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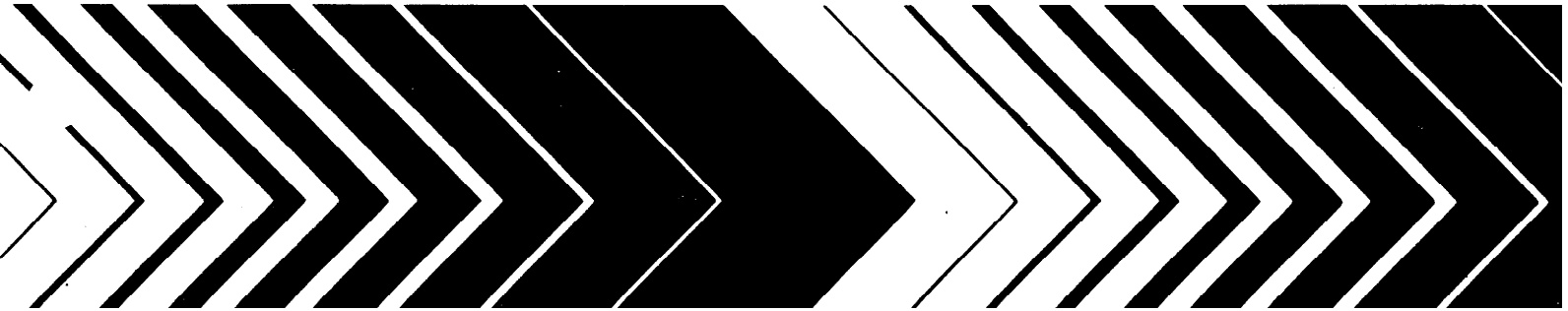
Methods for Aquatic Toxicity Identification Evaluations

Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity



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Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity

by

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Previous Phase III Methods
by D. I. Mount
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Foreword

This Phase III document is the last in a series of guidance documents intended to aid dischargers and their consultants in conducting aquatic organism toxicity identification evaluations (TIEs). TIEs might be required by state or federal agencies as the result of an enforcement action or as a condition of a National Pollutant Discharge Elimination System (NPDES) permit. These documents should aid individuals in overseeing and determining the adequacy of effluent TIEs as a part of toxicity reduction evaluations (TREs).

There are two major reasons to require the confirmation procedures. First the effluent manipulations used in Phase I characterizations (EPA, 1988; EPA, 1991 A; EPA, 1992) and Phase II identifications (EPA, 1989A; EPA, 1993A) might (with some effluents) create artifacts that might lead to erroneous conclusions about the cause of toxicity. Therefore in Phase III confirmation steps, manipulations of the effluent are avoided and/or are minimized, therefore artifacts are far less likely to occur. Sometimes, toxicants will be suspected through other approaches (such as the treatability route) which on their own are not definitive and in these instances, confirmation is necessary. Secondly, there is the probability that the substances causing toxicity might change from sample to sample, from season to season or some other periodicity. As toxicity is a generic measurement, measuring toxicity cannot reveal variability of the suspect toxicant whereas the Phase III confirmation procedures are designed to indicate the presence of variable toxicants. Obviously, this crucial information is essential so that remedial action may be taken to remove toxicity.

Confirmation, whether using the procedures described in this document or others, should always be completed because the risk is too great to avoid or eliminate this step. Especially for discharges where there is little control over the influent or for discharge operations that are very large or complex, the probability that different constituents will cause toxicity over time is great. Most of the approaches in Phase III are applicable to chronically toxic effluents and acutely toxic effluents.

In this confirmation document, guidance is included when the treatability approach (EPA, 19898; EPA, 1989C) is taken. Use of the treatability approach requires confirmation as much as or more than the toxicant identification approach (Phase II). The reader is encouraged to use both the acute Phase I characterization (EPA, 1991 A) and the chronic Phase I characterization (EPA, 1992) documents for details of quality assurance/quality control (QA/QC), health and safety, facilities and equipment, dilution water, sampling and testing. The TIE methods are written as general guidance rather than rigid protocols for conducting TIEs and these methods should be applicable to other aqueous samples, such as ambient waters, sediment elutriate or pore waters, and leachates

Abstract

In 1989, the guidance document for acutely toxic effluents entitled *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures* was published (EPA, 19890). This new Phase Ill manual and its companion documents (EPA, 1991 A; EPA, 1992; EPA, 1993A) are intended to provide guidance to aid dischargers in confirming the cause of toxicity in industrial and municipal effluents. The toxicity identification evaluation (TIE) starts with a characterization of the effluent toxicity using aquatic organisms to track toxicity; this step is followed by identifying a suspect toxicant(s) and then confirming the suspect toxicant as the cause of toxicity.

