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Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity

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16. ABSTRACT

This manual and its companion documents describe a three phase approach to provide guidance to dischargers in identifying toxicity in municipal and industrial effluents (Phase I, EPA, 1991A; EPA, 1992; and Phase III, EPA, 1993A). In 1989, the document titled *Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures* was published as a guidance document for identifying the cause of toxicity in acutely toxic effluents (EPA, 1989A). This new Phase II document provides details for more types of samples, tests and test procedures that can be used to identify the specific chemical(s) responsible for acute or chronic effluent toxicity when the cause of toxicity is related to non-polar organics compounds, ammonia, surfactants, chlorine, or metals. Phase I characterization and Phase III confirmation, the other manuals in the three phase TIE approach have also been produced or updated to include chronic toxicity information, and new developments made since the first set of documents were printed. The TIE approach is applicable to effluents, ambient waters, sediment pore waters or elutriates, and hazardous waste leachates.

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Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity

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Previous Phase II Methods
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EPA-600/3-88/035

National Effluent Toxicity Assessment Center
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Foreword

This document is one in a series of guidance documents intended to assist dischargers and their consultants in conducting acute or chronic aquatic toxicity identification evaluations (TIEs). TIEs might be required by state or federal agencies resulting from an enforcement action or as a condition of a National Pollutant Discharge Elimination System (NPDES) permit. The methods described in this document will also help to determine the adequacy of effluent TIEs when they are conducted as part of a toxicity reduction evaluation (TRE).

This Phase II document is the second of a three phase series of documents that provide methods to characterize and identify the cause of toxicity in effluents. The first phase of the series, Phase I (EPA, 1991A; EPA, 1992), characterizes the physical/chemical nature of the acute and chronic toxicant(s), thereby simplifying the analytical work needed to identify the toxicant(s). Phase II provides guidance to identify the suspect toxicants, and the last phase, Phase III (EPA, 1993A) provides methods to confirm that the suspect toxicants are indeed the cause of toxicity. The recent TIE documents (EPA, 1991A; EPA, 1992; EPA, 1993A; and this document) have been produced or revised to include chronic toxicity recommendations and additional information or experiences we have gained since the original methods were printed (EPA, 1988A; EPA, 1989A; EPA, 1989B).

This Phase II document provides identification schemes for non-polar organic chemicals, ammonia, metals, chlorine, and surfactants that cause either acute or chronic toxicity. The document is still incomplete in that it does not provide methods to identify all toxicants, such as polar organic compounds. This Phase II manual also incorporates chronic and acute toxicity identification techniques into one document.

While the TIE approach was originally developed for effluents, the methods and techniques directly apply to other types of aqueous samples, such as ambient waters, sediment pore waters, sediment elutriates, and hazardous waste leachates. These methods are not mandatory protocols but should be used as general guidance for conducting TIEs.

The sections of both Phase I documents (EPA, 1991A; EPA, 1992) which address health and safety, quality assurance/quality control (QA/QC), facilities and equipment, dilution water, testing, sampling, and parts of the introduction are applicable to Phase II. These sections, however, are not repeated in their entirety in this document.

Abstract

This manual and its companion guidance documents describe a three phase approach for dischargers to identify the causes of toxicity in municipal and industrial effluents (Phase I, EPA, 1991A; EPA, 1992; and Phase III, EPA, 1993A). In 1989, the document titled *Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures* was published as a guidance document for identifying the cause of toxicity in acutely toxic effluents (EPA, 1989A). This new Phase II document provides details for more types of samples, tests and test procedures that can be used to identify the specific chemical(s) responsible for acute or chronic effluent toxicity when the cause of toxicity is related to non-polar organic compounds, ammonia, surfactants, chlorine, or metals. Phase I characterization and Phase III confirmation manuals, the other guidance documents in the three phase TIE approach, have also been produced or updated to include both chronic toxicity information and new developments made since the first set of documents were printed. The TIE approach is applicable to effluents, ambient waters, sediment pore waters or elutriates, and hazardous waste leachates.

Contents

Contents (continued)

Tables

Figures

Abbreviations

AA	Atomic Absorption
C_{18}	Octadecylcarbon Chain
C_8	Octylcarbon Chain
CTAS	Cobalt Thiocyanate Active Substances
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
EDTA	Ethylenediamine Tetraacetic Acid
ERL-D	Environmental Research Laboratory-Duluth
GC	Gas Chromatography
GC/MS	Gas Chromatography/Mass Spectrometry
Heppso	N-(2-Hydroxyethyl) Piperazine-N'-2-Hydroxypropane Sulfonic Acid
HPLC	High Performance Liquid Chromatography
ICp	Inhibition Concentration Percentage
ICP-AES	Inductively Coupled Plasma-Atomic Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
K_{ow}	Octanol-Water Partition Coefficient
LAS	Linear Alkylbenzene Sulfonate
LC/MS	Liquid Chromatography/Mass Spectrometry
LC	Lethal Concentration
MBAS	Methylene Blue Active Substances
Mes	2-(N-Morpholino) Ethane-Sulfonic Acid
Mops	3-(N-Morpholino) Propane-Sulfonic Acid
NETAC	National Effluent Toxicity Assessment Center
NIST	National Institute of Standards and Technology
NOEC	No Observed Effect Concentration
NPDES	National Pollutant Discharge Elimination System
PBO	Piperonyl Butoxide
Popso	Piperazine-N,N'-bis (2-Hydroxypropane) Sulfonic Acid
QA/QC	Quality Assurance/Quality Control
SPE	Solid Phase Extraction
SS	Suspended Solids
Taps	N-tris-(Hydroxymethyl) Methyl-3-Aminopropane Sulfonic Acid
TDS	Total Dissolved Solids
TIE	Toxicity Identification Evaluation
TOC	Total Organic Carbon
TRE	Toxicity Reduction Evaluation
TU	Toxic Unit
YCT	Yeast-Cerophyl®-Trout food

Acknowledgments

This document presents additional methods and improvements made to the procedures of *Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures* (EPA-600/3-88/035) by Donald Mount and Linda Anderson-Carnahan. This manual reflects new information, techniques, and test procedures developed by the National Effluent Toxicity Assessment Center (NETAC) since the previous Phase II document was printed in 1989. This Phase II document is based on the efforts of both federal and contract staff of the NETAC group. We gratefully acknowledge the following individuals' contributions to the research and development of the methods for this document: Penny Juenemann and Shaneen Schmitt (federal staff); Joe Amato, Lara Andersen, Steve Baker, Tim Dawson, Joe Dierkes, Nola Englehorn, Doug Jensen, Correne Jenson, Jim Jenson, Liz Makynen, Phil Monson, Don Mount, and Greg Peterson (contract staff).

Through the support of Rick Brandes and Jim Pendergast (EPA, Permits Division) and Nelson Thomas (ERL-D), the NETAC staff members developed and revised the TIE series of documents.

Section 1
Introduction

1.1 General Overview

The major objective of Phase II is to identify the suspected toxicant(s) in effluent samples using toxicity identification evaluation (TIE) procedures. Some general guidance to achieve this goal might be furnished by the results of acute or chronic Phase I tests (EPA, 1991A; EPA, 1992), but for many effluents, such as those that contain non-polar organic toxicants, both separation and concentration steps will be needed to achieve the stated objective. If metals are the suspect toxicants, atomic absorption (AA) spectrometry should be sensitive enough to measure toxic concentrations directly in the sample, and the number of metals is small enough that toxicity can be attributed without separating one from another. The same principle applies to toxicants such as ammonia and chlorine; measurements can be made without separating or concentrating the effluent. However, if non-polar organic chemicals are suspected, separation is usually necessary for analytical and toxicological reasons.

Because there are often many constituents within the classes of chemicals (e.g., non-polar organics) identified in Phase I, initial efforts are most productively directed towards separating the toxic from the non-toxic constituents. With the need to identify the toxicant(s) quickly, comes the temptation to analyze too soon. Using methods such as gas chromatography/mass spectrometry (GC/MS) one can identify many non-polar organics that are present in the whole effluent mixture, but the association of toxicity with compound identification is very difficult to make for several reasons:

- There can be hundreds of compounds present in the mixture, and to investigate all of them would be very time consuming.
- Toxicity data for many of the chemicals identified are usually not available; chronic data are especially scarce.
- Separate constituents are often not commercially available; therefore, their toxicities cannot be measured and compared to effluent toxicity.
- Interactions (additivity, synergism, antagonism) are not known for the given mixtures and one must know interactions to apportion toxicity.

Therefore, it is suggested that the search for a separation technique to simplify the mixture into toxic and non-toxic subsamples be the first priority, rather than spending time investigating non-toxic components. If there is a single suspect toxicant such as ammonia, then separation needs are limited largely by the analytical requirements. If the toxicity is caused by one constituent, the number of other non-toxic constituents is irrelevant when attributing toxicity. However, Phase I results do not usually lead to a single suspect toxicant and, therefore, separation may be necessary.

When a method for separating the toxicant(s) is found, concentration might be an inherent part of the procedure (as in solvent extraction) which will simplify the problem of finding a method to concentrate the toxicity. At each stage of the separation and concentration process, measurement of toxicity is the best way to evaluate the success or failure of the manipulations.

The interpretation of TIE results can be different than in the classical research approach, where experiments are designed to either accept or reject a hypothesis. In TIE work, an experiment usually permits acceptance but not rejection of the hypothesis. For example, if ammonia is the suspect toxicant, it can be removed using zeolite resin. If the post-zeolite effluent is still toxic, you can conclude that there are additional toxicants present. If the post-zeolite effluent is not toxic, you cannot conclude that there are no additional toxicants because the zeolite might have removed other toxicants in addition to the ammonia.

The always present question of whether or not there is more than one toxicant immensely complicates data interpretation. Phase I results might not give an indication of multiple toxicants unless the toxicant classes change over time or from sample to sample. Phase II results are often such that one cannot tell whether the situation is one of partial removal of a single toxicant or

toxicity resulting from multiple toxicants. The issue might be resolved when one toxicant is identified and measured analytically. Experience shows that the best choice is to try to focus on the toxicant that appears easiest to identify. Usually that will be a toxicant that can be separated from the sample (e.g., extracted or recovered from a sorbent that reduces the toxicity) and for which there is a broad spectrum analytical identification method. Above all, data should always be interpreted under all probable scenarios, i.e., one toxicant, multiple toxicants, and even different toxicants from sample to sample.

Experience gained since the first Phase II (EPA, 1989A) document was printed has shown that effluent toxicants are not always strictly additive. When they are not additive, the toxicant present in the largest number of toxic units (TUs)[1] will determine the toxic units of the effluent. Non-additive toxicity will not be reduced by manipulations that remove toxicants present in fewer TUs than the major toxicant. Two or more toxicants might be present in approximately equal TUs, however, the ratio of TUs might change over different sampling times so that different chemicals determine the toxicity of the effluent. These important problems can be dealt with in Phase III (EPA, 1993A). In Phase II, the objective is to find which toxicants are present in toxic concentrations. However, failure of additivity may confuse Phase II results. Minor toxicants might not be noticed until the major one has been removed. In addition, additivity cannot be determined until at least one toxicant has been identified. Usually Phase II and Phase III merge and overlap, therefore such concerns regarding non-additivity must be incorporated in Phase II, at least in the latter stages.

As effluent constituents are identified, a sorting process begins in which a decision must be made as to whether or not each one identified contributes to the toxicity of the effluent. Usually, this is based on the estimated concentration and the constituents' toxicities. Analytical error in quantitation might be large (10-fold or more) because recoveries and instrument response factors probably will not yet have been determined on a particular chemical. Uncertainty about toxicological data is caused by differences in species sensitivity and water quality effects, when literature values are available. Confidence in an acute toxicity value (LC50) will vary depending on the quality of the test, the number of times it was repeated, and the completeness by which the results and conditions were described. Data on chronic effect levels are often scarce and rarely have tests been repeated. Species sensitivity frequently varies from 100-fold to 1,000-fold; an error will likely be introduced when the published toxicity data for species other than the test species are used. When the uncertainty of the toxicity data is high, a maximum of 100-fold difference between measured concentrations and literature effect would be acceptable to classify a chemical as a suspect. If one has good data for the test species being used, then this difference might be reduced (e.g., to 10-fold). Since these decisions are always subjective, they will sometimes be wrong no matter how carefully they are made. Perhaps most important is use of an iterative process to make these decisions. First evaluate candidates that have concentrations higher than or closest to their chronic or acute effect levels and if these prove to be negative, then examine those that have concentrations below their effect levels. Remember that the suspected toxicant concentrations at the dilution equal to the effect level concentration are the important concentrations to compare. At some point, a decision must be made whether the true toxicants have not yet been identified or measured and that different sample preparation or analyses must be used.

For some effluents, Phase I results might not have provided any guidance for selecting the appropriate Phase II procedures to follow. Other characterization steps that might be helpful are solvent extraction (acidic or basic), sample evaporation, size exclusion chromatography, lyophilization, and vacuum or steam distillation (Jop et al., 1991; Walsh et al., 1983). We have little experience upon which to recommend procedures in these cases. It is most important to realize that the more severe the effluent treatment, the more likely it is that toxic artifacts will be created. These toxic artifacts could then be confused with effluent toxicity; therefore, artifactual toxicity must be monitored for each technique by using blanks.

Phase II efforts should develop into Phase III confirmation (EPA, 1989B; EPA, 1993A) as soon as good evidence is obtained that one or more candidates are probable toxicants. The primary product of Phase II is the chemical identification of the suspected toxicants to furnish the basis for Phase III testing. The techniques described in this document are useful for TIE work with effluents as well as ambient waters (Norberg-King et al., 1991) and sediment pore water or elutriates (EPA, 1991B).

1.2 Biological Testing Considerations

The Phase I characterization documents (EPA, 1991A; EPA, 1992) provide detailed discussions of various issues that are important in decision making throughout the TIE. The guidance covers use of various species, test concentrations, effluent sample types, testing requirements for quality assurance (QA), test endpoints, frequency of changing the test solutions, and more. All of these issues will not be discussed at length here and the user is encouraged to refer to the acute Phase I or the chronic Phase I as companion documents for the TIE process. As the Phase II identification and Phase III confirmation steps are initiated, QA requirements should

[1] TU calculations are described in EPA, 1992. The TUs of whole effluent equals 100% divided by the LC50, NOEC, or ICp (IC25,IC50) of the effluent. The TU of a specific chemical equals the concentration of the compounded divided by the effect level of the compound.

be revisited and the types of tests modified as needed. Several of these testing concerns are addressed below.

During Phase I, the analyst is searching for an obvious alteration in effluent toxicity, which might be obtained by using modified acute or chronic test methods. Confirmation testing (Phase III) conducted according to the standard methodologies will confirm whether the suspect toxicant(s) detected in the characterization and identification steps (Phases I and II) is the true toxicant.

In characterizing the toxicity in Phase I, factors such as time requirements, number of tests and the test design had to be considered and weighed against the type of questions that are posed. EPA has published manuals that describe the acute or chronic test methods to determine the toxicity of effluent or receiving waters to freshwater and marine organisms (EPA, 1991C; EPA, 1993B; EPA, 1993C), and these tests are typically those that indicated the presence of toxicity which the TIE initiated. Deviations from these standard effluent testing protocols were discussed in both the acute Phase I (EPA, 1991A) and the chronic Phase I (EPA, 1992) manuals. For either the acute or the chronic Phase I procedures, the test volumes, number of test concentrations, and number of replicates were all reduced from the standard test methods (EPA, 1991C; EPA, 1993B). Additional modifications for the short-term chronic tests (EPA, 1993B) including shorter test duration and a reduction in the frequency of the test solution renewal are suggested.

Throughout this document the TIE procedures for acutely toxic samples are based on the following species: *Ceriodaphnia dubia, Pimephales promelas, Daphnia magna, Daphnia pulex, Hyalella azteca,* and *Chironomus tentans.* Almost all acute tests have been conducted using 10 ml of test solution in a 1 oz plastic cup (or 30 ml glass beaker). TIE procedures with chronically toxic effluents are based on tests using either *C. dubia* or larval fathead minnows (*P. promelas*). In our laboratory, the chronic tests with *C. dubia* generally are conducted using 10 ml of test solution in 1 oz cups and the chronic tests with fathead minnows are conducted using 50 ml of test solution in a 4 oz plastic cup (10 fish per cup). Use of other species is constrained only by availability, size, age, and adaptability to test conditions, and the threshold levels for additives and reagents for the other organisms must be determined.

As soon as good evidence is obtained to implicate a suspect toxicant(s), the procedures for performing the toxicity tests can be changed. Therefore in Phase II, the time to modify the tests from the way they were conducted in Phase I may depend on when the toxicant is identified, and generally there is more flexibility for this in Phase II than in Phase III. The quality control (QC) measures in Phase I were not very strict because the data are primarily informative rather than definitive. The identity of the suspect toxicant(s) furnishes the basis upon which Phase III testing will be conducted, which will require stricter QC measures.

Initially, the use of modified protocols in Phase II may continue; however, once specific toxicant(s) identification has been made, Phase II (and Phase III) testing conditions should be similar to the methods described in the protocol that was used to trigger the TIE. Although a shortened version of the 7-d *C. dubia* test (which is referred to as the 4-d test) may have been used in Phase I, the use of this test changes in Phase II (and Phase III). In order to use the 4-d test in Phases I and II of the TIE, the 4-d test must detect similar trends of toxicity as the 7-d test does. However, in Phase III the 7-d test is required because the toxicity as measured in the 7-d test (with additional replicates, more test concentrations, additional volume) was used to detect toxicity for the permit, and should be used to confirm the cause of toxicity. In the early Phase II chronic toxicity evaluation steps, the qualitative evaluation of toxicity might be useful and there is no reason why a toxicity test could not be terminated sooner than day seven, if the answer to a particular question has been found.

Information obtained from all toxicity tests should be maximized. For instance, in acute toxicity tests, monitoring time to mortality might be useful. In chronic toxicity tests, time to young production of the cladocerans or the lack of food in the stomach of the larval fish might be useful parameters. Observations such as these made during a test might be subtle indications and quite informative of small changes in toxicity. For example, if there is complete mortality on day four of the baseline effluent test, and in the EDTA addition test (Section 4) the animals either do not reproduce or grow yet they are alive at day seven of the exposure, the indication is that the toxicity was reduced. These results suggest that either an additional toxicant is present or the EDTA concentration was not sufficient to remove all cationic metal toxicity. These types of observations in the short-term tests might be just as useful as reductions in young production or growth. For evaluating whether any manipulation changed toxicity, the investigator should not rely only on statistical evaluations of test endpoints (see below and Phase I; EPA, 1992). 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.

In addition, for acute or chronic toxicity tests, randomization, careful exposure time readings, use of animals of uniform narrow-age groups (i.e., *C. dubia* neonates 0-6 h old rather than 0-12 h old) might assist in detecting smaller differences in tests. For example, in the chronic *C. dubia* tests, it is important to use organisms of known parentage (EPA, 1993B) when the number of replicates is reduced from ten to five. For *C. dubia*, daily renewals of the test media (as required in the chronic manual; EPA, 1993B) might not be necessary in Phase I or early Phase II testing as long as the toxicity of the

effluent can be measured with one or two renewals. However in Phase III, tests must be conducted with daily renewal of test solutions similar to the routine biomonitoring test.

Although reference toxicant tests are not recommended for each set of Phase I manipulations, when a toxicant has been identified in Phase II and some Phase III confirmation tests indicate it is the toxicant, that chemical should become the reference toxicant with the species used in the TIE. In Phase II, the reference toxicant data are useful for identification interpretation and provide information on the quality of the test organisms and general test procedures. Reference toxicant tests should be conducted routinely and control charts should be generated (EPA, 1991C; EPA, 1993B). If a toxicant has not been identified, standard reference toxicants should be used, but as soon as a toxicant is identified, that compound should be used as the reference toxicant for the TIE tests.

The Phase I procedures frequently rely only on one test species, but in Phase III of the TIE the use of more than one species is recommended. This will be useful in determining whether or not the cause of toxicity is the same for other species of the aquatic community. In Phase I, we recommended that the species that detected the toxicity be the first choice as the TIE species. If an alternative species is chosen one must prove in Phases II and III that the species that initially detected the toxicity is being impacted by the same toxicant as the alternate species. Both 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 two species are being affected by the same compound(s) is to test several samples of the effluent over time with both species. If the effluent possesses sufficient variability, and the two species LC50s or IC25s change in proportion one to another as would be expected, 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 Phases II and/or III. If the toxicant is the same for both species, then characterization manipulations that 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 to remove toxicity and the organism's sensitivity to the toxicant. Approaches used in Phase III will confirm whether the two species are indeed sensitive to the same toxicant in the effluent. If in Phase III, the organism of choice is not responding to the same toxicant as the species that triggered the TIE, extensive time and resources might have been wasted.

The type of sample to use in the Phase II identification stage most often will be similar to those used in Phase I. The use of multiple (daily samples for acute and chronic tests or the minimum of three for chronic tests) effluent samples for each chronic test should *not* be used in the early stages of Phase II (EPA, 1992). The use of one grab or composite sample for the Phase II identification procedures is needed until some suspects have been identified. For instance, if several effluent samples are used for renewals during the chronic Phase I and/or Phase II TIE and the toxicants are different or change in their ratios one to another, interpreting the results will be difficult. 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 even more important in Phase III, (EPA, 1993A) where toxicity data are correlated to the measured concentrations in the effluent. If multiple samples are used for one toxicity test, this correlation *cannot* be readily done because the same toxicant may not be present in each sample, it might be present in varying concentrations, or other toxicants may appear. For the acute TIE, one composite or grab sample has been used for identification and confirmation steps. However, since the permit test may require daily samples for an acute static renewal or 7-d short-term tests, once the toxicant is identified each daily sample may have to be analyzed for that toxicant.

Sample degradation is a concern that should be addressed. The toxicity of the whole effluent can be monitored by conducting toxicity tests upon sample arrival and at periodic intervals throughout the TIE. For some types of toxicants degradation or loss might be expected (i.e., chlorine, see Section 5) but for toxicants such as non-polar organic compounds, this may not readily be known. Since the toxicant identification stage might be lengthy, it is important to know that the toxicity remains in the effluent even at a lower concentration. When a toxicant is identified, further analyses and toxicity tests can be conducted on effluent samples and any toxicant degradation or loss evaluated.

As discussed in the Phase I manuals (EPA, 1991A; EPA, 1992) if the level of toxicity for any given effluent has been established with some degree of certainty from previous tests, it might be adequate to use four effluent dilutions and a control to follow toxicity changes of the sample to reduce the cost of the tests. As the toxicant is identified, test concentrations should be selected to detect small changes in toxicity. We are assuming that if effluents have inhibition concentration percentage (ICp) (or no observed effect concentration (NOEC)) values below 10%, the effluent is likely to show acute toxicity and if so, an acute TIE approach can be used. If chronic TIE work is to be done on a highly toxic effluent, the same recommendations given in the chronic Phase I manual should be followed; that is, use concentrations of 4x, 2x, 1x and 0.5x the IC25 or IC50 value. For example, if the IC25 is 5% effluent, we would suggest using a test concentration range such as 20%, 10%, 5% and 2.5% effluent for the various tests. With chronic toxicity data where the NOEC is 12% (or the IC25 is 10%), a concentration series such as 6.3%, 12.5%, 25%, and 50% would be logical. If closer concentration intervals are desired,

using 20% effluent as the high concentration and a dilution factor of 0.7, 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 concentration series to test might be either 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 precision required and practicality.

After conducting Phase I procedures on an effluent sample, the amount of effluent available for subsequent identification work can be sufficiently reduced so that it may be impractical to try to conduct each step as described in this manual. This is most likely to be a concern for the non-polar organic identification techniques and other methods that require large volumes of effluent to identify the toxicant. Therefore, when the volume of an effluent sample is limited, it might be possible to track toxicity through the non-polar identification steps without quantifying the amount of toxicity that is being tracked. Essentially, this means that the toxicity tests are done without dilutions and the results would indicate only that toxicity was present or absent; the degree of toxicity present would not be measured. Once a suspect toxicant is identified, it is important that the amount of toxicity removal is known (through the use of dilutions) because this information can be used to correlate a suspect toxicant to the effluent toxicity in the Phase III confirmation.

If the number of replicates per test concentration is reduced, one must assume that precision is sufficient to decipher changes in toxicity that must be measured. One problem in using reduced replicates and low numbers of test concentrations in chronic tests is that this smaller data set is not amenable to all statistical requirements as recommended for the short-term tests (EPA, 1989C; see Section 5.8). Use of more organisms and more replicates than in the Phase I modified tests might be preferable if Phase I and/or Phase II data are likely to be used in Phase III confirmation (See Sections 2.2 and 2.3).

For acute toxicity tests, usually the LC50 or EC50 is reported for the toxicity data (calculated as recommended in EPA, 1991C). Endpoints for the most commonly used freshwater short-term chronic tests are growth, reproduction, and survival. The no effect level (the NOEC), and the effect concentration (the lowest observed effect concentration (LOEC)) are determined using the statistical approach of hypothesis testing to determine a statistically significant response difference between a control group and a treatment group. The NOEC/LOEC are heavily affected by choice of test concentrations and test design (see Phase I; EPA, 1992). The linear interpolation method (EPA, 1993B) provides a point estimate of the effluent concentration that causes a given percent reduction based on organism response. To calculate the inhibition concentration percentage (ICp), a computer program (Norberg-King, 1993; DeGraeve et al., 1988; EPA, 1989C) is available and the assumptions for the method are not the same as the test design requirements for hypothesis-based analyses. This point estimation method is particularly useful for analyzing the type of data obtained from chronic TIE tests using dilutions (see Phase I; EPA, 1992). Confidence intervals are calculated using a bootstrap technique and might be useful in determining if significant toxicity alterations have been observed. A significant reduction in toxicity and the precision of reference toxicant tests must be determined by each laboratory for each effluent. The use of the IC50 for Phase I TIEs might be more useful in correlating the characterization test results to the effluent toxicity than an IC25. However, there are situations when an IC50 may not be able to be estimated while the IC25 can. Above all, it is most important to use a consistent effect level for TIE toxicity testing (EPA, 1992A; EPA, 1992B). When substantial toxicity reductions occur in the toxicity tests, it may not always appear to be a significant reduction when the IC25s are compared. In order to further evaluate whether toxicity reductions occurred, the sample size (number of replicates, number of concentrations) should be increased in subsequent testing in an effort to differentiate toxicity responses from the sample size limitations. The dose response curves should then be compared to see if responses are similar. Once the toxicant is identified, the number of replicates should be increased, the dilution sequence might be modified and more dilutions used (see Phase III; EPA, 1993A). This should increase the confidence in the IC25 (or any other ICp value chosen) estimate.

Section 2
Non-Polar Organic Compounds

2.1 General Overview

The procedures described in this section presume that the results of Phase I tests have implicated non-polar organic compounds as the cause of acute or chronic toxicity. Results of Phase I tests that clearly implicate a non-polar organic toxicant typically are (1) all toxicity is removed by the C_{18} Solid Phase Extraction (SPE) column and (2) toxicity was observed in the methanol eluate test (see Section 8.6, EPA, 1991A; and Section 6.7, EPA, 1992). In some instances, toxicity might not be removed completely by the SPE column, but sufficient toxicity is recovered in the methanol eluate to suggest a non-polar organic toxicant is present. While toxicants other than non-polar organic compounds might be removed by the column (e.g., metals), the elution for such toxicants is unlikely to be similar to that of the non-polar organic compounds. However, there is also the possibility that non-polar toxicants such as surfactants will be removed by the column and not recovered in the methanol eluate. The goal in this section is to separate the non-polar organic toxicants from the many non-toxic components of the sample to simplify the analytical work needed to identify the toxicant.

