

Marine Toxicity Identification Evaluation (TIE)

Phase I Guidance Document

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Abstract

During the last ten years Toxicity Identification Evaluation (TIE) methods have been used extensively with freshwater effluents, receiving waters, and sediments. TIEs may be required by state or federal agencies as a result of enforcement actions, as a condition of the discharger's National Pollutant Discharge Elimination System (NPDES) permit, or may be conducted voluntarily by permittees. This guidance document, using the freshwater TIE approach as a model, has been developed to aid in conducting acute and chronic marine TIEs. It focuses on Phase I of the TIE: Toxicity Characterization. Phase I of a TIE characterizes the classes of toxicants causing adverse biological effects. These classes may include metals, organics, pH dependent toxicants, volatile toxicants, filterable toxicants, and oxidants. In this document, information is provided for: (1) salinity adjustment of freshwater effluents with brine, (2) general guidance for the performance of small volume marine toxicity tests with Atlantic, Gulf, and Pacific Coast species used in NPDES permit or as a NPDES permit testing requirement, (3) tolerances to the chemicals added during a TIE, and (4) the conduct of TIE manipulations. These acute/chronic TIE procedures have been developed for a number of specific macroalgas, echinoids, mysids, bivalves, an amphipod, gastropods, and fishes. Recommended manipulations described in this document include filtration, aeration, EDTA chelation, oxidant reduction, graduated pH, C₁₈ solid phase extraction (SPE), cation exchange SPE, and sea lettuce *Ulva lactuca* addition.

Foreword

The Marine Toxicity Identification Evaluation (TIE): Phase I Guidance Document focuses on methods for characterizing toxicity associated with discharges to marine waters including effluents and receiving waters. Its purpose is to provide guidance to dischargers, testing laboratory staff, and local, state, and regional personnel in conducting Phase I of a marine TIE. Methods for conducting freshwater toxicity tests and TIEs have been produced (EPA 1991a, 1991b, 1993a, 1993b, 1993c); however, these methods were not directly applicable to marine samples. As stated in EPA 1993c:

These methods are not mandatory but are intended to aid those who need to characterize, identify or confirm the cause of toxicity in effluents or other aqueous samples such as ambient waters, sediments, and leachates. Where we lack experience, we have indicated this and have suggested avenues to follow. All tests need not be done on every sample; the tests are, in general, independent. However, experience has taught us that skipping tests may result in wasted time, especially in the early stages of Phase I. An exception to this is when one wants to know only if a specific substance, for example ammonia, is causing the toxicity or if toxicants other than ammonia are involved. Otherwise, we urge the whole battery of tests.

We assume the reader is familiar with the following documents describing (1) TIE methods: *Toxicity Identification Evaluation: Characterization of Chronically Toxic Effluents, Phase I* (EPA 1991a), *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures*, Second Edition (EPA 1991b), *Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA 1993b), *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA 1993c); 2) toxicity testing methods: *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA 1994), *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA 1993a), *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA 1995); and 3) Toxicity Reduction Evaluations (TREs): *Toxicity Reduction Evaluation Protocol for Municipal Wastewater Treatment Plants* (EPA 1989a), and *Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations (TREs)* (EPA 1989b). Methodologies for both acute and sublethal (chronic) toxicity testing have been included in this manual. We invite comments on this document in order to improve future editions.

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Abbreviations

AED	Atlantic Ecology Division, EPA, Narragansett, Rhode Island
C ₁₈	Octadecyl
CWA	Clean Water Act
DI	Deionized Water
DO	Dissolved Oxygen
EC ₅₀	Median Effect Concentration
EDTA	Ethylenediaminetetraacetic Acid
EPA	U.S. Environmental Protection Agency
GP2	General Purpose Medium Number 2
LC ₅₀	Median Lethal Concentration
MEOH	HPLC Grade Methanol
MED	Mid-Continent Ecology Division, EPA, Duluth, Minnesota
MSDS	Materials Safety Data Sheets
Na ₂ S ₂ O ₃	Sodium Thiosulfate
NPDES	National Pollutant Discharge Elimination System
QAP	Quality Assurance Plan
SDS	Sodium Dodecyl Sulfate
SLP	Standard Laboratory Procedure
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction
TIE	Toxicity Identification Evaluation
TRC	Total Residual Chlorine
TRE	Toxicity Reduction Evaluation
WQC	Water Quality Criteria

Section 1

Introduction

1.1 Background

The Clean Water Act (CWA 1972), in its original and all subsequent versions, established a "national policy that the discharge of toxic pollutants in toxic amounts be prohibited." The goal of the CWA is to eliminate the discharge of pollutants into waters in the U.S.; however, this goal is not immediately attainable. Consequently, the CWA allows for National Pollutant Discharge Elimination System (NPDES) permits for wastewater discharges. In order to insure that the CWA's prohibition on toxic discharges are met, an integrated system of testing procedures has been developed. This document presents additional methods for the conduct of Toxicity Identification Evaluation (TIE) which are part of this testing system.

During the last several years, TIE methods were developed and applied to freshwater effluents and receiving waters (Parkhurst et al. 1979; Walsh and Garnas 1983; Gasith et al. 1988; EPA 1991a, 1991b, 1993b, 1993c; Burkhard and Ankley 1989; Norberg-King et al. 1991). Methods for freshwater sediment TIEs have also been drafted (Ankley et al. 1992a). Implementation of these methods has demonstrated the regulatory and scientific utility of the TIE approach. For example, TIEs have identified specific 'problem toxicants' in effluents (Schimmel et al. 1988; Goodfellow et al. 1989; Ankley et al. 1990a; Jop et al. 1991a; Norberg-King et al. 1991; Amato et al. 1992; McCulloch et al. 1993; Ankley and Burkhard 1992; Burkhard and Jenson 1993; Schubauer-Berigan et al. 1993) receiving waters (Galassi et al. 1988; Schimmel et al. 1988; Norberg-King et al. 1991; Kszos et al. 1992), and freshwater sediments (Ankley et al. 1990b; Schubauer-Berigan and Ankley 1991; Ankley et al. 1992b; Hoke et al. 1992; Krantzberg and Boyd 1992; Schubauer-Berigan et al. 1993; Wenholz and Crunkilton 1995; Gupta and Karuppiah 1996). Furthermore, improvements have been incorporated as methods were applied (Doi and Grothe 1989; Ankley et al. 1990b; Durhan et al. 1990; Burkhard et al. 1991; Jop et al. 1991b; Mount and Mount 1992; Wong et al. 1996; Bailey et al. 1996; Hewitt et al. 1996).

1.2 Related Documents

As stated in the forward, this report assumes that the reader is familiar with several related documents. The report, *Methods for Aquatic TIEs: Phase I Toxicity Characterization Procedures*,

Second Edition (EPA 1991b), contains essential background information on Phase I TIE procedures that is not duplicated in this report; and in addition, that report describes the related freshwater TIE procedures. Also, this report assumes that the reader is familiar with the following related documents: *Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA 1993b), *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA 1993c), *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA 1994), *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA 1993a), *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA 1995), *Toxicity Reduction Evaluation Protocol for Municipal Wastewater Treatment Plants* (EPA 1989a), and *Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations (TREs)* (EPA 1989b), and that this report will be used in conjunction with these related documents. Methodologies for both acute and sublethal toxicity testing have been included in this manual.

1.3 Development of Marine TIE Methods

Research conducted at the U.S. Environmental Protection Agency's (EPA) Atlantic Ecology Division (AED) in Narragansett, RI has focused on the development of marine TIEs for saline samples using freshwater TIE methods as models. In addition, two new TIE manipulations are described: a cation exchange manipulation and macroalga *Ulva lactuca* addition (Burgess et al. submitted; Ho et al. in prep.). Marine TIEs are performed using marine species on waters discharging into or from marine environments. The marine TIE methods described in this document are designed specifically for use with the marine species listed in Table 1-1. Other TIE or toxicity testing directed fractionation studies performed in marine waters and sediments used mutagenic (Grifoll et al. 1988; Grifoll et al. 1990; Grifoll et al. 1992; Samiloff et al. 1983; Ho and Quinn 1993a; Ho and Quinn 1993b) and whole organism assays (Walsh and Garnas 1983; Quilliam and Wright 1989; Higashi et al. 1992; Svenson et

al. 1992; Weis et al. 1992; Burgess et al. 1993; Bailey et al., 1995; Burgess et al., 1995; Ho et al., 1995).

Table 1-1. Marine Species Discussed in This Document.

Region	Organism Type	Species
Atlantic and Gulf Coast	Macroalga	<i>Champia parvula</i>
	Echinoid	<i>Arbacia punctulata</i>
	Bivalve	<i>Mulinia lateralis</i>
	Mysid	<i>Mysidopsis bahia</i>
	Amphipod	<i>Ampelisca abdita</i>
	Fishes	<i>Menidia beryllina</i> <i>Cyprinodon variegatus</i>
Pacific Coast	Macroalga	<i>Macrocystis pyrifera</i>
	Echinoids	<i>Strongylocentrotus purpuratus</i> <i>Dendraster excentricus</i>
	Bivalves	<i>Crassostrea gigas</i> <i>Mytilus californianus</i> <i>Mytilus galloprovincialis</i>
	Gastropod	<i>Haliotis rufescens</i>
	Fish	<i>Atherinops affinis</i>

Two fundamental questions addressed during the development of this manual were: (1) can marine species tolerate the chemicals used in TIE manipulations and (2) are freshwater TIE chemical manipulations directly applicable to saline effluent samples? The tolerance of marine species was addressed with most of the species in Table 1-1 using TIE additives (e.g., ethylenediaminetetraacetic acid (EDTA), sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), and methanol). A series of Phase I TIEs, conducted with several marine species on four industrial (electrical equipment) and municipal effluents and several mock effluents and single chemicals, were used to address whether the freshwater manipulations were compatible with saline samples (Burgess et al. 1995; Ho et al. 1995; Ho et al. in prep.). It should be noted that the Atlantic and Gulf coast species in Table 1-1 have undergone fairly extensive TIE research with "real" effluents for the preparation of this document. The Pacific coast species have not undergone similar research; however, they have been used in the private sector for the past few years.

Results of tolerance tests for EDTA and $\text{Na}_2\text{S}_2\text{O}_3$ readily demonstrated that these marine species can tolerate TIE manipulations at concentrations sufficient to alter toxicant effects. Generally, the effect concentrations for various additives by these

marine species were similar to those for freshwater species (EPA 1991b).

The feasibility of using TIE chemicals and manipulations, such as EDTA, cation exchange solid phase extraction (SPE), and C_{18} , to characterize toxicity in a seawater matrix has been illustrated through several studies. For example, experiments with the chelator EDTA investigated the toxicity of metals in seawater (Sunda and Guillard 1976; Anderson and Morel 1978). Cation exchange has been used extensively for isolating divalent metals from seawater (e.g., McLaren et al 1985; Pai and Fang 1990). Similarly, C_{18} reverse-phase chromatography has been applied to measure the marine partitioning behavior of chemicals between dissolved organic carbon and aqueous phases (Mills et al. 1982; Hanson et al. 1988).

As the procedures in this manual illustrate, the majority of the freshwater methods (EPA 1991a, 1991b) functioned acceptably when used with marine samples. Two primary exceptions were the graduated pH procedures designed to characterize pH dependent toxicants and the conduct of each manipulation at pHs 9 and 11 (EPA 1991b). Seawater has a strong carbonate buffering system that makes any long-term pH adjustments difficult to maintain. Alteration of seawater pH with acids, bases, or organic buffers, while often initially successful, does not permanently repress the natural carbonate buffering and prevent the return to initial seawater pH. We found the most effective way to successfully adjust and maintain the pH of seawater samples (for the durations required for toxicity testing) was to conduct exposures in controlled atmospheric chambers. Unlike the variety of procedures used in the chronic and acute freshwater TIE methods (EPA 1991a, 1991b, 1993b, 1993c), we found that controlling pH in atmospheric chambers was the least intrusive, and only efficient, method of those we tested.

The use of 'closed chambers' was also investigated. In this approach, exposure chambers were completely filled with the sample, adjusted to the desired pH with acid or base, and the test organisms added. Tight-fitting lids sealed the chambers from the atmosphere. Closed chambers, while useful in some applications (i.e., where dissolved oxygen was not low) were not as universally applicable as the controlled atmospheric chambers.

Unlike the freshwater graduated pH procedure which is conducted at three distinctly different pHs (e.g., 6.0, 7.0 and 8.0 (EPA 1991b)), exposures on saline waters are performed at pHs 7, ambient seawater (8.2-8.4), and 9. These pH values were adopted because: (1) some marine test species demonstrated unacceptable control survival at pHs less than 7 and (2) maintaining sample pHs at levels two pH units above or below ambient pH levels was difficult and often ineffective. Additionally, shifting sample pHs to 11, resulted in the precipitation of some seawater hydroxides (Stumm and Morgan 1981) and severely altered seawater composition.

Section 2

Health and Safety

The following section has been reprinted, with minor modifications from *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures*, Second Edition (EPA 1991b).

Since TIEs involve, by definition, working with effluents of unknown composition, the accompanying safety measures must be adequate for a wide spectrum of chemical and biological agents. Often, one may be able to judge probable concerns from the type of treatment used. For example, extended aeration is likely to minimize the presence of volatile chemicals and chlorinated effluents are less likely to contain viable pathogens.

Exposure to water samples during collection and its use in the laboratory should be kept at a minimum. Inhalation and dermal absorption can be reduced by using laboratory hoods and wearing rubber gloves, laboratory aprons or coats, safety glasses, and respirators. Further guidance on health and safety for toxicity testing is described in Walters and Jameson (1984).

In addition to taking precautions with effluent samples, a number of the reagents that might be used during the tests described in this manual are known or suspected to be toxic to humans. Analysts should familiarize themselves with safe handling procedures for these chemicals (DHEW, 1977; OSHA 1976), as well as the manufacturer's Materials Safety Data Sheets (MSDS). Use of the compounds may also necessitate specific waste disposal practices.

Section 3

Quality Assurance

The following section has been reprinted, with minor modifications from *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures*, Second Edition (EPA 1991b).

Quality assurance is composed of two aspects, quality verification and quality control. Quality verification entails a demonstration that the proposed study plan was followed as detailed and that work carried out was properly documented. Some of the aspects of quality verification include chain of custody procedures, statements on the objective of the study and what is known about the problem at its outset, instrumental log books, and work assignments. This aspect of quality assurance ensures that a "paper trail" is created to prove that the work plan has been covered completely. The quality control aspect of quality assurance involves the procedures which take place such as the number of samples to be taken and the mode of collection, standard operating procedures for analyses, and spiking protocols.

No set quality assurance program can be dictated for a TIE; the formula to a successful study will be unique to each situation. However, adherence to some general guidelines in formulating a Quality Assurance Plan (QAP) may increase the probability of success.

In preparing a QAP, enough detail should be included so that any investigator with an appropriate background could take over the study at any time. Cross checking of results and procedures should be built into the program to the extent possible. Records should be of a quality that can be offered as evidence in court. Generally, the QAP should be provided in a narrative form that encourages the user to think about quality assurance. To be effective, the QAP must be more than a paper exercise simply restating standard operating procedures (SOPs). It must increase communication between clients, program planners, field and laboratory personnel and data analysts. The QAP must make clear the specific responsibilities of each individual. The larger the staff, the more important this becomes. While QAPs may seem to be an inconvenience, the amount of effort they require is commensurate with the benefits derived.

3.1 TIE Quality Control Plans

A successful TIE is dependent upon a strong quality control program. Obtaining quality TIE data is difficult because the constituents are unknown in contrast to quality control procedures for a standard analytical method for a specific chemical. In such an analysis, one knows the characteristics of the analyte and the implications of the analytical procedure being used. Without knowledge of the physical/chemical characteristics of the analyte, however, the impact of various analytical procedures on the compound in question is not known. Further, quality control procedures are specific to each compound; quality control procedures appropriate to one analyte may be completely inappropriate to another.