This Phase III confirmation document provides greater detail and more insight into the procedures described in the acute Phase III confirmation document (EPA, 1989D). Procedures to confirm that all toxicants have been correctly identified are given and specific changes for methods applicable to chronic toxicity are included. Adifficult aspect of confirmation occurs when toxicants are not additive, and therefore the effects of effluent matrix affecting the toxicants are discussed. The same basic techniques (correlation, symptoms, relative species sensitivity, spiking, and mass balance) are still used to confirm toxicants and case examples are provided to illustrate some of the Phase III procedures. Procedures that describe the techniques to characterize the acute or chronic toxicity (EPA, 1988) and to identify (EPA, 1989A) toxicants have also been rewritten (EPA, 1991 A; EPA, 1992; EPA, 1993A).

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Acknowledgments

This document presents additional information acquired since the document entitled *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures* (EPA-60013-88-036; EPA, 1989D) was prepared by Donald Mount and published in 1989. This manual reflects new information, techniques, and suggestions made since the Phase III confirmation methods for acute toxicity were developed. The suggestions, techniques and cautions contained in this document are based on a large database generated by the staff of the National Effluent Toxicity Assessment Center (NETAC) at the U.S. Environmental Protection Agency (EPA), Environmental Research Laboratory, Duluth (ERL-D), MN. NETAC staff that provided technical support consisted of Penny Juenemann and Shaneen Schmidt (ERL-D staff), Joe Amato, Lara Anderson, Steve Baker, Tim Dawson, Nola Englehorn, Doug Jensen, Correne Jenson, Jim Jenson, Elizabeth Makynen, Phil Monson, Greg Peterson, and Jo Thompson (contract staff). Their collective experience has made this document possible and the contributions are gratefully acknowledged. The support through EPA's Office of Research and Development (ORD) and Office of Water made this research possible at ERL-D.

Section 1
Introduction

The final confirmation phase of a toxicity identification evaluation (TIE) consists of a group of steps intended to confirm that the suspect cause(s) of toxicity is correctly identified and that all the toxicity is accounted for. Typically this confirmation step follows experiments from the toxicity characterization step (Phase I) and analysis and additional experiments conducted in toxicity identification (Phase II) (EPA, 1991A; EPA, 1992; EPA, 1993A). However, there often may be no identifiable boundary between phases. In fact, all three phases might be underway concurrently with each effluent sample and depending on the results of Phase I characterization, the Phase II identification, and Phase III confirmation activities might begin with the first sample evaluated. Phase III confirmation procedures should also follow after toxicants have been identified by other means or when treatability approaches are used. Rarely does one step or one test conclusively prove the cause of toxicity in Phase III. Rather, all practical approaches are used to provide the weight of evidence that the cause of toxicity has been identified. The various approaches that are often useful in providing that weight of evidence consist of correlation, observation of symptoms, relative species sensitivity, spiking, mass balance estimates and various adjustments of water quality.

The approaches described in this document have been useful in TIEs at ERL-D. While the guidance provided in this manual is based largely on experience with wastewater effluents, in general the methods discussed are applicable to ambient waters (Norberg-King et al., 1991) and sediment pore or elutriate water samples as well (EPA, 1991B). However, specific modifications of the TIE techniques might be needed (e.g., sample volume) when evaluating these other types of samples.

Confirmation is important to provide data to prove that the suspect toxicant(s) is the cause of toxicity in a series of samples and to assure that all other toxicants are identified that might occur in any sample over time. There may be a tendency to assume that toxicity is always caused by the same constituents, and if this assumption carries over into the data interpretation but the assumption is false, erroneous conclusions might be reached. That is why the correlation step (Section 2) is accompanied by other approaches (i.e., Sections 3-9) because each approach aids in revealing any changes in the toxicant(s) in the confirmation phase of the TIE.