This section provides the general background information on non-polar organic compounds along with methods for concentrating and separating the toxicants for samples with acute and/or chronic toxicity. While the method provides a stepwise procedure, there are instances where the investigator may have to modify the approach to achieve the best results.

Also provided in this section are procedures that might prove helpful in less common situations. Metabolic blockers can be used to reduce or eliminate certain organophosphate compounds from exhibiting their toxicity (Section 2.5.1). In the instance when toxicity is not recovered in the methanol eluate and toxicity is not evident in the post-column effluent, alternate SPE elution procedures might be needed (Section 2.6).

A flow diagram of the general procedures followed in identifying non-polar organic toxicants is shown in Figure 2-1. In this procedure, the C_{18} SPE column is used to extract non-polar organic compounds from effluent samples. These compounds are then selectively

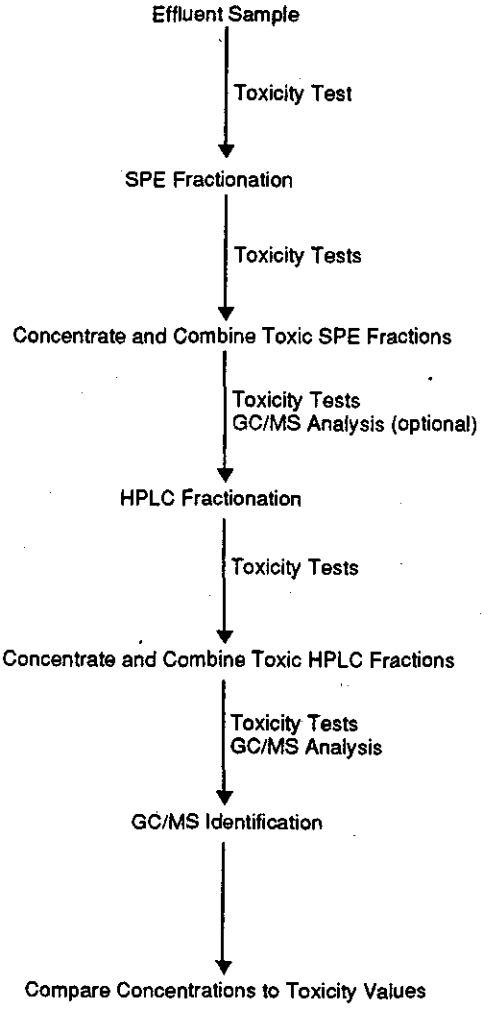

Figure 2-1. Phase II schematic for the identification of non-polar organic toxicants.

stripped off the column by eluting the C_{18} sorbent with solvent/water mixtures that are increasingly less polar. As a series, the "fractions" resulting from column elution contain analytes that are decreasingly polar and decreasingly water soluble. Each fraction is then tested for toxicity. The fractions that exhibit toxicity are concentrated, and chromatographed using reversed phase High Performance Liquid Chromatography (HPLC). The resulting HPLC fractions are collected and tested for toxicity. The toxic HPLC fractions are concentrated into methanol by using another C_{18} SPE column. The concentrates are toxicity tested as before and analyzed using gas chromatography/mass spectrometry (GC/MS). Those constituents that are identified by GC/MS are roughly quantitated, by assuming that the identified constituents and the internal standard have the same response factor, and the estimated concentrations are compared to available toxicity values for each chemical. If this process reveals strong suspect toxicants, mass balance testing (Phase III; EPA, 1993A) could be started to determine whether additional toxicants are present. If no suspect toxicants are identified by GC/MS, a longer analysis time on the HPLC might help the identification by increasing the separation between toxic and non-toxic components, especially if there are many constituents present. Also, additional constituents might be identified by increasing the concentration factor by using larger effluent samples. At some point, the probability that the toxicants are not chromatographing on the gas chromatograph or the mass spectrometer is not detecting the toxicants must be considered if no suspect toxicants are identified. Use of other types of mass spectrometry, such as liquid chromatography/mass spectrometry (LC/MS) or direct probe mass spectrometry may be useful. Some effluents might require the SPE fractionation of several different samples before good suspect toxicants are found (Burkhard et al., 1991; Lukasewycz and Durhan, 1992).

The sorbents that we recommend for use in SPE and HPLC columns are chemically identical. The column packing is composed of silica gel which has been reacted with octadecyl silane to produce a covalent bonded phase one layer thick. The mechanism of extraction with C_{18} sorbents is relatively simple. Extraction of effluent compounds occurs because the C_{18} sorbent competes for the non-polar compounds more strongly than the surrounding water molecules of the effluent. Sorption of non-polar organics is also influenced by ionic strength, pH, and total organic carbon (TOC) levels. The same compounds will partition on both SPE and HPLC columns and the order of elution of chemicals will be approximately the same. The major difference between the SPE and HPLC columns is the amount of resolution achieved. The particle size employed in HPLC columns is smaller, providing a greater surface area and better component resolution. Despite less resolution, SPE columns have the advantage of possessing a higher loading capacity in general than HPLC columns. The SPE column could be considered as a preparatory column for sample cleanup while the HPLC column gives far more refined and controlled separation of sample constituents.

To elute non-polar organic toxicants extracted by the C_{18} SPE column, the sorbed compounds must have a higher affinity for the eluting solvent than for the octadecyl functional group (C_{18}). Choosing a solvent for elution is complicated because the toxicants' identities are not known. In general, the solvent should be less polar than water and more polar than the C_{18} functional group. The degree of solvent strength required to elute the toxicants is also unknown. Since methanol is less polar than water, has a very low toxicity (EPA, 1991A; EPA, 1992) and elutes chemicals from C_{18} sorbents, it has been a good solvent choice for most TIE purposes to date.

During sequential column elutions with successively increasing methanol in water concentrations, the relatively hydrophilic, polar compounds are eluted first, and the more hydrophobic non-polar compounds are eluted last. Given the strength of methanol as a solvent for non-polar compounds, it is possible that very hydrophobic (octanol water partition coefficient (log K_{ow}) ≥4) effluent compounds will not be eluted from the C_{18} sorbent. If toxicity caused by a very hydrophobic compound is extracted by the SPE column but not eluted by methanol, less polar solvents might be used to elute the SPE column (Section 2.6.1).

Once toxicity is found in one or more C_{18} SPE effluent fractions, the toxic fractions can be concentrated, then fractionated using HPLC. HPLC separation is used to reduce the number of non-polar organic chemicals associated with the toxicant(s) and to simplify analytical identification. The toxic HPLC fractions are concentrated and then analyzed by GC/MS. The estimated concentrations of constituents in the final concentrate (based on an internal standard) are then compared to their toxicity values to decide which may be sufficiently high in concentration to cause toxicity. If none are found, higher concentration factors, other analytical methods (e.g., LC/MS), and better separation are recommended.

Fractionation and toxicity testing procedures for non-polar organic toxicants causing either acute or chronic toxicity are presented in different sections of this chapter. The acute toxicity (Section 2.2) and chronic toxicity (Section 2.3) sections, have similar outlines and were written so either section could be used independently. As a result, some details that apply to both acute and chronic toxicity are repeated. After using Section 2.2 or Section 2.3 the investigator should then follow the identification techniques described in Sections 2.4 and 2.5. Additional identification techniques are included in Sections 2.5.1 and 2.5.2, and alternate fractionation methods are found in Section 2.6.

2.2 Acute Toxicity: Fractionation and Toxicity Testing Procedures

In the initial stages of Phase II, toxicity tests may be conducted on C_{18} SPE effluent fractions and blank fractions to detect the presence of toxicants and not to quantify the magnitude of the toxicity in each. As in the toxicity tests conducted during Phase I, careful measure-

ment of test solution water chemistry parameters is not required, and duplicate exposures are not needed during initial stages of Phase II. The major purpose of this step is to assess whether or not acute toxicity is present in the effluent fractions and the blank fractions. However, as suspect toxicants are identified, quantitative toxicity measurements will be needed to compare with the analytical measurements. If Phase II data will be used to correlate effluent toxicity to toxicant concentrations (Phase III), then more replicates per concentration, randomization of test concentrations, careful observation of organism exposure times, and organisms of approximately the same age should be used (Section 1.2). Also, the amount of eluate that is collected from the SPE fractionation, SPE concentration, and the amount of eluate used for testing and GC/MS analysis should be measured at all steps. The volume of eluate must be measured to determine the actual toxicity concentration in each step of the procedure. If it is expected that the Phase II data will be needed later, it is prudent to measure the degree of toxicity in the eight SPE effluent fractions at the onset of testing. We rarely see blank fraction toxicity; therefore, there is little need to evaluate the toxicity of the blank fractions with dilutions.

2.2.1 Sample Volume

The volume of effluent needed depends on its toxicity, the toxicity of the chemicals causing effluent toxicity, and the sensitivity of the analytical method. Since only the first of these will usually be known when Phase II begins, trial and error will dictate volume size. For acutely toxic effluents with LC50 values in the range of 25-100%, 2,000 ml have usually been adequate to perform one complete Phase II procedure, i.e., C_{18} SPE and HPLC fractionations, and GC/MS identification. Examples of the variables that should be considered when deciding what volume of effluent to fractionate are provided in Appendix A, Tables A-1 and A-2.

2.2.2 Filtration

For acute tests, glass fiber filter(s) (1 µm nominal pore size) should be prepared as described in Section 8 of Phase I (EPA, 1991A). Both 45 mm and 90 mm diameter filters have been used routinely, the 90 mm filter allows about four times more effluent to be passed over the filter than one 45 mm filter. All filters and glassware should first be pre-rinsed with pH 3 high purity water (e.g., Milli-Q® Water System, Millipore Co., Bedford, MA) to remove any metal residues followed by a high purity water rinse which is discarded. The filter should then be rinsed with 200 ml of dilution water (rather than with high purity water) and a sample collected after most of the volume has been filtered, to provide the filter toxicity blank. In subsequent steps, a dilution water column blank will be collected after passing the filtered dilution water through the SPE column. The same type of dilution water should be used for the filter blank as for the column blank (Section 2.2.4). Usually, a reconstituted water is used for these procedures (EPA, 1991C).

The volume of effluent that can be passed through a single filter is sample specific. If more than one filter is needed (as is often the case) a single filter blank can be prepared by stacking three to eight pre-rinsed filters in one filter holder, followed by a dilution water rinse. The filters are then separated and used one at a time to filter the effluent sample. If samples are high in suspended solids additional pre-filtration may be needed. Centrifugation may also be useful for reducing solids in the sample. The decision to use a vacuum or a pressure system for filtering should have been made during the filtration tests of Phase I. If a volatile chemical is indicated in Phase I, pressure filtration should be used.

Filtration equipment should be thoroughly cleaned before use to prevent any toxicity carry-over or particle buildup from previous samples. We have found that glass vacuum filtering apparatus with stainless steel filter supports (for samples without pH adjustments), or plastic pressure filtering devices are the most useful. We have also found that if removable glass frits are used, they can be rigorously cleaned with aqua regia for 20-40 min followed by rinsing with copious amounts of water to remove residual effluent particles, since glass frits may act as a filter. The removable stainless steel filter supports do not require as rigorous cleaning as fritted glassware, and therefore are a good substitute.

A portion of the filtered sample must be reserved for toxicity testing while the rest is used for C_{18} extraction. If the filtration toxicity blank exhibits slight or complete toxicity, but the post C_{18} SPE column effluent is not toxic (and effluent toxicity was unchanged after filtration), the blank toxicity can be ignored since the effluent toxicity was removed (see Phase I). However, as the identification process continues, the blank toxicity will have to be eliminated, or it could lead to a misidentification of the cause of toxicity.

When effluent samples are readily filtered (~2,000 ml for one 90 mm 1 µm filter) it may be possible to filter the effluent for the filtration test of Phase I but then use unfiltered effluent with the C_{18} SPE column test and the methanol eluate test (Phase I). Once it has been demonstrated that filtration does not reduce toxicity, routine filtering of these effluents (before passing the effluent through the SPE column) can be eliminated. This will reduce the amount of toxicity testing required.

2.2.3 Column Size

Various sizes of C_{18} SPE columns are available ranging from 100 mg to 10,000 mg packing material. We routinely have used Baker® 1,000 mg columns for 1,000 ml of effluent (J.T. Baker Chemical Co, Phillipsburg, NJ). Volumes for a 1,000 mg C_{18} SPE column are used in the following description, since this is the size most often used for acutely toxic effluents. Other available column sizes and the appropriate volumes to be used in their preparation are listed in Table 2-1. Positive pressure pumps (EPA, 1991A) are convenient for the large volume

effluent samples because flow rate can be controlled. Vacuum manifolds can be used for drawing the small samples and solvents through the column. Whichever system is used, it must be made of materials that dilute acid and solvents do not destroy, or from which chemicals are not leached that are toxic or that interfere with analytical measurements. Teflon, glass, and stainless steel are all acceptable choices.

2.2.4 C_{18} SPE Column Conditioning

The 1,000 mg C_{18} SPE columns are conditioned by pumping 10 ml of 100% methanol through the column at a rate of 5 ml/min. The pumping rate can be increased to 40-50 ml/min when using the larger C_{18} SPE columns (e.g., 5 g or 10 g). The volumes of conditioning solvent recommended for other size columns are shown in Table 2-1. We most commonly use methanol as the conditioning solvent but other water miscible solvents such as acetonitrile, ethanol, or isopropanol may be substituted. *Before the packing goes dry, 10 ml of high purity water* must be added. As the last of the high purity water is passing through the column, 25 ml of filtered dilution water is added. The last 10 ml of dilution water is collected for a dilution water column blank. After the dilution water has been collected, pumping is continued until no dilution water emerges from the column.

2.2.5 Elution Blanks

To generate elution blanks from a 1,000 mg column, two successive 1.5 ml volumes of 25% methanol/water (%v/v) are pumped sequentially through the conditioned column and collected in one analytically clean, labeled glass vial to produce a 3 ml sample. This procedure is repeated with two successive 1.5 ml volumes of 50%, 75%, 80%, 85%, 90%, 95% and 100% methanol/water. The column should be allowed to dry for a few seconds between each elution with the different 3 ml volumes of methanol/water solutions. This will result in eight 3 ml SPE fraction blanks (Figure 2-2). The volume of methanol solutions used for elution will vary depending on column size as shown in Table 2-1.

2.2.6 Column Loading with Effluent

The same column is then reconditioned with 10 ml of 100% methanol and 10 ml of high purity water, as described in Section 2.2.4. *Without allowing the column to dry*, 1,000 ml of filtered effluent is pumped through the column at a rate of 5 ml/min (Figure 2-3). The pumping rate can be increased to 40-50 ml/min when using the larger C_{18} SPE columns (e.g., 5 g or 10 g). Three samples (~25 ml) of the post-C_{18} SPE column effluent are collected after 25 ml, 500 ml and 950 ml of the sample has passed through the column. Each post-column aliquot is toxicity tested to determine the presence of acute toxicity in the post-column effluent. This information can be used to determine whether the toxicant is removed from the effluent by the column. As Phase II progresses, the recommendation is to increase the volume of post-column effluent collected to 50-60 ml so that dilutions can be made and LC50 values obtained. Pumping is continued until no effluent emerges from the column.

The efficiency of the C_{18} SPE column is determined by the extraction efficiency (i.e., how well the column sorbent removes the effluent components) and the elution efficiency (i.e., how well sorbed effluent compounds are removed from the column by the solvent elution). For purposes of the TIE, *"efficiency" applies only* to recovery of those compounds causing or affecting effluent toxicity. Since most acute effluent tests do not require large volumes of post-column effluent, the question of extraction efficiency can be determined by measuring the toxicity of the post-C_{18} column effluent sample. The toxicity of each aliquot collected after different vol-

Table 2-1. Solid Phase Extraction (SPE) Column Fractionation Information[1]

C_{18} SPE Sorbent Amount[2] (mg)	Volume *Conditioning* Solvent (ml)	High Purity *Water* Volume (ml)	Maximum Volume Effluent (ml)	Minimum (500x) Elution Volume[3] (ml)	Suggested (333x) Elution[4] Volume[3] (ml)
100	2	2	100	2 x 0.1	2 x 0.15
500	5	5	500	2 x 0.5	2 x 0.75
1,000	10	10	1,000	2 x 1.0	2 x 1.5
5,000	50	50	5,000	2 x 5.0	2 x 7.5
10,000	100	100	10,000	2 x 10	2 x 15

[1]*The information is based on manufacturer's guidance and experimental data from* ERL-D.
[2]The smaller columns (100, 500, and 1,000 mg sorbent) are available pre-packed from J.T. Baker Chemical Co., the larger columns (5,000 and 10,000 mg sorbent) are available pre-packed from Analytichem International.
[3]Elution with two successive aliquots of the volume listed.
[4]The 333x concentration factor is most often used for acute work.

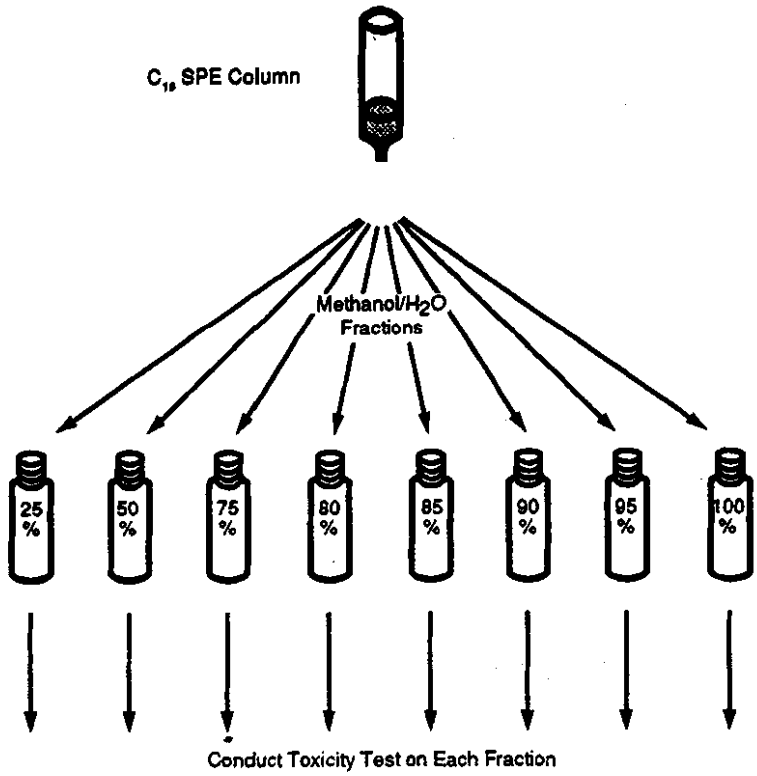

Figure 2-2. Procedures for eluting the SPE column with a gradient of methanol/water solutions.

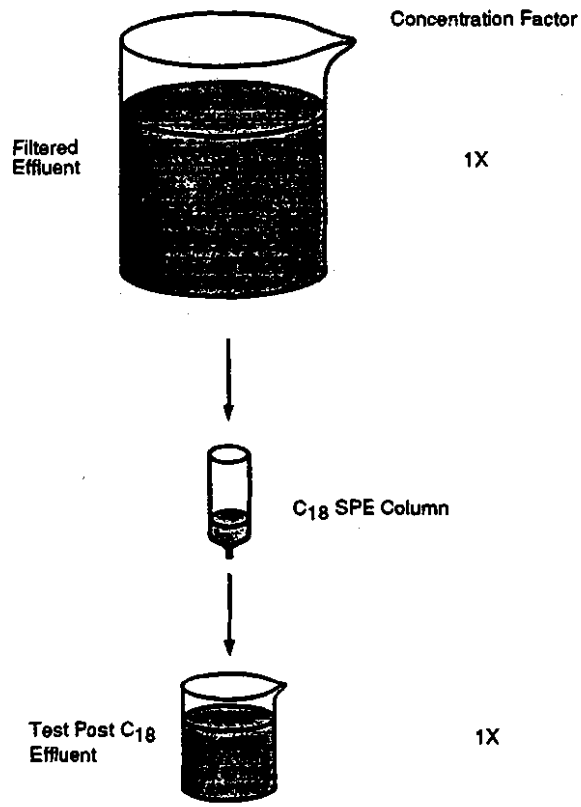

Figure 2-3. Concentrating effluent on the C_{18} SPE column.

umes of effluent have passed through the column can be compared. If there is toxicity in these aliquots, but it is independent of the volume of effluent previously passed through the column, then the post-column effluent toxicity is probably caused by toxicants that are not extracted by the column. If toxicity increases as the volume of post-column effluent passed through the column increases, the capacity of the column to sorb the toxicants was probably exceeded.

In some post-column effluent, a biological growth may occur during toxicity testing which may result in artifactual toxicity. Such growth can make it appear as if the toxicant is not removed 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 from the same preparation. The growth may appear to be filamentous and give a milky appearance in the test vessel. This effect has been linked to methanol stimulation of bacterial growth. Methanol is present in the post-column samples because a small amount of methanol is constantly released from the column during the sample extraction. Effluents from biological treatment plants may develop this characteristic more readily than industrial effluents.

Additional filtering of the post-column effluent sample through a 0.2 µm filter before testing to remove bacteria and eliminate the growth has been helpful. To avoid artifactual toxicity as much as possible in the post-column effluent, initiate the tests with the post-column samples on the same day the effluent is extracted even if fractions are not tested simultaneously. For those few effluents where we have not eliminated this type of artifactual toxicity, holding the post-column effluent is problematic in that more time is available for bacteria to cause problems in the post-column sample matrix. When post-column artifactual growth is not readily eliminated, a different solvent (e.g., acetonitrile) to condition the column (but not for eluting) may be useful in reducing the post-column artifactual bacterial growth. This artifactual growth has not occurred in the toxicity tests with methanol SPE fractions (Section 2.2.8).

2.2.7 C_{18} SPE Column Elution

Once the effluent sample has been loaded onto the column, elution can begin. To elute a 1,000 mg column, two successive 1.5 ml volumes of the 25% methanol/water mixture are pumped through the column and collected in one labelled, analytically clean vial to make a 3 ml sample. Subsequently, two successive 1.5 ml volumes of each of the 50%, 75%, 80%, 85%, 90%, 95% and 100% methanol/water are pumped through the column and collected in separate vials (Figure 2-2). The next elution volume should be added when no more of the preceding one is emerging from the column.

This entire procedure (conditioning through elution) is repeated using a second 1,000 mg C_{18} SPE column for the second 1,000 ml of filtered effluent. The dilution water column blank samples should be kept separate. The corresponding fractions from the blank and the sample from each 1,000 ml fractionation can be combined. For example, the 3 ml 100% methanol sample fraction from the first column and the 3 ml 100% methanol sample fraction from the second column are combined to produce a total of 6 ml. There will be eight 6 ml blank fractions and eight 6 ml effluent fractions.

The vials containing the methanol/water fractions are tested immediately or sealed with perfluorocarbon or foil-lined caps and stored under refrigeration. These fractions represent a "first cut" separation of effluent components. Elution volumes will vary if columns of different sizes are used or if the particular effluent under study or the research question being posed dictates method modification.

2.2.8 Blank and Effluent Fraction Toxicity Tests

The next step is to determine the toxicity of the blank and effluent fractions. While the choice of test concentration depends on the toxicity of the effluent in most instances, we have used a high test concentration of 2x or 4x (the LC50 or 100% effluent) for acutely toxic effluents. The methanol content in the fractions limits the concentration that can be tested, and at this point the amount of methanol is assumed to be 100% in all the fractions for dilution calculations; however, this is not assumed for add-back tests (described below). Usually 120 µl of each blank and sample fraction (333x) is injected into separate 10 ml aliquots of dilution water to test at 4x the 100% effluent[2]. This will give a 1.2% methanol concentration which is below the methanol LC50 for both *C. dubia* and fathead minnows in the 100% methanol fraction. The resulting methanol concentration must be adjusted for the species tested (see Section 8 of EPA, 1991A). During the initial stages, five animals in each 10 ml aliquot are used without duplicates. Using the above volumes, the tested solution is more concentrated (i.e., 4x) than 100% effluent, assuming 100% extraction and 100% elution in one fraction. These test solutions can be diluted to provide an LC50 for each sample fraction. Blank fractions need not be diluted, since hopefully they are nontoxic.

Individual chemicals in the fractions could be toxic even when they are not toxic in the whole effluent, since the concentration tested may be as high as 4x whole effluent. Therefore, to be toxic at whole effluent concentrations, an individual fraction must have an LC50 of 25% or less. Since there is no way to know whether the toxicant(s) eluted over more than one fraction or what the percent extraction and elution efficiency are, fraction tox-

[2] In the Phase II document published in 1989, testing at 5x was recommended, the methanol level was 1.5% at this concentration. In order to lower the methanol level, this was changed to 4x in this document.

icity up to 100% (4x whole effluent) should not be disregarded.

If toxicity occurs in any of the fraction blank tests and it is small relative to the toxicity in the corresponding sample fraction (e.g., 20% mortality versus 80%), the sample fraction results should not be dismissed. If all organisms die in the blanks and the effluent fractions, dilutions should be tested to make sure the sample fraction is substantially more toxic than the blank. In general, blanks should not have measurable toxicity.

If the SPE fractions are toxic at effluent concentrations of 1x or 2x and toxicity is reduced in two of the three post-column effluent samples, the toxicant could still be a non-polar organic compound. If the effluent fractions are not toxic individually and the post-column samples are non-toxic, it is possible that the toxicity has been spread across several fractions or has not been recovered from the column. Combining and concentrating fractions may be useful or other elution procedures may be necessary. If toxicity is observed in the fractions at 1x, 2x, or 4x and in the post-column effluent samples, it is possible that not all the toxicity is caused by non-polar compounds, that break-through of the toxicant has occurred, or that the toxicity is artifactual.

In addition to concentrating column artifacts to toxic levels, effluent constituents present at nonlethal levels may be concentrated to toxic levels in this test if they have a relatively high recovery value. Actual effluent toxicants with poor recovery may not be present in these test solutions at toxic levels. Spurious results of this nature will be identified in the later stages of Phase II and/or in Phase III.

Elution efficiency may be approximated by summing the amount of toxicity (i.e., TUs) in the toxic fractions (provided dilutions are tested) and comparing this value to whole effluent toxicity expressed as TUs. When summing acute toxicity, it is important that all values are for comparable endpoints (i.e., LC50s). Adding of TUs may be somewhat imprecise for several reasons. A single toxicant may occur in more than one adjacent fraction, in which case a small amount of the toxicant in one fraction may not be detectable because it is present below the effect concentration. For acute toxicity, this problem may be solved by combining a portion of each effluent fraction (and separately testing the corresponding blank fractions) and measuring total toxicity at 1x. If more than one toxicant is present, the effluent fraction toxicity may not be strictly additive in their toxicities, and when separated into different fractions the sum of the fraction toxicities may be low even if extraction and elution efficiencies were 100%. Table 2-2 illustrates a hypothetical example. The toxicity test results from the test with a portion of all fractions or a few of the fractions may show somewhat greater toxicities than those of the whole effluent. This may be caused by enhanced toxicity due to matrix effects. When this occurs, it may be possible to compensate for toxicity enhancement by methanol, by adding methanol to the whole effluent and evaluating the toxicity. This methanol addition may in turn stimulate

Table 2-2. Comparison of Toxic Units (TUs) in Each Toxic Fraction to TUs of All Fractions Combined and Whole Effluent

Toxic Fraction (% Methanol)	TUs
75	0.5
80	1.2
85	0.6
SUM	2.3
Combined Fractions	2.7
Whole Effluent	2.5

biological growth, and if this happens, the test is negated. We have rarely used this approach since the fractions have seldom caused more toxicity than the effluent itself. At this point in Phase II, the effluent fractions should also be tested in water with TOC and suspended solids which mimics the effluent to lessen matrix effects on toxicity. As the identification step moves into Phase III, it is better to use dilution water that mimics effluent or receiving water characteristics.

