water fractions should be tried as well as testing the eluate at higher concentrations than 2x (i.e., 4x or 8x). If those tests do not indicate toxicity present in the eluates (see below) alternate elution schemes (EPA, 1992A) must be tried to recover the toxicant. It is important to recognize that the toxicity removed by the C_{18} SPE column is not necessarily due to non-polar compounds. Metals can be removed from some effluents via the C_{18} SPE sorbent. However, metals are not efficiently eluted in methanol or other organic solvents. Acid adjusted (pH 3) dilution water may be needed to elute toxicant(s) from the column. If this is done, the pumping rate of the pH-adjusted water should be slowed (perhaps by one-fourth of original pumping rate) to allow adequate contact time to elute the compound from the sorbent. In addition, compounds such as polymers or surfactants may be sorbed onto the column and some will elute with methanol while others do not.

The column can act as a filter itself and the various solvents used do not elute the toxicant. To check whether the C_{18} column is acting as a filter, unfiltered effluent can be passed over the C_{18} column and toxicity test results compared to those from the filtered effluent sample simultaneously. When effluent samples are readily filtered (e.g., ≥1.5 L for one 1 µm filter) filter the effluent to conduct the *filtration test* and use unfiltered effluent for the *post C_{18} SPE column test* and *the methanol eluate test.* When toxicity can be recovered in the methanol eluate, the toxicant(s) is most likely to be non-polar and since filtration can be eliminated for subsequent identification steps the amount of testing is subsequently reduced.

If the post-column toxicity was reduced and/or removed but not recovered in the *methanol eluate test,* the possibility exists that the toxicant has degraded or decomposed during the manipulation and the toxicant(s) was not concentratable.

As mentioned above, when no toxicity occurs in the post-column effluent (or the toxicity is reduced), and yet the *methanol eluate test* did not exhibit toxicity, metals may be involved or a non-polar that was not recovered in the solvent may be involved (discussed above). To check for cationic metal toxicity, the *post C_{18} SPE column test* should be combined with the *EDTA addition test* and the *sodium thiosulfate addition test* to characterize the post-column toxicity (see Section 6.16, *multiple characterization tests*).

For effluents that have shown that the toxicant is C_{18} recoverable, but the degradation of toxicity occurs fairly rapidly (i.e., the effluent sample is non-toxic in 1-2 weeks), it may be prudent to concentrate additional volumes of effluent immediately after the effluent arrives at the testing laboratory. Non-polar toxicants may not degrade in the methanol fractions as quickly in the effluent samples. Collect the methanol fractions (three 100% fractions) or the various methanol/water fractions as described in Phase II (EPA, 1992A) and hold them at 4°C for analysis as the TIE proceeds. Similarly,

once the cause of toxicity has been determined to be non-polar (C_{18} extractable) it might be more appropriate to immediately concentrate 10 to 20 L of effluent and for the elution step, replace the three 100% methanol elutions with the methanol/water procedures (EPA, 1992A). For chronic work, we have been using seven water/methanol fractions (50%, 75%, 80%, 85%, 90%, 95%, and 100%) rather than the eight used in acute TIEs because the toxicity has never recovered in the 25% fraction and by eliminating it the testing workload is reduced. It may be prudent to try two additional 100% methanol fractions following the seven fractions as well or follow it with alternate elution schemes (cf., Phase II; EPA, 1992A). By immediately concentrating the effluent, it is possible to optimize the amount of methanol available for testing and subsequent concentration for analysis and the post-column samples can be tested at one time. This eliminates duplication of effort that is required when additional methanol eluate is needed for subsequent work in Phase II.

Artifactual toxicity in the test containers may appear as a biological growth in the 100% post-column effluent and the effluent dilutions during the test. Effluents from biological treatment plants may develop this characteristic more readily than physical-chemical treatment plant effluents. This growth can negate actual toxicant removal by the column. While this growth does not occur in all effluents, when it does occur with one post-column effluent sample, the growth often occurs in each subsequent post-column effluent sample. The growth appears as a filamentous growth and gives a milky appearance in the test vessel. This growth has been linked to methanol stimulation of bacterial growth. Methanol is present in the post-column samples because methanol is constantly released from the sorbent during the sample extraction. Additional filtering of the post-column effluent sample through a 0.2 µm filter before testing to remove bacteria and eliminate the growth, has not been particularly successful. Artifactual toxicity from the post-column effluent may be avoided if the tests with the post-column samples are initiated on the same day the effluent is concentrated. To date, when we have collected the post-column samples and tested them on the same day, we have not experienced less artifactual toxicity than we found in those effluents where artifactual toxicity consistently has been a problem. However, less time elapses before animals are exposed to the test solution, therefore less time is available for bacteria to cause problems in the post-column sample matrix. Another option is to perform daily concentration of the effluent and extraction of the column during the 7-d test, as fresh post-column samples may minimize the artifactual toxicity.

When post-column artifactual growth is not readily eliminated, then a different solvent (acetonitrile) to prepare the column (but not for eluting) may be useful in reducing the post-column artifactual bacterial growth. Acetonitrile causes narcotic effects in toxicity tests, and is recommended only to condition the columns to avoid toxic concentrations. This technique has been successful on a limited number of effluents.

Special Considerations/Cautions: Careful observations and judgement must be exercised in detecting problems in the *post C_{18} SPE column test.* Low DO levels can occur in these samples. Through testing experience, the investigator will know whether toxicity appears as artifactual (i.e., growth, low DO) as opposed to the presence of the sample toxicity. If artifactual toxicity is not recognized, then a conclusion that the C_{18} SPE column did not remove toxicity can erroneously be made. For this reason if the post-column effluent is toxic, the methanol eluate must be tested (Section 6.7). This avoids the artifactual toxicity issue and the error can be avoided by determining the toxicity of the eluate.

The methanol elution process does not always produce predictable results with the same effluent sample. When toxicity is removed by the column but no toxicity occurs in the 100% methanol eluates, it does not indicate that the toxicity is *not* due to a non-polar toxicant(s). To check this possibility, immediately test the series of methanol/water fractions at concentrations of 4x or 8x. Not all non-polar organic compounds elute into 100% methanol as well as they do into lower methanol/water concentrations. Also toxicants may smear across the fractions and when <100% recovery of toxicity from the column is not 100%, toxicity may not be observed at 2x or 1x.

General test conditions will be tracked (dilution water, health of test animals) by the *controls* in the *baseline test.* The post-column dilution water *blanks* should be compared to those *controls* to determine if the column imparted toxicity. If the post-column dilution water *blank* was toxic, but no toxicity or artifactual toxicity occurred in the post-column effluent sample, the toxic *blank* can be ignored.

Results of the *post C_{18} SPE column effluent test(s)* must be compared to the results of the *filtration test* to determine if the manipulations effectively reduced toxicity. When the *post C_{18} SPE column test* is plagued by artifactual toxicity, the importance of the *methanol eluate test* increases. The results of the *post C_{18} SPE column test* must also be compared to the *baseline test* to determine if toxicity was removed by the C_{18} SPE column.

6.7 Methanol Eluate Test

General Approach: In order to elute toxicants from the C_{18} SPE sorbent, a relatively non-polar solvent is used. Hexane, one of the most non-polar solvents, can be used to remove highly non-polar compounds from the C_{18} SPE column. Yet hexane is one of the most toxic solvents to aquatic organisms and has a low miscibility with water. Methanol is more polar than hexane, but is much less toxic and will elute many

compounds. The use of methanol has been adopted as the eluant for the acute TIE (EPA, 1991A; EPA, 1989A) and the chronic TIE because of its low toxicity (Table 6-9) and its usually adequate ability to elute chemicals from the C_{18} SPE column.

Methods: The conditioning and elution steps are described in detail in the *post C_{18} SPE column test* above (see Section 6.6). For this test, we assume that the column extraction efficiency and elution efficiency are 100%.

If a 1 g (6 ml) SPE column was used with 1 L of 100% effluent, and a 3 ml methanol eluate was collected, the methanol eluate is a 333x concentrate of the original effluent (Table 6-7). Depending on the amount of effluent toxicity, this eluate may have to be concentrated further in order to test at a sufficient concentration (i.e., 4x) and have methanol concentrations in the test lower than the methanol effect concentration. In Table 6-9 the toxicity data for methanol toxicity to *C. dubia* and fathead minnows are given. The toxicity of methanol is slightly greater for *C. dubia* when the test solutions were renewed daily but not significantly for this characterization stage of the TIE. From these data, one can decide how much methanol can be added and how concentrated the eluant must be to achieve 2x or 4x the original effluent concentration. The choice of test concentration depends on the toxicity of the effluent; for example, if the effluent is toxic at ~25%, one may not need to achieve a 4x concentration. Some methanol toxicity can be present, as long as sufficient toxicity from the effluent is present to be measurable. As discussed in the *post C_{18} SPE column test*, the fathead minnows can be tested at 4x using only 0.96 ml of a 417x methanol eluate but the methanol concentration is about 1%, which cannot be tolerated by *C. dubia*.