The problem of quality control is further aggravated because quality control procedures for aquatic toxicity test may be radically different from those required for individual chemical analyses. This additional dimension to quality control requires a unique framework of checks and controls to be successful. The impacts of chemical analytical procedures on sample toxicity must be included. Likewise, procedures used to insure quality toxicity test results should not impact chemical analyses. For example, in performing a standard aquatic toxicity test, samples with low dissolved oxygen (DO) are usually aerated. This practice may, however, result in a loss of toxicity if the toxicant is volatile or subject to oxidation.

3.2 Cost Considerations/Concessions

The quality control practices required in any given experiment must be weighed against the importance of the data and decisions to be based upon that data. The crucial nature of certain data will demand stringent controls, while quality control can be lessened in other experiments having less impact on the overall outcome.

Effluent toxicant identification evaluations require a large number of aquatic toxicity tests. The decision to use the standard toxicity test methods described in EPA 1993a, 1994, 1995 (involving a relatively high degree of quality control), must be weighed against the degree of complexity involved, the time required and number of tests performed; all of these affect the cost of testing. For this reason, toxicity tests used in the early phases of the evaluation generally do not follow these protocols, nor do they require exacting quality controls because the data are only

preliminary. Phase I, and to a lesser extent, Phase II results are more tentative in nature as compared to tests performed for confirmation of effluent toxicant(s) in Phase III.

The progressions towards increasing definitive results is also reflected in the use of only a few species in the initial evaluation studies and multiple species in the later stages. The use of several species of aquatic organisms to assure that the effluent toxicity has been reduced to acceptable levels is necessary because species may have different sensitivities to the same pollutant. Quality control must relate to the ultimate goal of attaining and maintaining the designated uses of the receiving water. For this reason, final effluent test results must be of sufficient quality to ensure ecosystem protection. The use of dilution water for the toxicity tests that mimics receiving water characteristics (i.e., salinity) will help to ensure that the effluent will remain non-toxic after being discharged into the environment. In the instances where the effluent dominates the receiving water, the dilution water should mimic the characteristics of the effluent. In addition, it is essential that variability in the cause of effluent toxicity be defined during the course of the TIE so that appropriate control actions provide a final effluent safe for discharge.

3.3 Variability

The opportunities to retest any effluent to confirm the quality of initial TIE results will be limited at best. In addition to the shifting chemical and toxicological nature of the discharge over time, individual effluent samples stored in the laboratory change. Effluent constituents degrade at unknown rates, as each toxicant has its own rate of change. The change in a sample's toxicity over time represents the cumulative change in all of the constituents, plus that variation resulting from experimental error. Some guidelines for assessing and minimizing changes in sample chemistry and toxicity are discussed in later sections. Regardless of the precautions taken to minimize sample changes, a sample cannot be retested with certainty that it has not changed.

3.4 Intra-Laboratory Communication

Quality control procedures in chemistry and biology can be quite different. For example, phthalates are a frequent analytical contaminant requiring special precautions that are not of toxicological concern. The toxicological problem presented by zinc levels typically associated with new glassware are of no concern to those performing organic analyses. The difference in glassware cleanup procedures is an example of one of many differences that must be resolved. Cleaning procedures must be established to cover the requirements of both. Time schedules for analyses must be detailed in advance. One cannot assume toxicant stability; therefore, time delays between the biological and chemical analysis of a sample cannot be tolerated.

3.5 Record Keeping

Throughout the TIE, record keeping is an important aspect of quality verification. All observations, including organism symptoms, should be documented. Details that may seem unimportant during testing may be crucial in later stages of the evaluation. Investigators must record test results in a manner such that preconceived notions about the effluent toxicants are not unintentionally reflected in the data. TIEs required by state or federal pollution control agencies may require that some or all records be reviewed.

3.6 Phase I Considerations

Effluent toxicity is "tracked" through Phases I, II, and III using aquatic organisms. Such tracking is the only way to detect where the toxicants are until their identity is known. The organism's response must be considered as the foundation and therefore, the toxicity test results must be dependable. System blanks (blank sample carried through procedures and analyses identical to those performed on effluent sample) are used extensively throughout the TIE to detect toxic artifacts added during the effluent characterization manipulations. With the exception of tests intended to make the effluent more toxic, or situations in which a known amount of toxicity has been intentionally added, sample manipulation should not cause the effluent toxicity to change.

There are many sources of toxicity artifacts in Phase I. These include: excessive ionic strength resulting from the addition of acid and base during pH adjustment, formation of toxic products by acids and bases, contaminated air or carbon dioxide sources, inadequate mixing of test solutions, contaminants leached from filters, pH probes, solid phase extraction (SPE) columns, and the reagents added and their contaminants. The appropriate toxicity data for the reagent chemicals used in Phase I and common aquatic test organisms are provided as needed in subsequent sections of this document.

Frequently, toxic artifacts are unknowingly introduced. For example, some pH meters with refillable electrodes can act as a source of silver which can reach toxic levels in the solutions being measured for pH. This is especially a problem where there is a need to carefully maintain or track solution pH. Using pH electrodes without membranes avoids the silver problem (which can only be detected by the profuse use of blanks).

Oil in air lines or from compressors is a source of contamination. Simple aeration devices, such as those sold for use with aquaria are better as long as caution is taken to prevent contamination of the laboratory air which is taken in by the pump.

Worst case blanks should be used to better ensure that toxicity artifacts will be recognized. Test chambers should be covered to prevent contamination by dust and to minimize evaporation. Since small volumes are often used, evaporation must be controlled. For some manipulations, plastic disposable test chambers are recommended to avoid problems related to the reuse of test chambers. Cups from the same lot should be spot-checked for toxicity.

Glassware used in various tests and analyses must be cleaned not only for the chemical analyses but so that toxicity is not introduced either by other contaminants or by residues of cleaning agents. Since the organisms are sensitive to all chemicals at some concentrations, all toxic concentrations must be removed and not just those for which analyses are being made.

Randomization techniques, careful observance of organism exposure times and the use of organisms of approximately the same age ensure quality data. Standard reference toxicant tests should be performed with the aquatic test species on a regular basis and control charts should be developed (EPA 1993a, 1994, 1995). During Phase I it will not be known how much the toxicity of the reference toxicants varies over time compared to the toxicant(s). When the toxicants are known, they should be used as the reference toxicant. Reference toxicant tests should be performed to coincide with the TIE testing schedule.

3.7 Phase II Considerations

In Phase II, a more detailed quality control program is required. Interferences in toxicant analysis are for the most part unknown initially but as toxicant identifications are made, interferences can be determined. Likewise instrumental response, degree of toxicant separation, and detector sensitivity can be determined as identifications proceed.

3.8 Phase III Considerations

In Phase III of a TIE, the detail paid to quality control and verification is at the maximum. This phase of the study responds to the compromises made to data quality in Phases I and II. For this reason, confidence intervals for toxicity and chemical measurements must be calculated. These measurements allow the correlation between the concentration of the toxicants and effluent toxicity to be checked for significance based on test variability. Effluent manipulations prior to chemical analyses and toxicity testing are minimized in this phase in an effort to decrease the chance for production of artifacts. Field replicates to validate the precision of the sampling techniques and laboratory replicates to validate the precision of analyses must be included in the Phase III quality control program. System blanks must be provided. Calibration standards and spiked samples must also be included in the laboratory quality control program. Because an attempt will be made to correlate effluent toxicity to toxicant concentration, spiking experiments are important in determining recovery for the toxicant(s). These procedures are feasible because the identities of the substances being measured are known.

The toxicants being analyzed can be tested for using pure compounds, thereby alleviating the need for a general reference toxicant. Because the test organism also acts as an analytical detector in the correlation of effluent toxicity with toxicant(s) concentration, changes in the sensitivity of the test organism must be known. This is best achieved by using the same chemicals identified for the reference toxicants.

Section 4

Equipment, Supplies, and Facilities

Equipment necessary to perform each of the Phase I procedures is listed in Section 9 under each manipulation. In addition, basic analytical laboratory equipment such as pH meters, pumps (vacuum and fluid), pipettors, and the capacity for maintaining compressed gas cylinders and regulators are required.

A reliable source for large numbers (hundreds) of test organisms is essential for TIE work. It is recommended that on-site culturing facilities be used to prevent TIE activity from being subject to seasonal availability of field collected organisms or delays in shipping from suppliers.

A supply of "clean" saline water is necessary as a diluent, a natural seawater control, a performance control for reference toxicant testing (EPA 1994), and as a source of hypersaline brine. Large supplies of brine solutions (100‰) can be prepared, stored,

diluted with deionized water (DI) to desired salinities, and used in batches to insure seawater consistency and to avoid seasonal fluctuations in water quality. At AED, saline water has been prepared from both natural seawater and GP2 synthetic seawater (e.g., EPA 1994). In addition, water used for test organism culturing should come from the same source (EPA 1994). For a discussion of acceptable source waters and their quality control, one should consult the reports: *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Marine and Estuarine Organisms*, Second Edition (EPA 1994) and *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to West Coast Marine and Estuarine Organisms* (EPA 1995). Further discussion will be found in Section 5.4: Salinity Adjustments and Dilution Water.

Section 5

Sample Collection, Handling, Salinity Adjustment, and Dilution

5.1 General Collection

Effluents should be collected in clean plastic or glass containers. Generally, the collection site should be the same as the monitoring site specified in the NPDES permit unless a specific concern suggests otherwise (cf. EPA 1994). Examples of when it would be appropriate to use alternate or additional collection sites include: (1) better access to a sampling point between the final discharge and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge and it is desired to obtain a sample prior to chlorination; or (3) there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants prior to their being combined with other wastewater streams or non-contact cooling water. It may be possible to collect enough additional sample at the time of compliance sampling if a TIE is to be done. EPA (1991b) provides further guidance on sample handling and includes a discussion of the choice between plastic and glass containers that is useful, since certain types of toxicants may absorb to certain surfaces. Additionally, the documents (EPA 1994, 1995) should be consulted for collection requirements.

The time, date, location, duration and procedures used should be recorded for effluent sample collection. During collection, aeration and transfer of effluents should be minimized to reduce the loss of volatile chemicals. Any additional observations such as color, turbidity, chlorine odor, or unusual sampling conditions (i.e., heavy rain) should be noted. If an industrial effluent is to be tested, it may be useful to record any available information on the current production levels and types of operating processes. The condition of the facilities treatment system should also be determined by the individual collecting the sample. In addition, it is recommended that total ammonia, total residual chlorine (TRC), pH, dissolved oxygen (DO), salinity/conductivity, and temperature be recorded upon arrival of the sample. At AED, salinity is usually measured using a refractometer for marine samples. Figure 5-1 provides a sample log book page for recording of sampling data.

Stored or shipped samples should be kept at 4°C and tested for toxicity within 36 hours. Limited observations on a single industrial effluent suggest that the timing of salinity adjustment (i.e., at time of collection or immediately before testing) was not critical. Parallel tests showed no toxicity differences over a 16 day

period (Ho et al. 1995). However, this observation is not universal and it is suggested that an initial toxicity test be conducted on the day that the sample arrives.

The volume requirements for performing Phase I of a TIE will vary according to the toxicity of the sample. The more toxic the sample, the less effluent sample will be needed. To a certain extent, the choice of tests to be performed may also affect the desired sample volume. Table 5-1 provides estimates of the volumes of sample needed for the Phase I marine TIE tests.

5.2 Composite versus Grab Samples

There are several factors to consider when designing a sample collection scheme (EPA 1994). A 24-hour composite sample is more representative of total effluent toxicity and is more likely to collect the toxic fraction if it is intermittent (i.e., timed with an industrial process). However, a composite sample may make the toxic fraction more difficult to detect because of dilution. In addition, compositing is expensive and time consuming. The simpler and less expensive grab sample is a "snap shot" of effluent toxicity at the time of collection. A grab sample, however, has the disadvantage that it may miss intermittent toxicity altogether, or conversely, collections synchronized to a suspected manufacturing process or seasonal discharge can result in a very toxic sample. The choice of sampling method consequently will depend on the goals of a given TIE and the nature of the plant from which it is being collected. For example, if the sample is being taken from a wastewater treatment plant with a two-day detention time, there is little need for the use of composite samples. Please consult EPA 1991b, 1993a for further discussion of this issue.

5.3 Pre- or Post- Chlorinated Samples

The decision to sample a municipal effluent before or after the addition of chlorine will depend on the objectives of the study. While addition of sodium thiosulfate helps determine how much of the toxicity is due to chlorine, it may also remove other oxidants and some metals, thus complicating the interpretation of results. Further, the presence of chlorine will often mask the effects of other less abundant toxicants. It is recommended to test both pre- and post- chlorinated samples to determine what portion of toxicity is attributable to chlorine.

Sample Log No.: _____
Date of Arrival: _____
Date and Time
of Sample Collection: _____
Facility: _____
Location: _____
NPDES No.: _____
Contact: _____
Phone Number: _____
Sampler: _____

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

Specific Sampling Information:

Sample Conditions Upon Arrival:

Temperature: _____
pH: _____
Total Alkalinity: _____
Total Hardness: _____
☐ Conductivity: _____
or
☐ Salinity: _____
Total Residual Chlorine: _____
Total Ammonia: _____
Dissolved Oxygen: _____

Conditions of treatment system at time of sampling:

Status of process operations/production (if applicable):

Comments:

Figure 5-1. Example Data Sheet for Logging in Samples.

Table 5-1. Estimated Volumes for Phase I Marine TIE Tests.*

Characterization Step	Volume Needed (ml)†	Total (ml)
Chemistry	~ 500 ‡	
Initial	~ 100	
Baseline	~ 120	
Filtration	~ 100	
Aeration	~ 100	
EDTA Addition	~ 100	
Na ₂ S ₂ O ₃ Addition	~ 100	
<i>Ulva lactuca</i> Addition	~ 200	
C ₁₈ Solid Phase Extraction	~ 100	
Cation Solid Phase Extraction	~ 100	
Graduated pH	~ 100	
pH 7	~ 100	
Ambient pH	~ 100	
pH 9	~ 100	
		~2000

* Values are for three replicates for initial and baseline tests and two replicates in the manipulations. Test volumes are assumed to be 20 ml/replicate. Values are directly applicable to Atlantic and Gulf Coast species, Pacific Coast species may require greater volumes.

† Assumed sample tested at 100% and diluted by 50% splits. Initial and baseline include five treatments, and manipulations include three treatments.

‡ Includes physical measures (e.g., temperature, salinity), pH, ammonia, chlorine, and dissolved oxygen.

5.4 Salinity Adjustments and Dilution Water

Dilution water for marine TIEs is hypersaline brine (100‰) adjusted to the desired salinity with DI water. Brine is made by slowly evaporating filtered natural seawater until the salinity reaches 100‰ (do not exceed this level), filtering it through a one micron filter, and storing it in 20 liter cubitainers® or polycarbonate water cooler jugs (EPA 1994). The seawater

should be of high quality and collected on an incoming tide to minimize the possibility of contamination. The brine and DI mixture is a very consistent dilution water as any given "batch" of brine can be used for a year or more.

Directions for the use of hypersaline brine for salinity adjustment is also described in EPA 1993a. Basically, for freshwater salinity adjustment (0‰), the volume of brine (V_{brine}) added is described by the relationship: $V_{\text{brine}} = (S_{\text{test}} \times V_{\text{test}}) / S_{\text{brine}}$, where S_{test} is the desired test salinity, V_{test} is the test sample volume, and S_{brine} is the brine salinity.

Using hypersaline brine for effluent salinity adjustment causes a degree of sample dilution that is dependent upon the initial sample salinity and the desired test salinity. For example, the greatest concentration of a freshwater effluent (i.e., 0‰) adjusted to 30‰ with 100‰ hypersaline brine is 70%. For purposes of continuity and simplicity, all further discussion of effluent concentration in this document refers to salinity adjusted samples. Therefore, 100% salinity adjusted sample means the effluent concentration is between 70% and 100%.