2.2.9 SPE Fractions: Concentration and Subsequent Toxicity Testing

The SPE fractionation provides a general separation of non-polar organics and except in relatively uncomplicated effluents, GC/MS analysis of the concentrates of toxic C_{18} SPE fractions will result in very complicated chromatograms from which the toxicant(s) cannot be distinguished from other effluent components. A secondary fractionation using HPLC is often needed to further simplify toxic effluent fractions prior to component identification by GC/MS analysis.

In order to maximize the chromatographic separation capability of the HPLC, the volume of the sample injected onto an analytical size HPLC column should be as small as possible (i.e., <0.5 ml); therefore, the toxic SPE fractions (usually >1 ml) must be concentrated prior to injection onto the HPLC column. This concentration step will provide the added benefit of an increase in concentrations of constituents in the HPLC fractions as well as rid the SPE fractions of water. The latter issue is important if GC/MS analysis will be performed on the concentrated SPE fraction prior to injection on the HPLC (Durhan et al., 1990).

The volume of the fractions from the initial SPE fractionation procedure and the number of fractions to be combined will determine the size of the SPE column to use for the concentration procedure. Table 2-3 contains information on the column sizes we have found to be most useful. In the procedure outlined below (Figure 2-4), we have used a 100 mg column which is the most commonly used size for concentrating SPE fractions of acutely toxic effluents. Most often the toxic effluent SPE

Table 2-3. Information for Concentrating SPE and HPLC Fractions[1]

C_{18} SPE Sorbent Amount (mg)	Volume Conditioning Solvent (ml)	High Purity Water Volume (ml)	Maximum Toxic Fraction Volume (ml)	Maximum Diluted Fraction Volume (ml)	Minimum Elution Volume[2] (ml)	Approximate Eluate Volume (ml)
100	1	1	20	100	3 x 0.1	0.22
200	2	2	40	200	3 x 0.2	0.44
500	5	5	100	500	3 x 0.5	1.10
1,000	10	10	200	1,000	3 x 1.0	2.20

[1]Concentration information is based on manufacturers guidance and experimental data from ERL-D.
[2]Elution with three successive aliquots of the volume listed.

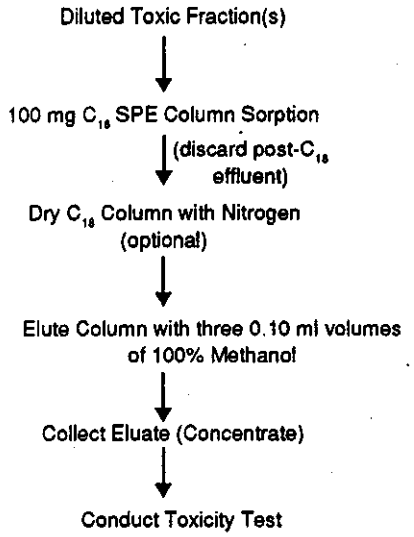

Figure 2-4. Procedure to concentrate toxic SPE fractions.

fractions are combined and diluted with high purity water and the corresponding blank fractions are treated similarly. In cases where there are multiple non-polar toxicants, and when toxicity occurs in several fractions, it may be more useful to concentrate each fraction separately for subsequent HPLC separation. The percent methanol in the diluted fraction sample should be ≤20% and the volume to which the fractions can be diluted is dependent on the amount of column packing. For example, the total volume of the diluted fractions should not exceed 100 ml for the 100 mg C_{18} SPE column (Table 2-3). No more than three toxic fractions of 6 ml each can be combined and concentrated on the 100 mg column. When the total volume of combined fractions or the individual fraction volume is above 20 ml, larger columns should be used; consult Table 2-3 for column size and elution volume information. The effluent and blank concentrates and the column blank are tested for toxicity to ensure that the toxicant is still in the concentrate and that artifactual toxicity was not introduced by the procedure. If there is not measurable toxicity in the concentrate, it is possible that the percentage of methanol in the diluted fraction was too high. The concentration procedure should then be repeated with a new set of toxicity tested fractions diluted to a lower methanol concentration, e.g., 10%.

Below is an example of how to prepare effluent and blank fraction concentrates. First, a 100 mg C_{18} SPE column is conditioned with 1 ml of methanol and 1 ml of high purity water similar to the procedures described in the SPE Column Conditioning Section (2.2.4). Column blanks for toxicity testing are obtained by rinsing the column with at least 20 ml of dilution water. After collecting the column blank, recondition the column with 1 ml of methanol and rinse with 1 ml of high purity water. The diluted blank fractions (for dilution guidance see Table 2-3) are then drawn through the 100 mg C_{18} SPE column under a pressure of 380 mm Hg using a vacuum manifold. Unlike the first fractionation step (Section 2.2.6) the post-column sample cannot be tested for toxicity because of its high methanol concentration (i.e., 10-20%). The column is then dried for 10 min using a gentle flow of nitrogen (10-20 ml/sec). Drying the column usually increases the recovery of toxicity, but sometimes toxicity is not recovered from the column, possibly as a result of volatilization. If this occurs the concentration procedure can be repeated without the nitrogen drying step.

After drying the sorbent, the luer tip of the column is fitted with a luer-lock needle and 100 µl of 100% methanol is placed into the column using a microliter syringe. Nitrogen is then applied to the column at a rate of ~4 ml/sec to force the methanol through the sorbent. The luer-lock needle is needed to ensure the collection of small volumes; when using larger column sizes (e.g., ≥500 mg) this is not necessary. The first 100 µl aliquot of methanol applied to the column will yield approximately 25 µl of eluate. Two more 100 µl aliquots of 100% methanol are also forced through the column. The final volume of eluate collected will be approximately 220 µl. If desired, measure the exact volume collected (using a µl syringe) to calculate concentration factors (Table 2-3). As in most chromatographic separations and extractions, three separate smaller elutions of methanol are more efficient than one large one.

The 100 mg C_{18} SPE column is reconditioned with 1 ml of methanol and rinsed with 1 ml of high purity water. It is then used to concentrate the diluted toxic SPE column fractions, using the same procedure used for the blank fractions (Figure 2-4). In lieu of reconditioning the same column, two columns can be conditioned, one used for the diluted blank fractions and the other used for concentrating the diluted toxic SPE fractions. The resulting column blanks should be toxicity tested separately.

The original effluent volume of 2,000 ml is now concentrated into a 220 µl sample or a nominal concentration of 9,091x (ignoring the amount used for testing). As work progresses and more quantitative results are needed, the eluate volume must be measured to provide the correct concentration factor. If 9 µl of concentrate is diluted to 10 ml in dilution water, the resulting test concentration will be 8x whole effluent. Additional test concentrations (e.g., 4x, 2x, 1x) can be prepared to determine an LC50 of the concentrate, and toxicity recovery can be calculated by comparing this LC50 to the LC50 of the effluent. The concentrate toxicity might be higher than the sum of the individual toxic fractions because some of the toxicant may have been in adjacent fractions that were concentrated in the first step (Section 2.2.7) but not detectable by the toxicity test of the single fraction. The concentrate toxicity may also be lower than expected because of low extraction and elution efficiencies. Where greater concentration factors are desirable, SPE fractionation should be repeated with additional volumes of effluent, followed by combining the toxic fractions before concentration. The size of the column used for concentrating may have to be increased, along with the appropriate changes in dilution and elution volumes (Table 2-3).

The important concern here is not 100% recovery of toxicants but enough recovery for GC/MS analyses and to obtain measurable toxicity in the HPLC fractions. If recovery is too low, changing or eliminating the column drying time may help. Sometimes recovery appears to increase with drying time while other compounds are volatilized from the column during the drying process. For concentrates analyzed using GC/MS, column drying to remove water is critical to GC column performance.

2.2.10 HPLC Separation

The same column packing functionality should be used in the HPLC column as the SPE column. At later stages, when more is known about the toxicants, other sorbent types may be used.

The HPLC conditions presented in this section are general. As more information on the effluent is gathered, HPLC conditions should be modified to achieve better separation and higher concentration factors. We use a flow rate of 1 ml/min on an instrument equipped with a 5 µm C_{18} reverse phase column (250 mm x 4.6 mm i.d.). The HPLC elution conditions will change depending on which SPE fractions have been concentrated. The HPLC conditions for the four most commonly toxic SPE fractions are listed in Table 2-4. Depending on the size of the HPLC injector and column, more than one HPLC fractionation run may be required to fractionate the entire blank concentrate. When multiple HPLC fractionations are conducted, collect all the corresponding HPLC fractions in the same set of vials. For example, if two HPLC fractionations were performed for the blank concentrate, 25-2 ml HPLC fractions would be obtained.

Table 2-4. Example HPLC Elution Gradients for Four Commonly Toxic SPE Fractions

75% or 85% SPE Fractions		85% or 90% SPE Fractions	
Time (min)	% Methanol/Water	Time (min)	% Methanol/Water
0	50	0	60
1	60	1	70
13	90	13	90
20	100	20	100
25	100	25	100

Using the HPLC equipment described above, all of the blank concentrate remaining after toxicity testing is injected (≤500 µl) and 25-1 ml fractions are collected in analytically clean glass vials (Figure 2-5). The same procedure is followed using the effluent sample concentrate. The vials should be sealed (e.g., with foil lined caps) and stored at 4°C after use. As soon as toxicant identification is obtained by GC/MS, then HPLC conditions (gradient, fraction size, and number of fractions) can be optimized for further fractionations.

2.2.11 HPLC Fraction Toxicity Tests

Before specific toxicants are identified, toxicity tests on each HPLC blank fraction and sample fraction are conducted using non-replicated exposures of five animals each. The amount of methanol in the HPLC fractions limits the concentration that can be tested. Assume that each fraction is 100% methanol to calculate the necessary dilution. A methanol concentration of 1.2% should not be exceeded for *C. dubia* and fathead minnow acute toxicity tests.

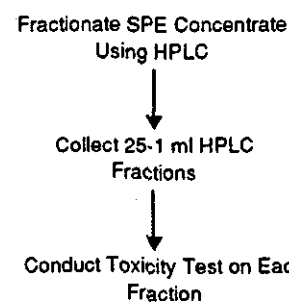

Figure 2-5. Procedure to fractionate acutely toxic SPE concentrates using HPLC.

For acute studies, when all of the SPE fraction concentrate remaining after toxicity testing is injected (one injection) on the HPLC (Figure 2-5), each resulting 1 ml HPLC fraction equals 2,000 ml of effluent (assuming no loss and toxicant elution in only one fraction) or a 2,000-fold concentration. If each HPLC fraction is then diluted for testing (80 µl to 10 ml) the resultant concentration is 16x the original effluent concentration. In preliminary Phase II testing the HPLC fractions are tested without dilutions. Only the toxic HPLC fractions are tested again with dilutions to generate an LC50. Some loss of toxicant tends to occur in each concentration step and the resulting toxicity may be decreased relative to the original effluent.

The blank fractions should not be toxic. If they are, then additional tests with dilutions must be conducted on both blanks and toxic fractions to find out whether there is enough additional toxicity in the sample fractions to warrant analysis.

The toxicity of the HPLC fractions should be tested at twice (at least) the concentration at which the original SPE column fractions were tested because recovery of toxicity and analytical measurements indicate that up to 50% of the initial concentration of toxic compounds may be lost in this step (Durhan et al., 1990). The amount of methanol should not exceed the amount used in the SPE fraction tests described above (Section 2.2.8).

2.2.12 HPLC Fractions: Concentration and Subsequent Toxicity Testing

The HPLC fractions that exhibit toxicity and their corresponding blank fractions must be concentrated in a solvent suitable for GC/MS or other analytical techniques. The procedure is identical to that described in Section 2.2.9 and is depicted in Figure 2-6. Judgement must be used to decide whether to concentrate each toxic fraction separately or to combine various toxic and adjacent fractions prior to concentration. If, for example, three successive fractions exhibit toxicity, there is a good chance that the same toxicant is in all three. If there are other fractions that show toxicity but they are separated from the first set by several non-toxic fractions, there is high probability that the second set contains a toxicant different from the first three. There is also a good chance that at least one non-toxic fraction on either side of the toxic fractions contains some of the toxicant(s). The advantage of combining fractions is to reduce the work load and increase concentration in the final concentrate. The disadvantage is that more constituents that are not the toxicant(s) will also be concentrated. This decision is not always straightforward and must be based on trial and error, and experience. Blank fractions corresponding to the toxic fractions are concentrated the same way.

The HPLC fraction and blank concentrates should be finally checked for toxicity before GC/MS analysis. This concentrate is now nominally 9,091x more concentrated than the effluent. If 18 µl is diluted to 10 ml, the resultant test concentration will be 16x the original sample

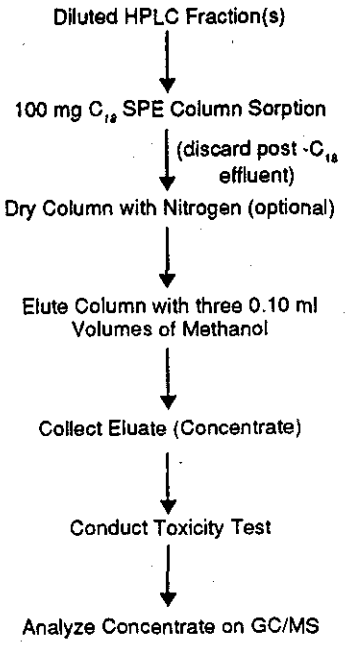

Figure 2-6. Procedure to concentrate toxic HPLC factions.

concentration. To quantitate toxicity and use the Phase II data later, additional lower concentrations should be tested (e.g., 8x, 4x, 2x); TUs of this concentrate can then be compared to previous toxicity test results. The HPLC concentrate should be tested at one to two times the test concentration of the HPLC fraction tests (i.e., 16x or 32x). This is the last opportunity to assure that the toxicant is still present in the concentrate before it is subjected to GC/MS analysis. Whether the toxicant is detected by the analytical detector (mass spectrometry in our laboratory) is always a question. Since GC/MS detects only about 20% of organic chemicals (EPA, 1989B), even such a broad spectrum method is not certain to identify the toxicant. As work progresses with more samples of the effluent and quantitative results are needed, the amount of eluate collected should be carefully measured and recorded to accurately calculate the concentration factors. In addition, the volume of concentrate removed for toxicity testing and analytical analyses should also be recorded.

2.3 Chronic Toxicity: Fractionation and Toxicity Testing Procedures

The chronic Phase II non-polar organic toxicity identification follows the same general approach and employs manipulations similar to those described for the acutely toxic non-polar organic compounds (Section 2.2). One major difference is that the concentration of the eluting solvent (e.g., methanol) must be lower in the chronic toxicity tests than in acute tests. In the initial stages of Phase II, toxicity tests may be conducted on C_{18} SPE effluent fractions and blank fractions to detect the presence of toxicants, and not to quantify the magnitude

of the toxicity in each. However, as suspect toxicants are identified, quantitative toxicity measurements will be needed to compare with the analytical measurements. If Phase II data will be used to correlate effluent toxicity to toxicant concentrations (Phase III), then more replicates per concentration, randomization of test concentrations, careful observation of organism exposure times, and organisms of approximately the same age should be used (Section 1.2). Also the amount of eluate that is collected from the SPE fractionation, SPE concentration, and the amount of eluate used for testing and GC/MS analysis should be measured at all steps. If it is expected that the Phase II data will be needed later, it is prudent to measure the degree of toxicity in the SPE effluent fractions (Section 2.3.5) at the onset of testing. We rarely see blank fraction toxicity, therefore, there is little need to evaluate the blank fraction toxicity with dilutions. Also, the volume of eluate must be measured to determine the actual toxicity concentration in each step of the procedure.

The following discussion is based on our experiences with *C. dubia* and fathead minnows (see Section 1.2). The use of other species will require reconsideration of the appropriate test volumes and methanol concentration for each step. Chronic testing is more labor intensive and generally requires more effluent sample volume than acute testing. For the most part, in the descriptions below, for *C. dubia* there are five replicates containing 10 ml of test solution and one animal per cup. For the fathead minnow tests, two replicates of 10 animals per 50 ml and the control are usually used (Section 1.2). Typically we use four concentrations and a control.

As soon as the cause of toxicity has been determined to be a non-polar organic compound (e.g., methanol eluate test; EPA, 1992) it is prudent to concentrate large volumes of effluent for the subsequent analyses. By concentrating large amounts of the effluent it is possible to plan the optimal usage of the amount of column eluate available for toxicity testing.

2.3.1 Sample Volume

The volume of effluent needed depends on its toxicity, the toxicity of the chemicals causing effluent toxicity, and the sensitivity of the analytical method. Since only the first of these will usually be known when Phase II begins, the volume of effluent to process should be considered at the beginning of the identification process to minimize the amount of re-fractionating and re-testing of effluent and fractions. Ideally, fractionation should provide enough volume of post-column effluent (Section 2.3.6), C_{18} SPE fractions (Section 2.3.8), SPE fraction concentrates (Section 2.3.9), HPLC fractions (Section 2.3.11), and HPLC fraction concentrates (Section 2.3.12) to conduct all chronic toxicity testing and chemical analyses. Because of the many factors affecting the amount of effluent needed, a significant amount of thought should be put into the volume of effluent to obtain and process at one time. It is prudent for the investigator to anticipate how many identification procedures will be done, and then calculate the volume of effluent needed using the particular test parameters desired, before extracting any effluent to ensure that sufficient volume of fractions, concentrates, and post-column effluent is available for the planned procedures. It may be best to perform these calculations with several different effluent volumes and test conditions to ascertain the optimal volume of effluent to fractionate. A worksheet to assist with these calculations and an example are provided in Appendix A.

The volumes of eluate needed for chronic toxicity testing at 2x, 1x, and 0.5x are provided in Table 2-5 for the *C. dubia* and fathead minnow short-term tests based on the methanol concentration that can safely be used for the chronic tests. The amount of SPE fractionation eluate needed for toxicity testing is presented for the range of tests that are commonly performed with *C. dubia* or fathead minnows, these volumes can be used in the calculation worksheets found in Appendix A (Table A-1). The approximate volume of effluent that will be needed for testing with *C. dubia* and fathead minnows is listed in Table 2-6 for various fractionation schemes and toxicity testing parameters. When only a portion of the TIE procedures will be used, obviously less effluent volume will be needed. In Table A-2, the example calculations are based upon the use of minimum elution volume for the SPE columns (Table 2-1), concentrating only one SPE fraction (Section 2.3.9), and taking into account the toxicity testing (Sections 2.3.8, 2.3.9, 2.3.11, and 2.3.12) and GC/MS analysis volumes (Section 2.5). These parameters are discussed in detail below. If additional eluate is needed, the chronic tests must be repeated for each fractionation. In Phase II and Phase III more confidence in the toxicity estimates is needed than in Phase I, therefore tests may require more replicates. The volumes needed for those tests are also presented in Table 2-5. When only limited amounts of effluent are available, one must be creative and plan its usage very carefully to obtain meaningful results.

2.3.2 Filtration

For filtration of chronically toxic effluents, the use of glass fiber filters (1 µm nominal pore size) is recommended. Both 45 mm and 90 mm diameter filters have been used routinely, but the 90 mm diameter filter allows about four times more effluent to be passed over one filter than the 45 mm filter. All filters and glassware should first be pre-rinsed with pH 3 high purity water to remove any residual metals followed by a high purity water (e.g., Milli-Q® Water System) rinse which is discarded. Low levels of metals (e.g., µg/l) from the filters may cause toxicity interferences and pre-rinsing the filters may provide cleaner blanks and less contamination in effluent samples. To collect the dilution water filter blank, first pass a volume (~200 ml) of dilution water over the filter and discard it. Next, collect the volume of dilution water needed to conduct the filtration blank test. It is a good idea to prepare excess volume, at least 500 ml for the *C. dubia* 7-d test and 800 ml for the fathead minnow 7-d test. A portion of the filtered dilution water is collected for testing and a portion is reserved for the solid phase extraction test blank (Section 6.6; EPA, 1992).

Table 2-5. Eluate Volumes Needed for Chronic SPE Fraction Toxicity Tests with *Ceriodaphnia dubia* and *Pimephales promelas*

Test Species	Test Duration	Original Sample& No. Renewals	High Test Conc. of SPE Fraction	No. Rep	Volume (ml) of 500x Eluate Needed for Testing[1]	Test Concentrations
C. dubia	4-d	2	2x	5	0.70	2x, 1x, 0.5x
C. dubia	4-d	4	2x	5	1.40	2x, 1x, 0.5x
C. dubia	7-d	3	2x	5	1.05	2x, 1x, 0.5x
C. dubia	7-d	7	2x	5	2.45	2x, 1x, 0.5x
C. dubia	4-d	2	2x	10	1.40	2x, 1x, 0.5x
C. dubia	4-d	4	2x	10	2.80	2x, 1x, 0.5x
C. dubia	7-d	3	2x	10	2.10	2x, 1x, 0.5x
C. dubia	7-d	7	2x	10	4.90	2x, 1x, 0.5x
P. promelas	7-d	7	2x	2	4.90	2x, 1x, 0.5x
P. promelas	7-d	7	2x	4	9.80	2x, 1x, 0.5x
P. promelas	7-d	7	4x	2	9.80	4x, 2x, 1x
P. promelas	7-d	7	4x	4	19.60	4x, 2x, 1x

[1]Test volumes per replicate are 10 ml/cup for *C. dubia* and 50 ml/cup for *P. promelas*. The fraction test solutions are prepared as one solution and divided into aliquots for the replicates. For the 500x eluate concentration, this volume is based on the assumption that the *C. dubia* test solutions are prepared as 200 µl of 500x into 50 ml for 2x, 100 µl into 50 ml for 1x, and 50 µl into 50 ml for 0.5x. More volume will be needed if serial dilutions are prepared (400 µl vs 350 µl). For the fathead minnow tests this assumes test solutions are prepared as 400 µl into 100 ml for 2x, 200 ml into 100 ml for 1x, and 100 µl into 100 ml for 0.5x. More volume will be needed if serial dilutions are prepared (800 µl vs 700 µl). For the 4x fathead minnow test, 800 µl per 100 ml can be prepared in a similar manner.

Table 2-6. Approximate Effluent Volumes Needed for the Chronic Non-Polar Organic Identification Procedures[1]

Test Species	Test Duration	Original Sample & Number of Renewals	High Test Conc. in SPE Fraction Test	No. Rep.	Are Dilutions Used ?	Volume Effluent (ml) Needed to Conduct SPE & GC/MS[2] Analyses	Volume Effluent (ml) Needed to Conduct SPE & HPLC & GC/MS[3] Analyses
C. dubia	4-d	4	2x	5	Yes	3,000	15,000
C. dubia	7-d	3	2x	5	Yes	2,000	15,000
C. dubia	7-d	7	2x	5	Yes	5,000	20,000
P. promelas	7-d	7	2x	2	Yes	10,000	50,000
P. promelas	7-d	7	2x	4	No	5,000	40,000

[1]Calculation of toxicity testing volumes assumes that: 4x high concentration for SPE concentrate test (Section 2.3.9), 8x high concentration for HPLC fraction test (Section 2.3.11), 16x high concentration for HPLC concentrate test (Section 2.3.12), concentration of only one toxic fraction (SPE and HPLC), the maximum amount of sample is concentrated on the SPE columns and all SPE columns are eluted with the minimum elution volume.

[2]TIE procedures used: SPE fractionation and GC/MS of SPE concentrate.

[3]TIE procedures used: SPE fractionation, GC/MS of SPE concentrate, HPLC fractionation, and GC/MS of HPLC concentrates.

After the filtration blank has been obtained, the effluent sample is filtered using the same filter, a portion of the filtrate is collected for toxicity testing, and a portion is set aside for concentrating on the C_{18} SPE column. For some effluents, one filter will often not suffice. A technique we use to prepare several filters at once is stacking three to eight filters together in one filter holder, followed by sequential rinses with pH 3 high purity water, high purity water and dilution water (using the same rinse volumes as above). Finally, the filters are separated and set aside, using one at a time for the effluent sample. If the samples have high suspended solids concentration, pre-filtering using a larger pore size filter may help, and the appropriate blanks should be used. If the sample cannot be effectively filtered due to the presence of many fine particles, centrifugation may be used (of course, blanks must be prepared).

The filter housing should be thoroughly cleaned before use to prevent any particle build-up or toxicity carry-over from previous samples. We have found large filtration apparatus (1,000 ml), removable glass frits, or plastic filtering apparatus (e.g., Millipore®) to be useful. The glassware cleaning procedure that is described in the acute Phase I TIE manual (EPA, 1991A) is sufficient for chronic TIE work. The glass frits may require rigorous cleaning (i.e., soak in aqua regia for 20-40 min) to remove residuals that may remain after filtering, since the glass frit may itself act as a filter. Also available are removable stainless steel filter supports in a glass vacuum filter apparatus (available from Millipore®). These filter supports do not require as rigorous cleaning as fritted glassware, and therefore are a good substitute.

When effluent samples are readily filtered (~2,000 ml for one 90 mm 1 µm filter) it may be possible to filter the effluent for the filtration test of Phase I but then use unfiltered effluent with the C_{18} SPE column test and the methanol eluate test (Phase I). Once it has been demonstrated that filtration does not reduce toxicity, routine filtering of these effluents (before passing the effluent through the SPE column) can be eliminated, which will reduce the amount of toxicity testing required.

2.3.3 Column Size

Available C_{18} SPE column sizes and the appropriate water and solvent volumes used in their preparation are listed in Table 2-1. Positive pressure pumps are the most convenient to use for the large volume effluent samples because the flow rate can be controlled. Pumps and vacuum manifolds can both be used for eluting C_{18} SPE columns. Whichever system is used, it should be made of materials that dilute acid and solvents do not destroy, or from which chemicals are not leached that are toxic or interfere with analytical measurements. Teflon, glass, and stainless steel are all acceptable.

When SPE is used for isolating non-polar organic toxicants, use the maximum volume of effluent and the minimum elution volume for the column size selected to optimize the concentration of toxicants in the methanol eluates. For example, if 6,000 ml is processed, it is best to use one 5,000 mg column with 5,000 ml and one 1,000 mg column with 1000 ml of effluent and elute both columns with the minimum elution volumes (Table 2-1) and combine eluates. It is always best to process the maximum volume of effluent on each column to achieve the highest concentration of toxicants in the eluate.

2.3.4 C_{18} SPE Column Conditioning

The 10,000 mg C_{18} SPE columns (Analytichem International, Harbor City, CA) are conditioned by pumping 100 ml of methanol through the column at a rate of 40-50 ml/min. This size column can process 10,000 ml of effluent and is the largest commercially pre-packed SPE column available at this time. The example presented in this section will be for 10,000 ml effluent using a 10,000 mg SPE column. The volumes of conditioning solvent will change when other size columns are used, as shown in Table 2-1. We most commonly use methanol as the conditioning solvent but other water miscible solvents such as acetonitrile, ethanol, or isopropanol may also be used to condition columns. *Before the packing goes dry*, 100 ml of high purity distilled water must be added. As the last of that water is passing through, filtered dilution water is added. The volume of dilution water needed may vary from 250 ml to 1,200 ml depending on the species tested. The first 100 ml is discarded and the remainder is collected for the dilution water column blank. After the dilution water has been collected, pumping is continued until no water emerges from the column.