Seasonal trends in toxicants have been observed in publicly owned treatment works (POTW) effluents and some sediment samples. For example, organophosphate pesticides have been observed to increase in concentrations in wastewaters during the late winter and spring months (Norberg-King et al., 1989). Therefore, the confirmation steps of Phase III might need to include seasonal samples. This effort cannot always be pre-determined. The presence of a different toxicant(s) must be considered throughout the TIE, and when samples are collected over several months the seasonality of a suspect toxicant should be carefully considered and studied. When remedial action requires treatment changes, one must be certain that toxicity from specific toxicant(s) is consistently present and that the suspect toxicant(s) accounts for all the toxicity. Treatment modifications will not necessarily result in removal of all toxicants to acceptable concentrations. If toxicity is caused by a variety of toxicants present at varying intervals, the remedial actions that are practical might differ from the remedial action required when toxicity is caused by the same constituents consistently.

TIEs conducted at ERL-D have shown that toxicants often are not additive or toxicants are present in ratios such that the toxicity contribution by one might be diluted out in the range of the effluent effect concentration (e.g., LC50 or ICp value). Thus, the toxicant present at lower yet toxic concentrations may not be readily discerned. The frequency of occurrence and impact on data interpretation of either of the above cases was not addressed previously (EPA, 1989D) but are now discussed in Section 2. Toxicants that do not express their toxicity because of the presence of other toxicants (either the toxicants are non-additive or the toxicants occur in disparate ratios) are referred to as hidden toxicants (Section 9). Detection of hidden toxicants is one of the most difficult aspects of confirmation. It is a mistake to search for a concentration of any chemical present in the effluent at a toxic concentration and to declare any found as the cause

of toxicity. Matrix effects of the effluent samples make conclusions such as these subject to error without further work as either the hidden toxicant(s) or the principal toxicant(s) are likely to be missed using such an approach.

There is a strong tendency to shorten or eliminate the confirmation steps because by the time Phase III confirmation has been reached, the investigators might be convinced of the cause of toxicity and the confirmation steps seem redundant. However, one cannot expect to concentrate the effluent on a C_{18} solid phase extraction (SPE) column and not change a complex mixture such as effluents, and arrive at some false conclusions about the toxicants in the earlier phases.

Not all approaches discussed in the following sections will be applicable to every effluent, and additional approaches might need to be developed during the TIE. The various approaches need not be performed in any particular sequence, and the list of possible approaches will get larger as experience is gained. To effectively evaluate effluent samples from one particular discharger to obtain a correlation, substantial calendar time could be required and any steps for correlation should be initiated at the beginning stages of Phase III. Judgement must be made as to how many of the approaches described in Phase III confirmation should be used and how many samples for each should he completed. How completely Phase III confirmation is done will determine the authenticity of the outcome. The amount of confidence in the results of the TIE that is required is dependent at least in part on the significance of the decision that will be based on the results. For example, if a suspect toxicant can be removed by pretreatment or by a process substitution, a higher degree of uncertainty may be acceptable than if an expensive treatment plant is to be built. Such considerations are subjective and cannot be reduced to a single recommended decision making process with a specified number of samples.

Time and resources might be conserved if identification (Phase II) and confirmation (Phase III) can be started on the very first effluent sample used in the Phase I characterization. However, this is only possible when the results from the Phase I characterization are definitive enough to allow the investigators to proceed to identification and confirmation. In the acute Phase III confirmation document (EPA, 1989D), although perhaps not explicitly stated, performing Phase I characterizations on several samples before attempting Phases II and III was implied. Initiating the Phase III confirmation steps earlier in the TIE is often particularly useful. In addition, many regulatory agencies have adopted a policy that requires that the previous TIE approach be modified. For some dischargers, action might be required after the first exceedence in toxicity, which means that each effluent sample collected for toxicity testing is of equal regulatory concern when the toxicity is greater than the permit allows. This regulatory practice was not in place in 1989 when the earlier TIE guidance was available (EPA, 1989D) and at that time we did not expect that the cause of toxicity in one sample could be sufficiently deduced as we have been able to do. The importance of confirmation on several samples is not reduced by the importance of conducting confirmation steps on single samples; rather, the cause of toxicity for each sample must be confirmed.