An alternative approach to adjust effluent salinity is the addition of artificial seawater salts like GP2. Although this method has not been tested at AED with Phase I Marine TIEs, this method has the advantage that it does not dilute the effluent sample, and consequently may be useful in certain circumstances. It is not recommended that the artificial seawater be substituted for brine as dilution water, as brine contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for the adequate growth, survival, and/or reproduction of marine and estuarine organisms (EPA 1994). Consequently, the use of artificial seawater salts may be problematic in some cases. Conversely, for a very weakly toxic samples, where brine dilution would be problematic, the addition of sea salts may be required. Finally, if a sample is hypersaline (i.e., >34‰), dilution with DI water may be needed. In general, a TIE should be performed using dilution waters similar to that used in the toxicity test(s) which triggered the TIE.

Concentrations selected for testing should be bracketed around known or estimated LC₅₀ and EC₅₀ values. Determining test concentrations for initial testing requires some estimations, unless the effluent has been previously tested. Starting at the highest possible concentration and using logarithmic splits results in a wide distribution of concentrations. Concentrations for the baseline and the manipulations testing should be established by bracketing the LC₅₀ or EC₅₀ values generated in the initial test.

Section 6 Toxicity Testing

6.1 Test Species

The toxicity testing species described in this document are listed in Table 6-1. The table indicates species recommended for use in Pacific, Atlantic, and Gulf Coast testing. The reader may note small changes to these methods compared to methods reported elsewhere (EPA 1993a, 1994, 1995). Changes were made to adapt methods for TIE use.

Both acute and chronic (i.e., sublethal) endpoints are presented. In the table, endpoints are labeled as "mortality" for acute toxicity tests while short-term chronic tests specify an endpoint other than

mortality. The chronic tests include the macroalga sexual reproduction and germination and growth test using *Champia parvula* and *Macrocystis pyrifera*, and the echinoid sperm cell test using sea urchins *Strongylocentrotus purpuratus* and *Arbacia punctulata*, and the echinoid fertilization test with the sand dollar *Dendraster excentricus*. Bivalve and gastropod development tests with *Mulinia lateralis*, *Crassostrea gigas*, *Mytilus californianus*, *Mytilus galloprovincialis*, and *Haliotis rufescens* are used. The acute tests include those for fishes: *Menidia beryllina*, *Cyprinodon variegatus*, and *Atherinops affinis*, the mysid *Mysidopsis bahia*, and the amphipod *Ampelisca abdita*.

Table 6-1. Marine Species Recommended for Use in Marine TIEs

Organism	Species	Region	Endpoint*	Exposure (hr.)
Macroalga	<i>Champia parvula</i>	Atlantic and Gulf Coasts	sexual reproduction	48
	<i>Macrocystis pyrifera</i>	Pacific Coast	germination/growth	48
Echinoid	<i>Arbacia punctulata</i>	Atlantic and Gulf Coasts	fertilization	1
	<i>Strongylocentrotus purpuratus</i>	Pacific Coast	fertilization	~1
			or development	72
	<i>Dendraster excentricus</i>	Pacific Coast	fertilization	~1
			or development	72
Bivalve	<i>Mulinia lateralis</i>	Atlantic and Gulf Coasts	mortality/development	24
	<i>Crassostrea gigas</i>	Pacific Coast	development	48
	<i>Mytilus californianus</i>	Pacific Coast	development	48
	<i>Mytilus galloprovincialis</i>	Pacific Coast	development	48
Gastropod	<i>Haliotis rufescens</i>	Pacific Coast	development	48
Mysid	<i>Mysidopsis bahia</i>	Atlantic and Gulf Coasts	mortality	48
Amphipod	<i>Ampelisca abdita</i>	Atlantic and Gulf Coasts	mortality	48
Fish	<i>Menidia beryllina</i>	Atlantic and Gulf Coasts	mortality	48
	<i>Cyprinodon variegatus</i>	Atlantic and Gulf Coasts	mortality	48
	<i>Atherinops affinis</i>	Pacific Coast	mortality/growth	48-168

* Acute tests are indicated by an endpoint of mortality, chronic tests by an endpoint other than mortality.

6.2 Test Methods

This section provides brief descriptions of the marine Phase I TIE toxicity tests. The TIE toxicity testing methods are very similar to conventional methods described in EPA 1993a, 1994, and 1995 except for minor changes to account for exposure volume reductions and feeding protocols. The Appendix provides test parameters of the methods.

In addition to the noted tests, we have conducted sediment interstitial water TIEs with the marine amphipod *Ampelisca abdita* and bivalve *Mulinia lateralis*. Further, we have used conventional NPDES toxicity tests, using the mysid *Mysidopsis bahia* and sea urchin *Arbacia punctulata*, in sediment interstitial water TIEs.

6.2.1 Macroalga Sexual Reproduction or Germination/Growth Tests

These methods use sexual reproduction of the macroalga *Champia parvula* and the germination and growth of the kelp *Macrocystis pyrifera* to measure toxicity. The *Champia parvula* procedure involves measuring the development of cystocarps on female plants. The *Macrocystis pyrifera* procedure quantifies the germination of settled zoospores and length of the germination tube.

Changes to the *Champia parvula* method (EPA 1994) for TIE purposes include a reduction in test solution volume from 100 mL to 20 mL and use of 50 mL petri dishes as the exposure chambers. Further, when conducting the Graduated pH Procedure, photosynthesis will increase pH by approximately 0.1 - 0.4 units. This is to be expected but should not exceed 0.5 pH units. Test parameters of these methods are presented in the Appendix.

6.2.2 Echinoid Sperm Cell Tests

The echinoid sperm cell tests have reduced fertilization of exposed gametes as an indication of toxicity. Dilute sperm solutions are exposed to test samples for 20 to 60 minutes. Following this exposure eggs are added to the samples and fertilization is allowed to occur. Twenty minutes after egg addition the test is terminated by the addition of a fixative. Fertilization is determined by microscopic examination of an aliquot from each treatment, and is shown by the presence of a membrane surrounding the egg.

Little has been changed in the sperm cell test methods to accommodate TIE applications. The existing method (EPA 1994, 1995) is extremely useful for TIE applications due to its use of very small exposure volumes (i.e., 5 mL), demonstrated sensitivity, and relatively rapid exposure. For conducting the Graduated pH Procedure, we have found it useful to keep the test scintillation vials in the atmosphere controlled chambers during the 20-60 minute sperm exposure to maintain desired pH values (cf. Section 9.9). Test parameters are presented in the Appendix.

6.2.3 Echinoid, Bivalve, and Gastropod Development Tests

The development tests involve several marine species and developmental endpoints (EPA 1994, 1995). Echinoid procedures assess the formation of the larval test. Bivalve and gastropod tests evaluate the growth of the larval shell. Microscopic analysis is used to determine test and shell condition. All tests are performed in small volumes (5-10 mL) and are amenable for TIEs. Test parameters of the methods are found in the Appendix.

6.2.4 Acute Mysid and Fish Tests

For TIEs, three Atlantic and Gulf Coast test methods are conducted similarly and use a mortality endpoint. Experimental designs consist of static 48-hour exposures with five organisms in 10 to 20 mL of test solution (i.e., 30 mL exposure cups). Mysid (*Mysidopsis bahia*) toxicity tests use 1-5 day animals. For fish testing, 9 to 14 day old *Menidia beryllina*, and 1-14 day old *Cyprinodon variegatus* are used. A TIE method for using 9-15 day old fish *Atherinops affinis* with small test volumes has not been fully developed. Test parameters are given in the Appendix.

Noteworthy changes to the standard marine acute methods (EPA 1993a) are the reduction in sample volume from approximately 100 mL to 10 or 20 mL and reduction in exposure period from 96 hours to 48 hours. When conducting the Graduated pH Procedure the organisms will add CO₂ to the exposure chambers resulting in decreases in sample pHs. Also, feeding test organisms *Artemia* will further reduce chamber pHs. To avoid drastic reductions in sample pH, especially in the pH 9.0 treatment, feed test organisms small rations. The Appendix details these and other changes to the standard methods.

6.2.5 Other Marine Species

Included in various sections of this document are references to other marine species, besides some of the common marine NPDES toxicity testing species, which can be incorporated into the marine TIE. Currently, these species are the amphipod *Ampelisca abdita* and the bivalve *Mulinia lateralis*. At AED, they have proven valuable in developing marine sediment TIE methods, but they can also be used to assess effluent toxicity. At the time this document was prepared, insufficient information was available to include the West Coast survival and growth method using the mysid *Holmesimysis costata*.

As with the other marine toxicity tests that use "whole organisms," major changes to the current methods with *Ampelisca abdita* (Scott and Redmond 1989) include reducing exposure volumes to approximately 10 mL and exposure duration to 48 hours. An evaluation of a 24-hour embryo-larval development test using the bivalve *Mulinia lateralis* is continuing.

Section 7

Statistical Methods

Test results are used to calculate point estimates (e.g., LC_{50} s and EC_{50} s). EPA recommends probit, Spearman-Kärber, trimmed Spearman-Kärber, and Inhibition Concentration (IC_p ; p is the percent effect, e.g., mortality, reduced growth, etc.) as means to calculate point estimates (EPA 1993a, 1994, 1995).

Conversion of point estimates to toxic units (e.g., Toxic Units = $100/LC_{50}$ or $100/IC_p$) eliminates the inverse relationship between

toxicity and LC_{50} or EC_{50} values making TIE interpretation easier. Furthermore, if the concentration of toxicants are known for a given sample, the toxic units for the individual toxicants can be compared to the total sample toxic units. The sum of the toxic units of the individual toxicants should be similar to the total toxic units of the sample, assuming they are all measured, bioavailable, and that their toxicities are additive.

Section 8

Ion Imbalance

The methods in this document do not directly address toxicity caused by ion imbalance as recorded in some types of effluents (e.g., McCulloch et al. 1993). If an ion imbalance is suspected in a sample, several studies are available that discuss how to characterize and identify such toxicity (McCulloch et al. 1993; Mount et al. in press; Douglas and Horne in press; Douglas et al. in press; Tietge et al. in press). It should be noted that although an ion imbalance may impart an apparent 'salinity' to a sample, in most cases the sample is not truly marine. Marine salinity has a specific composition of ions at relatively consistent proportions to one another. Effluents with ion imbalances seldom will have truly marine composition.

An approach for determining if an ion imbalance may be present in a given sample is to perform an anion and cation analysis for major elements (e.g., sodium, calcium, potassium, magnesium, chloride, sulfate, and bromide). Measured values can be compared to toxicity information (Douglas et al. in press), marine Water Quality Criteria (WQC), and known marine background levels (Millero and Sohn 1992) to assess if an imbalance may occur.

Section 9

Toxicity Identification Evaluation Procedures

A Phase I marine TIE characterization consists of the following recommended components (see also Figure 9-1):

- Initial Toxicity Test (§9.1, §6, Appendix)
- Baseline Toxicity Test (§9.2, §6, Appendix)
- Filtration Procedure (§9.3)
- Aeration Procedure (§9.4)
- EDTA Procedure (§9.5)
- $\text{Na}_2\text{S}_2\text{O}_3$ Procedure (§9.6)
- C_{18} Solid Phase Extraction (SPE) Procedure (§9.7)
- C_{18} SPE Methanol Elution Test (§9.8)
- Graduated pH Procedure (§9.9)
- Cation Exchange SPE Procedure (§9.10)
- Cation Exchange SPE Acid Elution Test (§9.11)
- *Ulva lactuca* Procedure (§9.12)

Figures 9-1 and 9-2 give an overview of the design of a typical marine Phase I TIE. One should note, however, that because of the varying durations of the toxicity tests used in a marine Phase I TIE that the indications of 'DAY 1' and 'DAY 2' may not always be appropriate.

While the Initial and Baseline Toxicity Tests are based on routine toxicity testing exposures, the other procedures (e.g., EDTA and $\text{Na}_2\text{S}_2\text{O}_3$) are specialized and require some knowledge of the sensitivity of the testing organisms to specific chemicals. The following sections describe the objectives and general procedures for conducting the TIE manipulations. Familiarity with the freshwater TIE procedures (EPA 1991a, 1991b) is recommended.

Specific information concerning numbers of treatments, types of species to test, volumes of effluent to prepare, and duration of exposures are only recommendations and may require modification depending upon each application. Blanks are described for each procedure and involve using the control seawater (often brine and DI) in the manipulations before the sample.

9.1 Initial Toxicity Test

9.1.1 General Approach

The objective of an Initial Toxicity Test for a TIE is to determine the toxicity of a given sample. The Initial Toxicity Test is performed on DAY 1 of the marine TIE process, while the Baseline Toxicity Test and procedures are generally conducted on DAY 2 (Figure 9-1 and Figure 9-2).

9.1.2 Materials

- Materials, organisms and apparatus necessary to conduct toxicity test (See Section 6 and Appendix).

9.1.3 Procedural Overview

Design of Initial Toxicity Test

Initial Toxicity Tests have a serial dilution design. We recommend five concentrations (post-salinity adjusted): 100%, 50%, 25%, 12.5%, 6.25% and a control (i.e., 0%) with one to three replicates (three preferred) per concentration (Figure 9-2). However, if a sample is very toxic, this range of concentrations will be too high and a set of lower concentrations will be needed. Therefore, if data from compliance testing suggests high toxicity, one should adopt a different set of concentration ranges including the necessary lower non-toxic concentrations.

Results of Initial Toxicity Test

Initial Toxicity Test results are used to judge how toxic the sample is and if a TIE on the given sample is warranted. If so, Initial Toxicity Test results will be used to establish effluent test concentrations for subsequent TIE manipulations.

From our experience, it may be difficult, but not impossible, to conduct a TIE when the toxic units of a sample from the Initial Toxicity Test using the most sensitive species are <2 (i.e., $\text{LC}_{50} > 50\%$). It is critical, however, to insure that the toxic units are <2 by repeating toxicity tests and using smaller concentration intervals (i.e., bracketing the effect concentrations more closely). Table 9-1 provides some other criteria as to when decisions can be made about proceeding with the Baseline Toxicity Test and TIE procedures.