Low dissolved oxygen (DO) in the post-column dilution water blanks (even in reconstituted waters) has occurred during some chronic tests; therefore, we discard the first 100-200 ml and collect the remainder of the post-column dilution water. Low DO has been a problem, particularly in the fathead minnow growth test, and is attributed to the small amount of methanol that bleeds into the post-column sample. This may be alleviated by discarding the first post-column aliquots.

2.3.5 Elution Blanks

For chronic work, we have been using seven methanol/water fractions (50%, 75%, 80%, 85%, 90%, 95%, and 100%) rather than the eight used in acute TIEs. By eliminating 25% methanol/water fraction used in acute work the toxicity testing workload is reduced, in turn a reduction in separation of toxic and non-toxic components can occur.

To collect the fraction blanks from the 10,000 mg column, two successive 10 ml volumes of 50% methanol in water are pumped through the conditioned column and collected in one analytically clean labeled vial, to make a 20 ml sample. This procedure is repeated six more times with two successive 10 ml volumes of 75%, 80%, 85%, 90%, 95% and 100% methanol/water solutions. The column should be allowed to dry for a few seconds between each elution with the different 20 ml volumes of methanol/water mixtures. This will result in seven 20 ml blank SPE

fractions. The volume of methanol solutions used for elution will vary depending on column size as shown in Table 2-1.

2.3.6 Column Loading with Effluent

The same 10,000 mg column is reconditioned with 100 ml of 100% methanol and 100 ml of high purity water, as described in Section 2.3.4. The sorbent must be reconditioned when the maximum volume of dilution water has been passed over the column, otherwise the sorbents' capacity will be exceeded. After the high purity water rinse and *without allowing the column to dry*, 10,000 ml of filtered effluent sample is pumped through the column at a rate of about 40 - 50 ml/min.

Discard the first 100-200 ml of post-column effluent, to reduce the possibility of higher concentrations of methanol in post-column samples, which may contribute to artifactual toxicity. To evaluate the post-C_{18} SPE column effluent for toxicity, collect at least two aliquots (e.g., beginning and end) separately. If only small quantities (<500 ml) of post-column effluent are needed for toxicity testing (e.g., *C. dubia* test), several separate post-column effluent samples may be more helpful in determining if the toxicants are retained by the column. About 800 ml of post-column effluent is needed for the fathead minnow test if only one concentration (100%) of post-column effluent is tested for toxicity. If two concentrations (100% and 50%) are used, then the required volume for that species increases to 1,200 ml for each post-column aliquot. As Phase II progresses, the recommendation is to collect enough post-column effluent to conduct toxicity tests with dilutions.

2.3.7 C_{18} SPE Column Elution

To elute the C_{18} SPE column, two successive 10 ml volumes of the 50% methanol/water mixture are pumped through the column and collected in one labelled, analytically clean vial. Subsequently, two successive 10 ml total volumes of each of the 75%, 80%, 85%, 90%, 95% and 100% methanol/water solutions are pumped through the column and collected in separate vials. The next elution volume should not be added until no more of the preceding one is emerging from the column. This results in seven 20 ml SPE fractions. If one 5 g column and two 1 g columns are used to concentrate 7,000 ml of effluent, the corresponding fractions can be combined. For example, the 10 ml eluate of the 80% fraction from the 5 g column can be combined with the two 2 ml 80% fractions from the two 1 g columns. This applies to both sample and blank fractions for a total of 14 ml.

This entire procedure (conditioning through elution) is repeated using a second 10,000 mg C_{18} SPE column for a second 10,000 ml of filtered effluent. The dilution water column blank samples should be kept separate. The corresponding fractions from both the blanks and the sample from each 10,000 ml fractionation can be combined as described above. There will be seven 40 ml blank fractions and seven 40 ml effluent fractions, representing 20,000 ml effluent.

The vials containing the methanol/water fractions are sealed with perfluorocarbon or foil-lined caps, and stored at 4°C if not tested immediately. These fractions represent a "first cut" separation of effluent components. Volumes will vary if columns of different sizes are used or if the particular effluent under study or the research question posed dictates method modification.

2.3.8 Blank and Effluent Fraction Toxicity Tests

While the choice of test concentration depends on the toxicity of the effluent (Section 1.2), in most instances we have used a concentration of 4x or 2x as the high test concentration for testing SPE fractions. The high test concentration of the SPE fraction is in part controlled by the tolerance of the organisms to methanol. For chronic testing the concentration of methanol should be less than 0.6% for *C. dubia*, and less than or equal to1% for fathead minnows (see Phase I; EPA, 1992).

If the minimum elution volumes are used, typically SPE eluates are 500x effluent concentration. For fathead minnow testing, eluates can be toxicity tested at 4x effluent concentration by diluting 80 µl to 10 ml, which results in a 0.8% methanol concentration. For *C. dubia*, eluates can be toxicity tested at 2x the 100% effluent concentration by diluting 40 µl to 10 ml which results in a methanol concentration of 0.4%. If there is the need to toxicity test the 500x eluate with *C. dubia* at 4x then the SPE eluates can be concentrated by gently airing the eluate down (using nitrogen) to half its original volume. However, by using this procedure you risk losing the toxicant because of evaporation or insolubility. Also realize that when a water and methanol mixture is aired down, the percent methanol composition changes, because methanol will evaporate faster than water.

If toxicity occurs in any of the fraction blank tests and it is small relative to the toxicity in the corresponding sample fraction, the sample fraction results should not be dismissed. If all organisms die in the blanks and effluent fractions, dilutions of each should be tested to make sure the sample fraction is substantially more toxic than the blank. In general, blanks should not have measurable toxicity.

When the post-column effluent sample is toxic and the fractions are toxic at effluent concentrations of 1x or 2x, the toxicant could still be a non-polar organic compound. If the fractions are not toxic individually and the post-column sample is non-toxic, it is possible that the toxicity is spread out among the fractions. Combining and concentrating these fractions may be useful or other elution procedures may be necessary (Section 2.6). If the fractions are toxic and the post-column effluent is toxic, it is possible that the toxicant(s) is in the fractions, and that either an additional toxicant(s) is present in the post-

column effluent, that break-through of the toxicant(s) occurred, or that the toxicity is artifactual. If toxicity is recovered at 1x, 2x, or 4x and in one of the post-column effluent samples, it is possible that not all the toxicity is caused by non-polar organic compounds or the possibility exists of break-through in the post-column sample.

For the chronic TIE, the question of extraction efficiency cannot be as readily addressed as it is for the acute TIE (Section 2.2.8). Measuring the chronic toxicity of the post-column effluent will be limited by the species tested, the test volumes required for the test and the frequency of sample replacement. Without a measure of the toxicity in the post-column effluent, conclusions regarding extraction efficiency are difficult to make. The limitations created by this concern are addressed in Phase III (EPA, 1993A). Artifactual toxicity in the post-column effluent has been a problem in chronically toxic effluents as it was in some acutely toxic effluents. For a detailed discussion of this artifactual toxicity that appears as a biological growth and suggestions to avoid it please refer to Sections 2.2.6 and 2.3.4., and EPA, 1992.

At this point in Phase II, the effluent fractions should also be tested in water with TOC and suspended solids that mimic the effluent to lessen matrix effects on toxicity. As the identification step moves into Phase III, it is better to use dilution water that mimics effluent or receiving water characteristics.

2.3.9 SPE Fractions: Concentration and Subsequent Toxicity Testing

The SPE fractionation provides a general separation of non-polar organics and except in relatively uncomplicated effluents, GC/MS analysis of the concentrates of toxic C_{18} SPE fractions will result in very complicated chromatograms from which the toxicant(s) cannot be distinguished from other effluent components. A secondary fractionation using HPLC is often needed to further simplify toxic effluent fractions prior to component identification by GC/MS analysis.

In order to maximize the chromatographic separation capability of the HPLC, the volume of the sample injected onto an analytical size HPLC column should be as small as possible (i.e., ≤0.5 ml); therefore the toxic SPE fractions (usually >1 ml) must be concentrated prior to injection onto the HPLC column. This concentration step will provide the added benefit of an increase in concentrations of constituents in the HPLC fractions as well as rid the SPE fractions of water. The latter issue is important if GC/MS analysis will be performed on the concentrated SPE fraction prior to injection on the HPLC.

The volume of the SPE fraction and the number of toxic fractions to be combined will determine which size SPE column will be used for the concentration procedure. Table 2-3 contains information on column sizes and the appropriate volume of conditioning and eluting solvents we have found to be most useful. In the procedures outlined below we have used a 200 mg SPE column to concentrate one 40 ml toxic fraction from two 10,000 mg SPE columns. Often the toxic effluent SPE fractions are combined and diluted with high purity water. If enough toxicity occurs in each fraction it may be more useful to concentrate each fraction separately for subsequent HPLC separation. The corresponding blank fractions are similarly treated. The percent methanol in the diluted fraction sample should be ≤20% and the volume to which the fractions can be diluted is dependent on the amount of column packing. For example, the total volume of the diluted fraction(s) should not exceed 200 ml for the 200 mg C_{18} column (Table 2-3).

A 200 mg C_{18} column is conditioned with 2 ml of methanol and rinsed with 2 ml of water similar to the procedures described in the SPE Column Conditioning Section (2.3.4). The diluted blank fractions are then drawn through the 200 mg C_{18} SPE column under a pressure of 380 mm Hg using a vacuum manifold. When processing larger volumes, or using larger columns, positive pressure can be used. The solution passing through the column cannot be tested for toxicity because of its high methanol concentration (e.g., 10-20% methanol). The column is then dried for 10 min using a gentle flow of nitrogen (10-20 ml/sec). Drying the column usually increases the recovery of toxicity, but sometimes toxicity is not recovered from the column possibly because of volatilization. If this occurs, the concentration procedure can be repeated without the nitrogen drying step.

After drying the sorbent, the luer tip of the column is fitted with a luer-lock needle (to ease collection of small volumes) and 200 µl of 100% methanol is placed into the column using a microliter syringe. Nitrogen is then applied to the column at a rate of ~4 ml/sec to force the methanol through the sorbent, which is then collected in a small glass vial. The first 200 µl aliquot of methanol applied to the column will yield approximately 125 µl of eluate. Two more 200 µl aliquots (applied separately) of 100% methanol are also forced through the column. The final volume of eluate collected will be approximately 440 µL. Measure the exact volume collected using a µl syringe or pipet. As in most chromatographic separations and extractions, three separate smaller elutions of methanol are more efficient than one large one.

The 200 mg C_{18} SPE column is reconditioned following the directions given above in Section 2.3.4. It is then used to concentrate the diluted toxic SPE fractions, using the same sequence used for the blank fractions (Figure 2-4). The concentrated blank fractions will serve as the dilution water column blank because it cannot be obtained for chronic toxicity testing as it can for acute testing.

When the total volume of fractions is above 40 ml, larger columns should be used; consult Table 2-3 for column size and elution volume information. The size of the column used for concentrating should be chosen to maximize concentration in the eluate. Therefore, choose the smallest column appropriate for the diluted fraction volume.

If there is a large toxicity loss after the concentration step, it is possible that the percentage of methanol in the diluted fraction was too high. The concentration procedure should then be repeated with a new set of toxicity tested SPE fractions diluted to a lower methanol concentration (e.g., 10%). Both the effluent and blank concentrates are toxicity tested at each step to track toxicity. Generally we suggest that this toxicity test be at least at two times higher than the concentration used in the first SPE fraction test. The tests are conducted exactly as the SPE fraction tests.

If an original effluent volume of 20,000 ml (using two 10,000 mg SPE columns) is now represented by a 440 µl concentrate, then the sample is 42,670x more concentrated than the effluent (accounting for volume removed for toxicity testing, see Table A-2 example). If 1 µl of concentrate is diluted to 10 ml in dilution water, the resulting test concentration will be about 4x whole effluent. However, the 4x test solution should be prepared as one sample before solutions are split among replicates. For example, 5 µl is diluted to 50 ml for five replicates with the *C. dubia* test described above (Table A-2). Additional test concentrations (e.g., 2x, 1x, 0.5x) can then be prepared to determine an IC25 or IC50 of the concentrate, and toxicity recovery can be calculated by comparing this value to the toxicity of the effluent. The concentrate toxicity might be higher than the sum of the individual toxic fractions because some of the toxicant may have been in adjacent fractions that were concentrated in the first step (Section 2.3.7) but not detectable by the toxicity test of the single fraction. The concentrate toxicity may also be lower than expected because of low extraction and elution efficiencies.

The important concern here is not 100% recovery of toxicants but enough recovery for GC/MS analyses to be successful and to obtain measurable toxicity in the HPLC fractions. If recovery is too low, changing or eliminating the column drying time may help. Sometimes recovery appears to increase with drying time while other compounds are volatilized from the column during the drying process. For concentrates analyzed using GC/MS, column drying to remove water is critical to GC column performance.

2.3.10 HPLC Separation

The same column packing functionality should be used in the HPLC column as is used in the SPE column, such as C_{18}. At later stages, when more is known about the toxicants, other sorbents might be more appropriate.

The HPLC conditions presented in this section are general. An important consideration of HPLC fractionation is the number of HPLC fractions to collect. Since chronic toxicity testing is very time consuming, deciding the appropriate number of fractions to collect is an important step. However, when choosing which collection scheme to use, keep in mind the trade-off between separation and toxicity testing load. When the fraction volume is increased (toxicity testing load decreases) the separation of the toxicants from the non-toxic components decreases. We have used a 20 min separation gradient with the collection of 20-1 ml fractions. There are many other collection options that could be used, such as 10-2 ml fractions or 4-5 ml fractions using the same separation gradient. As information on the effluent is gained, HPLC conditions should be modified from the general conditions described below, to achieve better separation and higher concentration factors.

We use a flow rate of 1 ml/min on an instrument equipped with a 5 µm C_{18} reverse phase column (250 mm x 4.6 mm i.d.). The HPLC elution conditions will change depending on which SPE fractions have been concentrated. An example of HPLC conditions for commonly toxic SPE fractions is listed in Table 2-7. First, the blank concentrate is injected (≤500 µl) and 20-1 ml fractions are collected in analytically clean glass vials. Depending on the size of the HPLC injector and column, more than one HPLC fractionation run may be required to fractionate the entire blank concentrate. When multiple HPLC fractionations are conducted, collect and combine all the corresponding HPLC fractions in the same set of vials. For example, if two HPLC fractionations were performed for the blank concentrate, 20-2 ml HPLC blank fractions would be obtained. The same procedure is followed using the effluent sample concentrate. The vials should be sealed (e.g., with foil lined caps) and stored at 4°C if not tested immediately. As soon as toxicant identification is obtained by GC/MS (Section 2.5), then HPLC conditions (gradient, fraction size, and number of fractions) can be optimized.

Table 2-7. Example HPLC Elution Gradient for SPE Fractions from Chronically Toxic Effluent Samples

80 or 85% SPE Fractions	
Time (min)	% Methanol/Water
0	80
10	90
12	100
20	100

2.3.11 HPLC Fraction Toxicity Tests

In the HPLC fraction toxicity tests for chronically toxic effluents, the methanol in the HPLC fractions is one of the limiting factors of the concentration of the fractions that can be tested. Each fraction is assumed to be 100% methanol to calculate the necessary dilution. A 0.6% methanol concentration or less can be tested with *C. dubia*, while a 1% or less methanol concentration can be tested with fathead minnows.

In a chronic TIE with *C. dubia*, when all of the SPE concentrate remaining after toxicity testing from 20,000 ml effluent is injected on the HPLC (one injection),

each resulting 1 ml HPLC fraction equals 15,575 ml of effluent (assuming the toxicant elutes in only one fraction, see Table A-2). If 11 µl of each HPLC fraction is then diluted to 10 ml, the test concentration is 16x the original effluent concentration. However, the 16x solution should be prepared as one sample before aliquots are split to provide replicates. For instance, in the example used above, 55 µl should be diluted to 50 ml, which is then equally distributed into five test cups. Additional concentrations are prepared in a similar fashion to estimate the IC25 or IC50 and to compare toxicity recovery to the toxicity of the sample. Of course, some loss of toxicant will occur in each step and the toxicity may be less.

The blank fractions should not be toxic. If they are, then additional tests with dilutions must be conducted on both blanks and toxic fractions to find out whether there is enough additional toxicity in the sample fractions to warrant analysis.

The toxicity of the HPLC fractions should be tested at twice (at least) the concentration at which the SPE fraction concentrates were tested because recovery of toxicity and analytical measurements indicates that up to 50% of the initial concentration of toxic compounds may be lost in this step (Durhan et al., 1990). The concentration of methanol should not exceed the amount used in the SPE fraction tests described above (Section 2.3.8).

2.3.12 HPLC Fractions: Concentration and Subsequent Toxicity Testing

The toxic HPLC fractions and their corresponding blanks must be concentrated in a solvent suitable for GC/MS or other analytical techniques. Use the procedure described in Section 2.3.9, Concentration of Fractions (Figure 2-6). Judgement must be used to decide whether to concentrate each toxic fraction separately or to combine various toxic fractions prior to concentration. If, for example, two successive fractions are toxic, there is a good probability that the same toxicant is present in both. If one toxic fraction is separated from the other by several nontoxic fractions, there is a high probability that they contain different toxicants. There is also a good probability that at least one nontoxic fraction on either side of the toxic fractions contains some of the toxicant. The advantage of combining fractions is to reduce the workload and to increase the amount of toxicant in the concentrate. The disadvantage is that more constituents that are not the toxicant will be included. The decision has to be based on trial and error and experience. Blanks corresponding to the toxic fractions are concentrated the same way.

The HPLC fraction and blank concentrates should also be checked for toxicity before analysis on the GC/MS. Generally, we suggest that these toxicity tests be done at concentrations at least 2x higher than the concentration used in the previous HPLC fraction tests. Hopefully, the amount of concentrate available will be enough to conduct the toxicity test and perform a GC/MS analysis. Dilutions of the concentrate may be useful to compare toxicity of this concentrate to each previous toxicity test result. The HPLC concentrate (of 20,000 ml effluent) is now 48,495x more concentrated than the effluent (see Table A-2). If 3 µl is diluted to 10 ml the resultant test concentration will be about 16x the original sample concentration. This 16x solution should be prepared as one solution before aliquots are removed for the replicates. For instance, 15 µl is diluted to 50 ml for use in the example given above, then split into five 10 ml test cups. It is prudent to verify toxicity in the HPLC concentrate before it is subjected to GC/MS analysis. Whether the toxicant is detected by the analytical detector is always a question. Since GC/MS detects only about 20% of organic chemicals (EPA, 1989B), even such a broad spectrum method is no guarantee that the toxicant will be identified.

2.4 GC/MS Analyses

Procedures and methods provided in this section are based upon our experience in performing GC/MS analyses on fractions from numerous effluents and are applicable to both acute and chronic toxicity identification. In general, these procedures should be used.

A GC/MS system equipped to perform standard chemical residue analyses is suggested; i.e., a 30 m capillary column, electron impact ionization, scan range of 50-500 amu, scan rate of 1 or 2 scans/sec, a GC temperature program of 50 to 300°C at 5°C/min, and a data system with library searching capability.

Prior to GC/MS analysis, the prepared blank and toxic fraction concentrates should be tested for toxicity (Figure 2-6). After verification of the toxicity in the methanol concentrate, inject 1 or 2 µl of the concentrate (to which an internal standard has been added) and collect the mass spectral data. Note, methanol is not a typical solvent for GC analysis and the injection of methanol on a capillary column will shorten the column's life. Therefore, routine GC/MS QA/QC procedures should be followed closely to monitor the performance of the column.

The mass spectral data should be collected, the chromatogram integrated, and all detected peaks library searched. Reverse search is preferred. Concentration estimates for all chromatographic peaks can be obtained by using the response factor of the internal standard. Usually the internal standard is added to a small aliquot (10-20 µl) of the concentrate prior to GC/MS analysis. The selection of internal standard to use is an individual choice, and many different standards are available. An external standard method could also be used for deriving concentration estimates.

The NIST (National Institute of Standards and Technology, Gaithersburg, MD) mass spectral library has been used in ERL-D for performing library searches. Other mass spectral libraries are available, but some of the larger libraries contain multiple spectra for some of the compounds in the database. Library searching results that contain multiple identifications of the same com-

pound are not as useful as those obtained using the NIST library.

Once the library search results are available, the search report for each peak must be examined to decide whether the identification by the search is valid and reasonable. The help of a trained GC/MS chemist is required to do this evaluation. Questions we consider in our laboratory when performing this process include: A) are all major ions present in the correct proportions?, B) is this identification consistent with other information about the fraction?, C) do forward and reverse searching provide similar fits? and D) are the library searching fits greater than 70%? Factor A *must* be met! Consistency, factor B, considers circumstances such as "has the identified chemical been found in vastly different fractions," or "has the same identification been given to numerous peaks in the same chromatogram?" Both factors C and D are somewhat relative and depend a great deal on the sample and its matrix. In addition, the toxicants are often very minor components in the GC/MS total ion chromatogram and thus, the quality of the mass spectral data even after background subtraction can lead to poor results for factors C and D.

After examination of the library search results, a list of identified chemicals is assembled and evaluated using the methods in the following section. For the confirmation analyses we suggest EPA method 625 (EPA, 1982).

2.5 Identifying Suspect Toxicants

If one toxicant is identified, then the goal of the rest of Phase II is to determine if there are any other toxicants contributing to effluent toxicity. Two parallel lines of investigation should be pursued to achieve that goal. The first is to determine whether or not the concentration of the suspect toxicant is sufficient to cause toxicity (EPA, 1993A). The second is to estimate the proportion of the effluent toxicity that is caused by the suspected toxicants, so that a decision can be made as to whether other toxicants are present in the effluent.

The first line of investigation should begin by comparing the estimated concentrations of identified chemicals in the SPE or HPLC concentrate to their known toxicity values. Recovery of 100% of each effluent toxicant in the C_{18} SPE fractions may not be crucial, because at this stage, only the estimated concentration of compounds in the fraction and the toxicity of the fraction are compared. Assumptions about the concentration of toxicant(s) in the whole effluent are not made at this point, nor is any statement made regarding recovery of whole effluent toxicity in C_{18} SPE column fractions. In later stages of Phase II, inferences regarding the relationship between the concentration of the suspected toxicant(s) in whole effluent and the observed toxicity in the SPE fractions are made. At this step, the compound quantification will have been performed using an internal or external standard response and since the compound's recovery is unknown, considerable error may be involved in the concentration estimate. Secondly, the toxicity data, if available, may be for a different species than that used in the TIE. Species differences are usually as large as 100-fold and often 1,000-fold. Given these two sources of uncertainty and the chance that they may reinforce one another, certainly if the estimated concentration of a chemical accounts for the toxicity within a factor of 100, the chemical should remain a suspect. To the extent that data for either quantitation or toxicity values of the compound are known to be better, concentration differences of smaller magnitude may be used to eliminate suspects.

Once a list of suspects is available, the measurements for both concentration and toxicity should be refined. This will usually require obtaining pure compound to make better analytical measurements and to establish acute or chronic toxicity estimates for the species of concern. This step requires as much separation as practical before analysis so that the list of suspects is small.

At this stage, only the concentration of the suspected toxicant(s) in the concentrate is known; until recovery through all the fractionation and concentration steps is complete, suspect compound concentrations in whole effluent are not known. Since the concentrate is virtually devoid of suspended solids and much of the effluent TOC, both of which may dramatically affect toxicity of non-polar organics, the toxicity of non-polar chemicals may be quite different in the fraction tests than in the effluent test. Therefore, the toxicity of suspects in the fraction test should be compared to the suspect's toxicity in a relatively pure water, such as reconstituted water.

During this same stage, the steps leading to the final concentrate should be checked for toxicity recovery. The objective is to place a good estimate on how much of the whole effluent toxicity is contained in the final concentrate. This is best done by testing the toxicity of the concentrate at concentrations near those of whole effluent, correcting for volume losses due to toxicity testing SPE column fractions (which was previously ignored). If the toxicity of the final concentrate is similar to that of whole effluent, allowing for losses, and if the concentration of the suspect(s) is sufficient to account for the concentrate's toxicity, it is time to begin Phase III (EPA, 1993A). If multiple toxicants occur, the toxic units of each are compared to the whole effluent toxic units.

If the concentrations from quantitation and toxicity measurements are close to one another, Phase III procedure should be started, recognizing that other toxicants may yet be identified. If no suspects are found, more concentration, more separation, and possibly different or more sophisticated analytical methods must be used. In some of the effluents we have tested, finding other candidates has taken months and concentration factors of >100,000 have been required. Since few laboratories will have all the needed analytical equipment, instrumentation from other sources should be considered.

Because artifactual toxicity that equals toxicity due to lost or unidentified toxicants can be created, as one progresses to Phase III the suspect toxicant should be identified. One purpose of Phase III is to identify such errors. Should this error occur, one must start again at the beginning of Phase II, or even return to Phase I. If several different effluent samples were evaluated during Phase II, redoing Phase I on additional samples may be time well spent since the effluent may have changed in the interim.

In practice there is no sharp boundary between Phases II and III. In general, as soon as a probable suspect is identified, confirmation procedures of Phase III should begin. If a toxicant has been assumed to have been identified when it has not, the identification of other suspected toxicants can be hampered.

A final suggestion is to investigate the additivity of toxicity for several constituents, if all toxicity is not accounted for. Enhancement of toxicity by methanol should also be checked.

2.5.1 *Identifying Organophosphate Pesticides*

Certain compounds must be metabolically activated by the test organism before they become toxic. These activation reactions consist of oxidative metabolism by a family of enzymes collectively known as cytochrome P-450. Compounds such as piperonyl butoxide (PBO) can block the toxicity of metabolically activated toxicants making it a useful tool in the TIE. PBO is a synthetic methylenedioxyphenyl compound that effectively binds to and blocks the catalytic activity of cytochrome P-450. Thus, when a nontoxic amount of PBO is coadministered with the effluent or the effluent fractions that exhibited toxicity, the toxicity of the compound requiring metabolic activation is greatly reduced or completely blocked (Ankley et al., 1991).

Phosphorothioates are organophosphates known to require cytochrome P-450 activation before expressing toxicity and include common insecticides such as diazinon, malathion, parathion, methyl parathion and fenthion. There also are a number of organophosphates that are toxic in the absence of metabolic activation; these include insecticides such as dichlorvos, mevinphos and chlorfenvinphos.

We have found organophosphate insecticides present in effluents and ambient waters at acute and chronic toxicity levels (Amato et al., 1992; Norberg-King et al., 1991). The toxicity of most organophosphates will be removed from the sample by the C_{18} SPE column, and they are typically recovered in the methanol eluates (see EPA, 1991A; EPA, 1992). The addition of PBO to the effluent before addition of the test organisms was used as a subsequent test in Phase I (EPA, 1991A; EPA, 1992). In addition to the C_{18} SPE column removing the toxicity, a reduction in toxicity with the addition of PBO would suggest the presence of metabolically activated compounds such as organophosphates. PBO has similar utility in Phase II of the TIE in that either SPE fractions (Sections 2.2.8 and 2.3.8) or HPLC fractions (Sections 2.2.11 and 2.3.11) can be tested for toxicity both in the presence and absence of PBO. A reduction in toxicity of the test fraction would suggest the presence of a metabolically activated chemical, and together with chemical analyses, can provide powerful evidence along with GC/MS data, for specific organophosphates as the toxicant(s). While PBO should be useful for both acute and chronic TIE work, most of our experience has been in the area of acute toxicity. Thus, guidance presented below is based mainly on acute tests.