Interpretation of Results/Subsequent Tests: If toxicity occurs in the *methanol eluate test* at any concentration tested, Phase II should be initiated. This step would include the use of a gradient of methanol/water eluant solutions to elute additional columns and conduct the toxicity tests on each fraction (Phase II; EPA 1989A; EPA, 1992A). Toxicants other than non-polar compounds may be retained by the SPE column but they are less likely to be eluted sharply or eluted at all (see Section 6.6). Non-polar toxicity can in some instances be distinguished from post-column artifactual toxicity if the eluate is checked for toxicity. Some toxicants (such as some surfactants) may not elute from the SPE column with methanol, but if toxicity is not recovered in the eluate, it does not exclude the possibility of a non-polar toxicant or metal (see Section 6.6 for additional discussion). Dilution water adjusted to pH 3 or pH 9 may be useful in eluting a toxicant(s) from the column. Some experimentation will be needed to determine the volumes of water to pump over the column. The pumping rate should be slowed considerably to allow sufficient contact time on the column (see details in Section 6.6 and Table 6-8).

At this time, we have not been successful in tracking chronic non-polar toxicity using the acute test endpoint with the methanol eluates, rather chronic tests have been needed to track the chronic toxicity.

A subsequent test that may be useful is to assess whether the toxicant must be metabolically-activated by the test organism before exhibiting toxicity. These activation reactions consist of oxidative metabolism by a family of enzymes collectively known as cytochrome P-450. Some toxicants require cytochrome P-450 activation before expressing toxicity. Piperonyl butoxide (PBO) is a synthetic methylenedioxyphenyl compound that effectively binds to, and blocks the catalytic activity of cytochrome P-450. When a non-toxic amount of PBO is added to an effluent test solution which contains a toxicant(s) that requires metabolic activation, the toxicity of the effluent can be reduced or completely blocked (EPA, 1991A). The relative specificity of PBO for blocking the toxicity of metabolically-activated organic compounds makes this test a useful part of the subsequent testing in the TIE. For example in the acute Phase I (EPA, 1991A) as a subsequent test, we suggest that PBO may be added directly to the effluent before adding the organisms. The 48 h LC50 of PBO is 1 mg/l for *C. dubia* and we have used 0.250 to 0.500 mg/l to effectively block the acute toxicity of metabolically-activated compounds for *C. dubia* in the effluent and the methanol eluate. The NOEC and the IC25 for PBO and *C. dubia* was determined as 63 µg/l and 89 µg/l, respectively. Low concentrations of PBO have reduced the chronic toxicity in the methanol eluate test and levels of 100 or 50 µg/l have been useful in chronic tests with *C. dubia*. The PBO should be added using a minimal amount of methanol as a carrier solvent since the level of methanol present in conjunction with the methanol eluate is present. Since PBO is not readily soluble in water, a superstock of 20 g/l is prepared by dissolving PBO in reagent grade methanol. An aliquot of the superstock is mixed in the standard laboratory dilution water to produce a stock solution at a concentration of 25 mg/l and aliquots of this stock solution are added to the test cups after addition of the methanol eluate, and the solution thoroughly mixed. This test should be conducted in similar fashion to the *EDTA addition test*. Appropriate *blanks* must be used, for example both the *methanol blank* and the *methanol eluate* must be tested with and without PBO. If toxicity occurs in the *methanol blank* fraction with the PBO additions, either PBO was present at toxic concentrations or the methanol concentration in the test was too high. If toxicity is observed in the *methanol eluate* with the PBO addition, but not in the *methanol eluate* without the PBO or either of the *blank eluates* (with PBO and without PBO), this result is not very informative. It is possible that the PBO has interacted in a synergistic fashion with another compound present in the test effluent that normally would not be toxic.

Compounds that are sparingly soluble in water may not be eluted from the column with methanol. If this

occurs, less polar solvents will have to be tried, but this technique will require solvent exchanges to avoid toxic solvent concentrations and other solvents may recover chemicals not toxic in the effluent due to solubility problems. At this time, we have not used solvent exchanges for chronic toxicity tests, but are exploring the use of methylene chloride. The 48 h LC50 of methylene chloride to *C. dubia* is 0.13% and the chronic toxicity to *C. dubia* is ≤0.03%. Therefore it cannot readily be used as the primary solvent, but rather as the exchange solvent and may be of limited use for this effort. Additional work on the appropriate solvent exchange for chronic TIEs is on-going (EPA, 1992A).

Special Considerations/Cautions: The *baseline test* serves as the *toxicity control*, and the *methanol blank* serves as a comparison of the effects of methanol alone in water. The health of the test animals, the viability of the dilution water and general test conditions are evaluated by the *baseline controls*. If the effluent *methanol eluate* is *non-toxic* at 2x or 4x but the *methanol blank* is toxic, the *blank* toxicity can be ignored since no non-polar toxicity is recovered.

If effluent dilutions are set at 100%, 80%, 60%, and 40%, it might be useful to test the eluate at a multiple of these concentrations, i.e., 2x, 1.6x, 1.2x, 0.8x or concentrate them to 4x, 3.2x, 2.4x, or 1.6x to compare the *baseline toxicity* with the toxicity in the *methanol eluate tests*. The artifactual growth observed in the *post C_{18} SPE column test* from the methanol has not occurred in our *methanol eluate tests*. This is most likely due to the differences in how the methanol degrades/behaves in dilution waters which are low in methanol-oxidizing bacteria and other organic matter in contrast to effluent samples (even post-column effluents).

6.8 Graduated pH Test

General Approach: This test will determine whether effluent toxicity can be attributed to compounds whose toxicity is pH dependent. The pH dependent compounds of concern are those with a pK_a that allows sufficient differences in dissociation to occur in a physiologically tolerable pH range (pH 6-9). The toxicity depends on the form that is toxic (ionized versus un-ionized). Metal toxicity can be affected by pH differences through changes in solubility and speciation. pH dependent toxicity is likely to be affected by temperature, DO and CO_2 concentrations, and total dissolved solids (TDS). The graduated pH test is most effective in differentiating substantial toxicity related to ammonia from other causes of toxicity.

Ammonia is an example of a chemical that exhibits different ionization states and subsequently pH dependent toxicity. Ammonia is also frequently present in effluents at concentrations of 5 mg/l to 200 mg/l (or higher). Measuring the total ammonia in the sample upon its arrival will be helpful to assess the potential for ammonia toxicity. pH has a great effect on ammonia toxicity. For many effluents (especially with municipal effluents) the pH of a sample rises upon contact with air, typically the pH of effluents at air equilibrium ranges from 8.0 to 8.5. Literature data on ammonia toxicity (EPA, 1985D) can be used only as a general guide because the pH values for most ammonia toxicity tests as reported in the literature are usually not measured or reported fully enough to be useful in TIE tests. Additional data on ammonia toxicity for *C. dubia* and *P. promelas* is provided in the revised Phase II (EPA, 1992A). The acute Phase I manual has a lengthy description of the toxicity behavior of ammonia (EPA, 1991A) and Phase II provides additional information (EPA, 1992A).

One might expect ammonia to be removed during the Tier 2 *aeration and pH adjustment test* at basic pH (described in Section 6.11). Based on our experience, however, ammonia is not substantially removed by the methods used to aerate the sample described in this manual. (If a larger surface to volume ratio is used, this manipulation can reduce ammonia levels; see *Interpretation of Results/Subsequent Tests* below and Phase II; EPA, 1992A.) Other techniques which can be used to remove ammonia may also displace metals or other toxicants with completely different physical and chemical characteristics. For example, ion exchange resins (e.g., zeolite) remove ammonia, cationic metals, and possibly organic compounds through adsorption.

Toxicity related to metals may also be detected by the *graduated pH test*, although these effects are less well documented in effluents (and for chronic toxicity) than those associated with ammonia toxicity. The toxicity may change for both pH increases and decreases from neutral pH (pH 7). Such behavior is characteristic of aluminum and cadmium. Acute toxicity test experiments with *C. dubia* in clean dilution waters indicate lead and copper were more acutely toxic at pH 6.5 than at pH 8.0 or 8.5 (in very hard reconstituted water), while nickel and zinc were more toxic at pH 8.5 than at 6.5 (EPA, 1991A). In recent experiments during a chronic TIE, we have found that chromium is pH dependent on an acute basis for *C. dubia*, but not water hardness dependent. The pH dependence was not observed in acute tests unless food (YCT) (EPA, 1992C) was added during the 48 h acute test at test initiation. Therefore, caution must be exercised in interpreting the chronic toxicity results with effluents, because the toxicant(s) may behave in certain ways that are not documented in the literature.

By conducting tests at different pHs, the effluent toxicity may be enhanced, reduced or eliminated. For example (at 25°C) where ammonia is the primary toxicant, when the pH is 6.5, 0.180% of the total ammonia in solution is present in the toxic form (NH_3). At pH 7.5, 1.77% of the total ammonia is present as NH_3 and at pH 8.5, 15.2% is present as NH_3. This difference in the percentages of un-ionized ammonia is enough to make the same amount of total ammonia about three times more toxic at pH 8.5 as at pH 6.5. Whether or not toxicity will be eliminated at pH 6.5 and the extent to which toxicity will increase at pH 8.5 will depend on the total ammonia concentration. If the graduated pH test is done at two pHs using the same dilutions, one

should see toxicity differences between pH 6.5 and 8.5. The effluent effect level (expressed as percent effluent) should be lower at pH 8.5 than pH 6.5 if ammonia is the dominant toxicant.

The most desirable pH values to choose to test for the *graduated pH test* will depend upon the characteristics of the effluent being tested. The graduation scheme that includes the air equilibrium (the pH the effluent naturally drifts to) will allow a comparison of treatments to unaltered effluent (i.e., *baseline test*). For example, if the air equilibrium pH of the effluent is pH 8.0, it may be more appropriate to use pHs 6.5, 7.3 and 8.0. The pHs of many municipal effluents rise to 8.2 to 8.5 (or higher), so pHs such as 6.5, 7.5 and 8.5 may be more appropriate. In any case, it will be necessary to conduct the test at more than one effluent concentration (e.g., 100%, 50%, 25%) to determine what role, if any, the pH dependent compounds play in toxicity.