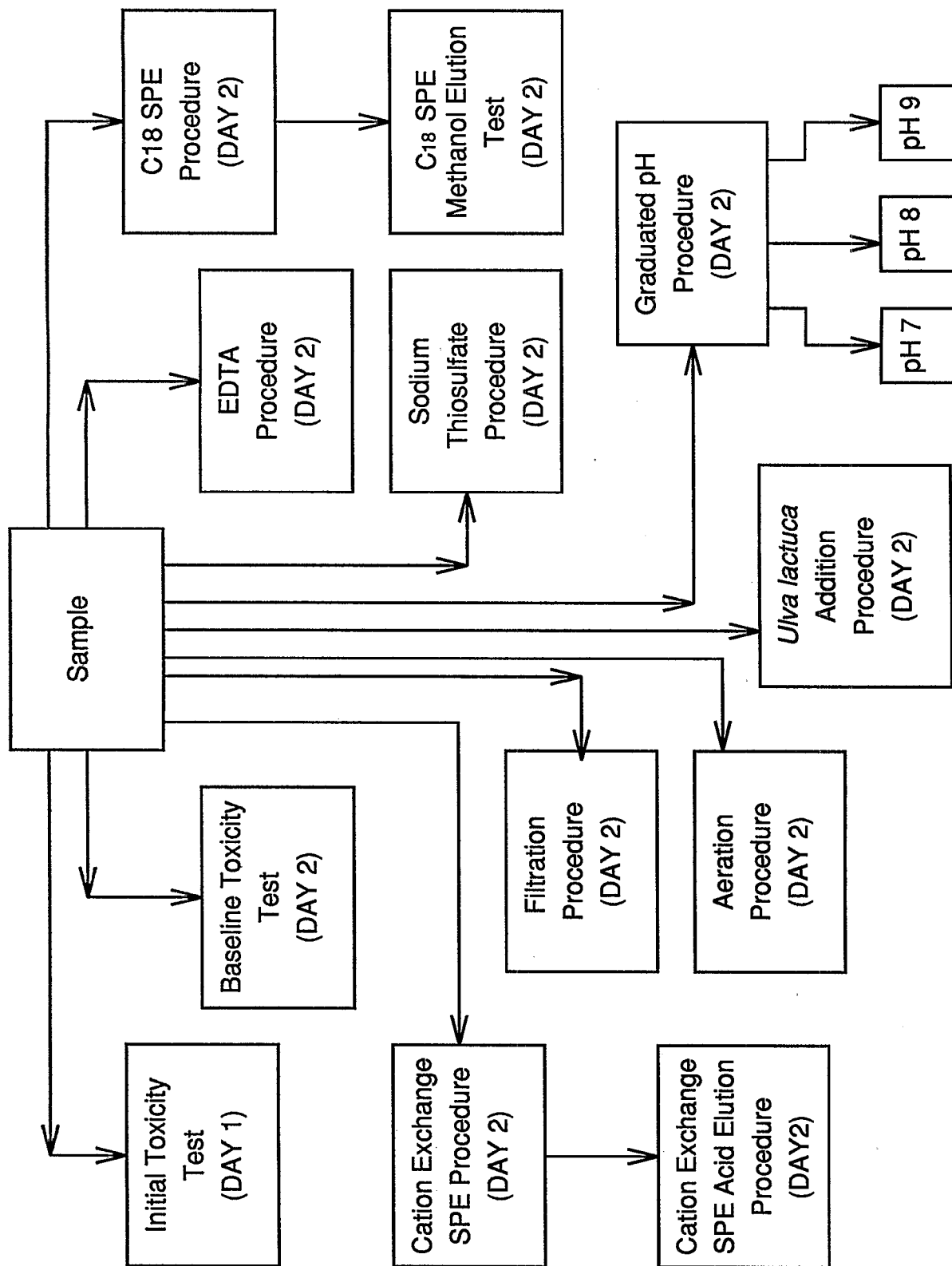


Figure 9-1. Overview Flowchart of a Typical Complete Phase I Marine TIE Characterization.
(NOTE: As a result of toxicity test durations, DAY 2 manipulations may occur later than the true DAY 2)

Table 9-1. Guidance on Conduct of Baseline Toxicity Test and TIE Procedures

Toxicity Test Species	Guidelines to Make Decision to Proceed
<i>Champia parvula</i>	Due to duration of exposure, one may have to use results of other tests or delay Initiation of TIE
<i>Arbacia punctulata</i>	Results of initial toxicity test (Day 1)
<i>Mulinia lateralis</i>	48 hr. results
<i>Mysidopsis bahia</i>	24 hr. results; if no toxicity, use 48 hr. results
<i>Ampelisca abdita</i>	24 hr. results; if no toxicity, use 48 hr. results
<i>Menidia beryllina</i>	24 hr. results; if no toxicity, use 48 hr. results
<i>Cyprinodon variegatus</i>	24 hr. results; if no toxicity, use 48 hr. results
<i>Macrocystis pyrifera</i>	48 hr. results
<i>Strongylocentrotus purpuratus</i>	fertilization: Day 1 results; development: 72 hr. results
<i>Dendraster excentricus</i>	fertilization: Day 1 results; development: 72 hr. results
<i>Crassostrea gigas</i>	48 hr. results
<i>Mytilus californianus</i>	48 hr. results
<i>Mytilus galloprovincialis</i>	48 hr. results
<i>Haliotis rufescens</i>	48 hr. results
<i>Atherinops affinis</i>	24 hr. results; if no mortality, use 48 hr. results, up to 168 hr.

Because of the long duration of the algal *Champia parvula* reproduction test, it is difficult to follow the standard TIE format. Therefore, it is necessary to use test results from other species to predict *Champia parvula*'s response or perform the initial test five to seven days earlier than the other species (assuming no alterations in toxicity due to storage). *Champia parvula* is often the most sensitive NPDES toxicity testing species when tested with municipal and industrial effluents (Schimmel et al. 1989) and therefore, a prediction of high toxicity is warranted. Conversely, because of the short duration of the sea urchin *Arbacia punctulata* sperm cell test, an entire TIE can often be conducted in two days, or even one day, if prior information about the toxicity of the sample is available and appropriate dilutions can be prepared. The fertilization endpoints of toxicity test using *Strongylocentrotus purpuratus* and *Dendraster excentricus* can be used similarly.

9.2 Baseline Toxicity Test

9.2.1 General Approach

Results of the Baseline Toxicity Test are used for comparison with the Initial Toxicity Test and TIE manipulations. Objectives are to: (1) determine if sample toxicity has changed relative to Initial Toxicity Test and (2) provide a baseline for comparison with results of TIE procedures. A Baseline Toxicity Test is performed following the Initial Toxicity Test, in conjunction with the TIE Manipulations (Figures 9-1 and 9-2). In Figure 9-2, we indicate the use of one replicate per test concentration and three concentrations per procedure. These values for the study design are not recommendations but must be determined according to study objectives, logistics and economic constraints.

9.2.2 Materials

- Materials, Organisms and Apparatus necessary to conduct toxicity test (See Section 6 and Appendix).

9.2.3 Procedural Overview

Design of Baseline Test

Baseline Toxicity Tests have a serial dilution design. Usually five concentrations: 100%, 50%, 25%, 12.5%, 6.25% and a control (0%) with three replicates/concentration are used. However, if the Initial Toxicity Test demonstrates greater toxicity, lower dilutions may be justified.

Results of Baseline Toxicity Test

Because of the variety of species potentially being tested, Baseline Toxicity Test results will be dependent on the toxicity test being used. However, regardless of species, the questions being answered are the same for each toxicity test, "Did sample toxicity change relative to the Initial Toxicity Test and did the TIE procedures decrease or increase toxicity compared to the Baseline Toxicity Test?" Quantitatively, these questions are answered by comparing toxic units between the various procedures. Sources of toxicity are implied from the magnitude of difference between the baseline and TIE procedures results. However, statistical evaluations of significance may be precluded, for most TIE tests, because of insufficient replication within TIE experimental designs. See Section 10 for further discussion of the interpretation of TIE results.

9.3 Filtration Procedure

9.3.1 General Approach

Filtration is used to determine whether toxicants pass through a filter or are associated with particles. Note for effluents, samples can be filtered before being passed through the C₁₈ column (See Section 9.7). However, filtration may create artifacts (e.g., toxicant sorption to filter) that may need to be addressed in evaluating results. Filtrates are the substances that pass through the filter.

9.3.2 Materials

- Oil-free air pump and tubing—to force sample through filtration apparatus.
- 0.45 μm (or similar size) glass fiber filters and filtration apparatus—to separate particles from sample. For samples that are suspected to contain toxic metals, organic membrane filters may be used instead of glass filters. However, a comparison of filter types may be necessary.

9.3.3 Procedural Overview

- (1) Filter brine and DI blank; remove brine and DI blank filtrate for testing (Figure 9-3).
- (2) Without changing filters, filter the effluent. Change filters as often as necessary to prevent clogging, repeating step 1 as needed. Save all filters for possible later analysis (i.e., wrap in aluminum foil or Parafilm® and store at 4°C). Remove filtrate for testing.
- (3) Use filtered brine and DI blank as diluent.

9.4 Aeration Procedure

9.4.1 General Approach

Samples are aerated to determine if toxicity is due to volatile toxicants (e.g., H_2S or volatile hydrocarbons).

9.4.2 Materials

- Oil-free air pump and tubing—to aerate sample.
- Graduated cylinders—to hold sample while aerating.
- 1-10 mL pipettes—attached to tubing and placed in sample during aeration. Fritted end on pipettor tubing will improve aeration.

9.4.3 Procedural Overview

- (1) Samples should be aerated in a hood.
- (2) Separately pour sample, and brine and DI blank into graduated cylinders (Figure 9-4).
- (3) Connect 1-10 mL pipettes to air pump tubing and place pipettes into graduated cylinders.
- (4) Turn pump on, adjust air flow to establish many small bubbles, and let sample aerate for 1 hour.
- (5) Test aerated sample using aerated brine and DI as diluent.

9.5 EDTA Procedure

9.5.1 General Approach

EDTA (ethylenediaminetetraacetic acid) is an organic chelating agent that preferentially binds with divalent cationic metals, such as copper, nickel, lead, zinc, cadmium, mercury, and other transition metals (Garvan 1964). Studies have demonstrated that when a metal is bound to the EDTA molecule, the toxicity of the metal is greatly reduced (e.g., Sunda and Guillard 1976). In this procedure, EDTA is added to samples to evaluate metal toxicity. Table 9-2 provides recommended exposure concentrations and Tables 9-3 and 9-4 report results of tolerance testing with Atlantic, Gulf, and Pacific Coast species.

9.5.2 Materials

- EDTA stock solution (25 g EDTA/L DI (74.4 mmols EDTA/L) refrigerated)
- Glass Erlenmeyer flask (100-250 mL), microbalance, weighing pan, and Teflon®-coated stirbar—for preparing EDTA stock solution.
- Adjustable microvolume pipetter (10-1000 μL range) and tips—for dispensing EDTA stock solution to exposure chambers.

Table 9-2. Volumes of EDTA Stock Solution for Additions (25g EDTA/L stock solution)

Replicate Volume (mL)	Volume (μL) EDTA Solution/Replicate	Volume (μL) EDTA Solution/Replicate for <i>M. pyrifera</i>
5	12	10
10	24	20
20	48	40
40	96	80
100	240	200
200	480	400

9.5.3 Procedural Overview

- (1) Prepare EDTA stock solution: weigh-out 2.78 g of $\text{EDTA} \cdot 2\text{H}_2\text{O}$ reagent (sodium salt) and add to 100 mL of DI. Mix with a Teflon®-coated stirbar until EDTA is completely in solution. This stock solution is stable and can be stored refrigerated (Figure 9-5).
- (2) Set-up dilution series with sample. Generally, a TIE dilution series consists of three effluent concentrations and a blank (brine and DI), however, the statistical design of the TIE should be based on the objectives of the study, logistics, and economic constraints. The concentrations tested should bracket observed toxicity, based on the Initial Toxicity Test. **Do not add the organisms yet!**
- (3) Tolerance testing of several Atlantic, Gulf, and Pacific Coast species indicates that most organisms can tolerate 60 mg EDTA/L (0.22 mmols EDTA/L) (Table 9-3, 9-4). Given the EC_{50} of 100 mg/L for *M. pyrifera*, it is advisable to use 50 mg/L (0.14 mmol/L) for the EDTA Procedure with that species. This concentration of EDTA is sufficient to chelate about 22 mg Total M^{2+} /L (equal molarity of metals). Use Table 9-2 to determine the volume of EDTA stock (25 g EDTA/L) to add to test containers:
- (4) Add specified volume, mix thoroughly and allow EDTA and sample to interact for about 3 hours. **Do not add the organisms yet!**
- (5) After 3 hours, add test organisms to dilution series.

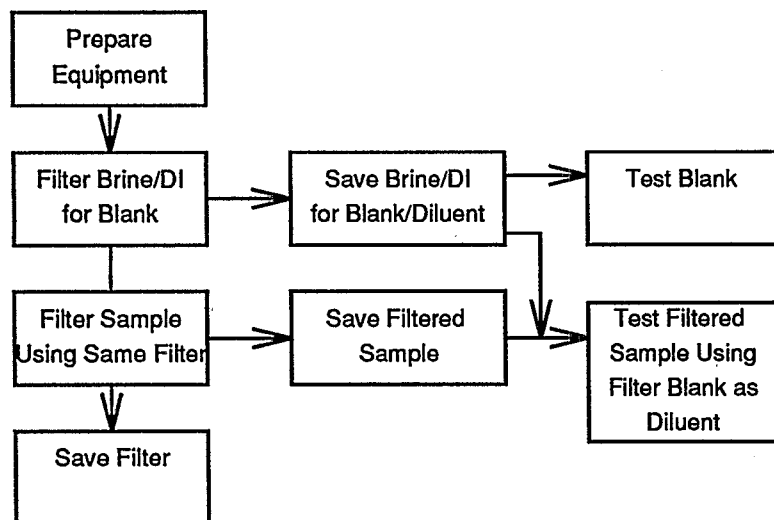


Figure 9-3. Overview Flowchart of Filtration Procedure.

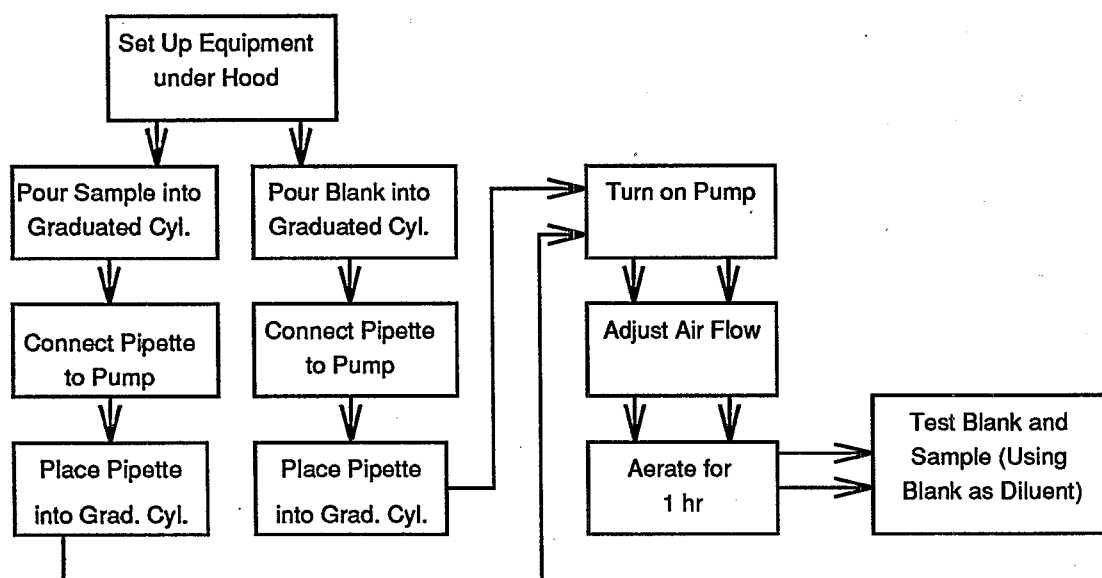


Figure 9-4. Overview Flowchart of Aeration Procedure.

Table 9-3. Atlantic and Gulf Coast Species Tolerance to EDTA (mg/L) (see Appendix for specific salinity and temperature).

Duration (hr)	Species LC ₅₀ or EC ₅₀ (± 95% Confidence Intervals)						
	<i>Champia parvula</i>	<i>Arbacia punctulata</i>	<i>Mulinia lateralis</i>	<i>Mysidopsis bahia</i>	<i>Ampelisca abdita</i>	<i>Menidia beryllina</i>	<i>Cyprinodon variegatus</i>
	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(LC ₅₀)	(LC ₅₀)	(LC ₅₀)	(LC ₅₀)
1.2	-	300 (300-300)	-	-	-	-	-
24	-	-	-	318 (309-323)	240 (150-350)	362 (354-369)	> 600 (-)
48	165 (94.2-240)	-	288 (283-295)	313 (300-326)	175 (65.6-205)	353 (347-359)	542 (534-547)
72	-	-	-	318 (303-327)	164 (50-200)	353 (344-359)	348 (345-349)
96	-	-	-	315 (298-325)	150 (28.2-188)	350 (344-359)	346 (344-349)

- Not Available

Table 9-4. Pacific Coast Species Tolerance to EDTA (mg/L) (see Appendix for specific salinity and temperature).