Toxicity values for PBO are presented in Phase I (EPA, 1991A; EPA, 1992). In acute toxicity tests, concentrations of PBO ranging from 250-500 µg/l have effectively blocked the acute toxicity of relatively large concentrations of metabolically activated organophosphates to cladocerans (Ankley et al., 1991). In chronic toxicity tests with *C. dubia*, PBO concentrations of 50 µg/l have been effective in blocking toxicity in the SPE fractions. Detailed information on stock solution preparation is presented in the Phase I documents and is not repeated here.

When toxicity tests are conducted on SPE fractions or HPLC fractions, aliquots of the PBO solution are added to the test solutions and mixed well before the test organisms are added. As for any TIE manipulation, the successful use of PBO is dependent upon the use of appropriate controls and blanks. Effluent fractions and blank fractions with and without the addition of PBO must be tested simultaneously. A reduction in toxicity of the effluent fraction occurring with the PBO added, and no toxicity exhibited in either of the blanks, indicates that the toxicant requires metabolic activation to exhibit toxicity. If toxicity associated with the PBO addition is observed in the blank fraction, either PBO was present at toxic concentrations or the methanol concentration (from fraction and/or PBO stock addition) in the test was too high. If toxicity is observed in the effluent fraction with PBO added, but not in the effluent fraction without the PBO or in either of the blank fractions, this result is essentially meaningless. In the latter situation 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.

2.5.2 *Identifying Surfactants*

The goal in this section of Phase II is to identify the toxicants when surfactants are implicated by Phase I and Phase II results. The Phase I procedures of filtration, aeration, and C_{18} SPE all affect surfactant toxicity, and effluent samples that exhibit several or all of these behaviors may contain toxic concentrations of surfactants (EPA, 1991A).

Surfactants are surface active agents that have a molecular structure that includes a polar, hydrophilic segment (either ionic or nonionic) and a relatively large nonpolar, hydrophobic, hydrocarbon segment. Surfactants are used for a variety of household and industrial purposes and therefore are ubiquitous in effluents, particu-

larly in untreated wastewater, and potentially could be present at toxic concentrations in effluents (Ankley and Burkhard, 1992). Some examples of surfactants are soaps, detergents, charged stabilization polymers, and coagulation polymers used in chemical manufacturing processes. The molecular structure of surfactants causes them to congregate at interfaces between water and other phases such as air, oily liquids and particulate matter. This congregative characteristic is responsible for the cleansing and dispersive properties of surfactants.

There are many different kinds of surfactants and they are classified by the nature of their polar segment. When in aqueous solution, the polar segment of a surfactant molecule can be either nonionic (not charged) or ionic (charged). The ionic polar segment can be either negatively charged (anionic), positively charged (cationic), or both negatively and positively charged (amphoteric). Based on this, surfactants are classified into the following major classes: nonionic, anionic, cationic, and amphoteric.

Surfactants physical/chemical properties set them apart from both strictly polar or non-polar organic compounds and these properties uniquely influence the results of Phases I and II procedures for surfactants.

Experiments were conducted with a small sample of surfactants from nonionic, anionic, and cationic categories with the Phase I procedures of filtration, aeration, and C_{18} SPE (Ankley et al., 1990A). In these experiments, filtration removed the toxicity of most of the surfactants tested to some degree, and the degree of removal is most probably dependent on sample matrix, especially solids concentration. Aeration removed the toxicity of all the surfactants tested to some degree while the C_{18} SPE column removed the toxicity completely for all surfactants regardless of class. Surfactants behave unpredictably with regard to elution from C_{18} SPE columns. For example, toxicity from surfactants of the nonionic and anionic classes, eluted in all fractions 80% to 100% methanol/water (Ankley et al., 1990A). Elution in several fractions rather than eluting in one or two fractions may be caused by the polar/non-polar nature inherent in surfactants. The toxicities from the cationic surfactants were either not recovered in any of the fractions or were recovered to only a small degree in the 100% methanol fraction.

Important indicators of surfactant toxicity are the toxicity test results from aeration experiments. If volatility can be eliminated and toxicity is reduced by aeration, this is strong evidence that a surfactant might be contributing to effluent toxicity (EPA, 1991A). During aeration, surfactants are most probably removed from solution by the process of sublation. Sublation occurs because surfactant molecules tend to congregate at the interface between the aqueous sample and the aerating nitrogen or air bubbles and are brought to the surface of the liquid sample by the bubbles. At the liquid surface the bubbles break releasing the surfactant, which then adheres to the aeration vessel walls. A compound that can be removed by sublation is by definition a surfactant. It might be possible to recover surfactants from glassware after the sublation process. The glassware can be rinsed with a solvent such as methanol, which can then be toxicity tested and analyzed in the same manner as methanol SPE fractions (Sections 2.2.8 and 2.3.8).

Overall, most surfactants exhibit some of the behavior that is common to non-polar organic compounds such as removal from the effluent by the C_{18} resin and recovery in the methanol/water SPE fractions. While surfactants in general can be considered to be non-polar organics, GC/MS analysis will probably not provide successful surfactant identification. Most surfactants are not readily chromatographed because of the polar segment of the surfactant molecule. One exception is a class of surfactants in common use that can be analyzed directly by GC/MS, the alkylphenol ethoxylates. Gieger et al. (1981), provides mass spectral data for the nonylphenol mono-, di- and tri-ethoxylates, which can be used to help identify these compounds. Techniques such as derivatization can make some other specific surfactants compatible with GC and GC/MS, but it is necessary to know the specific identity of the surfactant.

It is difficult to positively identify an unknown surfactant. Although there are many analytical methods available for accurately quantifying specific surfactants, these methods are useful only if the identity of the surfactant is known, or at least suspected. It is not reasonable or practical to analyze a sample using numerous intricate methods, in the hope that one of these methods will detect the surfactant in the sample. Unfortunately, there is no analytical technique available that can readily provide the identity and quantity of an unknown surfactant. Environmental samples (such as municipal and industrial effluents) contain numerous substances that can interfere with available analytical methods. Also, *pure* surfactants are actually mixtures of homologous and oligomers with varying chain lengths and, in the case of many nonionic surfactants, varying degrees of ethoxylation. The composition and therefore the toxicity of such a mixture might vary. In the course of a TIE, it might become necessary not only to identify the surfactant causing toxicity, but also to learn which particular homologue or oligomer is the most toxic.

One approach to reducing the complexity of identifying an unknown surfactant is to determine whether the unknown surfactant falls into the anionic or nonionic class. APHA (1989) describes a method for determining anionic surfactants as methylene blue active substances (MBAS). MBAS method can successfully measure the concentration of anionic surfactants of the sulfonate type, the sulfate ester type, and sulfated nonionics type. Unless the identity of the anionic surfactant is known, the analytical measurement is calculated and expressed in terms of the anionic surfactant linear alkylbenzene sulfonate (LAS). APHA (1989) also describes a method for determining nonionic surfactants as cobalt thiocyanate active substances (CTAS). This method is applicable to a wide

range of polyether nonionic surfactants, which includes the widely used alkyl and alkylphenol ethoxylated alcohols.

With these methods the relative amount of anionic or nonionic surfactant can be estimated, but the exact nature or molecular composition of the unknown surfactant will not be determined. These analyses can be conducted on the SPE fractions, HPLC fractions, fraction concentrates, and the whole effluent. Determining the class can be significant progress toward identifying the unknown surfactant. With the class known, specific analyses for the more common surfactants in that class can be performed as a subsequent effort. Unless the identity of the nonionic surfactant is known, the analytical measurement is expressed in terms of an arbitrarily chosen reference nonionic surfactant.

The type of discharge being processed by the wastewater treatment plant might provide information that would enable one to target specific surfactants for analysis. For example, industries feeding into the treatment plant might be discharging certain surfactants or a particular kind of surfactant that is being used in the manufacturing or housekeeping processes. An analytical method suitable for that particular surfactant could then be used to determine whether toxic concentrations can be found in the toxic effluent, fractions, or concentrates.

2.6 Alternate Fractionation Procedures

If toxicity is not recovered in the methanol procedures described above (Sections 2.2 and 2.3), and toxicity is not observed in the post-column effluent, alternative elution procedures can be used. These procedures are not as widely used as the methanol/water elutions discussed above but are effective for highly hydrophobic compounds.

2.6.1 Modified Elution Method

The current Phase II method for fractionating non-polar organic toxicants in aqueous samples does not effectively fractionate compounds that are highly hydrophobic. Modifications made to the method have been successful in overcoming this limitation (Schubauer-Berigan and Ankley, 1991; Durhan et al., 1993). Hydrophobic compounds probably are more prevalent in sediment pore waters than in treated effluents. Tracking toxicity caused by these kinds of compounds will be more difficult because of the potential for artifactual toxicity from the solvents required to elute them. An elution scheme incorporating water, methanol, and methylene chloride has been designed that effectively fractionates compounds over a log K_{ow} range from 2.5 to 6.9. The higher log K_{ow} compounds, however, elute in the same set of fractions. Further fractionation by HPLC might be necessary to achieve better resolution of these kinds of compounds. Substituting other sorbents for the currently used C_{18} SPE resin have also produced encouraging results. Both the C_8 SPE and XAD-7 (Rhom and Haas, Philadelphia, PA) sorbents might have utility with particular kinds of toxicants.

The modified elution scheme eliminates the 100% methanol fraction used in the original method, and replaces it with one 50% methylene chloride/methanol, and three 100% methylene chloride fractions (v/v). The composition of the resulting eleven 3 ml (when using a 1,000 mg C_{18} SPE column) fractions is shown in Table 2-8. The methylene chloride containing fractions are combined, then solvent exchange is conducted as described below. The modified elution scheme would be used when the original methanol/water elutions did not effectively elute toxicity in the SPE fractions. In addition, if the suspect toxicants were known to be highly hydrophobic, as in sediment pore water, then the modified elution scheme would be indicated. Blank toxicity should provide insight concerning artifactual methylene chloride toxicity; however, slight reductions in young production might occur in both the blanks and sample fractions. Development of this alternate procedure for chronic toxicity is underway for the *C. dubia* and should be used with caution at this time. If this procedure is used, it is important to accompany the solvent exchanged methanol blank with a methanol only blank.

When toxicity testing SPE fractions, it is always a concern that the matrix of the effluent has been changed and that chemicals might become bioavailable, whereas they were not in the original sample. If this were to happen, the fractions might be more toxic than expected and chemicals might be added to the suspect toxicant list erroneously. This kind of mistake should be caught by obtaining a good toxicity value for the suspect toxicant in an appropriate matrix. For instance, if the suspect toxicant is highly insoluble in water, then when tested in an effluent matrix it should have low toxicity because it is unavailable to the organism. The alternate solvent elution might enhance this problem because the solvent is more likely to solubilize the more hydrophobic compounds than

Table 2-8. Composition of 11 Recommended Fractions in Modified Elution Scheme

Fraction	Composition of Eluting Solutions (% v/v)		
	Water	Methanol	Methylene Chloride
1	75	25	0
2	50	50	0
3	25	75	0
4	20	80	0
5	15	85	0
6	10	90	0
7	5	95	0
8	0	50	50
9	0	0	100
10	0	0	100
11	0	0	100

methanol. Therefore, additional confirmation steps might be needed to eliminate the false suspects.

2.6.2 Solvent Exchange

Since methylene chloride is quite toxic to aquatic organisms, even at very low concentrations (NOEC for *C. dubia* is 0.03%), it must be removed from SPE fractions before the fractions can be tested for toxicity. The exchange of the methylene chloride fraction into methanol is a relatively easy process because of methylene chloride's volatility. The combined fractions to be exchanged (e.g., 15 ml) are placed in a centrifuge tube with a teflon stir bar and an additional 15 ml of methanol. The tube is placed in a 30°C water bath and stirred while a gentle stream of nitrogen is passed over the solution surface. When the volume of the solution reaches 3 ml, the sides of the tube are carefully rinsed with an additional 3 ml of methanol, and the solution is reduced again to a final 3 ml volume. Adjust the volume of methanol used in this procedure to reflect the total volume of combined fractions. The final volume of methanol may then be tested as suggested previously in Sections 2.2.8. and 2.3.8. It is important to obtain and toxicity test a methanol-only blank in addition to the solvent exchanged methanol blank.

2.6.3 Alternative SPE Sorbents and Techniques

In the SPE method described above, C_{18} bonded silica is used as the solid phase for fractionating and isolating non-polar organic toxicants. C_{18} bonded silica was selected because, with proper conditioning, it does not usually contribute artifactual toxicity to sample or sample fractions, it often achieves the required degree of separation and isolation of non-polar organic compounds, and it is commercially available in inexpensive, easy to use, disposable columns. There is, however, no restriction on the solid phase that is used in the TIE procedure, as long as it results in the isolation and separation of non-polar organic toxicants and at the same time does not contribute artifactual toxicity. We have evaluated several sorbents other than C_{18} bonded silica to use for this purpose (Durhan et al., 1993).

We evaluated two prepurified XAD sorbents, XAD-4 and XAD-7 (Rohm and Haas, Philadelphia PA) and a C_8 bonded silica sorbent. Of these sorbents, only XAD-4, a non-polar styrene-divinyl benzene copolymer performed as well as C_{18} bonded silica in the fractionation of non-polar organic compounds. One disadvantage of using an XAD sorbent such as XAD-4 is that it is not commercially available in prepacked disposable columns. In addition, it is important to obtain prepurified XAD-4 sorbent that is free of toxic artifacts, otherwise extensive, time consuming cleanup procedures are required before the sorbent can be used in a toxicity based fractionation. We found that on XAD-7, an acrylic ester copolymer, non-polar organic compounds were inadequately fractionated because of resolution and co-elution problems. The C_8 bonded silica yielded results that were similar but significantly inferior to those obtained with C_{18} bonded silica.

Traditionally, SPE is carried out with the solid phase particles packed in a cylindrical column or cartridge. An alternative form of SPE has been developed, the Empore™ Extraction Disk, in which C_{18} bonded silica particles are enmeshed in an inert PTFE matrix which is then formed into a disk. The manufacturer (3M, St. Paul, MN) claims good recovery of non-polar organics with flow rates as high as 100 ml/min, which would make this an attractive alternative form of SPE. We have evaluated this technique to a limited degree with acutely toxic effluents and sediment pore waters and feel it has great potential in a toxicity based fractionation scheme. Especially attractive is the high flow rate which would allow for large volumes of sample to be processed quickly. However, a procedure for eluting non-polar organics from the disk into several fractions has not yet been developed and could prove to be a challenge.

Section 3
Ammonia

3.1 General Overview

Unlike Phase II procedures for non-polar organic compounds or metals, the toxicant identification methods described in this section are specific for ammonia. The procedures used in this phase of the study assume that Phase I tests and ammonia measurements (see below) have implicated the pH sensitive toxicant, ammonia as causing the acute or chronic toxicity (see Phase I; EPA, 1991A; EPA, 1992). Other compounds with toxicities that increase directly with pH may lead to confounding results or may give results similar to ammonia. For instance, experiments at our laboratory have shown that *C. dubia* are more acutely sensitive to cadmium, nickel, and zinc in acute tests at high pH levels (Section 4). The testing in Phase II should help to discern the toxicity caused by ammonia from that caused by other compounds that might also become more toxic as pH increases. The methods described below can be used to identify ammonia as the toxicant and these data could also be used in Phase III confirmation.

Ammonia is relatively unique in its behavior as pH changes. When ammonia (NH_3) dissolves in water, some of the molecules react to form the ammonium ion NH_4^+, and the equilibrium between these two species is affected by both pH and temperature (EPA, 1985A). The term "total ammonia" refers to the sum of the un-ionized (NH_3) and the ionized (NH_4^+) forms and is referred to as N^+. The toxicity of ammonia to some aquatic species appears to be primarily caused by the un-ionized form. The equilibrium shifts to increase the un-ionized ammonia concentration with increasing pH and increasing temperature. In a constant temperature situation, Table 3-1 shows that as pH increases by one unit, there is nearly a 10-fold increase in the percent of un-ionized ammonia NH_3 present in aqueous solutions at pH 6.0-8.5. The data in Table 3-1 are calculated using the dissociation constants for ammonia (EPA, 1979). There are two effects to consider for ammonia as the pH increases; first, the concentration of NH_3 increases (Table 3-1) and second, the toxicity of NH_3 decreases (Tables 3-2 and 3-3). One possible explanation for the second effect is that NH_4^+ is contributing to the toxicity (EPA, 1985A). Measuring and maintaining the pH of the test solution and understanding the effect of pH on the toxicity of ammonia are very important.

As discussed in EPA's ammonia water quality criteria document (EPA, 1985A), the slope of the LC50-pH curve for acute toxicity is similar for different aquatic species (i.e., an average slope can be used for many species). A model was developed to describe the pH dependence of ammonia toxicity, primarily with data for fishes and cladocerans (i.e., daphnids, fathead minnows, rainbow trout, and coho salmon, see EPA, 1985A). This model has been used with acute toxicity data generated at a pH of 8 and a temperature of 25°C for both *C. dubia* and fathead minnows, to predict the LC50 of NH_3 at other pH values (Tables 3-2 and 3-3). It is apparent that the toxicity of NH_3 is about seven times less at pH 7.0 than at pH 6.0, but the amount of NH_3 is ten times greater at pH 7.0 than at pH 6.0. Similarly, at pH 8.0, NH_3 is three times less toxic than at pH 7.0 but ten times more is available at pH 8.0. Ammonia can be implicated as the cause of toxicity if the effluent toxicity and the suspect toxicant exhibit both of these pH effects. Acute toxicity test data generated at ERL-D indicate that this model is not appropriate for all species. For example, the trend of pH-dependence has not been observed in acute tests conducted with the amphipod, *Hyalella azteca*, over a range of pH values in reconstituted waters (EPA, 1991B) until the hardness is greater than 160 mg/l. In hard or very hard waters, *H. azteca* is more sensitive to NH_3 at higher pHs (P. Monson, personal communication, University of Wisconsin, Superior, WI). We recommend that the effect of pH on the toxicity of ammonia be characterized for the TIE organism, if it has not been done, so that accurate predictions can be made for the organism.

It has not yet been determined whether the pH dependence of ammonia toxicity described for acute toxicity is appropriate for chronic toxicity. The chronic toxicity of ammonia to species typically used in effluent tests, at temperatures similar to those used in TIEs and a variety of pHs is presented in Table 3-4. If chronic ammonia toxicity has not been characterized with respect to pH for the TIE species, it is prudent for the investigator to generate the ammonia toxicity data for at least three distinct pH levels.

Generally, three procedures are used to implicate ammonia in addition to measuring the ammonia in the effluent. These are 1) the graduated pH test (in place of

Table 3-1. Percent Un-Ionized Ammonia in Aqueous Solutions for Selected Temperatures and pH Values[1]

	Temperature (°C)								
pH	15	20	21	22	23	24	25	26	27
6.0	0.0274	0.0397	0.0427	0.0459	0.0493	0.0530	0.0568	0.0610	0.0654
6.1	0.0345	0.0500	0.0537	0.0578	0.0621	0.0667	0.0716	0.0768	0.0823
6.2	0.0434	0.0629	0.0676	0.0727	0.0781	0.0839	0.0901	0.0966	0.104
6.3	0.0546	0.0792	0.0851	0.0915	0.0983	0.106	0.113	0.122	0.130
6.4	0.0687	0.0865	0.107	0.115	0.124	0.133	0.143	0.153	0.164
6.5	0.0865	0.125	0.135	0.145	0.156	0.167	0.180	0.193	0.207
6.6	0.109	0.158	0.170	0.182	0.196	0.210	0.226	0.242	0.260
6.7	0.137	0.199	0.214	0.230	0.247	0.265	0.284	0.305	0.327
6.8	0.172	0.250	0.269	0.289	0.310	0.333	0.358	0.384	0.411
6.9	0.217	0.314	0.338	0.363	0.390	0.419	0.450	0.482	0.517
7.0	0.273	0.396	0.425	0.457	0.491	0.527	0.566	0.607	0.650
7.1	0.343	0.497	0.535	0.575	0.617	0.663	0.711	0.762	0.817
7.2	0.432	0.625	0.672	0.733	0.776	0.833	0.893	0.958	1.027
7.3	0.543	0.786	0.845	0.908	0.975	1.05	1.12	1.20	1.29
7.4	0.683	0.988	1.06	1.14	1.22	1.31	1.41	1.51	1.62
7.5	0.858	1.24	1.33	1.43	1.54	1.65	1.77	1.89	2.03
7.6	1.08	1.56	1.67	1.80	1.93	2.07	2.21	2.37	2.54
7.7	1.35	1.95	2.10	2.25	2.41	2.59	2.77	2.97	3.18
7.8	1.70	2.44	2.62	2.82	3.02	3.24	3.46	3.71	3.97
7.9	2.13	3.06	3.28	3.52	3.77	4.04	4.32	4.62	4.94
8.0	2.66	3.82	4.10	4.39	4.70	5.03	5.38	5.75	6.14
8.1	3.33	4.76	5.10	5.46	5.85	6.25	6.68	7.14	7.61
8.2	4.16	5.92	6.34	6.78	7.25	7.75	8.27	8.82	9.40
8.3	5.18	6.43	7.85	8.39	8.96	9.56	10.2	10.9	11.6
8.4	6.43	9.07	9.69	10.3	11.0	11.7	12.5	13.3	14.1
8.5	7.97	11.16	11.90	12.7	13.5	14.4	15.2	16.2	17.1
8.6	9.83	13.6	14.5	15.5	16.4	17.4	18.5	19.5	20.7
8.7	12.07	16.6	17.6	18.7	19.8	21.0	22.2	23.4	24.7
8.8	14.7	20.0	21.2	22.5	23.7	25.1	26.4	27.8	29.2
8.9	17.9	24.0	25.3	26.7	28.2	29.6	31.1	32.6	34.2
9.0	21.5	28.4	29.9	31.5	33.0	34.6	36.3	37.9	39.6

[1]Data from EPA, 1979.

the equitoxic solution test as described in the first Phase II document; EPA, 1989A); 2) use of the zeolite resin to remove the ammonia; and 3) air-stripping the ammonia from the sample at a high pH (i.e., pH 11). For both the zeolite resin method and the air-stripping method, subsequent toxicity tests and ammonia measurements are performed on whole effluent and the post-treatment samples.

Depending on the presence of other toxicants in the effluent, additional sample manipulations may be needed before proceeding with the three basic tests. For example, if toxic oxidants such as chlorine are also present in the effluent, sodium thiosulfate must be added to the sample before conducting the Phase II ammonia tests. To date we have not seen ammonia and chlorine as co-occurring toxicants in chronic tests, probably because chlorine degrades rapidly in a test system at 25°C, while ammonia does not. If the additional toxicant(s) can be removed by the C_{18} SPE column, it may be possible to conduct Phase II tests for ammonia on post-C_{18} SPE column effluent sample. However, the problem of artifactual toxicity associated with the post-C_{18} SPE column effluent may prevent the use of the graduated pH test (EPA, 1992) and/or the air-stripping test (see Section 8 of EPA, 1991A) on post-column samples.

The results of the graduated pH test, the post-zeolite column test, and the air-stripping test, all will be important in identifying ammonia as a toxicant in acutely or chronically toxic samples. Use of pH changes where graded responses are observed are particularly useful for data evaluation in Phase III correlation steps. Some of the Phase II tests for ammonia are the same steps that are used for Phase III confirmation procedures; therefore, tests such as spiking the effluent with ammonia and then performing the graduated pH test or spiking the post-zeolite effluent samples and then testing the samples simultaneously with the Phase II tests will support the confirmation steps in Phase III.

3.2 Toxicity Testing Concerns

A key issue in interpreting acute or chronic test results for a pH dependent toxicant such as ammonia is monitoring pH changes during the test period. Toxicity differences in Phase I manipulations may be misinterpreted simply because differences in NH_3 toxicity can occur with only a slight pH change. To illustrate, the change in pH from 8.0 to 7.9 lowers the concentration of NH_3 20%, as does a change in pH from 6.1 to 6.0, but a 20% difference is much more important to the toxicity

Table 3-2. Calculated Un-Ionized Ammonia LC50s (mg/l) Based on 24-h and 48-h Results of a *Ceriodaphnia dubia* Toxicity Test Conducted at pH 8.0 and 25°C[1]

pH	Percent Dissoc. at 25°C	Un-ionized Ammonia Expected 24-h LC50	Total Ammonia 24-h LC50	Un-ionized Ammonia Expected 48-h LC50	Total Ammonia 48-h LC50
6.0	0.0568	0.09	158	0.07	123
6.1	0.0716	0.12	168	0.09	126
6.2	0.0901	0.14	155	0.11	122
6.3	0.1134	0.18	159	0.14	123
6.4	0.143	0.22	154	0.17	119
6.5	0.180	0.27	150	0.21	117
6.6	0.226	0.33	146	0.25	111
6.7	0.284	0.40	141	0.31	109
6.8	0.358	0.48	134	0.38	106
6.9	0.450	0.58	129	0.45	100
7.0	0.566	0.69	122	0.53	94
7.1	0.711	0.81	114	0.62	87
7.2	0.893	0.93	104	0.72	81
7.3	1.12	1.06	95	0.82	73
7.4	1.41	1.21	86	0.93	66
7.5	1.77	1.34	76	1.04	59
7.6	2.21	1.48	67	1.14	52
7.7	2.77	1.61	58	1.24	45
7.8	3.46	1.73	50	1.33	38
7.9	4.32	1.83	42	1.42	33
8.0[2]	**5.38**	**1.93**	**36**	**1.49**	**28**
8.1	6.68	2.01	30	1.55	23
8.2	8.27	2.08	25	1.61	20
8.3	10.2	2.14	21	1.65	16
8.4	12.5	2.19	18	1.69	14
8.5	15.2	2.23	15	1.73	11
8.6	18.5	2.27	12	1.75	9.5
8.7	22.2	2.30	10	1.77	8.0
8.8	26.4	2.32	8.8	1.79	6.8
8.9	31.1	2.34	7.5	1.81	5.8
9.0	36.3	2.35	6.5	1.82	5.0

[1] LC50s for each pH interval were calculated using EPA's water quality criteria document formula (EPA, 1985A) shown below.

$$Formula \quad LC50 = \frac{(LC50[pH = 8.0])(1.25)}{1 + 10^{7.4 - pH}}$$

[2]The 24 h and 48 h LC50s to *C. dubia* are 1.93 mg/l and 1.49 mg/l, respectively, at pH 8.0. The formula was used to generated expected LC50s for pH values above 8, though the model is not recommended above pH 8, because generally we have found *C. dubia* data to track with these predictions.

expressed by the ammonia at pH 8.0 than at pH 6.0. For this reason frequent pH monitoring (at least daily) must be performed on tests conducted to determine the trend of ammonia toxicity. Ideally, continuous monitoring of pH is desired. The pH should be measured on each test concentration and each replicate. Experience has shown that the choice of pH meters and probes is critical to produce reliable results. The pH meter used must read accurately to two decimal places and should lock-on the stabilized reading after the rate of change has diminished to a specified rate. Routine cleaning of the probe and a standardized calibration procedure should be established. The pH values can also be recorded after an elapsed time of 60-90 sec. The pH readings should be made using a constant and reproducible stirring rate. The stirring should not result in excessive loss (or gain) of CO_2 which will of course change the pH. The choice of the pH electrode is important. We have found that the glass-bodied combination electrodes provide the most consistent pH readings. However, these should not be left in the test solutions for longer than is needed to obtain constant readings of pH because ions from the electrode reference solution can leak into the test solution, potentially causing artifactual toxicity.