The challenge of the graduated pH test is to maintain a constant pH in the test solution. This is a necessity if the ratio of ionized to the un-ionized form of a pH sensitive toxicant is to remain constant and the test results are to be valid. However, in conducting either acute or chronic toxicity tests on effluents, it is not unusual to see the pH of the test solutions change 1 to 2 pH units over a 24-h period.

Methods: To lower the pH of the samples, either CO_2/air mixtures or HCl additions (or the combination of both) are used. The pH should be maintained throughout the 4-d or 7-d test with little variation (±0.2 pH units).

When CO_2/air (without any acid addition) is used to control the pH, the pH of the effluent samples is adjusted by varying the CO_2/air content of the gas phase over the water or effluent samples. By using closed headspace test chambers, the CO_2 content of the gas phase can be controlled. The amount of CO_2/air needed to adjust the pH of the solution is dependent upon sample volume, the test container volume, the desired pH, the temperature, and the effluent characteristics (e.g., dissolved solids). The exact amount of CO_2/air to inject for a desired pH must be determined through experimentation (on day 1) with each effluent sample *before* the *graduated pH test* begins. Therefore, the test may have to be set up later than the other Phase I tests (e.g., day 3) unless experimentation was initiated on day 1. The amount of CO_2 added to the chamber assumes that the liquid volume to gas volume ratio remains the same. Generally, as the alkalinity increases, the concentration of CO_2 that is needed to maintain the pH also increases. For adjusting pH's downward from pH 8.5 to 6, 0.5-5% CO_2 has been used. If more than 5% CO_2 is needed, adjust the solutions with acids (HCl) and then flush the headspace with no more than 5% CO_2/air. With appropriate volumes of effluent, experiments with variable amounts of CO_2/air and equilibrated for about 2 h, are used to select the needed CO_2 concentration. More than 5% CO_2 is not recommended as CO_2 toxicity is likely to be observed. When dilutions of an effluent have the same hardness (or alkalinity) and initial pH as the effluent, the same amount of CO_2 is usually needed for each dilution, but sometimes different amounts are needed in the higher effluent concentrations. Use of a dilution water of similar hardness (or alkalinity) as the effluent makes the CO_2 volume adjustments easier. When tests are conducted in these CO_2 controlled environments, dilution water *controls* for each pH should be included.

Acid is used first to adjust pH's when the amount of CO_2/air needed to adjust to the desired pH is greater than 5% CO_2/air. Again experimentation is needed to determine how much CO_2/air is needed. Techniques for acid adjustment are described in Section 6.10 below and also in the acute Phase I manual (EPA, 1991A).

For adding a mixture of CO_2/air to the headspace of the test compartments, a 1 L gas syringe (Hamilton Model S-1000, Reno, NV) is used. In most instances, the amount of CO_2 produced by the invertebrates has not caused further pH shifts, but with larval fathead minnows, the pH may drop from the additional amount of CO_2 respired by the fish bacterial metabolic CO_2 released.

For the pH controlled tests, the pH should be measured at least two to three times for each 24 h period when readings of survival and/or young production are made. If samples are not renewed daily (as may be the case for the *C. dubia* tests), then the headspace should be re-flushed with CO_2/air after the animals are fed. Again, some experimentation may be needed to determine the amount of CO_2/air needed for this step. In all *graduated pH tests*, the pH should be measured in all the chambers. If the pH drifts as much as 0.2 pH units, the results may not be usable and better pH control must be achieved. However, if pH fluctuates more than 0.2 pH units and toxicity is gone at one pH and not another, the toxicity results may be useful (see *Interpretation of Results/Subsequent Tests* below).

Measurements of pH must be made rapidly to minimize the CO_2 exchange between the sample and the atmosphere. Avoid vigorous stirring of unsealed samples because at lower pH values, the CO_2 loss during the measurement can cause a substantial pH rise. In addition, measure the DO because toxicants such as ammonia have different toxicities when DO is decreased (EPA, 1985D). Keep in mind that if the test animals have been dead for awhile, the pH and/or DO of the test water most likely will have changed. Therefore, pH measurements should be made as soon as possible if animals die rapidly.

Methods that use continuous flow of a CO_2/air mixture, such as tissue cell incubators, may be preferable and give better pH control. A pH feedback system can be used to control the CO_2-mix to the incubators. At this time we have not attempted to use a continuous flow of CO_2 and cannot recommend a system to use.

Maintaining pH above the air equilibrium pH (generally above pH 8.3) is difficult to achieve because the

concentration of CO_2 must be very low, and microbial respiration can increase the CO_2 levels in the test chamber. Frequently we use a dilution water that has a higher pH (i.e., very hard reconstituted water) to prevent pH drift downward.

Interpretation of Results/Subsequent Tests: For the *graduated pH test*, the pHs selected must be within the physiological tolerance range for the test species used (which generally is a pH range of 6 to 9). In this pH range, the amount of acid or base added is negligible, and therefore the likelihood of toxicity due to increased salinity levels is low.

When ammonia is the dominant toxicant, the toxicity at pH 6.5, should be less than in the pH 8 test. However, ammonia is not the only possible cause of toxicity. Using the pH of the *baseline test*, the relative toxicity of each pH adjusted solution can be predicted if ammonia is the sole cause of toxicity (EPA, 1989A; EPA, 1992A).

However, if ammonia is only one of several toxicants in an effluent, this procedure will be hard to interpret. For this reason, if total ammonia concentrations in the 100% effluent are greater than 20 mg/l, include a pH 6 (rather than 6.5) and pH 7.3 (±0.2) effluent treatment interfaced with other Phase I tests. Complicating effects of metal toxicity may be reduced by adding EDTA to the test solutions. However, the ability of EDTA to detoxify metals may also change with pH, although we have not experienced this effect yet.

Other metals may exhibit some degree of pH dependence, but these are not as well defined. Whether the metal toxicity can be discerned will depend in large part on the concentration of other toxicants in the sample. In order to detect metal toxicity, one must be cautious when selecting a dilution water if the test solutions are low effluent concentrations. Artifactual toxicity due to metals may be created if the hardness of the dilution water is much different from that of the effluent (see Section 3). This effect may be magnified for metals when coupled with the pH change. A dilution water similar in hardness to that of the effluent must be used for this test to reveal metal-caused toxicity. If more than one pH dependent toxicant is present, the pH effects may either cancel or enhance one another.

In the acute TIEs, we have suggested the use of hydrogen ion buffers to maintain the pH of effluent test solutions and to compare these test results to those from CO_2 adjusted samples. Three hydrogen ion buffers were used by Neilson et al. (1990) to control pH in toxicity tests in concentrations ranging from 2.5 to 4.0 mM. These buffers were chosen based on the work done by Ferguson et al. (1980). These buffers are: 2-(N-morpholino) ethane-sulfonic acid (Mes) (pK_a = 6.15), 3-(N-morpholino) propane-sulfonic acid (Mops) (pK_a=7.15), and piperazine-N,N'-bis (2-hydroxypropane) sulfonic acid (Popso) (pK_a=7.8). We have replaced the Popso buffer with another buffer which is more readily soluble in order to achieve better pH control around the pH 8.0 range. This buffer is N-tris(hydroxymethyl) methyl-3-amino propanesulfonic acid (Taps) (pK_a = 8.4) and has been used primarily for the chronic *C. dubia* tests at this time.

The acute toxicity of these Mes, Mops, and Popso buffers is low to both *C. dubia* and fathead minnows (Phase I; EPA, 1991A) (48-h and 96-h LC50s for all buffers are ≤25 mM for both species). Sublethal levels of the buffer are added to hold the pH of test solutions for the acute Phase I tests (see EPA, 1991A). Chronic toxicity results using these three buffers indicated that 16 mM did not cause reduced survival or growth for the fathead minnow 7-d test. For *C. dubia*, 4mM of all four buffers has not caused reduced survival or reproduction in either the 4-d or 7-d tests. Use of the buffers is preliminary and the effects due to interferences from the buffers themselves have not been studied. It is possible that the buffers may reduce the toxicity of some toxicants.

The buffers must be weighed and then added to aliquots of the effluent dilutions and control water as batches. Then adjust to desired pH with acid and base to the selected values and add the test organisms. Solutions should be left for several hours to equilibrate, especially for the Popso buffer which has low solubility in water (in contrast to other buffers). While our experience with the buffers is limited, we have found the amount of any buffer needed to hold a pH is effluent specific. Once the pH is adjusted to the desired pH, the test solutions need not be covered tightly to maintain pH; however pH should be measured at each survival reading at all dilutions. The test results with the buffers should mimic those of the earlier *graduated pH test* if ammonia is the suspect toxicant.

The methods described in Phase II can be used to add identify ammonia as the pH sensitive toxicant. Use of the air-stripping method to remove ammonia from the sample at high pH's should help evaluate whether toxicant(s) other than ammonia are present (Phase II, 1992A). The results of this air-stripping test should be compared with the *aeration test* results of Phase I, the *baseline effluent test* and the *graduated pH test*. If the ammonia concentration is decreased and the toxicity is reduced or absent, more evidence that ammonia is playing a role in the toxicity of the effluent has been generated. Other compounds could precipitate with the pH adjustment and concentration during air-stripping and when water is added back into the solution, they may not be available.