Duration (hr)	Species LC ₅₀ or EC ₅₀ (± 95% Confidence Intervals)							
	<i>Macrocystis pyrifera</i> *	<i>Strongylocentrotus purpuratus</i> †	<i>Dendraster excentricus</i> ‡	<i>Crassostrea gigas</i>	<i>Mytilus californianus</i>	<i>Mytilus galloprovincialis</i>	<i>Haliotis rufescens</i>	<i>Atherinops affinis</i> ‡
	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)
<1.0	-	>750	>750	-	-	-	-	-
24	-	-	-	-	-	-	-	-
48	100 (-)	-	-	>750	>750	>750	300 (-)	-
72	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	300 (-)

- Not Available

* Germination Endpoint

† Fertilization Endpoint

‡ 7 Day Growth Endpoint

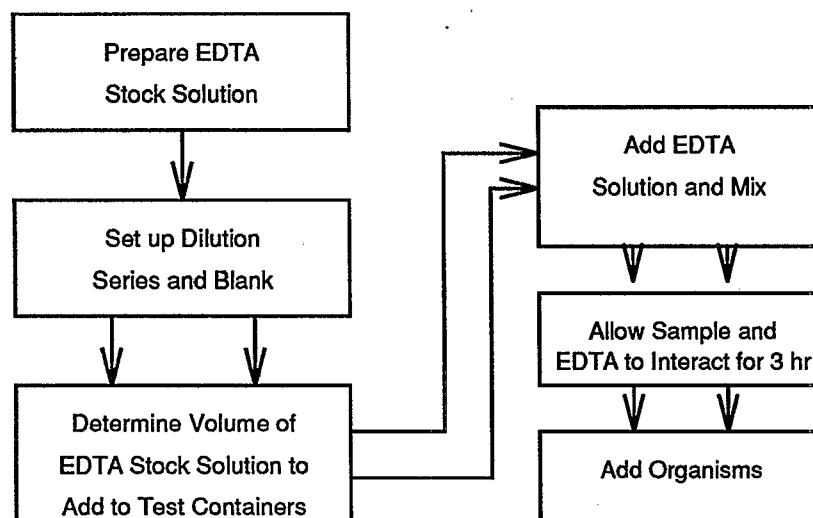


Figure 9-5. Overview Flowchart of EDTA Procedure.

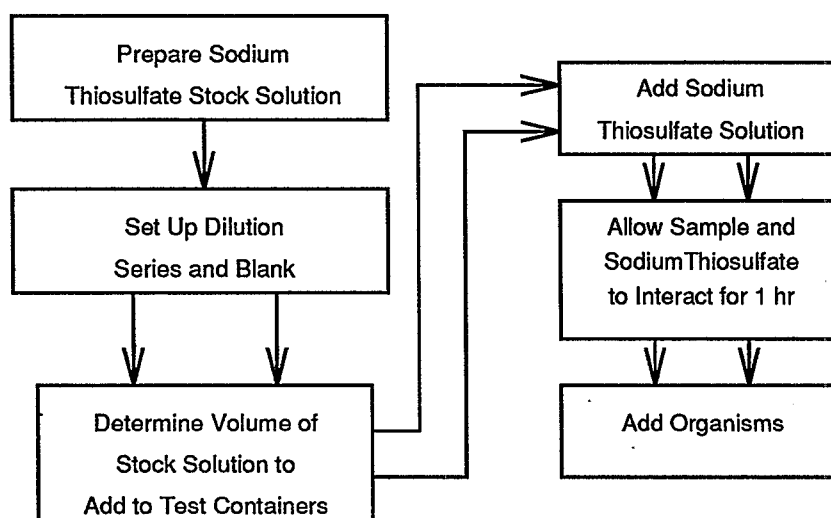
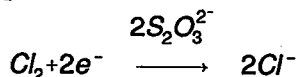


Figure 9-6. Overview Flowchart of Sodium Thiosulfate Procedure.

9.6 Sodium Thiosulfate Procedure

9.6.1 General Approach

Addition of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), a reducing agent, to a sample containing oxidants (e.g., chlorine or bromine), results in a reduction reaction (White 1972) that may decrease sample toxicity. For example, chlorine (Cl_2) added to sewage effluent prior to release would undergo the following reaction:



where the 2 electrons (e^-) provided by the thiosulfate (S_2O_3) reduce the toxic diatomic chlorine (Cl_2) to nontoxic chlorine ions (Cl^-). In this test, $\text{Na}_2\text{S}_2\text{O}_3$ is added to effluent samples to evaluate whether toxic oxidants are present. Table 9-5 provides recommended exposure concentrations and Tables 9-6 and 9-7 report the results of tolerance testing with Atlantic, Gulf, and Pacific coast species.

9.6.2 Materials

- $\text{Na}_2\text{S}_2\text{O}_3$ Stock Solution (15 g $\text{Na}_2\text{S}_2\text{O}_3/\text{L}$ DI (94.9 mmols $\text{Na}_2\text{S}_2\text{O}_3/\text{L}$)). **This solution cannot be stored. Make up prior to use.**
- Glass Erlenmeyer flask (100-250 mL), microbalance, weighing pan, spatula and Teflon[®]-coated stirbar—for preparing $\text{Na}_2\text{S}_2\text{O}_3$ stock solution.
- Adjustable microvolume pipetter (10-1000 μL range) and tips—for dispensing $\text{Na}_2\text{S}_2\text{O}_3$ stock solution to exposure chambers.

9.6.3 Procedural Overview

(1) Make-up $\text{Na}_2\text{S}_2\text{O}_3$ Stock Solution

- Weigh-out 2.35 g of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ reagent, add to 100 mL of DI in a flask with a Teflon[®]-coated stirbar, and allow to mix until all the $\text{Na}_2\text{S}_2\text{O}_3$ is completely in solution (Figure 9-6).

(2) Use of $\text{Na}_2\text{S}_2\text{O}_3$ in TIE

- (a) Set up dilution series with sample. Generally, a TIE dilution series will consist of three effluent concentrations and blank (brine and DI). Concentrations should bracket observed toxicity, based on the Initial Toxicity Test. **Do not add organisms yet!**
- (b) Use Table 9-5 to determine the volume of $\text{Na}_2\text{S}_2\text{O}_3$ stock to add to test chambers. Tolerance testing of several Atlantic, Gulf, and Pacific coast toxicity testing species indicates that all organisms can tolerate 50 mg $\text{Na}_2\text{S}_2\text{O}_3/\text{L}$ (0.32 mmol $\text{Na}_2\text{S}_2\text{O}_3/\text{L}$) (Table 9-5).

(3) Add $\text{Na}_2\text{S}_2\text{O}_3$ and allow to interact for about one hour. **Do not add organisms yet!**

(4) After one hour, add test organisms to exposure chambers.

Table 9-5. Volumes of $\text{Na}_2\text{S}_2\text{O}_3$ Stock Solution for Additions (15g $\text{Na}_2\text{S}_2\text{O}_3/\text{L}$ stock solution)

Replicate Volume (mL)	Volume (μL) $\text{Na}_2\text{S}_2\text{O}_3$ Solution/Replicate
5	17
10	34
20	68
40	136
100	340
200	680

9.7 C_{18} SPE Procedure

9.7.1 General Approach

The C_{18} solid phase extraction (SPE) column manipulation is used to determine if toxic components are nonionic organic compounds. In the manipulation, reverse phase liquid chromatography is applied to extract nonionic organic toxicants from the aqueous sample. Operationally, filtered test solutions (i.e., samples and controls) are passed through a disposable C_{18} column and the post-column effluent tested for toxicity (Figure 9-5). Absence of toxicity in the post-column effluent suggests that organic toxicants were active in the original sample. Elution of the column with methanol can return toxicants to aqueous solution to confirm toxicity (see Section 9.8).

Tables 9-8 and 9-9 provide information on the tolerance of Atlantic, Gulf, and Pacific coast species to methanol.

9.7.2 Materials

- Disposable C_{18} column(s)—for performing C_{18} manipulation (e.g., Waters (Sep-Pak Environmental Plus 1000 mg / 2.0 mL column))
- HPLC Grade Methanol (MEOH)—for activating C_{18} column(s).
- Low flow metering pump (~10 mL/min) and tubing—for forcing sample through C_{18} column.
- Separatory funnel—to serve as a sample reservoir.
- Erlenmeyer flasks—for collecting post- C_{18} effluent.

Table 9-6. Atlantic and Gulf Coast Species Tolerance to Na₂S₂O₃ (mg/L) (see Appendix for specific temperature and salinity)

Duration (hr)	Species LC ₅₀ or EC ₅₀ (± 95% Confidence Intervals)						
	<i>Champia parvula</i>	<i>Arbacia punctulata</i>	<i>Mulinia lateralis</i>	<i>Mysidopsis bahia</i>	<i>Ampelisca abdita</i>	<i>Menidia beryllina</i>	<i>Cyprinodon variegatus</i>
	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(LC ₅₀)	(LC ₅₀)	(LC ₅₀)	(LC ₅₀)
1.2	-	>15000 (-)	-	-	-	-	-
24	-	-	-	>200	>300 (-)	12200 (11300-13000)	>15000 (-)
48	181 (141-773)	-	9400 (8990-9760)	164 (155-169)	>300 (-)	12000 (11500-12600)	>15000 (-)
72	-	-	-	121 (116-126)	223 (122-283)	11500 (10700-12400)	>15000 (-)
96	-	-	-	119 (113-125)	150 (87.5-214)	9550 (8330-10600)	>15000 (-)

- Not Available

Table 9-7. Pacific Coast Species Tolerance to Na₂S₂O₃ (mg/L) (see Appendix for specific temperature and salinity).

Duration (hr)	Species LC ₅₀ or EC ₅₀ (± 95% Confidence Intervals)							
	<i>Macrocystis pyrifera</i> *	<i>Strongylocentrotus purpuratus</i> †	<i>Dendraster excentricus</i> ‡	<i>Crassostrea gigas</i>	<i>Mytilus californianus</i>	<i>Mytilus galloprovincialis</i>	<i>Haliotis rufescens</i>	<i>Atherinops affinis</i> ‡
	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)
<1.0	-	>1000	>1000	-	-	-	-	-
24	-	-	-	-	-	-	-	-
48	200 (-)	-	-	>500	>500	>500	10000 (-)	-
72	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	10000 (-)

- Not Available

* Germination Endpoint

† Fertilization Endpoint

‡ 7 Day Growth Endpoint

9.7.3 Procedural Overview

(1) Preparation of Tubing

- (a) Connect pump, sample reservoir and column with tubing. Do not attach column. Pump 25 ml of DI water followed by 25 ml of MEOH through the entire system to remove any contamination. Throughout this procedure a flowrate of 10 mL/min is used (Figure 9-7).

(2) Preparation of C₁₈ Column

- (a) Attach C₁₈ column to tubing (check manufacturer's recommendations for wetting volumes and total capacity of the column). Pass recommended volume of MEOH through the column. **Do not let the column dry out.**
- (b) Pass recommended volume of DI through the column. **Do not let the column dry out;** to avoid drying the column, leave a small volume of DI in the tubing.

(3) Blank Sample

- (a) Pass the brine and DI filtered blank through the wet prepped column.
- (b) Allow first 10-20 ml of brine and DI to pass into waste container before collecting sample. Collect enough post-column brine and DI to conduct toxicity tests (the column can now go dry).

(4) Re-prepare Column

- From Step 2, the same column may be used. **Do not let the column dry out** in between the preparatory steps or before adding the filtered sample.

(5) Sample

- (a) Pass the filtered sample through the wet prepped column.
- (b) Collect enough post-column sample to perform toxicity tests. Column can now go dry.

(6) Toxicity Testing

- (a) Prepare test dilutions using post-column sample and post-column brine and DI.
- (b) Add organisms.

9.8 Methanol Elution Test

9.8.1 General Approach and Materials

If following the C₁₈ Column SPE Procedure (Section 9.7), and the post-column effluent shows reduced toxicity, it is recommended that the column be eluted with methanol to attempt to verify sample toxicity is due to an organic toxicant. Tables 9-8 and 9-9 provide information on the tolerance of several marine species to methanol.

Materials are the same as in the C₁₈ Column SPE Procedure (Section 9.7.2) except the column is now "loaded."

9.8.2 Procedural Overview

(1) Preparation of Tubing

- Same as C₁₈ Column SPE Procedure, Section 9.7.3.(1).(a) (Figure 9-7).

(2) Elution of Column

- (a) The reader is advised to consult EPA 1993b for specific details of column elution. The information here is only cursory.
- (b) Attach loaded column to tubing. Pass at least one column bed volume of methanol through column twice using a flowrate of 10 mL/min. Volume reduce eluate if necessary.
- (c) Collect methanol in container and return to initial sample volume with clean brine and DI. Use only enough methanol to be well below toxicity values in Table 9-8 and 9-9.

(3) Toxicity Testing

- (a) Prepare test dilutions using reconstituted sample and brine and DI.
- (b) Add organisms.

9.9 Graduated pH Procedure

9.9.1 General Approach

The pH of marine waters is largely controlled by the concentration of dissolved CO₂ present:



As the concentration of CO₂ increases, the carbonic acid (H₂CO₃) and bicarbonate (HCO₃⁻) dissociate and the reaction goes to the right, generating an excess of hydrogen ions (H⁺) which decreases sample pH. Conversely, if CO₂ is absent the hydrogen ions are found in an associated form and sample pH increases. In this procedure, sample pH is manipulated to determine if pH dependent toxicants are responsible for observed toxicity. For example, if sample toxicity increases with increasing sample pH, toxicants such as ammonia (NH₃) are suspected (Miller et al. 1990). Conversely, if sample toxicity increases with decreasing sample pH, toxicants such as hydrogen sulfide (H₂S) are suspected. Also, in freshwater, the toxicity of some metals is known to change as a function of pH (Schubauer-Berigan et al. 1993). For marine samples, exposures are conducted at three pHs: 7, ambient (7.9-8.4), and 9 using atmosphere-controlled chambers (Figure 9-8).

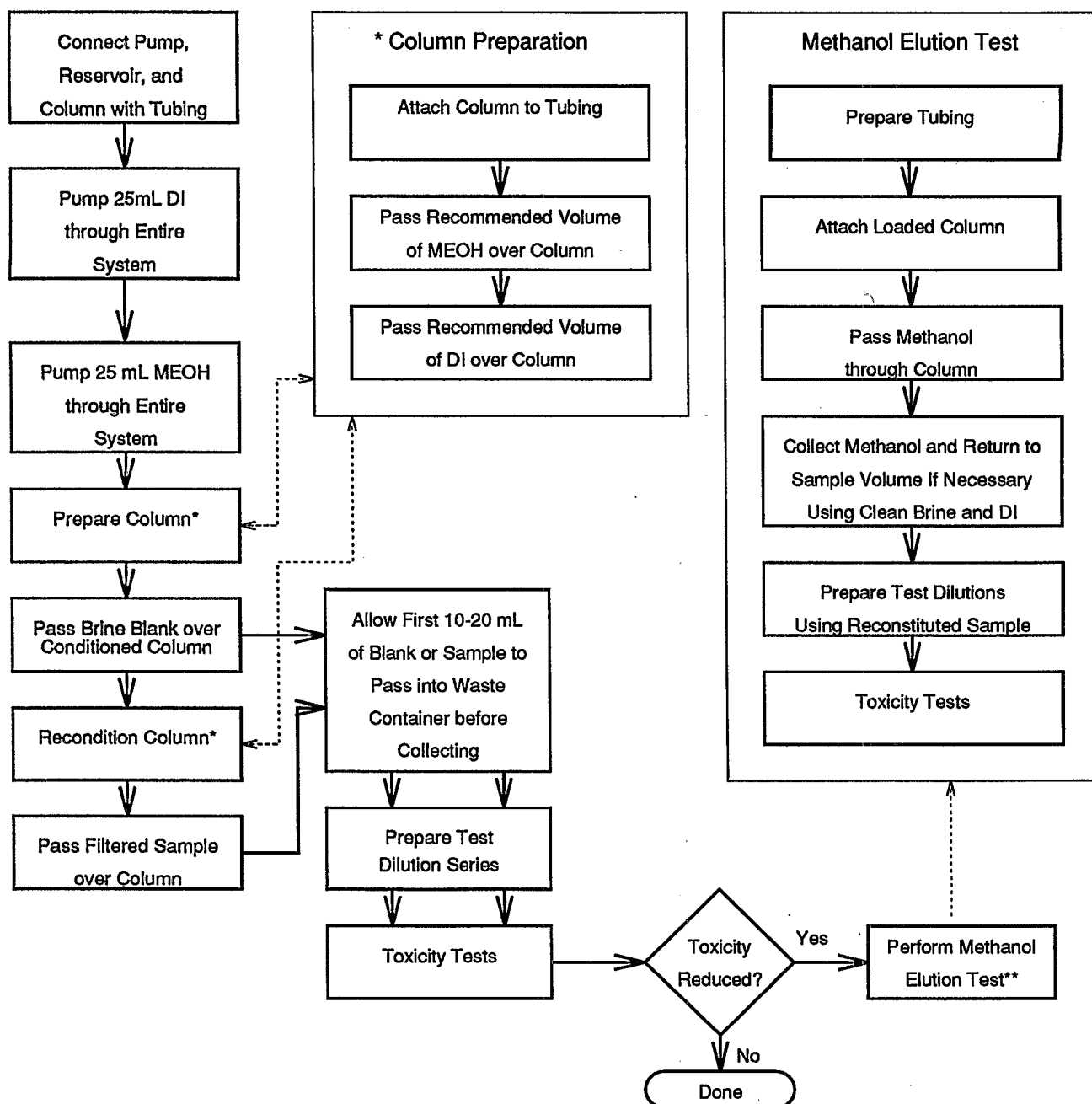


Figure 9-7. Overview Flowchart for C₁₈ SPE Procedure and Methanol Elution Test (** Consult EPA 1993b).