For the Phase II ammonia toxicity tests more replicates (at least double that used in Phase I) must be used and tighter QA/QC procedures must be adhered to than those described in the acute or chronic Phase I manuals (see Section 1.2). For example, a control and at least four effluent dilutions using concentrations that more closely bracket the effect and no effect concentrations (that were determined in Phase I) are used. While parameters such as time to mortality or onset of symptoms in the acute and chronic tests are not an integral part of the tests described below, these observations may be very

Table 3-3. Calculated Un-Ionized Ammonia LC50s (mg/l) Based on 24-h, 48-h, 72-h, and 96-h Results of a Fathead Minnow (*Pimephales promelas*) Toxicity Test Conducted at pH 8.0 and 25°C[1]

pH	Percent Dissoc. at 25°C	Un-ionized Ammonia Expected 24-h LC50	Total Ammonia 24-h LC50	Un-ionized Ammonia Expected 48-h LC50	Total Ammonia 48-h LC50	Un-ionized Ammonia Expected 72-h LC50	Total Ammonia 72-h LC50	Un-ionized Ammonia Expected 96-h LC50	Total Ammonia at 96-h LC50
6.0	0.0568	0.075	131	0.064	113	0.049	86	0.036	63
6.1	0.0716	0.093	130	0.080	112	0.061	85	0.045	63
6.2	0.0901	0.12	128	0.10	111	0.076	84	0.056	62
6.3	0.1134	0.14	127	0.12	109	0.094	83	0.069	61
6.4	0.143	0.18	124	0.15	107	0.12	81	0.086	60
6.5	0.180	0.22	121	0.19	104	0.14	80	0.11	58
6.6	0.226	0.27	118	0.23	102	0.18	77	0.13	57
6.7	0.284	0.32	114	0.28	98	0.21	75	0.16	55
6.8	0.358	0.39	109	0.34	94	0.26	72	0.19	53
6.9	0.450	0.47	104	0.40	90	0.31	68	0.23	50
7.0	0.566	0.56	98	0.48	85	0.36	64	0.27	47
7.1	0.711	0.65	91	0.56	79	0.43	60	0.31	44
7.2	0.893	0.76	85	0.65	73	0.50	56	0.36	41
7.3	1.12	0.87	77	0.75	67	0.57	51	0.42	37
7.4	1.41	0.78	55	0.67	48	0.51	36	0.38	27
7.5	1.77	1.09	62	0.94	53	0.72	40	0.53	30
7.6	2.21	1.20	54	1.03	47	0.79	36	0.58	26
7.7	2.77	1.30	47	1.12	40	0.85	31	0.63	23
7.8	3.46	1.39	40	1.20	35	0.91	26	0.67	19
7.9	4.32	1.48	34	1.27	29	0.97	22	0.71	17
8.0	**5.38**	**1.56**	**29**	**1.34**	**25**	**1.02**	**19**	**0.75**	**14**

[1]LC50s for each pH interval were calculated using EPA's water quality criteria document formula (EPA, 1985A) shown below. The 24-h, 48-h, 72-h, and 96-h LC50s to fathead minnows are 1.56 mg/l, 1.34 mg/l, 1.02 mg/l, and 0.75 mg/l, respectively, at pH 8.0.

$$Formula \quad LC50 = \frac{(LC50[pH = 8.0])(1.25)}{1 + 10^{7.4 - pH}}$$

Table 3-4. Un-Ionized Ammonia Toxicity Values for Species Frequently Used in Effluent Testing

Species	Method[1]	pH	Temp (°C)	LC50[2] (mg/l)	ChV[3]
		Acute Data			
C. dubia[4]	S, M	6.2	25	0.12	--
C. acanthia[5,6]	FT, M	7.1	24	0.77	--
Simocephalus vetulus[5,6]	FT, M	7.1	24	0.61	--
C. dubia[4]	S,M	7.2	25	0.78	--
C. dubia[4]	S,M	8.2	25	1.73	--
Daphnia magna[6]	S,M	8.2	25	2.08	--
P. promelas[6]	FT, M	7.8	25.6	1.87	--
P. promelas[6]	FT, M	8.0-8.3	25.2	1.65	--
P. promelas[6]	FT, M	8.1	26.1	2.55	--
		Chronic Data			
C. dubia[7]	4d-R, M	6.03	25	--	0.065
C. dubia[7]	4d-R, M	7.05	25	--	0.28
C. acanthia[5,6]	7d-FT, M	7.0-7.5	24-25	--	0.34
D. magna[6]	NR	7.6	20.2	--	0.63
C. dubia[7]	4d-R, M	8.03	25	--	0.62
P. promelas[6]	FT, M	8.0	24.0	--	0.13
P. promelas[8]	7d-R, M	7.5-7.6	25.0	--	0.48
P. promelas[8]	7d-R, M	7.5-7.7	25.0	--	0.45
P. promelas[4]	7d-R, M	8.4	25	--	0.66

[1]FT - flow-through; S = static; R = renewal of solutions at 24 or 48 h; M = measured concentration; NR = not reported.
[2]48-h LC50 for invertebrates and 96-h LC50 for fish.
[3]ChV = chronic value which is the geometric mean of the no observed effect concentration (NOEC) and the lowest observed effect-concentration (LOEC) or an IC25.
[4]Data generated at ERL-D.
[5]*C. acanthia* is equivalent to *C. dubia*.
[6]Data from EPA, 1985A.
[7]Data from 4-d *C. dubia* tests conducted at ERL-D; the effect level is an IC25 (mg/l)
[8]Data from Beigger, 1990.

useful in describing the identification steps used in confirming ammonia as the cause of toxicity.

3.3 Measuring Ammonia Concentration

We have found that the ammonia-selective electrode method has been satisfactory for measuring the ammonia concentrations in most samples, (EPA, 1983; APHA, 1992). Other methods for measuring ammonia are available (such as distillation, nesslerization, and titration) and can be used successfully for determining ammonia concentrations in effluents (EPA, 1983; APHA, 1992). The level of detection for total ammonia generally need not be below 0.5-1.0 mg/l, since concentrations of ≤1.0 mg/l of total ammonia have not been found to be toxic to fathead minnows and *C. dubia*. If ammonia measurements are below 1 mg/l and the sample is toxic, it is likely that the toxicant is not ammonia and other identification procedures should be pursued.

The most reliable ammonia measurements are obtained on fresh samples. However, samples can be preserved by adding concentrated sulfuric acid and storing the samples at 4°C. The pH of the preserved samples should be in the range of 1.5 to 2.0 (EPA, 1983; APHA, 1992). In recent experiments, we have used samples that were stored without acidification at 10°C or refrigerated at 4°C for short periods with good success.

During several effluent tests, the amount of ammonia in the test solutions (see test details below) has decreased over the duration of the test. When levels are in the range of 0-30 mg/l, it is prudent to measure the initial concentration of ammonia in the test solution and again after animals were exposed.

3.4 Graduated pH Test

The purpose of the Phase II graduated pH test is to provide more definitive toxicity test data to implicate ammonia as the toxicant in Phase II. In turn, this data may be used in Phase III to confirm the role of ammonia in the toxicity of the effluent. More stringent pH control and pH monitoring will be needed to interpret test results and more precise toxicity estimates (i.e., more replicates, more dilutions, larger number of organisms; see Section 1.2; EPA, 1991A; EPA, 1992) are needed in Phase II than in Phase I. When it is important to predict the impact of the toxicant in the receiving water, the pH of the dilution water should be maintained at receiving water pH. The test procedures discussed below provide good pH control for the graduated pH test. Greater detail is provided for some of the procedures in Phase I (EPA, 1991A; EPA, 1992).

The test chamber size, number of dilutions, species to be tested, type of test (acute or chronic), and the degree of toxicity of the effluent will dictate the volume of effluent needed for the graduated pH test. As a general guide for acute toxicity tests, 300 ml of effluent should suffice for any of three pH adjustment tests described below. The volume for chronic tests will vary based on the type of chronic test performed, the species used, the number of concentrations tested, and number of solution renewals in addition to the items discussed above (Section 1.2 and EPA, 1992).

The procedure for conducting the graduated pH test is to evaluate and determine the toxicity of the effluent at three different pHs (e.g., 6.0, 7.0, and 8.0). The pH should be measured in all of the chambers. If the pH drifts 0.2 pH units or more, 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 present only at one pH, the toxicity results may still be useful. The pH levels selected must be within the physiological tolerance of the test species used (which generally is a pH range of 6 to 9). We recommend use of two methods of pH control and comparison of these results to determine that the pH adjustment itself did not introduce an artifact of toxicity. This type of testing may be critical to explaining effects in Phase III (EPA, 1993A).

Regardless of the pH control method chosen, the selection of the appropriate blank is difficult. The change in pH of the dilution water or surface water is not comparable to that of the effluent because the composition of the solutions are different. For some effluents, the addition of either acid or base can be used to adjust and hold the pH within 0.1 pH unit. If this is possible, this technique can be used to compare the results with either the CO_2-pH controlled test or the buffer-pH controlled test. Test results should be similar and these comparisons can be used as a basis for identifying ammonia as a toxicant.

3.4.1 pH Control: Acid/Base Adjustments

The first method of pH adjustment is the acid/base adjustment described in Phase I (EPA, 1991A; EPA, 1992). For this manipulation, the adjustment of pH is relatively easy and quick, and the loss of volatile compounds is minimized. However, the drawbacks are that: toxicity enhancement from the additives may occur (especially in a chronic TIE), the addition of strong acid or base disrupts the carbonate system equilibrium, the effects of the pH change in the blanks may not serve as a toxicity control for the effluent, the pH stabilization time is lengthy, and pH tends to drift in longer term tests. In the pH range 6 to 9, the amount of high quality acid or base added is usually negligible, and the likelihood of toxicity caused by increased salinity levels is low.

The pH of each concentration and replicate must be frequently monitored because a constant pH during the toxicity test must be maintained. Larger test volumes may be useful to prevent rapid pH fluctuations. The amount of acid and/or base added should be recorded for each pH adjustment to track the additive amount in the effluent samples and the blanks. If toxicity increases dramatically, the concentrations of salts should be calculated to be sure the salinity has not increased above the tolerance level for the TIE species.

3.4.2 *pH Control: CO_2 Adjustments*

The second method uses CO_2 to adjust and control test solution pH. The pH is adjusted by varying and controlling the CO_2 concentration of the gas phase over the water or effluent sample in closed headspace test chambers. It is necessary to maintain a constant pH throughout the test period. The pH of most natural waters and some effluents is controlled by the bicarbonate buffering system and surface waters normally contain <10 mg/l of CO_2. Therefore, the amount of CO_2 to add depends on the desired pH and the chemistry of each test solution. The CO_2 adjustment has the advantages that the pH is controlled without placing additives directly into the effluent test solutions, the pH change is easy to make, and the pH is generally stable for at least 24 h if the gas-tight container is not opened. Frequent pH measurements are still possible because the headspace can be reflushed with a predetermined concentration of CO_2/air. The disadvantages are that toxicity can occur from the CO_2, the concentration of CO_2/air varies for each dilution and effluent (which requires sample specific experimentation) and the manipulations for chronic tests can be time-consuming relative to the acid/base adjustment method. We have not observed any increased toxicity from the addition of CO_2 unless the concentration in the chamber is over 10%.

Adjustments of the pH to 6.0 or 7.0 can be made by using CO_2 with or without first adding HCl to the test concentrations. The CO_2 is purchased in pure form through local commercial gas suppliers, and if particular concentrations of CO_2/air are frequently used, a cylinder of gas of the desired concentration may prove to be resource-efficient. The amount of CO_2 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 constituents (e.g., dissolved solids). Some preliminary work is needed to determine the concentration of CO_2 to add to achieve the desired pH. When dilutions of an effluent have the same hardness and initial pH as the effluent, about the same amount of CO_2 will usually be needed for each dilution. Sometimes, higher concentrations of CO_2 are needed for the higher test concentrations. Use of a dilution water of similar hardness as the effluent may make the CO_2 volume adjustments easier. A different dilution water may only be used in these tests if the toxicity has not been shown to be dependent on water hardness at any pH.

In our laboratory, we have found that glass canning jars with rubber seals and metal balers work well as a gas-tight testing chamber. The testing chamber should be large enough to hold the desired number of test cups, with sufficient headspace to ensure proper DO levels. For example, a 2-quart glass canning jar lying on its side will easily hold 6-1 oz cups. We simultaneously test *C. dubia* and fathead minnows in the same chamber using the test solution volumes described in Section 1.2. Since many plastics are permeable to CO_2, glass containers are recommended. When CO_2/air is flushed into the headspace of the test chamber, the pH of the test solutions will usually reach equilibrium in about 1 h and a reliable pH can be achieved. Generally, as the alkalinity increases, the concentration of CO_2 that is needed to maintain the pH also increases. After 1 h, check the pH of the solutions and flush the chambers again. Check the pH again after 2-3 h and from these data determine the concentration of CO_2 to add for initial pH adjustment for the actual toxicity test and the amount needed for reflushing after the chamber is opened for feeding or pH measurements. In most instances, the amount of CO_2 produced by the test organisms will not cause further pH shifts. When testing with fish, which usually increase in size during the test, a pH fluctuation may occur that would require flushing with different (e.g., slightly lower) concentrations of CO_2.

When the concentration of CO_2 to inject for the target pH values has been determined, prepare test solutions, add test organisms (and food if necessary) and inject the appropriate concentration of CO_2 in air using a 1-liter gas tight syringe, and quickly close the test chamber. The chambers should be flushed with the CO_2/air mixture several times to ensure the displacement of air currently in the chambers. Place the chamber out of direct laboratory light, as temperatures tend to rise out of the desirable test range in the closed chambers.

For effluents that have initial pH values from 7.8 to 8.5, 0-10% CO_2 concentration in the chamber has been used to lower the pH to 6.0. Experiments in hard reconstituted water have shown that up to 8% CO_2 can be tolerated by *C. dubia* and fathead minnows in acute tests, but 8% has been toxic to *C. dubia* and fathead minnows in the 7-d tests. About 2-3.5% usually will lower the pH of most effluents to 6.5-7.0. If more than 10% CO_2 for acute tests or 5% CO_2 for chronic tests is needed to lower the pH of the test solutions, before adding test animals adjust the pH with high quality acid (EPA, 1991A; EPA, 1992) and then flush the headspace with CO_2/air. The necessary concentration of CO_2 to use must first be determined experimentally with effluent test solutions adjusted to the appropriate pH with acid solutions. Sometimes >5% CO_2 cannot be used for the dilution water pH adjustment test without the CO_2 causing toxicity.

The use of a single enclosed test chamber for controlling the pH at all test concentrations may allow the transfer of volatile compounds among treatments. We have experienced volatilization of ammonia in tests and therefore, individual test chambers for each effluent concentration are preferable. Methods that use continuous flow of a CO_2/air mixture, such as tissue cell incubators, may be preferable and give better pH control provided that volatilization or cross contamination is not a problem. At this time we have not attempted to use a continuous flow of CO_2/air mixture and therefore cannot recommend a system to use.

Maintaining pH above the air equilibrium pH (generally above pH 8.3) is difficult without buffers (Section 3.4.3). The pH control in this high range is much more difficult because the concentration of CO_2 must be very low and microbial respiration can increase the CO_2 levels in the test chamber. Use of CO_2-free air in the headspace may work or flushing a mixture of CO_2-free air and normal air through the headspace or test solution may be successful. Because such small CO_2 concentrations are needed and because CO_2 evolution by microorganisms or test organisms can significantly alter the CO_2 concentration, frequently flushing (two to four times a day) of the headspace in static tests will probably be required to adequately control pH. For the chronic tests, we have not attempted to use the CO_2-free air bubbled through the test solution, because more CO_2 evolution tends to occur during the chronic tests and the need for reflushing makes the test labor intensive.

For the CO_2-pH controlled tests, the pH should be measured at least every 24 h for both acute and chronic tests and ideally, continuously during pH controlled tests. At each reading, flush the headspace with the CO_2/air mixture. A small amount of experimentation will confirm whether the concentration of CO_2 previously determined is adequate, or whether the amount required for flushing will be less than that used for the initial pH adjustment.

For chronic tests, daily renewal solutions should be prepared, pH adjusted with HCl if necessary, dispensed into test cups, and placed into a second glass jar chamber and flushed with appropriate concentration of CO_2. These should be left to equilibrate at least 1-2 h. Measure the pH quickly and transfer the animals to new test cups and place them into the glass jar. Flush the headspace again with the appropriate CO_2/air mixture.

Table 3-5. Percent Un-Ionized Ammonia in Aqueous Solutions at 25°C and Various TDS Levels[1]

TSD (mg/l)	pH			
	6.0	7.0	8.0	9.0
0	0.0568	0.566	5.38	36.2
250	0.0521	0.519	4.96	34.3
500	0.0505	0.503	4.81	33.6
750	0.0494	0.492	4.71	33.1
1000	0.0485	0.483	4.63	32.7
1500	0.0471	0.469	4.50	32.0
2000	0.0460	0.458	4.40	31.5
3000	0.0443	0.441	4.24	30.7

[1]Data from Skarheim (1973).

For the 7-d tests with fathead minnows, the chambers must be opened once more each day to accommodate the feeding schedule. The experimenter can take advantage of this by making a pH reading prior to placing food into the test cups. CO_2/air must again be flushed into the chamber. It is important to note that in the fathead minnow test, the pH most likely will be lower after 24 h than in the *C. dubia* test because of the food added and the respiration of the fish which is considerably greater than that of *C. dubia*.

Measurements of pH must be made rapidly to minimize the CO_2 exchange between the sample and the atmosphere. Avoid vigorously stirring unsealed samples because at lower pH values, the CO_2 lost during the measurement can cause a substantial pH rise. If possible, measure the DO at the same time because ammonia may have different toxicities when DO is decreased (EPA, 1985A). 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.

The controls in the CO_2 chamber 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 the pH of the adjusted test solutions (at their respective LC50s, IC25s or IC50s), the toxicity expressed in the two tests should be similar. Significantly greater toxicity in the pH-adjusted test may suggest interference from other factors such as the ionic strength related toxicity if the pH was adjusted with either HCl or NaOH, or possibly CO_2 toxicity. Dilution water blanks at the various pH levels may or may not be appropriate since the effluent matrix may differ from that of the dilution water. The dilution water blank will be useful in checking the acids and bases that are added for artifactual toxicity. Monitoring the acid and base additions may be useful in determining if artifactual toxicity resulted from the increase in salt content. Monitoring conductivity of the effluent solutions after the addition of the acids and bases may also be helpful in determining artifactual toxicity. The ionic strength of hardwaters or saline waters results in a decreased level of un-ionized ammonia (Table 3-5). For values of TDS from 0-500 mg/l, the dissociation constants are expected to be more accurate than values above 500 mg/l that were based on somewhat tenuous assumptions (Skarheim, 1973; see Table 3-5).

3.4.3 pH Control: Buffer pH Adjustments

The third method of pH control uses the addition of standard buffers to the effluent and dilution water to adjust the pH. This method has the advantage in that pH is stable with the buffer addition, the pH change during a test is slow, frequent pH measurements are possible because test vessels are not in air-tight chambers, and the test method set-up is rapid. The disadvantages are that toxicity enhancement or interference from buffers may occur, not all buffers can be used without additional

acid/base adjustments, and the pH stabilization time may be lengthy.

Hydrogen ion buffers are used to maintain the pH level in the graduated pH test (EPA, 1991A; EPA, 1992). 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 three 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 also used two additional buffers: N-(2-hydroxyethyl) piperazine-N'-2-hydroxypropanesulfonic acid (Heppso) (pK_a = 7.8) and N-tris-(hydroxymethyl) methyl-3-aminopropanesulfonic acid (Taps) (pK_a = 8.4). The Taps buffer is more frequently used than the Heppso buffer. We have experienced problems of having to add an excessive amount of base to obtain the desired pH with the Popso buffer. The Taps buffer effectively maintains the pH above 7.8. Keep in mind that pH is best maintained at or near the pK_a of the buffer.

The acute toxicity of these buffers is low to both *C. dubia* and fathead minnows (EPA, 1991A) and 4 mM concentration or less of all five buffers has not caused chronic toxicity to *C. dubia* or the fathead minnow. The buffers are added at sublethal (e.g., NOEC) levels to maintain the pH of test solutions. While these buffers serve to prevent the pH from drifting during the test, pH adjustment to the desired level is required in the preparation of the solution. A portion of the buffer compound is weighed out and added to the aliquots of whole effluent and dilution water, and both are then pH adjusted with acid or base solutions to the desired pH values. Serial dilutions are made, replicates prepared, and test organisms are added. Care should be taken to ensure equilibrium of buffered solutions, which may take at least 1-2 h. Dilutions should also be left to equilibrate and minor pH adjustments should be made. In certain situations, it may be desirable to prepare the solutions the day before tests begin. At present, we have found we can use batch solutions prepared ahead of time for solution renewals. Our experience also indicates that the amount of any buffer needed to hold any pH is effluent specific. Experimentation with effluents will be required to determine the lowest concentration of buffer needed to maintain the desired pH. The test solutions need not be covered tightly to maintain pH; however, pH should be measured at each test reading at all dilutions.

Use of the buffers is still being developed and the effects caused by interferences from the buffers themselves have not been fully studied. It is possible that the buffers may reduce the toxicity of some toxicants, but this has not generally been seen.

3.5 Zeolite Resin Method

Zeolite is composed of naturally occurring or synthetically created crystalline, hydrated alkali-aluminum silicates. The general formula is $M^{n+}O{\cdot}Al_2O_3{\cdot}ySiO_2{\cdot}zH_2O$; M = group IA or IIA element, n = +2 for group IA, +1 for group IIA, y>2, and z = the number of water molecules contained in the interconnected voids or channels within the zeolite (Windholz, et al., 1983). When zeolite is placed in aqueous solutions, the positively charged group IA or IIA elements (M^{n+}) of the zeolite are mobile and can undergo exchange with other cations in the water. As such, zeolite has frequently been employed as ion exchange resins to remove the ammonium ion (NH_4^+) from aqueous solutions in TIE work (Ankley et al., 1990B; Burkhard and Jensen, 1993). Because of its ability to exchange other cations such as heavy metals, and its use as molecular sieves, filter adsorbents and catalysts, zeolite was not suggested for use in Phase I, except as a subsequent test (EPA, 1991A). Zeolite can be effective in Phase II, if Phase I results implicate ammonia as the toxicant and establish that other types of toxicants (such as non-polar organics and metals) play no role in the effluent toxicity.

For the acute TIE procedure, zeolite particles should be screened to be in the range of 32 to 95 mm, to ensure efficient ion exchange while preventing channeling or excessive resistance to flow. Extremely large or small particles can be removed by screening the zeolite with sieves or mesh screens. The zeolite column can be prepared by taking 30 g of aquarium zeolite (Argent Chemical Laboratories, Redmond, WA) and adding it to 60 ml of high-purity water. The zeolite slurry is poured into a chromatography column (11 mm i.d. x 15 cm) and three bed volumes of dilution water are passed through the column. The last 10 ml of dilution water is collected for use as a zeolite blank and should not be toxic. Next, 200 ml of 100% effluent is passed through the column at a rate of 2 ml/min. The post-column effluent that is collected will be toxicity tested and its ammonia concentration measured. Temperature and pH should be recorded at test initiation to provide the means to calculate both total and un-ionized ammonia in the sample.

For chronic toxicity tests larger amounts of zeolite should be used. This can be scaled up proportionally from the amounts used in the acute zeolite work. The amounts of solution needed for testing and ammonia measurements will dictate the amount of sample to prepare. Typically a slurry of 60 g of zeolite and 120 ml of high purity water is sufficient for levels of ammonia in the range of 5-50 mg/l and for processing 2,000 ml of effluent. The post-zeolite effluent is collected in aliquots, then each is toxicity tested. In this manner, break-through of ammonia can be measured and toxicity of the various samples with different ammonia levels can be estimated.

Toxicity tests and ammonia measurements are conducted on the effluent and post-zeolite column effluent. Removal of toxicity by the zeolite column and removal of the ammonia concentration will add to the evidence implicating ammonia as the toxicant. An aliquot of the effluent sample (not having passed through the zeolite column) is used for ammonia analysis and the baseline toxicity test. These data will be compared with the same data for the post-zeolite column effluent to determine if the post-column reduction in effluent toxicity is consistent with ammonia removal by the zeolite. The control for test organism survival, dilution water quality and other test conditions will be provided through toxicity tests on dilution water. Dilution water (at the same hardness as the effluent) should be passed through the zeolite column, and will act as a blank for toxic artifacts leached from the zeolite. Increased toxicity in the post-zeolite effluent, relative to the whole effluent, indicates the presence of toxic artifacts. Since many cations will be exchanged, adding solids in the acute tests, such as the YCT food (yeast-Cerophyl®-trout food) fed to *C. dubia*, might improve control survival. Additional clean-up techniques for the zeolite (such as Soxhlet extraction) or alternate uncontaminated sources of zeolite might be needed. Column packing, effluent pH, ammonia levels, and flow rate through the column can all affect the efficiency of the cation exchange process. Lowering effluent pH prior to zeolite treatment and/or lowering flow rate through the column might also result in greater removal of ammonia. Occluded gas between zeolite particles might also impair the column's capacity to remove ammonia. If this appears to be a problem, the zeolite slurry should be degassed by using a vacuum prior to pouring it into the column.

Zeolite columns can be regenerated, but fresh zeolite should be used to pack columns the first time. If the graduated pH test and the zeolite test results are consistent with ammonia toxicity, Phase III confirmation procedures should be started.

Once ammonia is identified and confirmation is initiated, the post-zeolite samples can be spiked with ammonia at the same concentrations as are present in the effluent. These tests are an integral part of the Phase III confirmation process (EPA, 1993A).

3.6 Air-Stripping of Ammonia

This method of ammonia removal takes advantage of the fact that the relatively volatile un-ionized ammonia (NH_3) predominates in a solution with a pH above 9.3. For this reason, one might expect that ammonia would be removed during the Phase I pH 11 adjustment/aeration test (acute testing) or the pH 10 adjustment and aeration test (chronic testing). Based on our experience ammonia is not removed by this method, most likely because the Phase I aeration manipulation is done in a graduated cylinder, which has a low surface-to-volume ratio. By stirring the sample for a longer period of time (>1 h) at a high pH (pH 9.0 or higher) in a container that allows a large surface area to volume ratio, most of the ammonia can be removed from aqueous samples.

A measured amount of effluent for subsequent analysis and testing is pH adjusted to 10 or 11 and placed into a large shallow glass container (e.g., 1000 ml crystallizing dish). The solutions are then agitated (stirred) continuously. The length of time the sample must be stirred is dependent on the concentration of total ammonia in the sample. We have found that for most samples of 10-100 mg/l of total ammonia, 1-6 h is adequate to remove most of the ammonia. After air-stripping is completed, the volume of effluent should be measured and any appreciable loss replaced with high purity water or toxicity might be caused by the concentration of other components in the effluent. The ammonia concentration should be measured immediately after air-stripping and after volume adjustment is complete to ensure ammonia levels are reduced before toxicity tests are initiated. Toxicity tests on the air-stripped solution can then be conducted for both acute and chronic TIE work. Dilution water blanks at the various pHs may or may not be appropriate since the effluent matrix will probably differ from that of the dilution water. Monitoring the acid and base additions may be useful to determine if artifactual toxicity resulted from the increase in salt content and subsequent evaporation that occurred during the air-stripping process. Monitoring conductivity of the effluent solutions after the addition of the acids and bases may also be helpful in determining artifactual toxicity. The dilution water blank should be treated in the same manner as the effluent although it may not serve as a true toxicity control for the effluent.