Special Considerations/Cautions: The *controls* in the CO_2 controlled chambers for each pH and the *baseline test* act as checks on the general health of the test organisms, the dilution water and most test conditions. If the effluent pH in the *baseline test* is close to that of the pH adjusted test solutions, the toxicity expressed in the two tests should be similar. Significantly greater toxicity may suggest interference from other factors such as the ionic strength related toxicity (if the

pH was adjusted with HCl) or CO_2 toxicity. Dilution water tested at the various pH's does not serve as *blanks*, as the effluent matrix may differ from that of the dilution water. However, if acids and bases are added (with or without CO_2 additions) then *toxicity blanks* with the same amounts of acid/base added need to be tested to determine the cleanliness and effects of the acids and bases. Other compounds with toxicities that increase directly with pH may lead to confounding results or may give results similar to ammonia. Monitoring the conductivity of the effluent solutions after the addition of the acids and bases may also be helpful in determining artifactual toxicity.

6.9 Tier 2 Characterization Tests

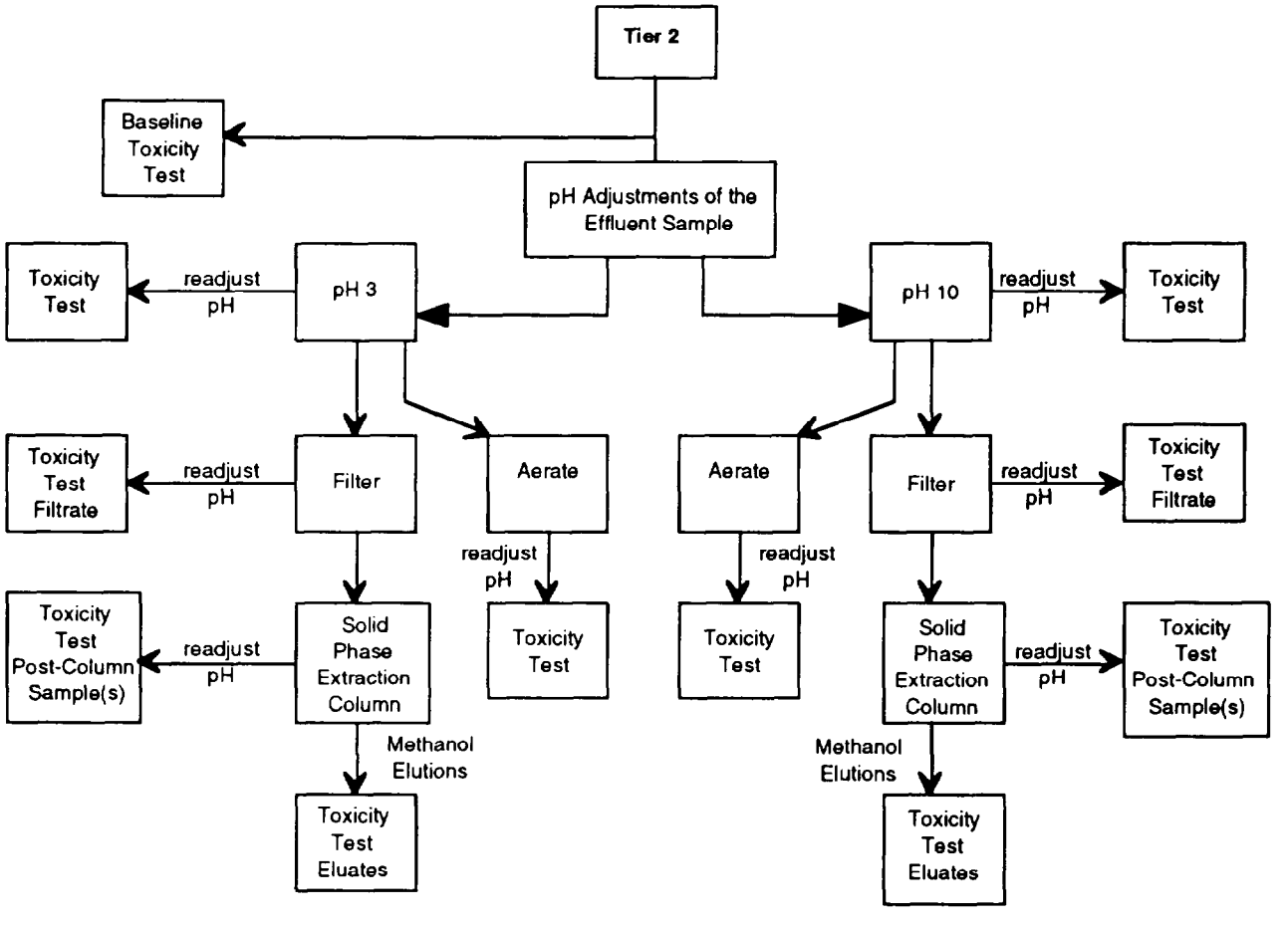
Two tiers are used in the chronic TIE approach primarily because in our experience, radical pH adjustment often is not needed. Only when the manipulations in Tier 1 do not indicate clear patterns is Tier 2 conducted. Tier 1 manipulations do not involve the use of drastic pH manipulations to characterize the toxicity of the sample. The pH adjustments are used to affect toxicity when the Tier 1 tests are not adequate or to assist in providing more information on the nature of the toxicants (Figure 6-3).

Changes in pH can affect the solubility, polarity, volatility, stability, and speciation of a compound. These can change the bioavailability of the compounds, and also their toxicity. The Phase I acute manual (EPA, 1991A; EPA, 1988A) discusses the effect of pH on groups of compounds at length, therefore only an abbreviated discussion of pH effects will be covered in this document.

Un-ionized forms of chemicals are generally less polar than the ionized form, and the ionized forms interact with water molecules to a greater extent. Compounds may be more toxic in the un-ionized form, as was discussed above in Section 6.8 *graduated pH test*. Un-ionized forms may be easily stripped from water using aeration, or extracted with SPE techniques and subsequent elution with non-polar solvents. Also, changes in solubility with pH change may cause compounds to be removed by filtration. The form of metals can be altered by pH and organic compounds can be degraded at extreme pH values.

Even if the chemical species are unchanged, changes in the pH of the solution may affect the toxicity of a given compound. The cell membrane permeability and the chemistry of the toxicant may be affected.

Figure 6-3. Tier 2 sample preparation and testing overview.



Changing pH and returning it to pH *i* after a short time (~1 h) will not always change the toxicity. However, this adjustment may result in a reduction, loss or increase in the toxicity. Sometimes only the pH adjustment in combination with a manipulation (e.g., filtering, solid phase extraction) changes toxicity when the same pH unadjusted manipulation test did not.

6.10 pH Adjustment Test

General Approach: For this Tier 2 test, the effluent is adjusted to either pH 3 or pH 10, and left at those pHs until other manipulations (aeration, filtration, and C_{18} SPE post-column effluent samples) are ready to be readjusted to pH *i*. The pH adjustment alone may not change toxicity, if equilibrium is slow. Satisfactory *blanks* in chronic tests with various reconstituted waters adjusted to pH 11 have not been consistently produced, but acceptable *blanks* have been obtained at pH 10 (and pH 3), while pH 11 adjustments have not been problematic in some effluent matrices. Since pH 11 was subjectively chosen, we recommend adjustment to pH 10 for chronic TIE's. The *pH adjustment test* serves as a *toxicity control* for the pH adjustments combined with aeration, filtration and the C_{18} SPE column manipulation. As described in Tier 1 and the acute Phase I manual, pH may drift very differently during the toxicity tests following these more severe pH manipulations. Therefore, monitoring and control of test pH is necessary.

Methods: An aliquot of effluent is pH adjusted to pH 3 and another aliquot is adjusted to pH 10, along with dilution water samples which will serve as *blanks*. Enough sample and dilution water are pH adjusted to provide the necessary volumes for the *aeration and pH adjustment test*, the *filtration and pH adjustment test*, and the *post C_{18} SPE column and pH adjustment test*. Minimal dilution of the effluent should occur, and the use of 0.01 N, 0.1 N, and/or 1.0 N solutions of acids/bases (Suprapur®, E. Merck, Darmstadt, Germany) to adjust pH are suggested. The volumes and strengths of the acid/base additions should be recorded as this information may be useful in determining if artifactual toxicity should be expected. This information can be helpful when subsequent testing is conducted and knowledge of the volumes of acid/base added to the previous samples assists in making the pH adjustments more rapidly.

Interpretation of Results/Subsequent Tests: A decrease in toxicity compared to the *baseline test* should be pursued to detect the mechanism of toxicity reduction. Often precipitation occurs after drastic pH change. If precipitation does occur, then the *filtration and pH adjustment test* will likely remove the toxicant and efforts should be focused on recovery and identification from the filter. Similarly, if the C_{18} SPE column or aeration changes toxicity, these manipulations should be pursued. If toxicity is only reduced by pH change, (which is not common) not much can be made of the information, and clustering of several manipulations as well as adding additional techniques such as ion exchange should be explored. Dilution from the acid and base additions should also be checked. Degradation of toxicity is a possibility also, but is nearly impossible to detect at this stage.