In addition to the importance of each sample with toxicity greater than the allowable amount specified in a permit, a sample that is quite different from the previous samples must be evaluated to determine if the data point must be included in the Phase III correlation final data analyses. For each effluent sample, the data points must be explainable. If one sample is quite different than other samples it can cause the correlation to be less useful; however, if it can be shown to have a different toxicant the data point for that sample can be eliminated from the correlation. For example, suppose five consecutive samples during a Phase III evaluation exhibited toxicity that correlated well with a suspect toxicant. Then a sixth sample exhibits greater toxicity than previous samples while the measured concentration of the suspect toxicant is much lower than measurements on previous samples. In this sixth sample, the greater toxicity is thought to be caused by a different toxicant. Now in plotting the data for the correlation (Section 2), the datum point for the sixth sample will not be similar to the points for the existing regression and could render the correlation non-significant. If however, when the sixth sample is then subjected to intensive study using Phase I characterization and Phase II identification techniques, and if another toxicant is identified (or even if Phase I only shows that the toxicity has very different characteristics), datum for the sixth sample can legitimately be excluded from the correlation. This preserves the worth of the data for the previous five samples. In confirmation, every effort should be made to determine why a particular sample shows different responses in the various TIE steps from other samples.

This is not to imply that multiple effluent samples need not be subjected to Phase I manipulations, even if Phase II and/or Phase III are initiated on the first sample. Most effluent samples tend to be representative of the routine effluent discharge. However, determining what is the characteristic discharge for each effluent is important to the final success and completeness of the TIE.

When Phase III is completed, all results that were obtained during the TIE should be explainable. Unless the results make sense for all samples (aside from an occasional aberrant data point) something has been missed or is wrong. If so, the confirmation is not complete. Many techniques used in Phase III require keen observations and extensive or broad knowledge of both chemistry and toxicology but above all the ability to synthesize small bits of evidence in a logical sequence is essential. This TIE work is most effective when scientists interact daily.

A note of caution. If data obtained on early samples during Phase I are to be used for Phase III purposes, quality control will have to be suitable to provide defensible data (cf., EPA, 1991A; EPA, 1992; EPA, 1993A). In Phases I and II, the permissibility of using small numbers of animals and replicates, and omitting measurements such as pH, DO, and temperature that are required for routine monitoring tests or single chemical tests was discussed (EPA, 1989E; EPA, 1991 A; EPA, 1992; EPA, 1993A). These modifications were made to reduce cost and allow more testing, but at this point shortcuts must be avoided because definitive data that constitute the basis for important decisions are generated in Phase III. For Phase III testing, the effluent test protocols that triggered the TIE (EPA, 1991C; EPA, 1993B) should be followed, paying careful attention to test conditions, replicates, quality of test animals, representativeness of the effluent samples tested, and strict QA/QC analytical procedures including blanks and recovery measurements. Analytical work must be selective for the identity of the toxicant and its concentration measurement. When small differences in toxicity must be detected, concentration intervals should be smaller to obtain partial effects (e.g., use dilution factors of 0.60 or 0.65 versus 0.5). Remember, all of the data from Phases I and II (for either acute or chronic toxicity) are considered preliminary relative to Phase III data. However, if a suspect toxicant is identified and Phases I and II data may be necessary for confirmation, stricter QA/QC can be applied for each of the subsequent Phases I and II techniques so that the data can be used in Phase III.

For samples exhibiting chronic toxicity, modifications or 'changes to some of the TIE procedures are required for confirming the cause of chronic toxicity. Remember that for confirmation (as well as for Phases I and II), only a single sample of effluent should be used for each renewal in any chronic test (cf., EPA, 1992; EPA, 1993A). This is important because one cannot correlate a measured concentration of a toxicant with the toxicity measured in a test if multiple samples are used for each renewal and the toxicant is not present in some samples but other toxicants appear. Even more likely, the ratios of the toxicants, when more than one is present, might change from sample to sample. In these instances, there is no valid way to calculate the toxicity of a given toxicant. Overall, considerations for chronic toxicity tests in Phase III are not much different than acute toxicity tests in Phase III. At present, permit requirements specify the 7-d test and unless data are gathered to show that the 4-d and 7-d tests yield the same results and that the same toxicants are involved, the 7-d test should be used for confirmation (cf., EPA, 1993A). If the 4-d *Ceriodaphnia dubia* test has been used instead of the 7-d *C. dubia* test (see EPA, 1992) during Phases I and II, serious consideration should be given to returning to the 7-d test for Phase III.