If the ammonia is decreased and the toxicity is reduced or absent after air-stripping, ammonia is strongly implicated as a contributing factor to the toxicity of the effluent. The results of this test should be compared with the aeration test results of Phase I, the baseline effluent test and the other graduated pH tests. Other compounds could precipitate as a result of the pH adjustment and during the air-stripping procedure. Precipitates may form during the air-stripping process and not dissolve after the volume is readjusted, leaving these compounds unavailable.

Section 4
Metals

4.1 General Overview

This section contains procedures that can be used to identify suspect metal toxicants. The initial evidence used to implicate metallic toxicants is obtained from the Phase I characterization tests, with the results of the EDTA (ethylenediamine tetraacetic acid) addition test providing the best indication of the presence of a metal toxicant. When certain cationic metal toxicants are present, a reduction in sample toxicity with the addition of EDTA should be observed. Other Phase I manipulations that remove or reduce sample toxicity and suggest the presence of a cationic metal include the sodium thiosulfate addition test, the use of a C_{18} SPE column, and filtering the sample when combined with minor pH adjustments. One additional indication of metal toxicity may be when the organisms' response in the toxicity test is atypical of the expected dose response relationship (i.e., partial mortalities in several test concentrations, Schubauer-Berigan et al., 1993B).

Subsequent Phase I tests such as using ion exchange resins might also lead one to the conclusion that a metal is the toxicant. Toxicity removal or reduction after a sample is treated with an anion exchange resin might implicate toxicants that exist as anionic oxides in water, such as arsenic, chromium, and/or selenium. These anionic oxides will not be specifically removed or rendered biologically unavailable by the routine Phase I tests. Therefore, when the Phase I tests do not seem to show any toxicity reduction, toxicants such as these might be suspected and subsequent tests as discussed above could be useful (see Phase I, EPA, 1991A; EPA, 1992). These situations should be approached on an individual basis since other classes of toxicants might demonstrate the same behavior in Phase I (e.g., total dissolved solids (TDS)).

Further discussion and interpretation of the Phase I results which would lead to the conclusion that a cationic metal toxicant was present in a sample are provided in the Phase I TIE documents (EPA, 1991A; EPA, 1992).

Other information, such as process details from the discharger and information from past TREs and/or TIEs, might also help to implicate cationic metals as the toxicants. However, this type of information should be interpreted and used with caution as it might bias the TIE efforts.

If the EDTA addition test in Phase I showed that toxicity was removed or reduced one should proceed to the metal analysis section (Section 4.2). This section provides guidance and recommendations for analyzing samples for metals so that a list of suspect metal toxicants can be obtained. This section also discusses clean metal techniques, detection limits, a prioritization process for analyzing for specific metals, dissolved vs biologically available metals, and provides the rationale for assembling the list of suspect metal toxicants. Prioritizing metals to analyze from Phase I results is strongly recommended in order to save money and time in the TIE process.

If other Phase I tests implicate a metal but EDTA does not, it may be helpful to acquire additional test information through the use of EDTA addition tests, sodium thiosulfate addition tests, graduated pH tests, and ion-exchange resins. This additional toxicity testing (Section 4.3) may be useful in certain situations before analyzing for metals, even when EDTA additions reduced toxicity. These situations include: when the addition tests of EDTA and sodium thiosulfate in Phase I were performed using a single sample concentration (i.e., no dilutions), when the time it takes to obtain results of metal analyses is lengthy, or when Phase I results indicate another type of toxicant (non-metal) is present. The data obtained from the additional testing can then be included in the prioritization process for metals analysis. Professional judgement is required to decide when you have sufficient and appropriate toxicity testing data to proceed to metals analysis.

After processing one sample, a list of suspects may be generated. As future samples are evaluated, the correlation between toxicity of a sample and the concentration(s) of metal(s) over time may also be used to narrow the list of suspect toxicant(s). In Phase III, the suspect metal toxicant is implicated based upon the correlation of effluent toxicity and metal concentrations, reference suspect metal toxicity data, the use of additives that chelate metal toxicants, and changes in toxicity observed during manipulations of water quality characteristics.

The procedures in this chapter are generally applicable for both acute and chronic toxicity. The main differences between the acute and chronic procedures are the concentrations of additives used in the EDTA and sodium thiosulfate addition tests, lower analytical detection limits, and generating non-toxic blanks for the ion exchange resins for chronic toxicity testing. The use of species other than *C. dubia* or fathead minnows will require consideration of appropriate test volumes and additive concentrations.

4.2 Analysis of Metals

4.2.1 Prioritizing Metals for Analysis

Many cationic metals can be analyzed in a specific sample, but to simplify the amount of analytical effort needed for metals analysis, we suggest a prioritizing process be performed before analyzing any samples. The prioritization process is more valuable when the metal analyses are performed by AA instrumentation since each metal requires an individual analysis. Conversely, with ICP (inductively coupled plasma) instrumentation, numerous metals can be analyzed at once, and the prioritization process is less valuable in this instance. With both ICP and AA methods, a list of metals and required levels of detection will be needed before the samples are analyzed.

This prioritization is based primarily upon acute toxicity data with *C. dubia*. Its applicability to chronic toxicity and other species is expected to be similar but has not yet been determined. The toxicity test results from the EDTA additions, sodium thiosulfate additions, and graduated pH tests performed in Phase I form the basis for prioritization. When available, Phase II results from using the procedures in Section 4.3, should be included in this evaluation. Because we do not have a complete understanding of the effects of these procedures for each metal, the following should be taken as a starting point for metals analysis.

Information regarding historical discharge monitoring data, past or current TRE and/or TIE information, or process information may be useful in prioritizing metals for analysis. For example, if a discharger uses zinc in their manufacturing process and EDTA removed the toxicity, it would be logical to analyze for zinc first. If zinc was present at nontoxic concentrations or at concentrations too low to cause the observed toxicity, analysis for additional metals would be performed. If zinc was present at concentrations high enough to cause the observed toxicity, Phase III procedures (EPA, 1993A) should then be started to confirm zinc as the identified suspect toxicant.

When EDTA additions reduce or remove the toxicity of the sample, initially copper, lead, cadmium, nickel, and zinc should be measured. When sodium thiosulfate additions reduce or remove the toxicity of the sample, copper, cadmium, and silver should be measured.

Phase I results would not normally lead to the conclusion that an anionic toxicant was present (i.e., cationic metals that exist in aqueous samples as anionic oxides). If additional Phase I tests had been performed which characterized anionic toxicants or other specific discharger information was available, measurements of arsenic, chromium, and selenium should be made.

As stated above, these metals should be a starting point for metals analysis. Further interpretation of the Phase I results could be done by including the results of the graduated pH test and by jointly examining the results of the EDTA addition, thiosulfate addition, and graduated pH tests.

When multiple toxicants of different classes are present, Phase I data are often difficult to interpret. One should try to identify and confirm as soon as possible the role of one toxicant when multiple toxicants are present. By defining the role of one toxicant, efforts can be better focused on the remaining unidentified toxicants.

4.2.2 Metal Analysis Methods

There are three types of chemical instrumentation available for the analysis of cationic elements; these are AA, inductively-coupled plasma-atomic emission spectroscopy (ICP-AES), and inductively-coupled plasma-mass spectrometry (ICP-MS).

EPA methods using ICP-AES, ICP-MS, and AA (EPA, 1983; EPA, 1991D) are available for quantifying cationic metals in aqueous samples. Tables 4-1 and 4-2 summarize method detection limits for the analysis of cationic metals in aqueous samples using AA with direct aspiration, AA with the furnace procedure, ICP-AES, and ICP-MS.

The detection limits required in Phase II for the identification of suspect cationic metal toxicants will be determined by the toxicity of metals for the TIE species. In some cases, especially for chronic toxicity, the effect level might be lower than the detection limits listed in Tables 4-1 and 4-2. Detection limits should be improved to obtain optimal levels of detection (i.e., at least two times lower than the effect level).

Toxicity data for some species and test types for many metals have not been determined, especially for 7-d chronic toxicity tests. Therefore, to determine the needed levels of detection, effect levels for specific metals may have to be determined.

The required level of detection will often dictate the method needed for performing the metal measurement. It will be beneficial for laboratories to compile a database containing method detection limits and toxic effect levels for cationic metals using data from their organisms, analytical methods, and toxicity testing conditions. These data are not necessary in advance but this

Table 4-1. Atomic Absorption Detection Limits and Concentration Ranges[1]

Metal	Direct Aspiration		Furnace Method[2]	
	Detection Limit (mg/l)	Optimum Concentration Range(mg/l)	Detection Limit (µg/l)	Optimum Concentration Range (µg/l)
Aluminum	0.1	5 - 50	3	20 - 200
Antimony	0.2	1 - 40	3	20 - 300
Arsenic[3]	0.002	0.002 - 0.02	1	5 - 100
Beryllium	0.005	0.05 -2	0.2	1 - 30
Cadmium	0.005	0.05 -2	0.1	0.5 - 10
Calcium	0.01	0.2 -7	-	-
Chromium	0.05	0.5 - 10	1	5 - 100
Cobalt	0.05	0.5 -5	1	5 - 100
Copper	0.02	0.2 -5	1	5 - 100
Lead	0.1	1 -20	1	5 - 100
Magnesium	0.001	0.002 - 0.5	-	-
Manganese	0.01	0.1 -3	0.2	1 - 30
Mercury[4]	0.0002	0.0002 - 0.01	-	-
Molybdenum (p)	0.1	1 - 40	1	3 - 60
Nickel(p)	0.04	0.3 - 5	1	5 - 50
Potassium	0.01	0.1 - 2	-	-
Selenium[2]	0.002	0.002 - 0.02	2	5 - 100
Silver	0.01	0.1 - 4	0.2	1 - 25
Sodium	0.002	0.03 - 1	-	-
Tin	0.8	10 -300	5	20 -300
Vanadium (p)	0.2	2 - 100	4	10 - 200
Zinc	0.005	0.05 - 1	0.05	0.2 - 4

[1]The estimated detection limits and concentration ranges were taken from EPA, 1983.
[2]The listed furnace values are those expected when using a 20 µl injection and normal gas flow except in the case of arsenic and selenium where gas interrupt is used. The symbol (p) indicates the use of pyrolytic graphite with the furnace procedure.
[3]Gaseous hydride method.
[4]Cold vapor technique.

Table 4-2. Estimated Instrumental Detection Limits for ICP-MS and ICP-AES

Element	Estimated Detection Limit, ICP-MS[1] (µg/l)	Estimated Detection limit, ICP-AES[2] (µg/l)
Aluminum	0.05	45
Arsenic	0.9	53
Antimony	0.08	32
Beryllium	0.1	0.3
Cadmium	0.1	4
Calcium	-	10
Chromium	0.07	7
Cobalt	0.03	7
Copper	0.03	6
Lead	0.08	42
Magnesium	-	30
Manganese	0.1	2
Molybdenum	0.1	8
Nickel	0.2	15
Potassium	-	[3]
Selenium	5	75
Silver	0.05	7
Sodium	-	29
Vanadium	0.02	8
Zinc	0.2	2

[1] The estimated instrumental detection limits are taken from EPA, 1991D. They are given as a guide for instrumental limits, the actual detection limits are sample dependent and may vary as the sample matrix varies.
[2]The estimated instrumental detection limits as shown are taken from EPA, 1983. They are given as a guide for an instrumental limit. The actual method detection limits are sample dependent and may vary as the sample matrix varies.
[3]Highly dependent on operating conditions and plasma.

type of information will be very useful for future TIE efforts.

When toxicity effect levels appear to be below the detection limits of current analytical methods, the use of "clean" analytical techniques may be required through all steps in the analysis of the sample because background contamination is the major cause of inadequate levels of detection. Some general principles of clean metal techniques include the use of contamination free reagents, acid cleaned plastic labware, acid cleaned membrane filters (not glass fiber), class 100 benches for sample preparation, proper sample collection, preservation, and storage procedures; and proper QA/QC procedures using blanks, spiked matrixes, and replicate analyses. A summary of clean metal techniques and procedures for lowering the levels of detection can be found in Nriagu et al., 1993; Patterson and Settle, 1976; and Zief and Mitchell, 1976.

Some cationic metals, such as arsenic, selenium, and chromium, have different stable oxidation states in aqueous samples and more importantly the different oxidation states may have different toxicities. In Section 4.2.3, procedures to determine the concentration of the different oxidation states are provided.

In some TIEs, a measurement of the metals associated with the suspended solids may be needed

(Section 4.2.4). Procedures for preparing suspended solids removed by filtration for metals analysis are available; see EPA method 200 (EPA, 1983) and EPA method 200.2 (EPA, 1991D).

4.2.3 Metal Speciation

The procedures suggested above (Section 4.2.2) are used to determine the total concentration of a metal in an effluent. Many metals exist in water in different forms due to the various stable oxidation states of the metal. Arsenic (As^{3+}, As^{5+}), chromium (Cr^{3+}, Cr^{6+}), and selenium (Se^{4+}, Se^{6+}) are important metals that exist in different forms in water. Determining the speciation for these metals may be important in the TIE since the toxicities are different for the various forms of each metal. For example, Cr^{6+} is the form that is of toxicological concern while Cr^{3+} is generally not toxic (EPA, 1985D).

For chromium, methods for measuring the hexavalent form (Cr^{6+}) such as method 218.5 (EPA, 1983) are available. The amount of the trivalent form of chromium (less toxic form) is determined by taking the difference between the concentrations for total and hexavalent chromium.

For arsenic, the method of Ficklin (1983) is suggested for speciation measurements. This method uses an anion-exchange resin to separate the arsenite (As^{3+}) and arsenate (As^{5+}, more toxic form) species. Graphite furnace atomic-absorption spectroscopy is then used to measure the concentrations of each form.

For selenium, the method of Oyamada and Ishizaki (1986) is recommended for speciation. This method (like that for arsenic) uses column chromatography with an anion-exchange resin to separate the selenite (Se^{4+}) and selenate Se^{6+} (more toxic form) and graphite furnace atomic-absorption spectroscopy to measure each form.

Ion chromatography can also be used to determine the different forms of the above metals (EPA, 1991D), but we have not used this technique to date.

4.2.4 Identification of Suspect Metal Toxicants

Initial implication of suspect metals based on a comparison of total metal analyses data and effluent toxicity test results should be made. Then analysis for dissolved and suspended metals can be made if necessary. These metal values should be compared to available toxicity values, but tests on reference metals might have to be conducted with matching effluent conditions, such as pH and hardness to obtain comparable toxicity values. Side-by-side tests with individual reference metal standards and effluent samples might prevent being mislead by different test designs and are worth the effort. Literature summaries of metal toxicity data are also available (EPA, 1980; EPA, 1985B; EPA, 1985D; EPA, 1985E; EPA, 1985F; EPA, EPA, 1986; EPA, 1987; EPA, 1988B; and AQUIRE, 1992). In addition to matching the hardness and pH of the dilution water to the effluent sample by the addition of the appropriate ratios of magnesium carbonate and calcium carbonate, it might be possible in some cases to mimic the wastewater total suspended solids (SS) and total organic carbon (TOC) in the water used to test the metal. For example, TOC and SS from the addition of the YCT food can be at levels such that the total SS level in the dilution water might be similar to that found in the effluent. TOC may also be modified by the addition of humic acid. If the dilution water does not closely match the effluent, nonstandard dose-response relationships are observed in the toxicity test, i.e., several test concentrations exhibit partial mortality. In addition, a trend is noticed that as metal concentrations decrease at the effluent LC50s or IC25s, toxicity of the effluent increases.

If a sample is to be filtered, a membrane filter(s), such as a 0.45 µm polycarbonate filter should be prepared by rinsing with high purity water, followed by an appropriate volume of dilution water needed for blank toxicity tests and analysis. The toxicity test guidance is described in Section 1.2 and in the Phase I documents (EPA, 1991A; EPA, 1992). An appropriate quantity (<50 ml) of the last portion of the high purity water passing through the filter should be collected as an analytical blank to check for metals contamination from the filter and the filtration apparatus. An aliquot of the effluent is then filtered through the 0.45 µm membrane filter(s). If more than one filter is required for the effluent, a portion of each can be combined for testing.

The filtered and unfiltered effluent samples and the filtration blank should be tested for toxicity to measure the effect of filtration on sample toxicity. The toxicity test techniques are described in the non-polar organic section (Sections 2.2.3 and 2.3.3) unless data are needed for Phase III confirmation and then, greater replication and randomization will be needed (see Section 1.2). The toxicity tests should be performed for the test species using a dilution water (e.g., reconstituted water) of a similar hardness and pH to that of the effluent. If toxicity is reduced or removed upon filtration (and effluent toxicity has not previously been affected by C_{18} SPE or filtration through a glass fiber filter), it is possible that metals were retained by the 0.45 µm filter. Analysis for metals retained by the filter may help in interpreting sample data.

Metals analyses should be performed on the analytical blank collected from the filter and on the filtered and unfiltered effluent samples. The choice of metals to measure will be determined by the prioritization process described above. As stated previously, the level of detection for the metal of interest should be lower than the effect concentration for the metal.

Biologically Available Metals: Traditionally, dissolved metals for aqueous samples have been defined as those that pass through a 0.45 µm membrane filter, i.e.,

polycarbonate filter. The dissolved metals are in no way synonymous with the *biologically available metals*. Other than the use of an aquatic organism there is no technique to determine the biologically available fraction of the total metal. Furthermore, only rudimentary techniques are available to specifically identify the individual species of a metal (e.g., free charged metal ions [M^{m+}], inorganic ion pairs or complexes such as aquoions, [$M(H_2O)^{n+m}$], hydroxoions [$M(OH)_p^{n-p+}$], oxoions [MO_q^{n-2q+}], organic complexes and chelates [M x EDTA], metal species bound to high molecular weight organic material [M x lipid] or metal species in the form of highly dispersed colloids or sorbed on colloids [M x clay]). Stumm and Morgan (1981) have listed some general methods for assisting in identification of individual species. In some cases, binding of metals to inorganic and organic ligands in effluents will reduce the bioavailability of the metals and cause the metal concentration at the effluent LC50, IC50, or IC25 to be larger than the metal concentration determined in the metal dilution water toxicity test. For a set of effluent samples with a wide range of toxicities, better agreement should occur between the effect concentration of the metal in a dilution water toxicity test and the more toxic effluent samples (where the toxicity testing matrix of the effluent more closely matches that of the dilution water). Methods for determining the bioavailable fraction of the total metal are limited.

Some indication of the binding of metals to organics in the effluent may be arrived at through hexane extraction of an aliquot of the sample (Stary, 1964). Theoretically, metals bound to organic materials that are soluble in hexane should be extracted from the effluent. The hexane can then be evaporated and the residue reconstituted and analyzed for metals. Additionally, the loss of metals can be estimated by repeating the metal analysis on the extracted effluent and comparing this result to the hexane extract results. The toxicity attributed to metals associated with organics might be estimated by performing a toxicity test on the solvent extracted effluent. Traces of hexane must be removed from the extracted effluent by aeration prior to toxicity testing. The effects of aeration on sample toxicity must also be considered in this analysis. In any case, metals strongly suspected of causing or contributing to sample toxicity should be tested in dilution water as described above with the TIE test species.

The effects of variable water quality characteristics on metal toxicity must be evaluated over the effluent sampling period. One way to assess this is to collect several samples over a short time span. As an example, for an acutely toxic effluent, collect six grab samples in 24 h, and calculate the correlation coefficient for sample metal concentration (or summed toxic units of metals) versus sample toxicity for each sample. The set of correlation coefficients for multiple sampling events might give results less affected by hardness, SS, and TOC, assuming that water quality characteristics affecting metal toxicity will vary less during short time periods. For chronic toxicity, it might be useful to measure concentrations of metals in several daily samples and conduct separate chronic tests on each sample. Obviously, metal concentrations must vary enough to provide a sufficient range for correlation. When one reaches this stage, Phase III work should start using Phase III methods. Symptoms, species sensitivity, spiking, water quality adjustments and correlation are all applicable Phase III approaches to confirm the cause of toxicity.

4.3 Additional Toxicity Testing Methods

Guidance on EDTA addition tests, sodium thiosulfate addition tests, graduated pH tests, and the use of ion-exchange resins for use in Phase II are presented in this section. These procedures might be used before performing analyses for cationic metals, but most often they will be used to refine a list of suspect metal toxicants and to provide data to support the identified suspect in Phase III.

In the acute Phase I, EDTA and sodium thiosulfate addition tests can be conducted by adding incremental amounts of EDTA or sodium thiosulfate to a single effluent concentration. To provide further evidence in Phase II, these two tests should be conducted with effluent dilutions to assess the toxicity reduction (see EPA, 1992). The data generated from these procedures provide a powerful tool for identifying the cause of toxicity in samples containing mixtures of cationic metals. For example, toxicity caused by either copper or zinc could be determined by using the following test information: toxicity of both metals would be removed by EDTA addition (Section 4.3.1), sodium thiosulfate can remove toxicity caused by copper but not zinc (Section 4.3.2), and copper is more toxic at higher pH levels while zinc is not (Section 4.3.3). Depending on how the toxicity of the sample changes with these tests, one could eliminate one of these metals from the list of suspect metal toxicants.

Results of this type of testing will be used to develop evidence implicating the identified suspect metal. These tests would be performed on a number of samples over time to demonstrate the consistency of the cause of toxicity. In addition, when a mixture of toxicants is present, additions of EDTA or thiosulfate could be used to remove the cationic metal toxicity after performing other Phase II manipulations, e.g., C_{18} SPE.

4.3.1 EDTA Addition Test

Any reduction in effluent toxicity effected by the addition of EDTA suggests that certain cationic metals might be present in the effluent at toxic levels. Background information and discussion of the behavior of EDTA and cationic metals can be found in Phase I (EPA, 1991A; EPA, 1992).

Ideally, the amount of EDTA added would be just enough to chelate the toxicant(s) without causing EDTA

toxicity or substantially changing the water quality. For either *C. dubia* or fathead minnows, we have found it useful to add two different EDTA concentrations to two separate effluent tests (with dilutions). Controls without EDTA must be included. The EDTA stock solution is added after the effluent dilutions are prepared so that the EDTA concentration is the same at each effluent dilution (see Phase I, EPA, 1991A; EPA, 1992).

In Phase II, conducting simultaneous EDTA addition tests on effluent and the suspect metal in matching test water can provide evidence supporting the suspect metal as the toxicant if the results of these two tests are similar. If the metal is chelated by EDTA in the dilution water test but not in the effluent test then either there is a strong matrix effect from the effluent or it is the incorrect suspect metal. It is important to use the same pH in both tests in case there is any pH effect on the metal's toxicity.

In addition to removing toxicity caused by metals, EDTA reduces the acute toxicity of some cationic surfactants. This reduction of toxicity might also occur in chronically toxic effluents, and the toxicity reduced by EDTA should not be assumed to be due only to cationic metals.

4.3.2 Sodium Thiosulfate Addition Test

The acute Phase I oxidant reduction test (EPA, 1991A) or the chronic sodium thiosulfate addition test (EPA, 1992) is used to determine to what extent constituents reduced by the addition of sodium thiosulfate ($Na_2S_2O_3$) are responsible for the effluent toxicity. Although the use of the sodium thiosulfate test was designed to determine if oxidative compounds (such as chlorine) were responsible for effluent toxicity, experience has also shown that thiosulfate can also form a stable non-toxic complex with some metals. Since the complexing ability of thiosulfate is more metal specific than EDTA, this reagent can be used to determine if a specific metal is responsible for the effluent toxicity. Recent work by Mount (1991) has shown that in acute toxicity tests with *C. dubia* in moderately hard water that Cu^{2+}, Cd^{2+}, Hg^{2+}, Ag^{+}, and Se^{6+} can be complexed using sodium thiosulfate (see EPA, 1991A for more details). This complexing ability might not be applicable to chronic toxicity. For example, in a *C. dubia* 7-d test with copper, the toxicity was not reduced with sodium thiosulfate addition but was reduced with EDTA addition.

Table 4-3. Metal LC50s with Respect to Test pH[1]

Metal	Species	LC50 (µg/l) pH 6.2	pH 7.2	pH 8.2
Zn	*C. dubia*	>530	360	95
	P. promelas	830	333	502
Ni	*C. dubia*	>200	137	13
	P. promelas	>4000	3360	3080
Pb	*C. dubia*	280	>2700	>2700
	P. promelas	810	>5400	>540
Cu	*C. dubia*	10	28	201
	P. promelas	15	44	>200
Cd	*C. dubia*	563	350	121
	P. promelas	54	74	<5

[1]LC50 values were determined at 48-h for *C. dubia* and 96-h for *P. promelas*. Data taken from Schubauer-Berigan et al., 1993A.

If the addition of sodium thiosulfate does not reduce the effluent toxicity thought to be related to metals, the use of SO_2 (EPA, 1991A) additions followed by the addition of sodium thiosulfate is recommended. In some situations, the thiosulfate concentration may be reduced by non-toxic oxidants and thus, not be available for complexing the toxic metal. The addition of SO_2 should preferentially reduce these non-toxic oxidants which will allow the now available thiosulfate to complex the toxic metals. Depending upon the complexation ability of sodium thiosulfate for a specific metal, it might or might not complex the toxic metal. If the suspected metal toxicant can be complexed (e.g., cadmium, copper, selenium (as selenate), mercury; see EPA, 1991A; EPA, 1992), then a reduction in sample toxicity should occur with the addition of sodium thiosulfate. If the suspected metal cannot be complexed (e.g., zinc, lead, manganese, and nickel), then no reduction in sample toxicity should occur with the addition of the sodium thiosulfate.

As with the EDTA addition test, sodium thiosulfate additions should be conducted concurrently on the effluent and on dilution water spiked with the suspect metal toxicant. Care must be used to conduct these tests at similar pH levels. When toxicity test results are consistent with the expected behavior, strong evidence relating the suspected metal toxicant to the cause of the effluent toxicity has been obtained. These results in conjunction with the ion exchange test, analytical measurements for toxic metals, and the EDTA addition test provide evidence sufficient for one to proceed to toxicant confirmation, Phase III, of the TIE.

Both sodium thiosulfate and EDTA can reduce the toxicity of some metals and this information can be helpful in identifying the toxicant. However to date, this effect of thiosulfate/metal complexation has not been demonstrated for chronic toxicity. Knowing which metals are bound by both sodium thiosulfate and EDTA and which metals are complexed with only one or the other additive can be very helpful in narrowing down the possible toxicant.

4.3.3 Metal Toxicity Changes with pH

In Phase I, the graduated pH test is performed to evaluate the presence of compounds whose toxicity varies with pH. For ammonia, toxicity is greatest at pH 8.5 and least at pH 6.5 for some species. Therefore, as suggested in the first Phase II document, that for samples in which toxicity is enhanced at elevated pH, the identification effort should focus on ammonia. However, some

effluent and sediment pore water TIEs have indicated that some toxicity caused by metals can be affected by pH within the range of pH 6 to 9 (Schubauer-Berigan et al., 1993A). Some metals, notably zinc, nickel and cadmium, exhibit greater toxicity at elevated pH, which could confuse their characterization with that of ammonia (Table 4-3), while copper and lead show elevated toxicity at pH 6.2. These pH-dependent toxicities can be used as a tool for the identification (and confirmation) of toxicity caused by these metals. For example, toxicity to *C. dubia* in a sediment pore water sample was completely removed by additions of EDTA. The sample also exhibited greater toxicity at pH 6.5 than at 8.5, and metal concentrations indicated that only copper was present at toxicologically significant concentrations. The pH dependent toxicity of the sample along with the EDTA addition results and metal analysis supported the identification of copper as the toxicant.