The adjustment of pH (to pH 3 or pH 10 and back to pH *i*) may cause toxicity problems. Just the addition of the NaOH or HCl may be the cause of the toxicity and may also occur in the dilution water *blanks* or only in the effluent sample. The effect on effluent toxicity of the Na^+ and Cl^- additions, depends on the TDS concentration of the effluent. The acid/base additions are typically more toxic in dilution water than in effluent, unless the effluent TDS concentration is high, and the additional concentrations of acid/base result in toxic TDS concentrations. These effects are of more concern in chronic TIE's. The effect of NaCl additions on TDS can be tracked by measuring conductivity. Appreciable increases in conductivity should be a warning to evaluate TDS toxicity caused by acid and base addition.

Increases in toxicity compared to the *baseline test* may be a result of either an increase in TDS or toxicant changes. TDS as a toxicant may be eliminated by calculating the TDS at the ICp value. Effluents that have high toxicity require high dilution to determine the ICp, and at such great dilution the TDS is subsequently diluted sufficiently to remove TDS as a candidate. If this is not the case, NaCl can be added to an aliquot of effluent to see if the acid/base additions could have caused the increased toxicity. Table 6-10 provides chronic toxicity information for NaCl in various hardness waters for *C. dubia* and fathead minnows.

Precipitates can remove toxicity through sorption of such chemicals as non-polar organics. In this case the precipitate is only the mechanism of removal, not the toxicant itself. The C_{18} SPE column is likely to remove the toxicity in such cases; however, in Tier 2 a pH change can also desorb toxicants from particles and make them bioavailable and therefore toxic.

Different pH drift during the *baseline toxicity test* and those after manipulations has been discussed (EPA, 1991A). For a valid test, the pH during the test must be known and maintained the same as in the pH *i* test. If the drift of the pH varies considerably, confusion in interpreting the results can arise if a compound whose toxicity is pH dependent is present in the sample. If good pH control is not maintained incorrect conclusions are likely to be made and mislead the TIE process.

Special Considerations/Cautions: The addition of acids and bases to the effluent does not give comparable results of acids and bases added to the dilution water. The amount of acid and base added to each sample will more than likely be dissimilar. However, dilution water toxicity *blanks* to assess the additions of the acid and base are needed to determine whether toxic concentrations of ions have been reached and to determine the cleanliness of the acid and base solutions that are used in this manipulation and subsequent pH manipulation tests. The *controls* from the *baseline test* provide information on the health of the test organ-

Table 6-10. Chronic toxicity of sodium chloride (g/l) to *C. dubia* and *P. promelas* in various hardness waters using the 7-d tests.

Species	Water Type	IC50 95% C.I.	IC25 95% C.I.	NOEC	LOEC
C. dubia	SRW	1.3 1.2-1.5	0.93 0.76-0.96	0.63	1.3
	MHRW	1.6 1.4-1.7	1.3 0.24-1.3	1.0	2.0
	HRW	1.5 1.3-1.6	1.2 1.0-1.3	1.0	2.0
	VHRW	1.4 1.1-1.6	1.0 0.58-1.2	1.0	2.0
P. promelas	SRW	0.84 0.76-1.1	0.67 0.63-0.77	0.50	1.0
	SRW	1.3 1.2-1.5	0.93 0.76-0.96	0.63	1.3
	MHRW	1.5 1.4-1.6	1.2 1.1-1.2	1.0	2.0
	HRW	3.2 2.9-3.3	2.3 2.0-2.5	2.0	4.0
	VHRW	4.5 3.9-4.9	3.2 2.4-3.5	2.0	4.0

Note: C.I. = confidence interval; SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water. laboratory test conditions. The *pH adjustment test* serves as the *toxicity control* (or perhaps the "*worst case*" *toxicity control*) for the subsequent pH adjustment/characterization tests.

isms, dilution water, and laboratory test conditions. The pH adjustment test serves as the toxicity control (or perhaps the "worst case" toxicity control) for the subsequent pH adjustment/characterization tests.

6.11 Aeration and pH Adjustment Test

General Approach: Aeration at pH 3 or pH 10 may make toxicants oxidizable, spargeable or sublatable, that are not so at pH *i*. If this does occur, avenues are then available to characterize and identify, similar to the procedures described for aeration at pH *i* in Tier 1. For this test, two effluent aliquots which were adjusted to pH 3 and pH 10 in the *pH adjustment test* are each aerated for a period of time, for example, 1 h. The aeration process can concentrate compounds due to loss of volume, and caution should be exercised in this aeration process and lost water may need to be replaced with dilution water.

Methods: The steps for this procedure should be identical to those used in the non-pH adjusted sample aeration (Section 6.4). The pH of the effluent may drift during the aeration, and it should be checked at 30 min intervals and readjusted to the original pH (pH 3 or 10) if it has drifted more than 1 pH unit. The amount of NaCl added from the acid/base additions may be different in aerated samples than for *pH adjustment test* and proper compensation for this difference must be made as described above. The volume of effluent aerated should be compared to the amount of original sample volume prepared.

After aeration is completed, adjustments back to pH *i* should be made on all samples at the same time. The formation of any precipitates should be noted, but the importance of precipitates (if any) will not be known at this point in the characterization.

Interpretation of Results/Subsequent Tests: If aeration with either pH adjustment removes or reduces the toxicity, additional tests must be performed to identify whether sparging, sublation, or oxidation removed the toxicity, as described in Tier 1 (Section 6.4). If toxicity is reduced because of precipitation, the results for this test and the *filtration and pH adjustment test* should be similar, but if oxidation is a problem, pH adjustment and filtration will not affect the toxicity of the effluent. At pH 10 the total ammonia levels can be reduced by aeration. However, the geometry of the aeration technique (i.e., small surface area) for this *pH adjustment and aeration test* described here is not particularly conducive to ammonia removal. However, if aeration at pH (10) reduces toxicity compared to the toxicity in the *aeration test* at pH *i* and the *baseline test*, measure the total ammonia level in the sample to determine if it was stripped from the effluent.

Special Considerations/Cautions: The results of this test should be compared to the *toxicity control* (*pH*

adjustment test) and the *baseline test*. The *aeration and pH adjustment blank* should be compared to the pH adjustment *blank*. If the effluent toxicity is reduced in the effluent following pH adjustment/aeration, and the *blank* is toxic, the *blank* can be ignored and the results indicate toxicity removal. However, if toxicity is the same or greater, artifactual toxicity cannot be ruled out and further tests must be conducted. Compare the results of the *aeration and pH adjustment blank* to the *filtration and pH adjustment blank* and the *pH adjustment blank* (Sections 6.10 and 6.12). If all have toxicity, then artifactual toxicity occurred from the pH adjustment, while if only the *aeration and pH adjustment blank* has toxicity, then the artifactual toxicity crept in during the aeration manipulation and the test should be repeated.

6.12 Filtration and pH Adjustment Test

General Approach: Since a pH change can cause toxicants to precipitate or cause solubilized toxicants to sorb on particles, filtration at altered pH values can be used as a tool in characterizing the effluent. Therefore, by filtering pH adjusted effluent, compounds that were in solution without a pH adjustment may no longer be in solution or any toxicants associated with particles may be removed by the filtration process. Differences in the toxicity caused by filtering (at pH *i*) compared to the *pH adjustment test* (Section 6.10) may imply toxicity associated with suspended solids. If pH affects the filterability of the toxicants, solubility changes are implied at those pH values. Once the toxicants are filtered, the particles may be recoverable from the filter if toxicity has not degraded.

Methods: Details of preparing filters are generally the same as described in Tier 1 (Section 6.5), except the high purity water used to rinse the filters must be pH adjusted to the appropriate pH, as should the dilution water for the *blank*.

Effluent samples adjusted to pH 3 or pH 10 (Section 6.10) are filtered, readjusted to pH *i*, and the filtrate toxicity tested. Stainless steel filter housings are not to be used for this step, because stainless steel will frequently bleed metals when a pH 3 solution being filtered is in contact with the stainless steel. An inert plastic or properly cleaned glass housing should be used.

Interpretation of Results/Subsequent Tests: The results of the *filtration and pH adjustment test* are compared to the *toxicity controls*—the *baseline test* and the *pH adjustment test*. If the effluent is more toxic after filtration and contamination is not the cause, the breaking of an emulsion might be involved. If the toxicity is removed or reduced by the filtration step and dilution is not the cause, then toxicants have been separated from the whole effluent and efforts should focus on identifying the compounds filtered out. The next step is to recover the toxicity as described in Tier 1 *filtration test*. This may be accomplished using a pH adjusted sample of water, perhaps using the pH opposite of that used in the filtration process.

Special Considerations/Cautions: The pH adjusted and filtered dilution water serves as a *blank* and the pH adjusted and filtered effluent sample serves as a *toxicity control* for the solid phase extraction step (Section 6.13). The results of the *filtration and pH adjustment test* should be compared to the effluent *pH adjustment test* and the *baseline test*. The *filtration blank* should be compared to the *baseline control*, the *aeration blank*, and *pH adjustment blank*. Toxicity in the *filtration blank* implies toxic artifacts from the filtration process, the glassware, the pH adjustment or a dilution water problem. If the *baseline control* performance is acceptable, the *blank* toxicity was most likely created during the pH adjustment or filtration. If the *aeration and pH adjustment blank* is non-toxic, and if the *filtration blank* is toxic, and the filtered effluent sample is still toxic or more toxic, artifactual toxicity cannot be ruled out. To check if it occurred during the manipulation, the experiment must be repeated. If the *filtration blank* is toxic, yet the filtered pH adjusted effluent indicates that toxicity is reduced/eliminated, the toxicity in the *blank* can be ignored.