When identification of the toxicant(s) causing chronic toxicity is desired, and the effluent also exhibits acute toxicity, it might be possible to use acute toxicity as a surrogate measure to characterize the toxicity in Phase I and assist in an identification in Phase II. It must be demonstrated that the cause of the acute toxicity is the same toxicant(s) as the toxicant(s) causing the chronic toxicity. Yet for confirmation, use of chronic toxicity endpoints to confirm the cause of the chronic toxicity is strongly recommended to avoid misleading the TIE results when using acute toxicity as a surrogate for chronic toxicity. As discussed in the chronic Phase I manual (Section 5.8; EPA, 1992), effect levels for chronic tests should be calculated using the linear interpolation method rather than the hypothesis test (EPA, 1992). In order to get more precise estimates of endpoints, test concentration intervals might have to be narrowed (see above). However, when point estimation techniques for other than survival endpoints (such as the inhibition concentration (ICp); EPA, 1993B) are used, a point estimate effect concentration can be estimated. The effect concentration estimates will also be more accurate when intermediate concentrations are used (i.e., use dilution factors of 0.6 or 0.65).

Section 2
Correlation Approach

2.1 Correlation

The purpose of the correlation approach is to show whether or not there is a consistent relationship between the concentration of suspect toxicant(s) and effluent toxicity. For the correlation approach to be useful, the toxicity test results with the effluent must demonstrate a wide range of toxicity with several effluent samples to provide an adequate range of effect concentrations for the regression analysis. For sediment samples, spatial variability might be used to perform correlation analyses (EPA, 19916).

The effluent effect concentration (i.e., LC50 or ICp) data and the measured toxicant concentration data must be transformed to toxic units (TUs) for the regression analysis to evaluate whether or not a linear relationship exists. Effluent TUs are obtained by dividing 100% by the effect concentration expressed in percent of the effluent (cf., EPA, 1991A; EPA, 1992). The suspect toxicant concentration is converted to TUs by dividing the measured toxicant concentration by the LC50 or ICp for that toxicant (data to make this comparison might have to be generated; EPA, 1993A). If more than one toxicant is present, the concentration of each one is divided by the respective LC50 or ICp value and the TUs can then be summed (cf., discussion below for non-additive toxicants).

Most of the effluents we have tested have exhibited a wide range of toxicity with several different samples and therefore the data can be used in the correlation approach. Typically for the correlations that we have conducted, the data used are from toxicity tests without any manipulations and from chemical measurements on the effluent samples for the concentrations of the suspect toxicant. However for effluents where ammonia was the cause of the toxicity, the effluent toxicity. results have not varied in toxicity enough, nor have the ammonia concentrations fluctuated enough to use the data in a correlation. Also, when the effect concentration is greater than 1 00%, this information is not useful since the data point cannot be included in the regression analysis. However, when samples are marginally toxic or when the suspect toxicant concentrations do not vary enough from sample to sample (i.e., ammonia is cause of toxicity), changes in toxicity can be induced by sample manipulation (cf., EPA, 1993A) and this toxicity data can be used to develop a different type of correlation. For example, the toxicity of a given amount of total ammonia can be changed by over an order of magnitude by altering the pH of aliquots of the effluent within an acceptable physiological range (e.g, pH 6 to 9). For some metals and some species, the toxicity can also be changed by adjusting the pH and using dilution waters of varying hardness. This type of data is useful in the correlation step as providing additional weight of evidence. Therefore, the idea of minimal manipulation(s) and any risk of creating artifactual toxicity are off set by the utility of the data.