4.3.4 Ion-Exchange Test

Ion-exchange resins have been used in TIEs for generating supporting evidence for identifying the cause of toxicity in effluents (Doi and Grothe, 1989; Phase II zeolite test). For cation exchange resins, removal of toxic cations such as NH_4^+, Cd^{2+}, and Pb^{2+} from the effluent occurs with the corresponding release of cations (i.e., counter ions) such as H^+ and Na^+ into solution. Similarly, for anion exchange resins, removal of toxic anions such as $Cr_2O_7^{2-}$ and AsO_4^{2-} from the effluent occurs with the corresponding release of anions such as OH^- and Cl^- into solution. For both cation and anion exchange resins, charge neutrality exists between the resin and aqueous phase and therefore, if the resins remove 5 µmoles of Cd^{2+} from solution, 10 µmoles of H^+ would be released into solution. The exchange process is concentration dependent and is reversible. Cations removed from the solution may then be recovered from the exchange resin by passing an acidic solution over the resins (e.g., 1 N HCl for analysis of metals).

We have had limited experience with ion exchange resins but the following general guidance can be provided. First, ion exchange resins are not chemical specific but rather remove a wide range of cations or anions, metallic and non-metallic. We have observed that anion exchange resins can remove cations (e.g., Zn^{2+}) from solution quite efficiently. The reasoning that only cationic materials are removed by cation ion exchange resins is not always reliable. Experimental verification of which materials were removed by the resin will be necessary on a case-by-case basis. Second, wide changes in the pH of the post-column effluent can occur depending upon the type of cation or anion released by the resin. These changes in pH will cause problems in interpreting toxicity tests if the pH is not adjusted prior to the toxicity test. Third, many of the ion exchange resins are based upon a styrene or acrylic divinylbenzene backbone and this material can remove other types of toxicants such as non-polar organics. Consequently, because of its non-specificity the removal of toxicity by an ion exchange column should not be used as the only piece of evidence to implicate a metal as the toxicant.

Resins under evaluation and/or those which have been used include IRA-35, IRA-68, IRA-94, IRA-900, IRC-718, and GT-73 (Rohm and Haas, Philadelphia PA) and aquarium zeolite (Argent Chemical Laboratories, Redmond WA). The key to obtaining useable data from an ion exchange test is to obtain non-toxic blanks. Since numerous ion exchange resins exist, guidance for preparing all resins for TIE work cannot be provided. A variety of procedures have been used in our laboratory to condition the columns and to obtain non-toxic blanks.

Effluent volumes ranging from 1,000 to 10,000 ml have been used, and the volume is dependent on the hardness of the dilution water, bed volume of the column, strength and type of the ion change resin, which ions were being exchanged, the toxicity of the effluent, and the species being tested. For example, for acutely toxic effluents, glass chromatography columns (11 mm i.d.) are packed with about 10 cm of resin and the solutions are pumped up through the column at a flow rate of 4 to 5 ml/min. First, a small volume of high purity water (e.g., 200 ml) is passed through the column, and discarded. Next, the dilution water (volumes are variable, i.e., 1,000-5,000 ml) is passed through the column until the pH of the post-column dilution water is above 7.0.

Following this procedure, the necessary volume of dilution water to use for toxicity testing is passed through the column and collected. The type of dilution water to use is effluent specific and in general, should be the same as the dilution water used in the toxicity test for the effluent. The pH of the post-column dilution water should be monitored and the pH adjusted to return the water solution to its original pH. Toxicity tests are then performed on the post-column dilution water sample (column blank). After obtaining non-toxic blanks for a particular batch of resin, the conditioning process can be used on other aliquots of the resin with a similar procedure; however, column toxicity blanks must always be tested.

To identify acute toxicity, we generally begin by using 200 ml of effluent (filtered or unfiltered) and collect the post-column effluent. The pH of the post-column effluent is checked and if necessary the pH is adjusted to that of the baseline test, and tested for toxicity. For chronic toxicity, the volume of effluent needed for the toxicity test will dictate the amount of resin and the size of the column. When evaluating a new resin, use proportions of water, effluent, and resin, similar to those described above for acutely toxic effluents. New aliquots of resins should be prepared and used for each ion exchange test. By doing so, artifactual toxicity problems

from other effluents and sample manipulations can be avoided.

We have had limited success in the elution of the ion exchange resin to recover the exchanged toxicant(s); therefore, we cannot provide specific guidance. In theory, cations and anions can be eluted from ion exchange resins using a strong acidic (HCl) or basic (NaOH) solution. Performing successful toxicity testing on these solutions is extremely difficult because of artifactual toxicity problems.

When toxicity is removed by the ion exchange test, useful information about the toxicant(s) may be obtained. However, as discussed above, the removal of a toxicant by the column may not be as straightforward as first perceived. The use of other manipulations and analytical measurements on the pre- and post-column effluents will be required to establish the significance of the results of the manipulation.

When toxicity is not removed by the ion exchange test and non-toxic blanks are obtained, the conclusion that the toxicant is not a cation or anion can be made. However, the slight possibility exists that the resin may not be able to exchange the toxicant because of steric and size considerations.

Section 5
Chlorine

5.1 General Overview

One of the first analytical measurements recommended in the Phase I documents (EPA, 1991A; EPA, 1992) upon arrival at the laboratory is for total residual chlorine (TRC) in each effluent sample. Chlorine is a commonly used biocide and oxidant and is frequently found at acutely toxic concentrations in municipal effluents (EPA, 1985C). Sublethal chronic toxicity from chlorine in effluent samples is not as likely to occur due to the degradation of chlorine (see below) with holding of the sample. Chlorine is unstable in aqueous solutions and decomposition is more rapid in solutions when chlorine is present at low concentrations. From the TRC measurement and the Phase I tests (sodium thiosulfate addition and aeration tests), further steps to identify the effects that might be due to chlorine can be taken. Oxidants other than chlorine occur in effluents and the removal of toxicity by the addition of sodium thiosulfate does not prove that chlorine was the cause of effluent sample toxicity.

Molecular chlorine or hypochlorite dissociates into free aqueous chlorine, hypochlorous acid, and hypochlorite ion when added to effluents. Chlorine can also combine with ammonia to form chloramines, i.e., mono-, di-, and tri-chloramines and with organic compounds, especially organic nitrogen (APHA, 1992). The measured total residual chlorine (TRC) of an effluent is the concentration of free and combined forms (mentioned above) added together. The portion of the TRC associated with an individual form is matrix dependent. Chlorinated industrial and wastewater effluents normally contain only the combined form of chlorine (APHA, 1992).

These various forms of combined chlorine may have different effect concentrations for toxicity, and the toxicities of these individual forms are not all known for acute or chronic toxicity to *C. dubia* or fathead minnows. However, while the TRC level in the effluent samples may be the same, the concentration of the various forms may be different because of the matrix inherent to the effluent. This matrix of TRC may also be variable from sample to sample for the same discharger.

Another complication is that current analytical methods for measuring TRC are not chlorine specific. Other oxidizing compounds, e.g., bromine, iodine, hydrogen peroxide, ozone, and manganese, will be quantified as chlorine by the analytical methods for measuring TRC and may provide the analyst with a false positive for chlorine.

5.2 Tracking Toxicity and TRC Levels

Several methods are available for measuring total TRC (EPA methods 330.1, 330.2, 330.3, 330.4, and 330.5 (EPA, 1983)). Measurements of TRC in the effluent upon arrival of the sample at the laboratory should always be made. If TRC is not detected, chlorine should not be considered a suspect toxicant since the analytical methods do not yield false negatives.

For acutely toxic effluents, grab samples both before and after the chlorination process should be collected simultaneously (i.e., within minutes of each other). Upon arrival of these samples at the laboratory, a baseline toxicity test should be initiated and at pre-determined intervals after day 1 (e.g., day 2, day 3, day 5, day 8) to evaluate whether the toxicity is degrading. TRC determinations should be performed in conjunction with each toxicity test.

Generally the TRC in most effluent samples stored at 4°C degrades in 2 to 5 d after collection. Therefore, if residual chlorine is a toxicant the toxicity of the post-chlorination sample should decrease as TRC levels decrease, and pre- and post-chlorination samples should have the same toxicity after the decay of TRC.

The toxicity of chlorine in an effluent sample will be dependent on the matrix of the effluent and the species tested. If chlorine toxicity data does not exist for the species being used, it will be necessary to measure the LC50 or IC25 of chlorine using the TIE organisms and dilution water. Using those LC50 and/or IC25 values, the comparison of TUs of the effluent to the TUs of residual chlorine is useful to evaluate the effects of the TRC. When the TU comparison data and pre- and post-chlorination toxicity data indicate TRC as a suspect toxicant Phase III procedures should be initiated.

With the measurable levels of TRC at sample collection, the loss of toxicity with the corresponding decreasing levels of TRC, and the pre- and post-chlorina-

tion samples exhibiting similar toxicity with the decrease in TRC, Phase III confirmation should begin (EPA, 1989B; EPA, 1993A). However, these steps will not insure that the toxicant is chlorine since other oxidants will be detected by the TRC measurement techniques.

Section 6
Identifying Toxicants Removed by Filtration

6.1 General Overview

If the results of Phase I tests indicate that the filtration manipulation removed or reduced toxicity, the investigator should carefully compare these results to those of the other manipulations before trying to identify the toxicants that might be on the filter. We have observed that metals, non-polar organic compounds and volatile compounds can all be removed under certain filtering conditions, but these observations have been dependent on the individual effluent or the sediment pore water samples. Other Phase I manipulations (e.g., EDTA, C_{18} SPE extraction) can lead to subsequent Phase II identification steps. However, for toxicity reductions effected by filtration, more intermediate steps of Phase I type manipulations must be done before analytical procedures are used to identify the toxicant(s). In addition, some other manipulations may provide specific information regarding the identity of toxicants that may have been removed by filtration; these include additions of PBO (Section 2.5.1), the graduated pH test (Phase I tests for determining toxicity caused by ammonia, metals and ionizable organic compounds), and the sodium thiosulfate test (Phase I test for detecting toxicity caused by volatile oxidants such as chlorine or metals). If one or more of these manipulations removes toxicity, then identification work should proceed as described in the previous sections to identify the cause of toxicity.

It is important to consider that all toxicity removed by filtration may not be actually removed by the process of filtering. For example, when the pH of the sample is altered, the mechanism(s) for removal by filtration can change. While ammonia is predominantly ionized at a sample pH of 8.3, the ammonia would not tend to be removed through volatilization if a vacuum was applied for filtration purposes. Yet by adjusting the sample pH to 11, the ionized ammonia concentration decreases to 1.7% at 25°C. When a sample is adjusted to a pH of 11, volatilization of a toxicologically significant amount of the un-ionized ammonia could occur and the toxicity results would indicate that filtration removed toxicity. Also, changes in speciation at elevated pH render many metals insoluble, which could result in their removal by the filter at pH 11 (Schubauer-Berigan et al., 1993B).

If the toxicant is thought to be a non-polar organic toxicant, and filtration partially removes toxicity, it may be useful and save toxicity testing time to eliminate the filtration step altogether before applying the sample to the C_{18} SPE column (discussion in Section 2.2.2 and Section 2.3.2).

6.2 Filter Extraction

When filtration has been the only manipulation to affect the toxicity, then extraction of the filters and tracking the toxicity of the extracts should be attempted. In addition, the use of other types of filters should be evaluated (i.e., nylon, teflon, and polycarbonate) to see if toxicity removal is a function of the filter type. In using the extraction procedures, the idea is to separate the toxic compounds associated with the filter by extracting them into a solvent. Next, efforts are made to concentrate the toxic compounds in the filter extract and test them at a concentration that can be related to the original sample and evaluate the efficiency of the extraction. Identifying the filter-removable contaminants without additional information can be difficult because of the lack of specificity of the filtration process. But once a suspect candidate has been discovered, then measurements can be made to determine whether a toxicologically significant concentration of the suspect toxicant(s) had indeed been removed by filtration. If this is the case, then it may not be necessary to consider further extractions of the filters. If, however, the concentrations of the suspect toxicant(s) are not decreased after filtration then it may be useful to attempt additional identifications by solvent extraction of the filters as described below.

One technique we have used with filterable toxicity is to extract the filters with either polar or non-polar solvents. To remove toxicants from the filter we have used either organic solvents (methanol, methylene chloride) or pH 3 high purity water as the extraction solvent. The solvent is then toxicity tested to track toxicity (methylene chloride must first be exchanged into methanol), additional Phase I tests are performed to characterize the filter extract, and then chemically analyzed using Phase II procedures. It is important to remove all of the methylene chloride before toxicity testing a filter extract and these procedures are described in detail in Section 2.6.2. To date, methanol has been used to extract toxicity from filters used with effluent samples and methylene chloride/ methanol solutions have been used to extract filters from

sediment pore water. The experiences described below are based on acute toxicity experiments, and efforts to recover filterable toxicity for chronically toxic effluent have not yet been needed.

To isolate a toxicant removed through filtration, several filters can be combined and extracted simultaneously if necessary. The volume of sample passed through the filters is important for calculating concentration factors, and should be recorded. The filtrate should also be reserved for toxicity comparisons and analytical testing. Sufficient sample should be passed through the filter to allow for both toxicity testing and chemical analysis on both the filtered sample and the filter-extract solution (generally >200 ml). Carefully move the filters to a glass (acid leached) or plastic beaker, then soak the filters (1-5) in 20 ml of solvent for 1 h. Cool water sonication is optional to attempt to recover particle-associated compounds. Carefully remove the filters and save (store at 4°C) in case additional extractions are necessary. If pH 3 high purity water is used as the extraction solvent, the extract should be readjusted to the initial pH of the sample, then toxicity tested. If methanol is used, it is evaporated to ~2 ml under a stream of nitrogen. Be careful to rinse the sides of the containers with methanol to ensure complete solubilization of organic compounds. This methanol solution can then be toxicity tested using SPE fraction testing procedures (Section 2.2.8). Alternatively, if a methylene chloride/methanol solvent is used, the solvent should first be exchanged into pure methanol (Section 2.6.2), then treated as the methanol extract described above. The concentration of the solvent extract will depend on the volume originally passed through the filters, which depends on the desired high test concentration, and the volume of extract and filtered sample required for analytical purposes. Blank filters (through which has been passed a volume of dilution water) should be extracted and tested identically to the sample filters to ensure that the solvents do not introduce artifactual toxicity.

For any of the extraction techniques, the solutions should be tested at the same time as the baseline test (unfiltered) and filtered sample test to compare the toxicity recovered by the filter extraction with that removed from the sample.

Another option for toxicants removed by filtration is to try other techniques to remove the toxicants which avoid filtration. For example, sediment pore water samples have been centrifuged at relatively high speeds (10,000-20,000 g) for 30 min prior to passing the sample over the C_{18} SPE column and filtration could thus be eliminated.

Filter extractions (EPA, 1991A) have been used in several sediment TIE studies, with procedures suggested for both non-polar organics and metals (Schubauer-Berigan et al., 1990; Schubauer-Berigan and Ankley, 1991). In some effluent and pore water samples, toxicity thought to be caused by non-polar organic compounds (e.g., PAHs and polymers) has also been removed by filtration. These compounds may be associated with particulate material, and be physically filtered from the sample, or removed by association with oil and grease that sorbs to the filter.

In some cases, binding of metals or organic compounds to inorganic and organic ligands in effluents or sediment pore waters will reduce their bioavailability and when toxicity testing filter extracts, it is always a concern that the matrix of the sample has been removed and that chemicals might become available when they were not in the original sample. If this were to happen, the extracts might be more toxic than expected and chemicals might be added to the suspect toxicant list erroneously. This kind of mistake should be caught by obtaining a good toxicity value for the suspect in an appropriate matrix (more detailed discussion in Section 2.6.1). Therefore, additional confirmation steps might be needed to eliminate the false suspects.

Section 7
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Appendix A
Effluent Volume Calculation Worksheets

Table A-1. Effluent Volume Calculation Worksheets

SPE Fractionation of the Effluent

1) Volume of effluent: See Table 2-6 for initial suggestions for the volume of effluent.		**ve**	_____ ml
2) SPE fractionation:			
Eluate volume from the SPE column: See Table 2-1 for approximate eluate volume or measure volume.		**a**	_____ ml
Concentration factor for eluate: $\mathbf{b_1 = ve \div a}$		$\mathbf{b_1}$	_____ x
3) Testing organism and conditions:			
Toxicity test volume/replicate:		**c**	_____ ml
C. dubia	10-15 ml/replicate (acute/chronic)		
D. magna	10-15 ml/replicate (acute)		
D. pulex	10-15 ml/replicate (acute)		
P. promelas	10-200 ml/replicate (acute)		
P. promelas	50-250 ml/replicate (chronic)		
Number of replicates:		**d**	_____
Initial sample + number of renewals		**e**	_____
Highest test concentration:		**f**	_____ x
Is the methanol concentration okay?[1] ml eluate = **f** x **c** ÷ $\mathbf{b_1}$ %methanol = ml eluate x 100 ÷ **c**			
4) Volume of eluate needed for toxicity testing:			
If no dilutions:	$\mathbf{g = c \times d \times e \times f \div b_1}$	**g**	_____ ml
If using 0.5 dilution factor: $\mathbf{h = 2 \times c \times d \times e \times f \div b_1}$		**h**	_____ ml
If using dilutions by spiking each concentration[2] directly: $\mathbf{i = f + f\div2 + f\div4 + f\div8}$ $\mathbf{j = i \times c \times d \times e \div b_1}$		**j**	_____ ml
Total volume of eluate used: **k** = **g**, **h**, or **j**		**k**	_____ ml
5) Volume of eluate remaining after toxicity testing:			
	$\mathbf{m_1 = a - k}$	$\mathbf{m_1}$	_____ ml

[1]Acceptable levels of methanol for *C. dubia* and fathead minnows are ≤0.6% and 1%, respectively.
[2]An example of using four test concentrations, the number of dilutions may vary.

Table A-1. Continued

Toxicity Testing and GC/MS Analysis of the SPE Concentrate

1) SPE Concentration of the SPE fraction:
   Eluate volume from the SPE column: **a** _____ ml
   See Table 2-3 for approximate eluate volume or measure volume.
   Concentration factor for the eluate: $b_2 = b_1 \times m_1 + a$ b_2 _____ x

2) Testing organism and conditions:
   Toxicity test volume/replicate: **c** _____ ml
   - *C. dubia* 10-15 ml/replicate (acute/chronic)
   - *D. magna* 10-25 ml/replicate (acute)
   - *D. pulex* 10-25 ml/replicate (acute)
   - *P. promelas* 10-200 ml/replicate (acute)
   - *P. promelas* 50-250 ml/replicate (chronic)

   Number of replicates: **d** _____
   Initial sample + number of renewals: **e** _____
   Highest test concentration: **f** _____ x

3) Volume of eluate needed for toxicity testing:
   If no dilutions: $g = c \times d \times e \times f \div b_2$ **g** _____ ml

   If using 0.5 dilution factor:
   $h = 2 \times c \times d \times e \times f \div b_2$ **h** _____ ml

   If using dilutions by spiking each concentration directly:
   $i = f + f \div 2 + f \div 4 + f \div 8$
   $j = i \times c \times d \times e \div b_2$ **j** _____ ml

   Total volume of eluate used: **k** = **g**, **h**, or **j** **k** _____ ml

4) Amount of eluate used for GC/MS analysis: **l** _____ ml

5) Volume of eluate remaining after toxicity testing:
   $m_2 = a - k - l$ m_2 _____ ml

Table A-1. Continued

HPLC Fractionation of the SPE Concentrate

1) HPLC Fractionation of the SPE concentrate:

HPLC Fraction volume: See Sections 2.2.10 and 2.3.10		**a**	_____ ml
Concentration factor for the eluate: $b_3 = b_2 \times m_2 + a$		b_3	_____ x

2) Testing organism and conditions:

Toxicity test volume/replicate:		**c**	_____ ml
C. dubia	10-15 ml/replicate (acute/chronic)		
D. magna	10-25 ml/replicate (acute)		
D. pulex	10-25 ml/replicate (acute)		
P. promelas	10-200 ml/replicate (acute)		
P. promelas	50-250 ml/replicate (chronic)		
Number of replicates:		**d**	_____
Initial sample + number of renewals:		**e**	_____
Highest test concentration:		**f**	_____ x

3) Volume of eluate needed for toxicity testing:

If no dilutions:	$g = c \times d \times e \times f + b_3$	**g**	_____ ml
If using 0.5 dilution factor: $h = 2 \times c \times d \times e \times f + b_3$		**h**	_____ ml
If using dilutions by spiking each concentration directly: $i = f + f{+}2 + f{+}4 + f{+}8$ $j = i \times c \times d \times e + b_3$		**j**	_____ ml
Total volume of eluate used: **k** = **g**, **h**, or **j**		**k**	_____ ml

5) Volume of eluate remaining after toxicity testing:

	$m_3 = a - k - i$	m_3	_____ ml

Table A-1. Continued

Concentration of the HPLC Fraction for Toxicity Testing and GC/MS Analysis

1) SPE Concentration of the SPE fraction:			
Eluate volume from the SPE column:		**a**	_____ ml
See Table 2-3 for approximate eluate volume or measure volume.			
Concentration factor for the eluate: $b_4 = b_3 \times m_3 + a$		b_4	_____ x
2) Testing organism and conditions:			
Toxicity test volume/replicate:		**c**	_____ ml
C. dubia	10-15 ml/replicate (acute/chronic)		
D. magna	10-25 ml/replicate (acute)		
D. pulex	10-25 ml/replicate (acute)		
P. promelas	10-200 ml/replicate (acute)		
P. promelas	50-250 ml/replicate (chronic)		
Number of replicates:		**d**	_____
Initial sample + number of renewals:		**e**	_____
Highest test concentration:		**f**	_____ x
3) Volume of eluate needed for toxicity testing:			
If no dilutions:	$g = c \times d \times e \times f + b_4$	**g**	_____ ml
If using 0.5 dilution factor:			
	$h = 2 \times c \times d \times e \times f + b_4$	**h**	_____ ml
If using dilutions by spiking each concentration directly:			
	$i = f + f{+}2 + f{+}4 + f{+}8$		
	$j = i \times c \times d \times e + b_4$	**j**	_____ ml
Total volume of eluate used: **k** = **g**, **h**, or **j**		**k**	_____ ml
4) Amount of eluate used for GC/MS analysis:		**l**	_____ ml
5) Volume of eluate remaining after toxicity testing:			
	$m_4 = a - k - l$	m_4	_____ ml

Table A-2. Effluent Volume Calculation Worksheets (Example)

SPE Fractionation of the Effluent

1) Volume of effluent: See Table 2-6 for initial suggestions for the volume of effluent.		**ve**	20,000 ml
2) SPE fractionation:			
Eluate volume from the SPE column: See Table 2-1 for approximate eluate volume or measure volume.		**a**	40 ml
Concentration factor for eluate: b_1 = **ve** ÷ **a**		b_1	500 x
3) Testing organism and conditions:			
Toxicity test volume/replicate:		**c**	10 ml
C. dubia	10-15 ml/replicate (acute/chronic)		
D. magna	10-15 ml/replicate (acute)		
D. pulex	10-15 ml/replicate (acute)		
P. promelas	10-200 ml/replicate (acute)		
P. promelas	50-250 ml/replicate (chronic)		
Number of replicates:		**d**	5
Initial sample + number of renewals		**e**	7
Highest test concentration:		**f**	2 x
Is the methanol concentration okay?[1]			
ml eluate = **f** x **c** ÷ b_1			
%methanol = ml eluate x 100 ÷ **c**			
4) Volume of eluate needed for toxicity testing:			
If no dilutions: **g** = **c** x **d** x **e** x **f** ÷ b_1		**g**	ml
If using 0.5 dilution factor:			
h = 2 x **c** x **d** x **e** x **f** ÷ b_1		**h**	ml
If using dilutions by spiking each concentration[2] directly:			
i = **f** + **f**÷2 + **f**÷4			
j = **i** x **c** x **d** x **e** ÷ b_1		**j**	2.45 ml
Total volume of eluate used: **k** = **g**, **h**, or **j**		**k**	2.45 ml
5) Volume of eluate remaining after toxicity testing:			
m_1 = **a** - **k**		m_1	37.55 ml

[1]Acceptable levels of methanol for *C. dubia* and fathead minnows are ≤0.6% and 1%, respectively.
[2]Example uses three test concentrations.

Table A-2. Continued

Toxicity Testing and GC/MS Analysis of the SPE Concentrate

1) SPE Concentration of the SPE fraction:			
Eluate volume from the SPE column:		**a**	0.44 ml
See Table 2-3 for approximate eluate volume or measure volume.			
Concentration factor for the eluate: $b_2 = b_1 \times m_1 \div a$		b_2	42,670 x
2) Testing organism and conditions:			
Toxicity test volume/replicate:		**c**	10 ml
C. dubia	10-15 ml/replicate (acute/chronic)		
D. magna	10-25 ml/replicate (acute)		
D. pulex	10-25 ml/replicate (acute)		
P. promelas	10-200 ml/replicate (acute)		
P. promelas	50-250 ml/replicate (chronic)		
Number of replicates:		**d**	5
Initial sample + number of renewals:		**e**	7
Highest test concentration:		**f**	4 x
3) Volume of eluate needed for toxicity testing:			
If no dilutions: $g = c \times d \times e \times f \div b_2$		**g**	ml
If using 0.5 dilution factor: $h = 2 \times c \times d \times e \times f \div b_2$		**h**	ml
If using dilutions by spiking each concentration directly: $I = f + f \div 2 + f \div 4$ $j = I \times c \times d \times e \div b_2$		**j**	0.057 ml
Total volume of eluate used: **k** = **g**, **h**, or **j**		**k**	0.057 ml
4) Amount of eluate used for GC/MS analysis:		**l**	0.018 ml
5) Volume of eluate remaining after toxicity testing: $m_2 = a - k - l$		m_2	0.365 ml

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Table A-2. Continued

Concentration of the HPLC Fraction for Toxicity Testing and GC/MS Analysis

1) SPE Concentration of the SPE fraction:			
Eluate volume from the SPE column: See Table 2-3 for approximate eluate volume or measure volume.	a	0.22	ml
Concentration factor for the eluate: $b_4 = b_3 \times m_3 \div a$	b_4	48.495	x
2) Testing organism and conditions:			
Toxicity test volume/replicate:	c	10	ml
C. dubia 10-15 ml/replicate (acute/chronic)			
D. magna 10-25 ml/replicate (acute)			
D. pulex 10-25 ml/replicate (acute)			
P. promelas 10-200 ml/replicate (acute)			
P. promelas 50-250 ml/replicate (chronic)			
Number of replicates:	d	5	
Initial sample + number of renewals:	e	7	
Highest test concentration:	f	16	x
3) Volume of eluate needed for toxicity testing:			
If no dilutions: $g = c \times d \times e \times f \div b_4$	g		ml
If using 0.5 dilution factor: $h = 2 \times c \times d \times e \times f \div b_4$	h		ml
If using dilutions by spiking each concentration directly: $i = f + f \div 2 + f \div 4$; $j = i \times c \times d \times e \div b_4$	j	0.202	ml
Total volume of eluate used: k = g, h, or j	k	0.202	ml
4) Amount of eluate used for GC/MS analysis:	l	0.018	ml
5) Volume of eluate remaining after toxicity testing: $m_4 = a - k - l$	m_4	0	ml

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