6.13 Post C_{18} Solid Phase Extraction (SPE) Column and pH Adjustment Test (pH 3 and pH 9)

General Approach: Shifting the ionization equilibria at high and low pHs may cause the C_{18} SPE column to extract different compounds than at pH *i*. pH adjusted and filtered effluent is passed over a prepared C_{18} SPE column to remove non-polar organic compounds (cf., *post C_{18} SPE column test*, Section 6.6 above). Organic acids and bases may be made less polar by shifting their equilibrium to the un-ionized species. By adjusting the effluent samples to a low pH and a high pH, some compounds that are in the un-ionized form should sorb onto the column. However, the C_{18} packing degrades at high pH, so pH 9 (rather than pH 10 or pH 11) is used in this manipulation. Specific manufacturer's data should be checked for acceptable pH range. We have had no experience in eluting toxicants off the C_{18} SPE column that would be sorbed only at an altered pH, and therefore we can only provide general rules to follow in these cases except those inferred from how ionizable compounds behave in regard to pH change.

Methods: All of the procedures for this manipulation and the use of the C_{18} SPE column are the same as is described in Tier 1 for the SPE extraction at pH *i* (Section 6.6) with one exception. All water passed through the column (rinse, *blank* and effluent) should be acidified or rendered basic depending on which pH is under investigation (see Section 6.12). The potential for bacterial growth and artifactual toxicity in the post-column samples remain the same as for pH *i*.

Interpretation of Results/Subsequent Tests: The extraction efficiency of the column is assessed by comparing the results of the *post C_{18} SPE column and pH adjustment test* (pH 3 and pH 10) to the *filtration and pH adjustment test*, and the *pH adjustment test*. Again post-column test results are the most interpretable when there is no artifactual toxicity and toxicity was removed.

When the toxicity is removed, compare the results of the test with the *methanol eluate test* below (Section 6.14). If toxicity is removed that was not removed under pH *i* and recovered in the methanol eluate, efforts to identify the toxicants should be started. If methanol does not recover toxicity, a pH adjusted water should be tried. For further discussions of the interpretation of the results, see Section 6.6 above.

Special Considerations/Cautions: Careful observations and judgement must be exercised in detecting problems in the *post C_{18} SPE column and pH adjustment test*. Low DO levels can occur in these samples (cf., Section 6.6). Through testing experience, the investigator will know whether toxicity appears as artifactual (i.e., growth, low DO) or as lack of toxicity removal. If artifactual toxicity is not recognized, then an erroneous conclusion that the C_{18} SPE column did not remove toxicity can be made.

General test conditions (dilution water, health of test animals) will be tracked by the *controls* in the *baseline test*. The post-column dilution water *blanks* should be compared to those *controls* to determine if the column imparted toxicity. If the post-column dilution water *blank* was toxic, but no toxicity or artifactual toxicity occurred in the post-column effluent sample the toxic *blank* can be ignored.

Results of the *post-column effluent test(s)* must be compared to the results of the *filtration and pH adjustment test* to determine if the manipulations effectively reduced toxicity. When the *post C_{18} SPE column test* data is plagued by artifactual toxicity, the importance of the *methanol eluate test* increases.

6.14 Methanol Eluate Test for pH Adjusted Samples

General Approach: This test is essentially the same as the *methanol eluate test* in Section 6.7, except that the columns were prepared with pH adjusted waters/effluents (see Section 6.13).

Methods: These are identical to those in Section 6.7, except the pH of the rinse water, blank and effluent sample has to be adjusted to pH 3 or pH 9 (lowered from pH 10).

Interpretation of Results/Subsequent Tests: If the toxicity is recovered in the eluate, identification should be initiated. Refer to Sections 6.6, 6.7, and 6.13 for more information.

Special Considerations/Cautions: The *baseline test* serves as the *toxicity control*, and the *methanol blank* (for *pH adjusted samples*) serves as the *toxicity control* for the effects of methanol in water. The health of the test animals, the viability of the dilution water and general test conditions are evaluated by the *controls*.

The artifactual growth observed in the *post C_{18} SPE column test* (with and without pH adjustments) from the methanol has not occurred in *methanol eluate tests*. This is most likely due to the differences in how the methanol degrades/behaves in dilution waters which are low in bacteria and other organic matter in contrast to effluent samples (even post-column effluents).

6.15 Toxicity Characterization Summary

Phase I will not usually provide information on the specific toxicants. If effluent toxicity is consistently reduced, for example, through the use of the C_{18} SPE column, this does not prove the existence of a single toxicant because several non-polar organic compounds may be causing the toxicity in the effluent over time, but use of the C_{18} SPE technique in Phase I detects the presence of these compounds as a group. This lack of specificity is very important to understand for subsequent Phase II toxicant identification. Efforts should concentrate on those manipulations affecting toxicity in which the toxicant is isolated from other effluent constituents, such as the SPE column, filtration and aeration.

After the Tier 1 group of Phase I tests has been completed, the results will usually show that some manipulations increased toxicity, some decreased it, and others effected no change. In some instances, Tier 1 results allow the researcher to proceed immediately into the Phase II identification, and sometimes Phase I (Tier 1 and/or Tier 2) and Phase II combinations are needed to determine the cause of toxicity (cf., EPA, 1992A). Of course, new approaches are frequently devised as more Phase I TIEs are completed.

Toxicity may be changed by two or more tests, and if so, then more conclusive inferences might be possible than when only one manipulation changed the toxicity.

If all of the toxicity is not removed, it is possible that other toxicants could be present in the effluent so that only partial removal was obtained. Frequently more than one manipulation affects toxicity but only infrequently is there no effect from any manipulation. Even if toxicity is affected by only one manipulation, one still does not know whether or not there are multiple toxicants. When several manipulations affect toxicity, it still does not ensure that there are multiple toxicants. There is also no way to tell at this stage if there are multiple toxicants, whether or not they are additive, partially additive or independent. In our experience with acutely toxic effluents, we have not found synergism, but independent action has commonly been found. Some toxicants identified in effluents have been additive, but more often these have been only partially additive.

The two objectives which usually move the TIE along more rapidly are to separate and concentrate the toxicant(s). Therefore, the first step in Phase II (EPA, 1989A) will often be to reduce the number of constituents accompanying the toxicants. These efforts may reveal more toxicants than are suggested by Phase I testing. In Phase II one may discover that toxicants of quite a different nature are also present but were not in evidence in Phase I and if this is the case, different Phase I characterizations may then be needed. Once the analytical methods to identify one or more of the toxicants is found, efforts to confirm the cause should be initiated immediately (EPA, 1989B; EPA, 1992B).

As discussed earlier, the amount of time necessary to adequately characterize the physical/chemical nature and variability of the toxicity will be discharge specific. For a given discharge, the factors that will affect the length of time it takes to move through Phase I is the appropriateness of Phase I tests to the toxicants, the existence of long- or short-term periodicity in individual toxicants and the variability in the magnitude of toxicity. An effluent which consistently contains toxic levels of a single compound that can be neutralized by more than one characterization test should be identified and moved into Phase II more quickly than an ephemerally toxic effluent with highly variable constituents, few of which or none of which are impacted by any of the Phase I tests. Several samples should be subjected to the Phase I characterization tests but not all manipulations have to be done on all subsequent samples. The decision to do subsequent tests on these samples to confirm or further delineate initial results is a judgement call and will depend on whether or not the results of Phase I are clear-cut. Sometimes it may be reasonable to start Phase II and Phase III on the first sample.

If the Phase I characterization tests that remove or neutralize effluent toxicity vary by the sample, the number of tested samples must be increased and the frequency of testing should be sufficient to include all major variability. The differences seen among samples can be used to decide when further differences are not being found. Phase I characterization testing should continue until there is reasonable certainty that new types of toxicants are not appearing. No guidance can be given as to how long this may take—each problem for every discharger is unique. While the toxicity of samples can be very different, the same characterization tests must be successful in removing and/or neutralizing effluent toxicity.

Often the next step of the TIE is obvious; at other times the outcome of Phase I will be confusing and the next step will not be obvious. In our experience with acutely and chronically toxic effluents, once one toxicant is identified, identification of subsequent toxicants becomes easier because: (a) the toxicity contribution of the identified toxicant can be established for each sample; (b) the number of Phase I manipulations that will affect the toxicity of the known toxicant can be determined; (c) one can determine whether the identified and the unidentified toxicant(s) are additive; (d) if some manipulations affect the toxicity due only to the unidentified toxicants, some of their characteristics can be inferred; and (e) one can determine if the relative toxicity contributions of identified and unidentified toxicants varies by sample. Such information can be used to design tests to elucidate additional physical/chemical characteristics of the toxicants that cause chronic toxicity.

6.16 Use of Multiple Characterization Tests

Type and amount of testing is dependent on the toxicity persistence in the effluent, the nature of the toxicity, and reassessment of previous Phase I results (observed trends in the characteristics can be very important). Several tests could each partially remove the effluent toxicity because several compounds are causing the toxicity, or that one toxicant can be removed by several Phase I steps. For example, if several toxicants are acting to cause the toxicity, then the *graduated pH test* and the *post C_{18} SPE column test* both might result in a partial toxicity reduction. If sodium thiosulfate and EDTA both reduce toxicity, cationic metals might be suspect.