An example of the regression from an effluent from a POTW in which the suspect toxicant was diazinon is given in Figure 2-l. The independent variable (x-axis) is the TUs of diazinon and the dependent variable (y-axis) is the effluent TUs. The solid line is the observed regression line obtained from the data points, and the dashed line is the expected or theoretical regression line. If there is 1 .O TU of the toxicant in 100% effluent, then the effluent should have 1 .0 TU (i.e., the LC50 =100%). Likewise for 2.0 TUs of suspect toxicant, the effluent TUs should be 2.0, et cetera. Thus, the expected line has a slope of one and an intercept of zero. In Figure 2-1, the intercept (0.19) is not significantly different from zero and the slope is very close to 1 (1.05). The r^2 value is 0.63 which, while not high, indicates that the majority of the effluent toxicity is explained by the concentration of the toxicant. As the r^2 becomes lower, less confidence can be placed on slope and intercept. In a small data set such as this, one datum point that had 5.0 TUs for the effluent toxicity lowered the r^2 value substantially. As discussed in Section 1, if an intensive effort had been expended on that sixth sample and another toxicant(s) had been found, this particular datum point could have been excluded and the r^2 value would have been higher.

In another POTW effluent, diazinon was also the suspect toxicant. For these data (Figure 2-2), the slope is 1.38, the intercept is 1.24 and the r^2 value is only 0.15, which all indicate poor fit for diazinon as the only toxicant. The low r^2 value indicates a large amount of scatter, therefore little can be inferred from the slope and the intercept. Based on this correlation, we returned to Phase II analytical procedures and identified two other organophosphates (chlorfenvinphos (CVP) and malathion). Toxicity data indicated that CVP was present at toxic

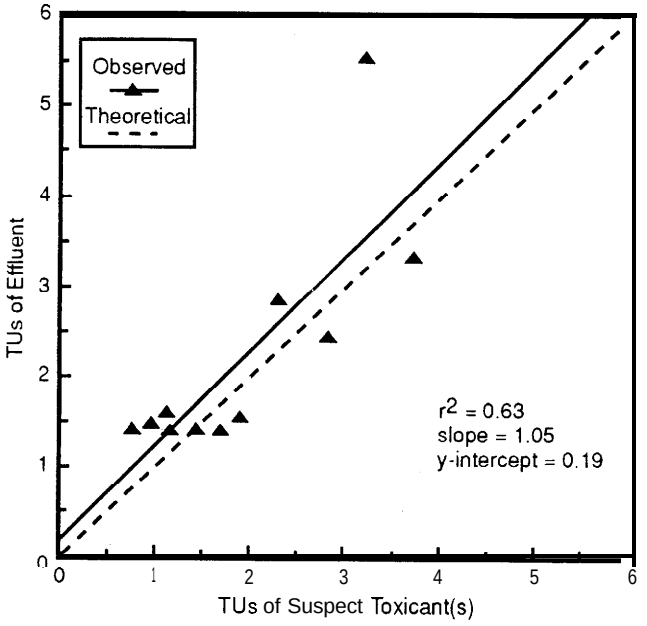

Figure 2-1. Correlation of toxic units (TUs) for an effluent and one suspect toxicant in POTW effluent.

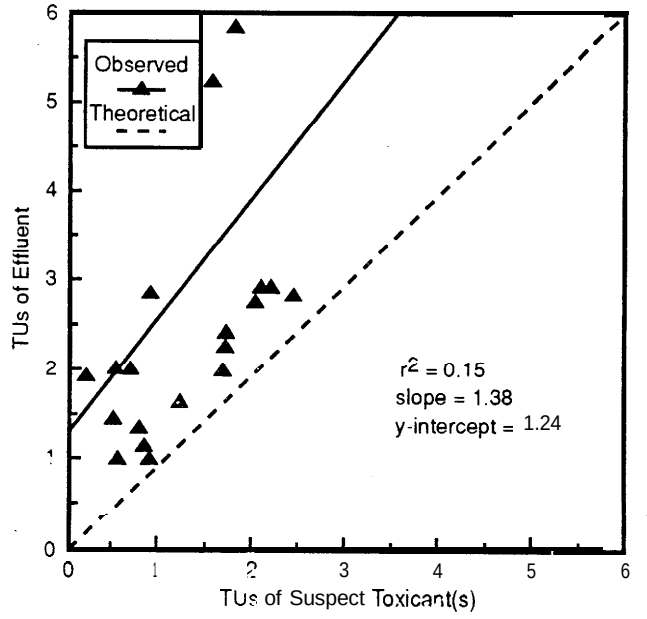

Figure 2-2. Correlation of toxic units (TUs) for an effluent and cne suspect toxicanr in a POTW effluent when two toxicants are the cause of toxicity.