Table 9-8. Atlantic and Gulf Coast Species Tolerance to Methanol (%v/v) (see Appendix for specific temperature and salinity)

Duration (hr)	Species LC ₅₀ or EC ₅₀ (± 95% Confidence Intervals)						
	<i>Champia parvula</i>	<i>Arbacia punctulata</i>	<i>Mulinia lateralis</i>	<i>Mysidopsis bahia</i>	<i>Ampelisca abdita</i>	<i>Menidia beryllina</i>	<i>Cyprinodon variegatus</i>
	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(LC ₅₀)	(LC ₅₀)	(LC ₅₀)	(LC ₅₀)
1.2	-	9.31 (9.30-9.33)	-	-	-	-	-
24	-	-	-	2.43 (2.37-2.46)	3.75 (3.75-3.75)	2.56 (2.44-2.63)	3.89 (3.81-3.95)
48	0.13 (0.10-0.26)	-	2.18 (2.14-2.25)	2.35 (-)	3.21 (3.01-3.33)	2.33 (2.14-2.50)	3.67 (3.38-3.94)
72	-	-	-	2.35 (-)	1.25 (0.98-1.91)	1.77 (1.50-2.17)	3.39 (2.90-3.93)
96	-	-	-	2.30 (2.26-2.33)	0.75 (0.59-0.86)	1.55 (1.32-1.81)	3.33 (2.85-3.75)

- Not Available

Table 9-9. Pacific Coast Species Tolerance to Methanol (%v/v) (see Appendix for specific temperature and salinity).

Duration (hr)	Species LC ₅₀ or EC ₅₀ (± 95% Confidence Intervals)							
	<i>Macrocystis pyrifera</i> *	<i>Strongylocentrotus purpuratus</i> †	<i>Dendraster excentricus</i> †	<i>Crassostrea gigas</i>	<i>Mytilus californianus</i>	<i>Mytilus galloprovincialis</i>	<i>Haliotis rufescens</i>	<i>Atherinops affinis</i> ‡
	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)	(EC ₅₀)
<1.0	-	3.78 (3.47-4.11)	3.50 (3.32-3.69)	-	-	-	-	-
24	-	-	-	-	-	-	-	-
48	-	-	-	3.14 (2.69-3.59)	2.26 (2.02-2.57)	3.55 (3.34-3.74)	-	-
72	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-

- Not Available

* Germination Endpoint

† Fertilization Endpoint

‡ 7 Day Growth Endpoint

9.9.2 Materials

- pH 7.0 and pH 9.0 atmospheric chambers—for maintaining sample pHs at desired levels. Our atmospheric chambers were constructed from plexiglass in two sizes: 30 cm wide x 25 cm deep x 16 cm high and 80 cm x 40 cm x 30 cm. These chambers are not completely sealed from the ambient atmosphere but do maintain a positive pressure ensuring atmospheric gases do not enter. Locating the gas ports in the center of the chambers is advised to improve gas mixing.
- pH meter, stir plate, Teflon®-coated stirbars and calibration buffers—for monitoring sample pHs.
- Cylinders of CO₂, air, low CO₂ or low hydrocarbon air (e.g., Zero-Grade® or CO₂-Free®, (M.G. Industries, Valley Forge, PA)), and regulators for above cylinders (CGA 320 (CO₂), CGA 346 (Air) & CGA 590 (low CO₂))—to flow into pH chambers.
- CO₂ Scrubber—to remove CO₂ contamination from low CO₂ air (e.g., Merck, Darmstadt, Germany).
- Precision flow meters (CO₂ meter should be capable of 2 mL/min)—for metering gas flow to chambers.

9.9.3 Sample Preparation

Samples are prepared for testing as described in the other TIE procedures, but with the following special preparations (Figure 9-9).

pH 7

(1) Approximately 24 hours before the manipulations are to be conducted, initiate CO₂ and air flow into the pH 7.0 chamber. Adjust the CO₂ flow to approximately 2% of the air flow (e.g., ~2 mL/min CO₂ to 98 mL/min of air).

(2) Approximately 18 hours before toxicity testing is to begin, check gas flow and place separate containers of the sample and blank (brine and DI) into the chamber. Let equilibrate overnight.

pH 8 (Initial)

Generally, pH 8 is the blank (brine and DI) and sample under initial atmospheric conditions. Because of the strong carbonate buffering capacity of seawater, the pH of these samples will usually range from 7.90 to 8.40. Set up this series at the same time as the pH 7 and 9.

pH 9

(1) Approximately 24 hours before manipulations are to begin, adjust the low CO₂ air flow to the pH 9.0 chamber to 150 - 300 mL/min.

(2) Adjust needed volumes of blank (brine and DI) and sample with 1 M sodium hydroxide (NaOH) to pH 9.0±0.3. CAUTION! The amount of NaOH needed varies based on the sample; overshooting pH 9.0 can result in excessive toxicity due to high salinity from excess sodium addition. After adjusting the pH, place the blank and sample volumes into the pH 9 chamber and close tightly.

(3) Approximately 18 hours before toxicity testing is to begin, check the pHs of the blank and sample to ensure that pH 9 is being maintained.

9.9.4 Procedural Overview

(1) Before conducting the toxicity test, check pHs of test solutions. For tests with marine animals (except for bivalves), pHs should be 7.0±0.3 for pH 7, ambient pH for pH 8, and 9.0±0.3 for pH 9 (Table 9-10). When testing marine plants, pHs should be 7.5±0.2 for pH 7, ambient pH for pH 8 and 9.0±0.3 for pH 9 (Table 9-10). Adjusted pH samples can be maintained outside of the chambers for short time periods (e.g., 5 - 10 minutes) to allow for preparing and monitoring the test.

(2) Set up toxicity test with test solutions and place dilution series in the appropriate chambers for the duration of test. Table 9-10 provides acceptable pH ranges for exposing Atlantic, Gulf, and Pacific coast marine organisms. Note that bivalve species are particularly sensitive to low pHs.

(3) Check gas flow and pH at least every 24 hrs. NOTE: Because of organism respiration or photosynthesis, pHs in the respective chambers will decrease or increase from nominal values, but changes should not exceed ± 0.3 pH units. If necessary, adjust gas flow to maintain desired pHs.

Table 9-10. Operational Species Tolerance Ranges to pH*

Species	pH Range
Atlantic and Gulf Coasts	
<i>Champia parvula</i>	7.4-9.2
<i>Arbacia punctulata</i>	7.2-9.1
<i>Mulinia lateralis</i>	8.0-8.8
<i>Mysidopsis bahia</i>	6.8-8.8
<i>Ampelisca abdita</i>	7.1-9.0
<i>Menidia beryllina</i>	Insufficient Data
<i>Cyprinodon variegatus</i>	6.6-8.8
Pacific Coast	
<i>Macrocystis pyrifera</i>	7-9
<i>Strongylocentrotus purpuratus</i>	~7.8-8.5
<i>Dendraster excentricus</i>	Insufficient Data
<i>Crassostrea gigas</i>	7.5-8.5
<i>Mytilus californianus</i>	8.0-8.5
<i>Mytilus galloprovincialis</i>	7.5-8.5
<i>Haliotis rufescens</i>	7-9
<i>Atherinops affinis</i>	7-9

* See Appendix for specific salinity and temperature.

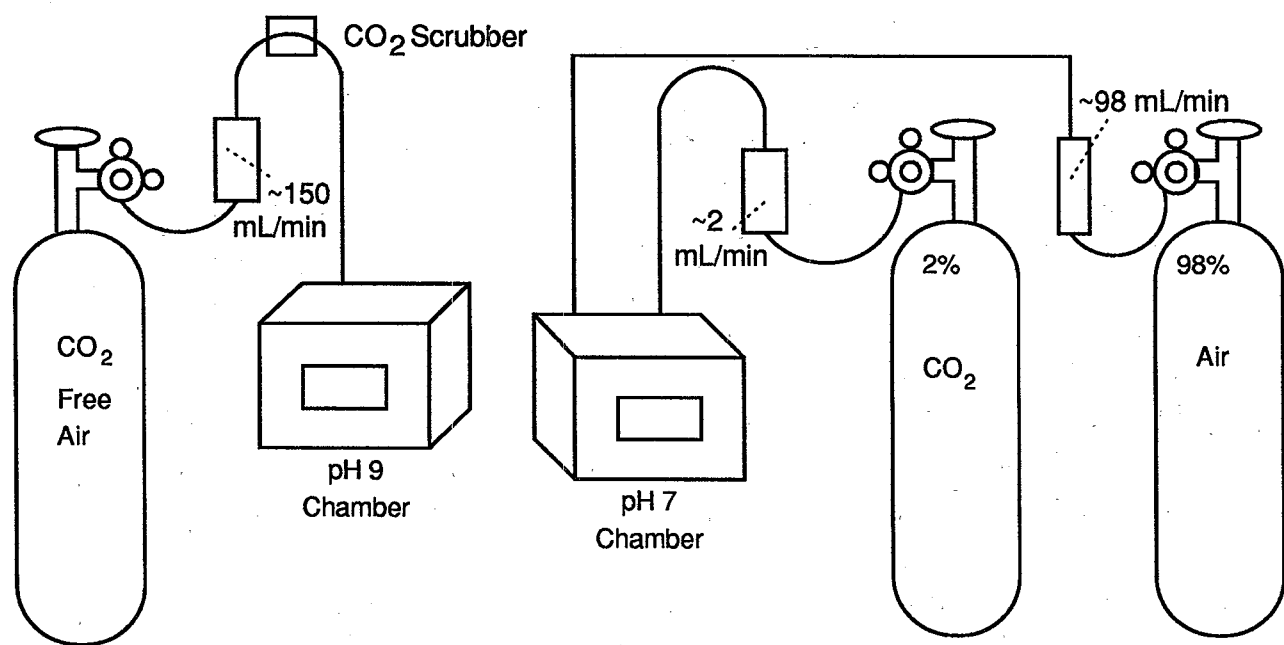


Figure 9-8. Apparatus Schematic for Graduated pH Procedure.

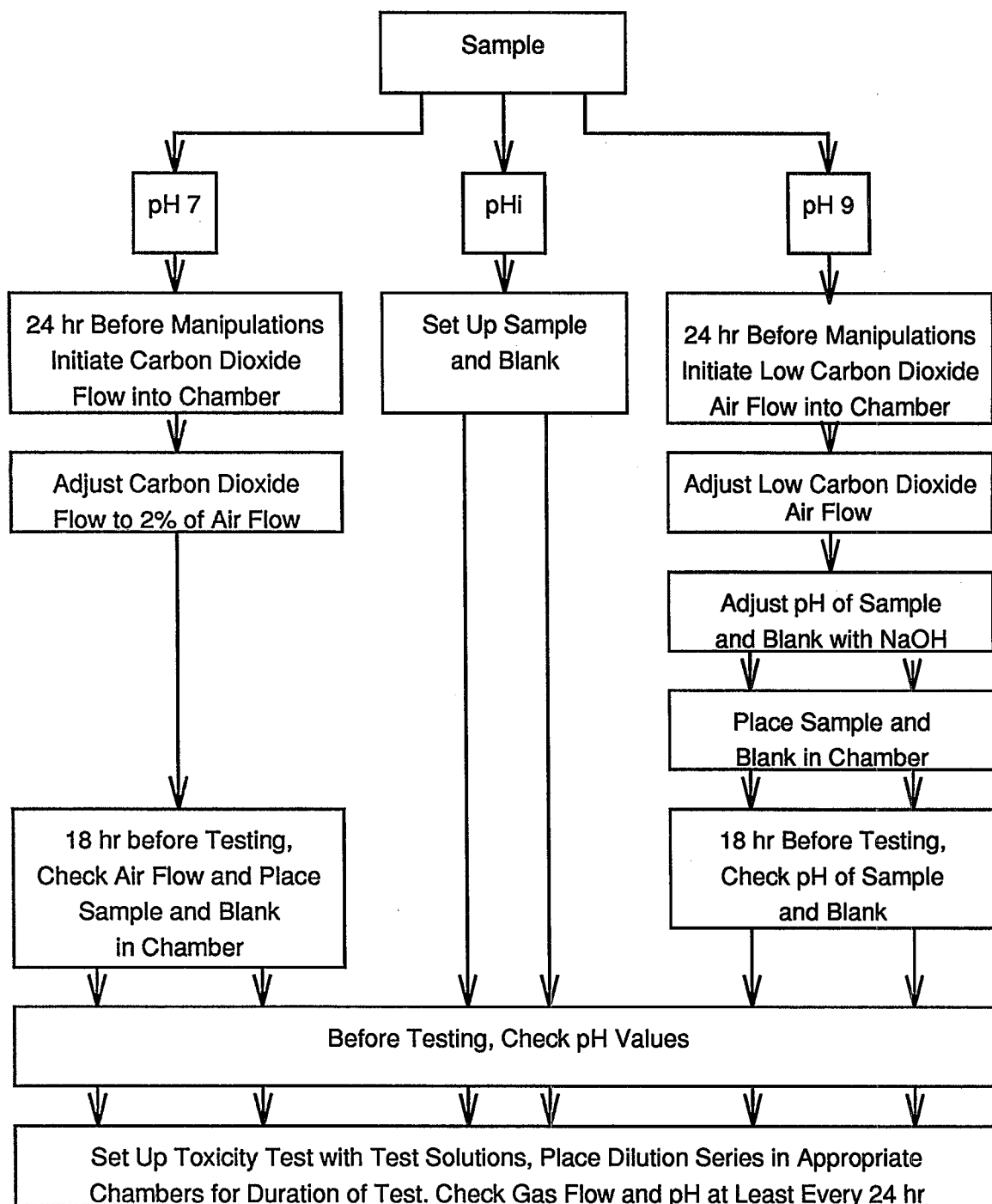


Figure 9-9. Overview Flowchart for Graduated pH Procedure.

9.10 Cation Exchange SPE Procedure

9.10.1 General Approach

The cation exchange manipulation is used to determine if toxic components are cationic in nature (e.g., metals). Cation exchange chromatography is applied to remove cationic toxicants from the aqueous sample. This manipulation can be used to support the EDTA manipulation (cf. Section 9.5) and with elution verify potential metal toxicity. Operationally, filtered test solutions (i.e., samples and controls) are passed through a disposable cation exchange column and the post-column sample tested for toxicity (Figure 9-10). Reduced toxicity in the post-column sample suggests that cationic toxicants are active (Burgess et al. submitted). Not all interferences with the cation exchange SPE procedure have been identified; therefore, it is important to perform the acid elution to verify metal toxicity.