In the acute Phase I (EPA, 1991A), the use of multiple manipulations (combining two of the Phase I tests) was advocated and this same concept is also useful for the chronic TIE as well. For effluents with multiple toxicants, especially if they are not additive, multiple manipulations are helpful. Especially when no single manipulation removes all the toxicity, multiple manipulations should be tried.

When the C_{18} SPE column only partially removes toxicity, Phase I manipulations with the post-column sample should be tried. For this multiple manipulation, the post C_{18} SPE column effluent can be treated as whole effluent, and several of the Phase I steps can be conducted on the post-column effluent such as the *EDTA addition test*, the *thiosulfate addition test*, and the *graduated pH test*. However, these combinations are useful only with the post-column effluent provided that no artifactual toxicity is present.

If the C_{18} SPE column partially removes toxicity, pass an aliquot of the post-column effluent over an ion exchange column to determine the characteristics of the remaining toxicity. If a non-polar toxicant and ammonia are suspected, then passing the sample over the C_{18} SPE column and then over zeolite may assist in accounting for all of the toxicity. Likewise, passing the effluent over zeolite and then over the C_{18} SPE column may provide additional insight. To gain this knowledge toxicity tests must be performed after each manipulation and not just on the multiple manipulated sample.

Effluent characterization must be approached without any preconceived notion or bias about the cause of

toxicity because many constituents are present in effluents and their chemistry is often unknown, resulting in circumstantial evidence that is frequently misleading. Certainly all available information and experience should be used to guide the investigative effort but temptations to reach conclusions too soon must be resisted. Sometimes the answer being sought is only whether or not a specific substance is causing toxicity. Obviously in such cases testing is specifically selected to answer that question and therefore not all manipulations need to be performed.

Section 7
Interpreting Phase I Results

After Phase I on one sample or several samples is completed, the investigator must carefully evaluate the data, draw conclusions, and make decisions about the next steps that are needed. Sometimes the next step is obvious, at other times the outcome will be confusing and the next step will not be obvious. Several general suggestions, based on our experience to date, may provide some help.

In this section, various examples of Phase I results are given with interpretation suggestions. This discussion is repeated from the acute Phase I characterization manual (EPA, 1991A), and not all aspects have been evaluated for chronic TIEs yet. These examples should be used only as guides to thinking and not as *definitive* diagnostic characteristics. Since almost any toxicant can be present in effluents, clear-cut logic is not totally dependable in interpreting results. Rather, one must use the weight of evidence to proceed, and be aware that artifacts cannot at this point always be identified.

One should avoid making categorical assumptions to every extent possible. For example, to assume that the toxicity is due to a non-polar toxicant(s) because the toxicity in the post C_{18} SPE column effluent was removed, often is an error. Metals may also be the toxicant adsorbed by the SPE column; we have observed zinc, nickel, and aluminum concentrations remaining on the C_{18} SPE column. However, if the toxicity can be recovered in the methanol fraction, then the theory that a non-polar toxicant(s) is causing the toxicity is better substantiated, because metals do not elute with methanol and therefore do not produce toxicity in the methanol fraction toxicity test (cf., Phase II).

Example 1. Non-polar toxicant(s). The Phase I results implicating non-polar toxicants are:

- Toxicity in the *post C_{18} SPE column test* was absent or reduced.
- Toxicity was recovered in the *methanol eluate test.*

Toxicants other than non-polar compounds may be retained by the SPE column but they are less likely to be eluted sharply. Also, artifactual post-column toxicity can occur, but non-polar toxicity is typically distinguished from the artifactual toxicity when the eluate is checked for toxicity. Some toxicants (metals, some surfactants) may not elute from the SPE column with methanol and so failure to recover the toxicity in the eluate does not exclude the possibility of a non-polar toxicant. Recovery of toxicity in the eluate at pH *i* is less likely to be an artifact than recovery only at pH 3 or pH 9. For those instances where methanol does not recover C_{18}-removable toxicity, other solvents may be needed to elute the toxicants (see Phase II; EPA, 1992A).

Example 2. Cationic Metals. This group of metals has varied chemical/physical behaviors which result in less definitive Phase I results. The following characteristics can be used only in a general way to point to metals as the cause of the toxicity:

- The toxicity is removed or reduced in the *EDTA addition test.*
- The toxicity is removed or reduced in the *post C_{18} SPE column test.*
- The toxicity is removed or reduced in the *filtration test,* especially when pH adjustments and filtration are combined.
- The toxicity is removed or reduced in the *sodium thiosulfate addition test.*
- Erratic dose response curve observed.

No single characteristic is definitive, with the possible exception of EDTA. In addition, toxicity may be pH sensitive in the range at which the graduated pH test is performed but may become more or less toxic at low or high pH depending on the particular metals involved. This characteristic for chronic toxicity has not yet been demonstrated to the extent it was for the acute toxicity of several metals (EPA, 1991A).

Example 3. Total dissolved solids (TDS). TDS consists of a group of common cations and anions (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, SO_4^{-}, NO_3^{-}, Cl^{-}, CO_3^{-}) and in parts of the United States, this group is called "salinity." TDS is usually measured by conductivity, density or refraction, none of which measure specific compounds or ions. The toxicity of any given amount of TDS will depend on the specific composition. TDS behaves as a mixture of toxicants, which do not cause toxicity through osmotic stress. Evidence of this is that the LC50s of the individual salts expressed in moles, are quite different. If osmotic stress were the mode of action, the concentration in moles at the LC50s would be similar (EPA, 1991A). One cannot use marine organisms to circumvent TDS unless NaCl is by far the

dominant TDS. Marine organisms regulate Na^+ and Cl^- but like freshwater organisms, they too are sensitive to non-NaCl TDS.

For these reasons, only very general relationships exist between toxicity and TDS. Because of their varied nature, they do not sort out clearly in Phase I. Rather, unless conductivity is very high (e.g., 10,000 µmhos/cm), one might suspect TDS when nothing else is indicated. For example, if high TDS were present and caused by calcium sulfate ($CaSO_4$), toxicity is likely to be removed in the *pH adjustment test* at pH 10 or in the *filtration and pH adjustment test* at pH 10, whereas if the TDS were due to NaCl, toxicity would likely not be affected.

As a general guide, when conductivity exceeds 1,000 and 3,000 µmhos/cm at the effect concentration for *Ceriodaphnia* and fathead minnows, respectively, TDS toxicity might be suspect. The conductivity of 100% effluent is not the relevant reading, but rather the conductivity at the concentrations bracketing the effluent no effect and effect concentrations.

Following are some Phase I general indicators that TDS might be a suspect:

- No pH adjustments changed the toxicity, unless a visible precipitate occurs upon pH adjustment, pH adjustment and filtration, and pH adjustment and aeration.
- No loss of toxicity in the *post C_{18} SPE column test*, or a partial loss of toxicity but no change in conductivity measurements.
- No change in toxicity with the *EDTA addition test*, *sodium thiosulfate addition test* or in the *graduated pH test*.

In addition, there are two tests that can be used that are not included in Phase I but may help to characterize the toxicity:

- Use acid/base ion exchange resins (EPA, 1992A). When toxicity is removed or reduced, the toxicity could be due to TDS.
- Use of activated carbon to remove toxicity (EPA, 1992A). When no toxicity is removed by passing the effluent over carbon, TDS could be responsible for toxicity.

An additional caution is that where TDS is marginally high, the addition of NaCl from pH manipulations can increase TDS enough to produce artifactual TDS toxicity. The conductivity of the solutions before and after the pH adjustments should be monitored closely to avoid this.

Example 4. Surfactants. There are three main groups of surfactants and/or flocculants (anionic, cationic and nonionic) that may occur in effluents. The Phase I behavior of these types of compounds may vary depending on which particular groups are present.

The general Phase I results implicating a surfactant(s) as the toxicant(s) are:

- Toxicity is reduced or removed in the *filtration test*.
- Toxicity is reduced or removed by the *aeration test*. In some cases, the toxicity is recoverable from the walls of the aeration vessel after removing the aerated effluent sample.
- Toxicity is reduced or removed in the *post C_{18} SPE column test*. The toxicity may or may not be recovered in the *methanol eluate test*.
- Toxicity is reduced or removed in the *post C_{18} SPE column test* using unfiltered effluent. Toxicity reduction/removal is similar to that observed in the *filtration test* and toxicity may or may not be recovered in *methanol eluate test* or the extraction of the glass fiber filter.
- Toxicity degrades over time as the effluent sample is kept in cold storage (4°C). Degradation is slower when effluent is stored in glass containers rather than plastic containers.

Example 5. Ammonia. Ammonia concentrations can be measured easily, and because it is such a common effluent constituent, determining the total ammonia concentration in the whole effluent is a good first step (see Section 4). If more than 5 mg/L of total ammonia is present, additional evaluations should be done. Sole dependence on analyses is not advisable because the chronic effects of ammonia and some other toxicants (e.g., such as surfactants) is not well known. Even though the ammonia concentration is sufficient to cause toxicity, other chemicals may be present to cause toxicity if the ammonia is removed. Three indicators of ammonia toxicity are:

- The concentration of total ammonia is 5 mg/L or greater.
- In the *graduated pH test* the toxicity increases as the pH increases.
- The effluent is more toxic to fathead minnows than to *Ceriodaphnia*.

Example 6. Oxidants. In effluents, oxidants other than chlorine may be present. Measurement of a chlorine residual (TRC) is not enough to conclude that the toxicity is due to an oxidant. In general, oxidants are indicated by the following:

- The toxicity is reduced or removed in the *sodium thiosulfate addition test*.
- Toxicity is removed or reduced in the *aeration test*.