concentrations while malathion was no?. After testing each compound both separately and as a mixture, the toxicity from all three chemicals was determined to be additive, so a new correlation was begun with analytical measurements made for all three chemicals. CVP and diazinon have nearly identical LC50 values for the species (C. *dubia*) used in this TIE. Malathion is about one-fourth as toxic as CVP or diazinon. Since the measured concentrations of malathion were lower than its toxicity, it was not included in the regression analysis. In a new correlation with data for the TUs summed for CVP and diazinon versus the effluent TUs, the data show a much better fit to the expected slope and intercept and a high r^2 value (Figure Z-3). Malathion TUs could also have been included in the regression (although its contribution to toxicity was minimal) because it was additive with other toxicants. This type of situation is discussed below.

In addition to slope and intercept, some judgement of the scatter about the regression line must be made. This can be done statistically, but when the sample size is large, the scatter can be very large and yet not negate the relationship. A-suggested approach to avoid the effect of sample size on the significance of scatter is to set a lower limit on r^2. This value (often expressed as percent) provides the measure of how much of the observed effluent toxicity is correlated to the measured toxicant. It is not dependent on choosing the correct effect concentration of the toxicant. The specific choice of the minimum value of r^2 should be made based upon the consequences of the decision. It is important to recognize that experimental error makes an r^2 value greater than 0.80 or 0.85 difficult to obtain. Therefore, where minimal chance of an incorrect decision is required, an r^2 value of nearly 0.80 may be used. Where an increased risk of an incorrect decision (i.e., a lesser amount of the toxicity accounted for) is acceptable, a lower value such as 0.60 may be used.

Since <1.O TU cannot be directly measured in the effluent, such values are, of necessity, excluded from the regression. (This comment is exclusive of the use of concentrates such as the C_{18} SPE fractions' where TUs of <1.O are possible.} However in some instances, when the TUs based on chemical analyses are <1.O TU and effluent effect values are <1.0 TU, the data support the validity of the regression provided a suspect toxicant has been found in several previous samples. In the correlation for the effluent toxicity depicted in Figure 2-2, toxicity was present in a different fraction (Phase II non-polar organic identification) than where the pesticides were identified. A specific toxicant was not identified in that fraction and toxicity was not always measurable in that fraction. However, this additional toxicity may have decreased the r^2 value.

Correlation might be more definitive when two or more toxicants are present. For example, suppose three toxicants are involved. If each toxicant has the same LC50 and each is strictly additive with the ratio of their concentrations remaining the same, the slope will be the expected but the intercept will be positive if all toxicants

[1]TUs can be calculated from toxicity tests with the fractions, the concentrate or the HPLC fractions as described in Phase II (EPA,' 1993A).

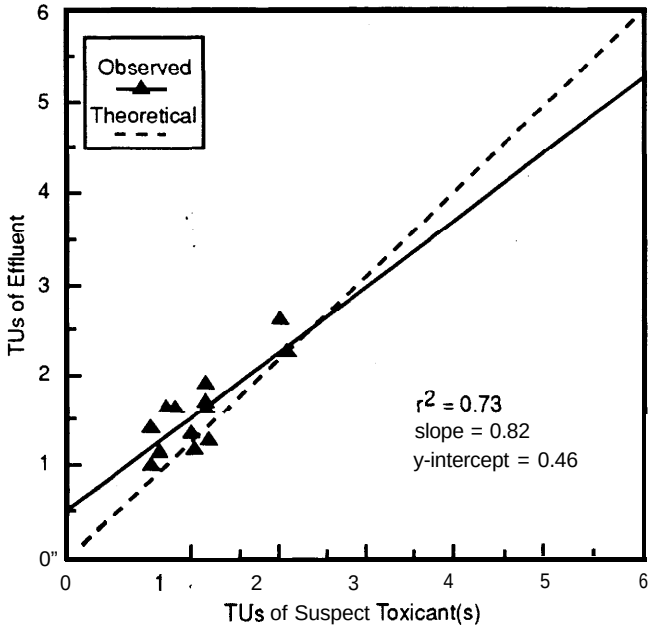

Figure 2-3. Correlation of toxic units (TUs) for an effluent and two toxicants in a POTW effluent.