Resulting post-cationic exchange column effluent is then tested to determine if the toxicity has been removed. The cation exchange column is activated with a combination of methanol and DI.

9.10.2 Materials

- Disposable cation exchange column(s)—for performing cation exchange manipulation (e.g., Supelco LC-WCX (500 mg/3 mL tube))
- 1M HCl Acid
- 1M NaOH
- Low flow metering pump (~0.5-10 mL/min) and tubing—for forcing sample through cation exchange column.
- Separatory funnel—to serve as effluent sample reservoir.
- Erlenmeyer flasks—for collecting post-column effluent.

9.10.3 Procedural Overview

(1) Preparation of Tubing

- (a) Connect pump, sample reservoir and column to tubing. Do not attach column. Pump 10 mL of 1 M HCl followed by 25 mL of DI through the entire system to remove any contamination. Throughout column preparation a flow of 7-10 mL/min is used.

(2) Preparation of Cation Exchange Column

- (a) Attach cation exchange column to tubing. For Supelco LC-WCX (3 mL/500 mg) column, the following procedure is recommended; for other types, check manufacturer recommendations. Using a flow rate of 2.5 mL/min, pass 2 mL of methanol through column. **Do not let the column dry out.**
- (b) Pass 6 mL of DI through the column. **Do not let the column dry out.** To avoid drying the column, leave a small volume of DI in the tubing.

(3) Blanks

- (a) Pass the brine and DI filtered blank through the wet prepared column.
- (b) Allow first 5 mL of brine and DI to pass into a waste container before collecting blank. Collect enough post-column brine and DI to conduct toxicity tests. Check pH to insure residual acid is not contaminating the sample. **Do not let the column dry out.**

(4) Effluent Sample

- (a) Pass the filtered sample through the wet prepared column.
- (b) Collect enough post-column sample to perform toxicity test. Column can now go dry. Check pH to insure residual acid is not contaminating sample.

(5) Toxicity Testing

- (a) Prepare test dilutions using post-column sample and post-column brine and DI.
- (b) Add organisms.

9.11 Cation Exchange SPE Acid Elution Test

9.11.1 General Approach and Materials

If following the Cation Exchange SPE procedure (Section 9.10), the post-column sample is non-toxic, it is recommended that the column be eluted with 1 M HCl to verify sample toxicity due to metal toxicants.

Materials for this test are the same as the Cation Exchange SPE Procedure (Section 9.10.2).

9.11.2 Procedural Overview

(1) Preparation of Tubing

- Same as Cation Exchange SPE Procedure, Section 9.10.3.(1).(a).

(2) Elution of Column

- (a) Attach loaded column to tubing. Pass 6 mL 1 M HCl through column using a flowrate of 0.5 mL/min.
- (b) Collect HCl in container and return sample to original volume with clean brine and DI and adjust pH with sodium hydroxide (Figure 9-10).

(3) Toxicity Testing

- (a) Prepare test dilutions using reconstituted sample and DI.
- (b) Add organisms.

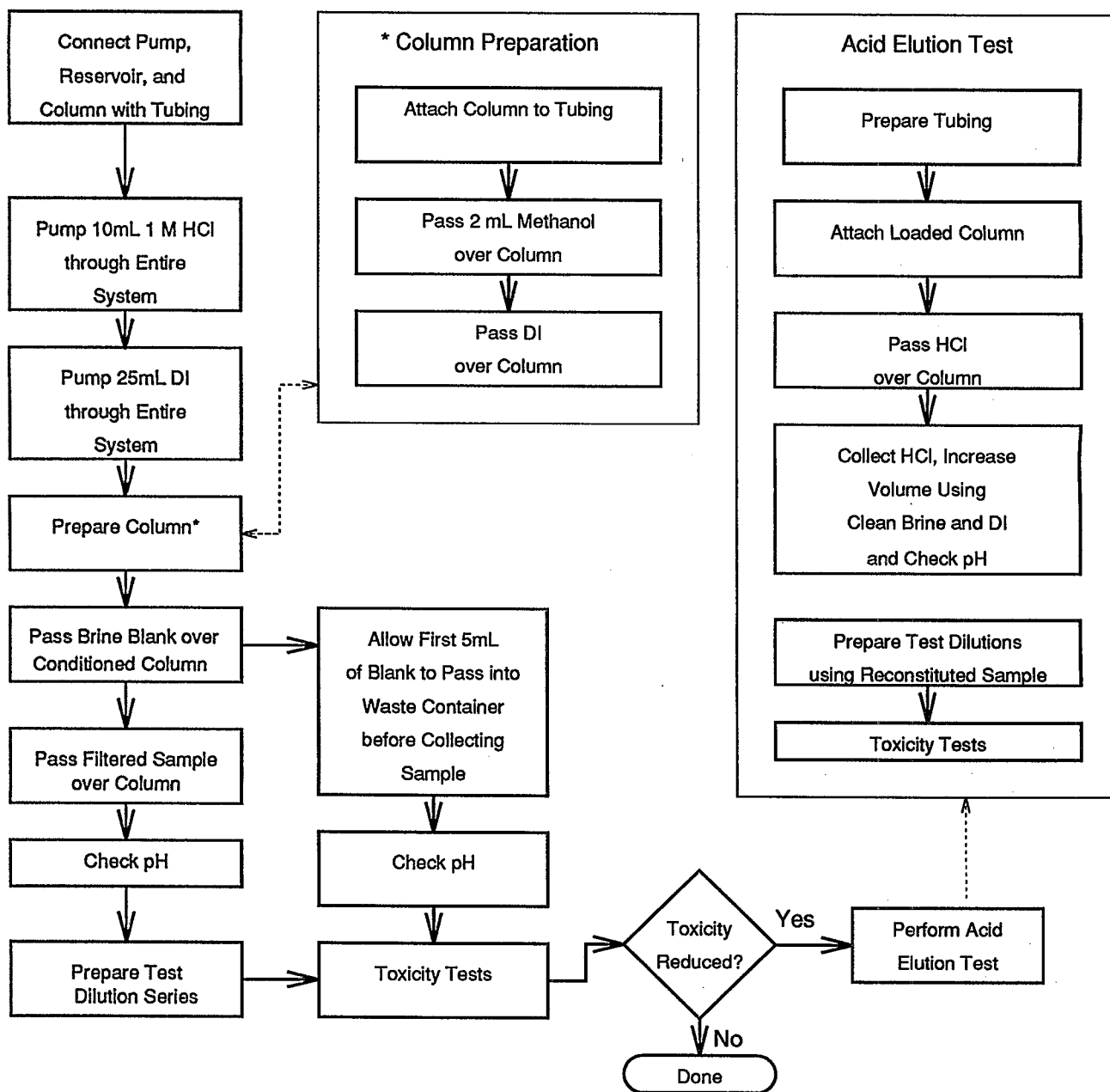


Figure 9-10. Overview Flowchart for Cation Exchange SPE Procedure and Acid Elution Test.

9.12 *Ulva lactuca* Procedure

9.12.1 General Approach

The objective of this manipulation is to remove ammonia from seawater samples by addition of a marine macrophyte *Ulva lactuca*, commonly known as sea lettuce. *Ulva lactuca* is a macrophyte that has the ability to uptake, store, and utilize large amounts of ammonia. *Ulva lactuca* has historically been used to clean-up effluents in aquaculture (Cohen and Neori 1991; Neori et al. 1991) and has proven effective in removing environmental concentration of ammonia from seawater (Ho et al. in prep.).

9.12.2 Materials

- *Ulva lactuca* 5g/60mL of sample
- Oil-free air pump, tubing, and pipettes
- Containers—to hold 60 mL sample, *Ulva lactuca*, and allow for aeration
- Light source ($\sim 75 \mu\text{E}/\text{m}^2/\text{s}$)
- Temperature 15-20°C. Temperatures over 20°C hasten the degradation of *Ulva lactuca* during storage.

9.12.3 Procedural Overview

(1) *Ulva lactuca* Collection and Storage

- Collect *Ulva lactuca* from a clean site. Sort through plants and discard any with white or yellowing tips. Remove any surficial organisms and hold static in 30‰ clean seawater in aerated jars under 16:8 light:dark condition until use. Sea lettuce is held in static systems, not flow-through conditions to minimize the exposure of the plant to nutrient concentration. Presumably, if the plant is “starved”, it will uptake ammonia more quickly when placed in the sample. Maximum holding time for *Ulva lactuca* is four days but should be used within 24 hr for optimal results (Figure 9-11).

(2) *Ulva lactuca* Addition

- Remove *Ulva lactuca* from holding jars using forceps, gently pat dry and place in salinity adjusted sample under lights with gentle aeration for five hours.
- (b) Remove *Ulva lactuca* from sample.

(3) *Ulva lactuca* Removal

- (a) Remove *Ulva lactuca* from sample.
- (b) Prepare toxicity dilutions with *Ulva lactuca* treated brine and DI and sample.

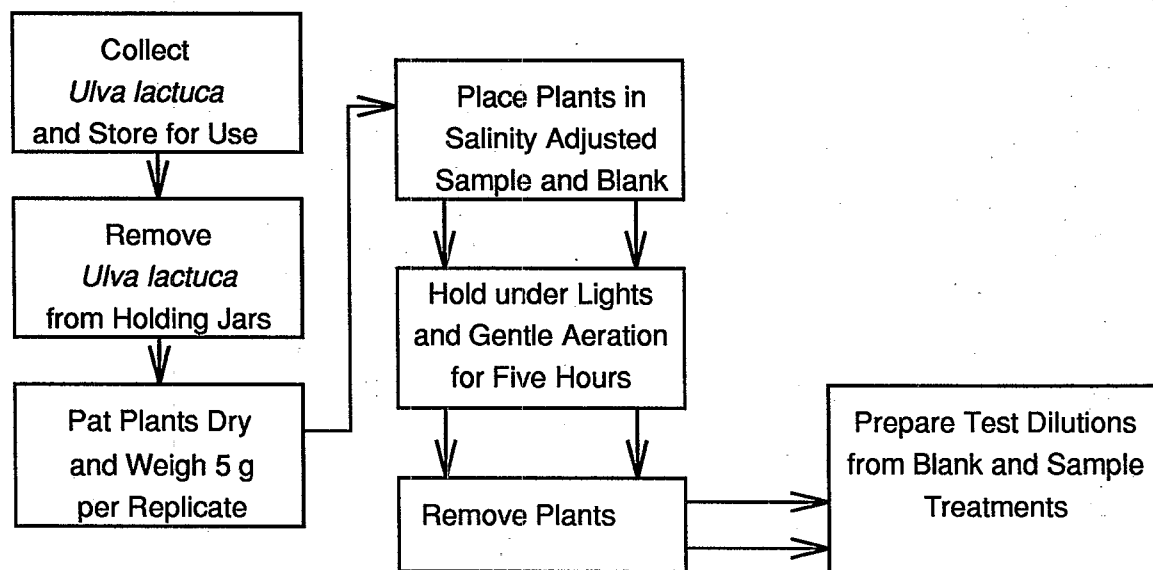


Figure 9-11. Overview Flowchart for *Ulva lactuca* Procedure.

Section 10

TIE Interpretation

To determine the efficacy of these methods in characterizing unknown toxicants, we performed some marine TIE manipulations on two spiked brine and DI samples (i.e., mock effluent). One sample contained 40 mg/L of the reference toxicant sodium dodecyl sulfate (SDS) and the other copper sulfate (1.0 mg copper/L). Results from these TIEs conducted on very simple samples provide insight into the complexity of interpreting marine TIE data.

10.1 Sodium Dodecyl Sulfate (SDS)

In this TIE, tests were conducted with the mysid *Mysidopsis bahia*. Results are presented in Table 10-1.

Table 10-1. Results of Toxicity Test with Sodium Dodecyl Sulfate-Spiked Brine and DI Using Mysid, *Mysidopsis bahia*. Conditions: 30‰, 21°C.

Manipulation	Toxic Units
Initial	Not Performed *
Baseline	6.8
EDTA Addition	6.7
Na ₂ S ₂ O ₃ Addition	7.5
Filtration	6.7
Post C ₁₈	No Toxicity †

* Historic data used to determine baseline exposure concentration.

† 0% Mortality in highest concentration (40 mg SDS/L)

As these data demonstrate, the C₁₈ column removed all toxicity, and there was no significant change in toxicity in the other manipulations except for the possible increase in toxicity caused by sodium thiosulfate. These results should be interpreted that organic compounds are responsible for all or most of the toxicity. Although C₁₈ column elution data for this example analysis is not available, the reader is reminded that that procedure is highly recommended (cf. Section 9.8).

10.2 Copper

Copper toxicity tests were conducted with the sea urchin *Arbacia punctulata*, mysid *Mysidopsis bahia*, and fish *Menidia beryllina*. Results are presented in Table 10-2

Table 10-2. Results of Toxicity Test with Copper-Spiked Brine and DI Using Sea Urchin, *Arbacia punctulata*, Mysid, *Mysidopsis bahia*, and Fish, *Menidia beryllina*. Conditions: 30‰, 21°C.

Manipulation	Toxic Units		
	<i>Arbacia punctulata</i>	<i>Mysidopsis bahia</i>	<i>Menidia beryllina</i>
Initial	5.0	2.4	8.6
Baseline	11.9	1.7	5.3
EDTA Addition	<2.0 *	<2.0 †	<4.0 §
Na ₂ S ₂ O ₃ Addition	2.2	5.3	<4.0 #
Filtration	5.0	2.1	<4.0 **
Aeration	14.5	5.8	6.4
Post C ₁₈	3.1	<2.0 ‡	<4.0 §

* 100% Fertilization at 50% effluent.

† 100% Survival at 50% effluent.

‡ 60% Survival at 50% effluent.

§ 100% Survival in 25% effluent.

90% Survival in 25% effluent.

** 60% Survival in 25% effluent.

Results of this TIE are not as easily evaluated as was SDS; clearly, EDTA removed the most toxicity in all cases with all three species, but other manipulations removed toxicity as well. Toxicity to *Arbacia punctulata* increased between the Initial Toxicity Test and the Baseline Toxicity Test by 6.9 toxic units. This significant variability in the response of the sea urchin sperm cell test is not uncommon when measuring copper toxicity. Morrison et al. (1989) reports a coefficient of variation of 46% for *Arbacia punctulata* in reference toxicant tests with copper.

All manipulations removed some amount of toxicity to *A. punctulata* except aeration, which increased toxicity about 2.5 toxic units. Toxicity to the mysid was fairly low but both the sodium thiosulfate and aeration manipulations increased toxicity. Exposures to the fish demonstrated a small reduction in toxicity between the Initial and Baseline Toxicity Tests and all manipulations reduced toxicity except for aeration.

Possible reasons for these results are: 1) sodium thiosulfate reduces the toxicity of some metals (EPA 1991b; MED, Duluth, personal communication), 2) filtration of metals through a glass fiber filter may result in adsorption of copper to the filter surface, and 3) C_{18} chelates some metals like copper. Aeration results that were consistent for all species suggest that the sample volume was reduced, and consequently, metal concentrations increased. However, it has been observed that EDTA seldom reduces the toxicity of any other toxicants except metals (MED, Duluth, personal communication); therefore, Table 10-2 results strongly support the presence of metals toxicity. If this sample had been a complex mixture of toxicants from an industrial or municipal plant, evaluation of these initial results would have suggested a combination of metals and organics as being the sources of toxicity.

10.3 Summary of Results

Phase I as described in this guidance document is dedicated to toxicity characterization. In Phases II and III, the TIE includes more advanced approaches: for example, the use of analytical chemistry (EPA 1993b, 1993c). For the exercise with copper above, analytical chemistry would progress the characterization from types of toxicants to specific toxicants by demonstrating the presence of elevated levels of copper. In general, comparison of these concentration data for various contaminants to the

sensitivities of the test species in the scientific literature, including EPA WQC, may help to elucidate which types of toxicants to include or exclude from consideration. Specifically, toxicity information on toxic metals, organics and ammonia are readily available from these sources. Use of this information will help individuals conducting marine TIEs to establish sensitivity patterns for the various marine species (e.g., *Arbacia punctulata* is very sensitive to most divalent transition metals and insensitive to most organics and ammonia). These sensitivity patterns in turn become diagnostic TIE tools contributing to the determination of what toxicants are active. Any complementary data (e.g., historical, collection, site) will assist in the characterization.