- The sample is less toxic over time when held at 4°C (and the type of container does not affect toxicity).
- *Ceriodaphnia* are more sensitive than fathead minnows.

Of course, TRC greater than 0.1 mg/L in 100% effluent might indicate chlorine as the oxidant causing the toxicity. In addition, the dechlorination with SO_2 provides evidence of chlorine toxicity in the same manner as the sodium thiosulfate addition test.

Section 8
References

Aquatic Habitat Institute. 1992. Proceedings of A Workshop on Chronic Toxicity Identification Evaluation (TIEs) in the San Francisco Bay Region. Workshop sponsored by the San Francisco Bay-Delta Aquatic Habitat Institute, Bay Areas Discharger's Association and San Francisco Bay Regional Water Quality Control Board, March 17 and 18, 1992, Richmond, CA.

APHA, 1989. Standard Methods for the Examination of Water and Wastewater, 17th Edition. American Public Health Association, Washington, D.C.

Clean Water Act, Public Law 92-500, October 18, 1972, 86 Stat. 816, U.S.C. 1251 et seq.

EPA. 1978. Methods for Measuring the Acute Toxicity of Effluents to Aquatic Organisms. EPA/600/4-78/012. Environmental Monitoring and Support Laboratory, Cincinnati, OH.

EPA. 1979. Aqueous Ammonia Equilibrium - Tabulation of Percent Un-ionized Ammonia. EPA/600/3-79/091. Environmental Research Laboratory, Duluth, MN.

EPA. 1985A. Technical Support Document for Water Quality-Based Toxics Control. EPA/440/4-85/032. Office of Water, Washington, D.C.

EPA. 1985B. Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms. Third Edition. EPA/600/4-85/013. Environmental Monitoring and Support Laboratory, Cincinnati, OH.

EPA. 1985C. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. EPA/600/4-85/014. Environmental Monitoring and Support Laboratory, Cincinnati, OH.

EPA. 1985D. Ambient Water Quality Criteria for Ammonia. EPA/440/5-85/001. Environmental Research Laboratory, Duluth, MN, and Criteria and Standards Division, Washington, D.C.

EPA. 1988A. Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures. EPA/600/3-88/034. Environmental Research Laboratory, Duluth, MN.

EPA. 1988B. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. EPA/600/4-87/028. Environmental Monitoring and Support Laboratory, Cincinnati, OH.

EPA. 1989A. Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures. EPA/600/3-88/035. Environmental Research Laboratory, Duluth, MN.

EPA. 1989B. Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures. EPA/600/3-88/036. Environmental Research Laboratory, Duluth, MN.

EPA. 1989C. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Second Edition. EPA/600/4-89/001 and Supplement EPA/600/4-89/001A. Environmental Monitoring and Support Laboratory, Cincinnati, OH.

EPA. 1989D. Toxicity Reduction Evaluation Protocol for Municipal Wastewater Treatment Plants. EPA/600/2-88/062. Water Engineering Research Laboratory, Cincinnati, OH.

EPA. 1989E. Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations (TREs). EPA/600/2-88/070. Water Engineering Research Laboratory, Cincinnati, OH.

EPA. 1991A. Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures. Second Edition. EPA/600/6-91/003. Environmental Research Laboratory, Duluth, MN.

EPA. 1991B. Technical Support Document for Water Quality-Based Toxics Control. Second Edition. EPA/505/2-90/001. Office of Water, Washington, D.C.

EPA. 1991C. Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms. Fourth Edition. EPA/600/4-90/027. Environmental Monitoring and Support Laboratory, Cincinnati, OH.

EPA. 1991D. Toxicity Identification Evaluation: Characterization of Chronically Toxic Effluents, Phase I. EPA/600/6-91/005. Environmental Research Laboratory, Duluth, MN.

EPA. 1992A. Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification

Procedures. EPA/600/R-92/080. Environmental Research Laboratory, Duluth, MN.

EPA. 1992B. Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures. EPA/600/R-92/081. Environmental Research Laboratory, Duluth, MN.

EPA. 1992C. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Third Edition. EPA/600/4-91/002. Environmental Monitoring and Support Laboratory, Cincinnati, OH.

EPA. 1992D. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. Second Edition. EPA/600/4-91/003. Environmental Monitoring and Support Laboratory, Cincinnati, OH.

DeGraeve, G.M., J.D. Cooney, B.H. Marsh, T.L. Pollock and N.G. Reichenbach. 1989. Precision of the EPA-Developed Seven-Day *Ceriodaphnia dubia* Survival and Reproduction Test: Intra- and Interlaboratory Study. Electric Power Research Institute, (EPRI), Palo Alto, CA, Report EN-6469.

Denny, J.S. 1988. Guidelines for Culture of Fathead Minnows (*Pimephales promelas*) for Use in Toxicity Tests. EPA/600/S3-87/001. Environmental Research Laboratory, Duluth, MN.

Federal Register. 1984. U.S. EPA: Development of Water Quality Based Permit Limitations for Toxic Pollutants; National Policy. EPA, Volume 49, No. 48, Friday, March 9, 1984.

Federal Register. 1989. U.S. EPA: National Pollutant Discharge Elimination System; Surface Water Toxics Control Program. EPA, Volume 54, No. 105, Friday, June 2, 1989.

Ferguson, W.J., K.I. Braunschweiger, W.R. Braunschweiger, J.R. Smith, J.J. McCormick, C.C. Wasmann, N.P. Jarvis, D.H. Bell, and N.E. Good. 1980. Hydrogen Ion Buffers for Biological Research. Anal. Biochem. 104: 300-310.

Flaschka, H.A. and A.J. Barnard, Jr. (Eds.) 1967. Chelates in Analytical Chemistry. Marcel Dekker, Inc., New York, NY. 418 p.

Giles, M.A. and R. Danell. 1983. Water Dechlorination by Activated Carbon, Ultraviolet Radiation and Sodium Sulphite. Water Res. 17(6): 667-676.

Hockett, J.R. and D.R. Mount. In Preparation. Use of Metal Chelating Agent to Differentiate Among Sources of Toxicity. Manuscript.

Masters, J.A., M.A. Lewis, D.H. Davidson and R.D. Bruce. 1991. Validation of a Four-Day *Ceriodaphnia* Toxicity Test and Statistical Considerations in Data Analysis. Environ. Toxicol. Chem. 10:47-55.

McKim, J.M. 1977. Evaluation of Tests with Early Life Stages of Fish for Predicting Long-Term Toxicity. J. Fish. Res. Board Can. 34:1148-1154.

Mount, D.R. 1991. A Toxicity-Based Approach to Pollutant Identification. In: Proceedings of the Thirtieth Annual EPA Conference on Analysis of Pollutants in the Environment, May 9 and 10, 1990. 21W-7005. Environmental Protection Agency, Office of Water, Washington, D.C.

Neilson, A.J., A.S. Allard, S. Fischer, M. Malmberg, and T. Viktor. 1990. Incorporation of a Subacute Test with Zebra Fish into a Hierarchical System for Evaluating the Effect of Toxicants in the Aquatic Environment. Ecotox. and Environ. Safety 20: 82-97.

Norberg-King, T.J. 1988. An Interpolation Estimate for Chronic Toxicity: The ICp Approach. National Effluent Toxicity Assessment Center Technical Report 05-88, U.S. Environmental Protection Agency, Environmental Research Laboratory, Duluth, MN.

Norberg-King, T.J. 1989A. An Evaluation of Relative Sensitivity of the Fathead Minnow Seven-Day Subchronic Test for Estimating Chronic Toxicity. Environ. Toxicol. Chem. 8(11):1075-1089.

Norberg-King, T.J. 1989B. Culturing of *Ceriodaphnia dubia*: Supplemental Report for Video Training Tape. EPA/505/8-89/002a. Office of Water, Washington D.C.

Norberg-King T.J, and J.S. Denny. 1989. Culturing of fathead minnows, (*Pimephales promelas)*: Supplemental Report for Video Training Tape. EPA/505/8-89/002b. Office of Water, Washington D.C.

Oris, J.T., R.W. Winner, and M.V. Moore. 1991. A Four-Day Survival and Reproduction Toxicity Test for *Ceriodaphnia dubia*. Environ. Toxicol. Chem. 10:217-224.

Smith, R.M. and A.E Martell. 1981. Critical Stability Constants. Volume 4: Inorganic Complexes. Plenum Press, NY. p. 87.

Stephan, C.E. and J.W. Rogers. 1985. Advantages of Using Regression Analysis to Calculate Results of Chronic Toxicity Tests. Aquatic Toxicology and Hazard Assessment: Eighth Symposium, ASTM STP 891, R.C. Bahner and D.J. Hansen, Eds., American Society for Testing and Materials, Philadelphia, pp. 328-338.

Stumm, W. and J.J. Morgan. 1981. Aquatic Chemistry - An Introduction Emphasizing Chemical Equilibria in Natural Waters. John Wiley & Sons, Inc., New York, NY. 583 p.

Woltering, D.M. 1983. The Growth Response in Fish Chronic and Early Life Stage Toxicity Tests: A Critical Review. Aquat. Toxicol. 5:1-21.

Zuiderveen, J.A. and W.J. Birge. 1991. A Comparison of Metal Chelators for Use in the TIE/TRE Chronic Toxicity Tests with *Ceriodaphnia dubia*. Poster, 12th Annual Meeting of the Society for Environ. Toxicol. and Chem., November 1991.

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