The investigator needs to keep in mind potential interferences to the TIE manipulations; although the methods are designed to be specific to single classes of toxicants, they may not be so in practice. Documented interferences or 'side effects' include: the pH manipulations changing the toxicity of both metals and ionic organic toxicants (Schubauer-Berigan et al. 1993; Spehar et al. 1984); and the C_{18} SPE can sorb certain metals from seawater; filtration may remove metals and nonionic organic toxicants from solution while *Ulva lactuca* removes nonionic toxicants (Ho et al. in prep.). Also, not all possible interferences associated with the cation exchange SPE have been determined. Despite the problems interferences can create when interpreting a TIE, advantage may be taken of interferences to aid in the characterization of toxicants.

Following the Phase I of a marine TIE are Phases II (Identification) and III (Confirmation). The reader is advised to refer to EPA 1991b, 1993b, and 1993c for guidance in performing these phases.

Section 11

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Appendix

Summary of Test Conditions and Acceptability

The tables in this appendix summarize test conditions and acceptability for the Phase I Marine TIE characterization tests. Because routine TIE toxicity testing methods are not currently available for all Pacific Coast species, the standard test conditions are provided. Tables correspond to those in EPA 1993a, 1994, 1995. Readers should refer to these references for detailed procedural outlines of the toxicity tests, and use the tables in this appendix for Marine Phase I TIE-specific variations.

Table A.1. Summary of TIE Test Conditions and Test Acceptability Criteria for Amphipod, *Ampelisca abdita*, Acute Toxicity Tests.

1. Test Type	Static non-renewal
2. Salinity	30±2‰
3. Temperature	20±2°C
4. Light quality	Ambient laboratory light
5. Light intensity	10-20 µE/m ² /s (50-100-ft-c) (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	25 mL chambers
8. Test solution volume	10-20 mL
9. Size of test organisms	0.5-0.7 mm
10. No. of organisms per chamber	5
11. No. replicate chambers per concentration	1-3 (TIE manipulations) 3 (Initial and Baseline)
12. Feeding regime	none
13. Dilution water	Natural seawater or hypersaline brine
14. Test concentrations	6 (Initial and Baseline toxicity tests) 4 (TIE procedures)
15. Dilution series	0.5
16. Test duration	24, 48, or 96 h
17. Endpoints	Mortality (LC ₅₀)
18. Test acceptability criteria	≥90% survival in controls

Table A.2. Summary of TIE Test Conditions and Test Acceptability Criteria for Sea Urchin, *Arbacia punctulata*, Fertilization Test.

1. Test Type	Static
2. Salinity	30±2‰
3. Temperature	20±1°C
4. Light quality	Ambient laboratory light during test preparation
5. Light intensity	10-20 µE/m ² /s, or 50-100 ft-c (ambient laboratory levels)
6. Test chamber size	Disposable (glass) liquid scintillation vials (20 mL capacity), presoaked in control water
7. Test solution volume	5 mL
8. No. of sea urchins	Pooled sperm from four males and pooled eggs from four females are used per test
9. No. egg and sperm cells per chamber	About 2000 eggs and 5,000,000 sperm cells per vial
10. No. replicate chambers per concentration	4 (minimum of 3)
11. Dilution water	Uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW Marinemix®, FORTY FATHOMS®, GP2, or equivalent)
12. Effluent concentrations	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Test dilution factor	Effluents: ≥0.5 Receiving waters: None, or ≥0.5
14. Test duration	1 hour and 20 min
15. Endpoints	Fertilization of sea urchin eggs
16. Test acceptability criteria	70%-90% egg fertilization in controls

Table A.3. Summary of Standard Test Conditions and Test Acceptability Criteria for the Topsmelt, *Atherinops affinis*, Larval Survival and Growth Test. (NOTE: for Phase I TIE, conditions may need to be altered (e.g., test volume)).

1. Test Type	Static-renewal
2. Salinity	5 to 34‰ (± 2‰ of the selected test salinity)
3. Temperature	20±1 °C
4. Light quality	Ambient laboratory illumination
5. Light intensity	10-20 µE/m ² /s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	600 mL
8. Test solution volume	200 mL/replicate
9. Renewal of test solutions	Daily
10. Age of test organism	9-15 days post hatch
11. No. of larvae per test chamber	5
12. No. replicate chambers per concentration	5
13. Source of food	Newly hatched <i>Artemia</i> nauplii
14. Feeding regime	Feed 40 nauplii per larvae twice daily (morning and night)
15. Cleaning	Siphon daily, immediately before test solution renewal and feeding
16. Aeration	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min.
17. Dilution water	Uncontaminated 1 µm-filtered natural seawater or hypersaline brine prepared from natural seawater
18. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
19. Dilution factor	effluents: ≥0.5 Receiving waters: None, or ≥0.5
20. Test duration	7 days
21. Endpoints	Survival and growth (weight)
22. Test acceptability criteria	≥80% survival in controls, 0.85 mg average weight of control larvae (9 day old), LC ₅₀ with copper must be ≤205 µg/L, <25% MSD* for survival and 50% MSD for growth†

* MSD Mean Standard Deviation

† Provisional, check with appropriate Region or State for latest guidance.

Table A.4. Summary of TIE Test Conditions and Test Acceptability Criteria for the Red Macroalga, *Champia parvula*, Sexual Reproduction Test.

1. Test type	Static, Static non-renewal
2. Salinity	30±2‰
3. Temperature	23±1°C
4. Light source	Cool-white fluorescent lights
5. Light intensity	100 µE/m ² /s (500 ft-c)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	50 mL polystyrene or borosilicate petri dishes or 125 mL Erlenmeyer flasks
8. Test solution volume	20 mL (minimum)
9. No. of organisms per test chamber	5 female branch tips and 1 male plant
10. No. replicate chambers per concentration	4 (minimum of 3)
11. No. of organisms per concentrations	24 (minimum of 18)
12. Dilution water	Uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW Marinemix®, FORTY FATHOMS®, GP2, or equivalent)
13. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
14. Dilution factor	Effluents: ≥0.5 Receiving waters: None, or ≥0.5
15. Test duration	Two day exposure to effluent, followed by 5 to 7 day recovery period in control medium for cystocarp development
16. Endpoints	Reduction in cystocarp production compared to controls
17. Test acceptability criteria	80% or greater survival, and an average of 10 cystocarps per plant in controls

Table A.5. Summary of Standard Test Conditions and Test Acceptability Criteria for Oyster, *Crassostrea gigas* and Mussels, *Mytilus californianus* and *Mytilus galloprovincialis*, Embryo-Larval Development Test.

1. Test type	Static non-renewal
2. Salinity	30±2‰
3. Temperature	20±1 °C (oysters) 15 or 18 ±1 °C (mussels)
4. Light quality	Ambient laboratory illumination
5. Light intensity	10-20 µE/m ² /s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	30 mL
8. Test solution volume	10 mL
9. No. of larvae per chamber	150-300
10. No. replicate chambers per concentration	4
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor	effluents: ≥0.5 Receiving waters: None, or ≥0.5
14. Test duration	48 hours (or until complete development up to 54 hours)
15. Endpoints	Survival and normal shell development
16. Test acceptability criteria ^a	Control survival must be ≥70% for oyster embryos or ≥50% for mussel embryos in control vials; ≥90% normal shell development in surviving controls; and must achieve %MSD* of <25%†

* MSD Mean Standard Deviation

† Provisional, check with appropriate Region or State for latest guidance.

Table A.6. Summary of TIE Test Conditions and Test Acceptability Criteria for Fish, *Cyprinodon variegatus*, Acute Toxicity Tests.

1. Test type	Static non-renewal
2. Salinity	25±10
3. Temperature	20±2°C
4. Light quality	Ambient laboratory light
5. Light intensity	10-20 µE/m ² /s (50-100-ft-c) (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	25 mL chambers
8. Test solution volume	10-20 mL
9. Age of test organisms	1-14 days old at start
10. No. replicate chambers per concentration	1 (TIE manipulations) 3 (Initial and Baseline)
11. No. organisms per chamber	5
12. Feeding regime	Feed one drop of concentrated <i>Artemia</i> nauplii suspension daily (approximately 100 nauplii per mysid)
13. Dilution water	Natural seawater or hypersaline brine
14. Test concentrations	6 (Initial and Baseline toxicity tests) 4 (TIE procedures)
15. Dilution series	0.5
16. Test duration	24, 48, or 96 h
17. Endpoints	Mortality (LC50)
18. Test acceptability criteria	≥80% survival in controls

Table A.7. Summary of Standard Test Conditions and Test Acceptability Criteria for Albalone, *Haliotis rufescens*, Larval Development Test. (NOTE: for Phase I TIE, conditions may need to be altered (e.g., sample volume)).

1. Test Type	Static non-renewal
2. Salinity	34±2‰
3. Temperature	15±1 °C
4. Light quality	Ambient laboratory illumination
5. Light intensity	10 µE/m ² /s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	600 mL*
8. Test solution volume	200 mL/replicate*
9. Larvae density per chamber	5-10 per mL
10. No. Replicate chambers per concentration	5
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine plus reagent water
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor	Effluents: ≥0.5 Receiving waters: None, or ≥0.5
14. Test duration	48 h
15. Endpoint	Normal shell development
16. Test acceptability criteria	≥80% normal shell development in the controls; must have statistical significant effect at 56 µg/L zinc; must achieve a %MSD† of <20%‡

* Successful tests performed at 10 mL volume in 20 mL scintillation vials (Hunt et al. In press).

† MSD Mean Standard Deviation

‡ Provisional, check with appropriate Region or State for latest guidance

Table A.8. Summary of Standard Test Conditions and Test Acceptability Criteria for Giant Kelp, *Macrocystis pyrifera*, Germination and Germ-tube Length Test. (NOTE: for Phase I TIE, conditions may need to be altered (e.g., sample volume)).

1. Test Type	Static non-renewal
2. Salinity	34±2‰
3. Temperature	15±1 °C
4. Light quality	Ambient laboratory light during test preparation
5. Light intensity	50±10 µE/m²/s
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	600 mL
8. Test solution volume	200 mL/replicate
9. Spore density per test chamber	7500 /mL of test solution
10. No. Replicate chambers per concentration	5
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor	Effluents: ≥0.5 Receiving waters: None or ≥0.5
14. Test duration	48 h
15. Endpoints	Germination and germ-tube length
15. Test acceptability criteria	≥70% germination in the controls; ≥10µm germ-tube length in the controls and the NOEC must be below 35 µg/L in the reference toxicant test; must achieve a %MSD* of <20 for both germination and germ-tube length in the reference toxicant.†

* MSD Mean Standard Deviation

† Provisional, check with appropriate Region or State for latest guidance.

Table A.9. Summary of TIE Test Conditions and Test Acceptability Criteria for Fish, *Menidia beryllina*, Acute Toxicity Test.

1. Test Type	Static non-renewal
2. Salinity	25±10‰
3. Temperature	20±2°C
4. Light quality	Ambient laboratory light
5. Light intensity	10-20 µE/m ² /s (50-100-ft-c) (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	25 mL chambers
8. Test solution volume	10-20 mL
9. Age of test organisms	9-14 days old at start
10. No. replicate chambers per concentration	1 (TIE manipulations) 3 (Initial and Baseline)
11. Organisms per chamber	5
12. Feeding regime	Feed one drop of concentrated <i>Artemia nauplii</i> suspension daily (approximately 100 nauplii per mysid)
13. Dilution water	Natural seawater or hypersaline brine
14. Test concentrations	6 (Initial and Baseline toxicity tests) 4 (TIE procedures)
15. Dilution series	0.5
16. Test duration	24, 48, or 96 h
17. Endpoints	Mortality (LC ₅₀)
18. Test acceptability criteria	≥80% survival in controls

Table A.10. Summary of TIE Test Conditions and Test Acceptability Criteria for Bivalve, *Mulinia lateralis*, Embryo-Larval Development Test.

1. Test type	Static non-renewal
2. Salinity	30±2‰
3. Temperature	20±2°C
4. Light quality	Ambient laboratory illumination
5. Light intensity	10-20 µE/m²/s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	30 mL
8. Test solution volume	10 mL
9. No. of larvae per chamber	~300
10. No. Replicate chambers per concentration	3-4
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor	Effluents: ≥0.5 Receiving waters: None, or ≥0.5
14. Test duration	48 hours
15. Endpoints	Survival and normal shell development
16. Test acceptability criteria	> 70% Survival; >90% Development

Table A.11. Summary of TIE Test Conditions and Test Acceptability Criteria for Mysid, *Mysidopsis bahia*, Acute Toxicity Tests.

1. Test type	Static non-renewal
2. Salinity	25±10‰
3. Temperature	20±2°C
4. Light quality	Ambient laboratory light
5. Light intensity	10-20 µE/m ² /s (50-100-ft-c) (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	30 mL chambers
8. Test solution volume	10-20 mL
9. Age of test organisms	48 h old at start
10. Number of organisms per chamber	5
11. No. Replicate chambers per concentration	1 (TIE manipulations) 3 (Initial and Baseline)
12. Feeding regime	Feed one drop of concentrated <i>Artemia nauplii</i> suspension daily (approximately 100 nauplii per mysid)
13. Dilution water	Natural seawater or hypersaline brine
14. Test concentrations	6 (Initial and Baseline toxicity tests) 4 (TIE procedures)
15. Dilution series	0.5
16. Test duration	24, 48, or 96 h
17. Endpoints	Mortality (LC ₅₀)
17. Test acceptability criteria	≥80% survival in controls

Table A.12. Summary of Standard Test Conditions and Test Acceptability Criteria for the Purple Urchin, *Strongylocentrotus purpuratus*, and Sand Dollar, *Dendraster excentricus*, Fertilization Tests.

1. Test Type	Static non-renewal
2. Salinity	34±2‰
3. Temperature	12±1°C
4. Light quality	Ambient laboratory light during test preparation
5. Light intensity	10-20 µE/m ² /s (ambient laboratory levels)
6. Test chamber size	16 x 100 or 16 x 125 mm
7. Test solution volume	5 mL
8. Number of spawners	Pooled sperm from up to four males and pooled eggs from up to four females are used per test.
9. No. Egg and sperm cells per chamber	About 1,120 eggs and not more than 3,360,000 sperm per test tube
10. No. Replicate chambers per concentration	4
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater or artificial sea salts
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
12. Dilution factor	Effluents: ≥0.5 Receiving waters: None or ≥0.5
13. Test duration	40 min (20 min plus 20 min)
14. Endpoint	Fertilization of eggs
15. Test acceptability criteria	≥70% egg fertilization in controls; %MSD* of <25%; and appropriate sperm counts†

* MSD Mean Standard Deviation

† Provisional, check with appropriate Region or State for latest guidance.

Table A.13. Summary of Standard Test Conditions and Test Acceptability Criteria for the Purple Urchin, *Strongylocentrotus purpuratus*, and Sand Dollar, *Dendraster excentricus*, Embryo-Larval Development Test.

1. Test Type	Static non-renewal
2. Salinity	34±2‰
3. Temperature	15±1 °C
4. Light quality	Ambient laboratory illumination
5. Light intensity	10-20 µE/m ² /s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	30 mL
8. Test solution volume	10 mL
9. No. Replicate chambers per concentration	4
10. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
11. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
12. Dilution factor	Effluents: ≥0.5 Receiving waters: 100% receiving water and a control
13. Test duration	72±2 h
14. Endpoint	Normal development; mortality can be included
15. Test acceptability criteria	≥80% normal shell development in the controls; must achieve a %MSD* of <25%†

* MSD Mean Standard Deviation

† Provisional, check with appropriate Region or State for latest guidance.