are not identified. If the relative amounts (ratios) of each toxicant vary from sample to sample, the slope, intercept and r^2 will be different from the expected if only one toxicant is identified. If the toxicity of one of the toxicants is substantially different, and if the ratios of the three toxicants vary from sample to sample, then the slope, intercept, and r^2 value will all be different from expected if all are not identified. Much can be learned from studying the interrelationship of slope, intercept and the r^2 value. For example, a high r^2 value and an intercept near zero with a slope larger than 1 can be caused by using an effect concentration for the toxicant that is not appropriate for the toxicant in the effluent matrix (e.g., suspect toxicant is more toxic in effluent matrix than in single chemical test). This error causes the toxicant TUs to be too few relative to the effluent TUs (Figure 2-4) (cf., discussion below on non-additive toxicants). If toxicant concentrations and effluent toxicity show a wide distribution, a significant correlation will be easier to demonstrate than for a narrow range.

Great care must be taken to understand whether or not toxicants are additive or if the TUs for each toxicant are so different that only one toxicant determines the effect level. For either situation, the resulting data will have to be interpreted as though the toxicants are non-additive. For example, suppose the ratio of TUs is so disparate that at the effluent effect concentration, the toxicant with fewer TUs is always present at a fraction of a TU (e.g., 0.25 of a TU). Whether the two toxicants are additive or not is irrelevant because the major toxicant will set the effluent effect concentration. While 0.25 TUs of the minor toxicant appear to be relatively unimportant in view of experimental variability, this affects the regression. If in one sample the effect concentration is 25% and the 4 to 1 ratio of toxicants occurs, there are 4 TUs of the major toxicant and 1 TU of the minor toxicant. If the toxicant concentrations are summed, 5 TUs will be plotted against 4 effluent TUs, and this results in a 25% error. When secondary toxicants are present in concentrations that will not contribute to the effect concentration of the effluent, they should not be included in the correlation data set. Obviously if an effluent had several toxicants in dissimilar ratios, the error of including the minor TUs in a correlation plot could be large and may negate the correlation significance. The investigator should evaluate the data in regression plots to consider the significance of the contribution of the secondary toxicant especially if the toxicants appear to be additive.

Unfortunately the minimum fraction of a TU that is detectable will depend on the precision of the laboratory performing the testing. And of course the precision of the testing is not only dependent on the quality of the work, but the inherent precision of measuring specific toxicant TUs. That is, the toxicity measurement for some chemicals is more precise than for some other chemicals. In general, a chemical such as NaCl whose toxicity is generally not affected by pH, alkalinity, hardness, total organic carbon (TOC), suspended solids or solubility, can be measured more precisely than a chemical whose toxicity is affected by these factors, such as lead or copper. Therefore, each laboratory must determine which fractional value of a TU at the effect concentration is unmeasurable, thus indicating which TUs contributed by the minor toxicant should be deleted from the correlation data set.

Clearly, if two or more toxicants are strictly non-additive, then only the major one (the one present in the most TUs) should be included in the correlation data set. Since additivity might be easier to measure than the minimum measurable contribution of a fraction of a TU, it may be preferable to first determine if additivity occurs. If substances appear to be partially additive, then very careful work is required to properly add TUs.

Some very unusual decisions are required in accepting data into the correlation database when toxicants are strictly non-additive. For example, consider zinc and ammonia in the same effluent sample; we have found them to be strictly non-additive. Also consider that in some samples zinc and ammonia occur in TU ratios of 3 to 1 and in other samples the ratio is 1 to 2. In the regression for the 3 to 1 ratio samples, only zinc TUs should be plotted. In the regression for the 1 to 2 ratio samples, only ammonia TUs should be plotted. For this particular example, 3 TUs for the first sample and 2 TUs for the second sample would be used if the data is interpreted correctly (i.e., plotting total TUs) or 4 and 3 TUs would be used respectively, if the data is interpreted

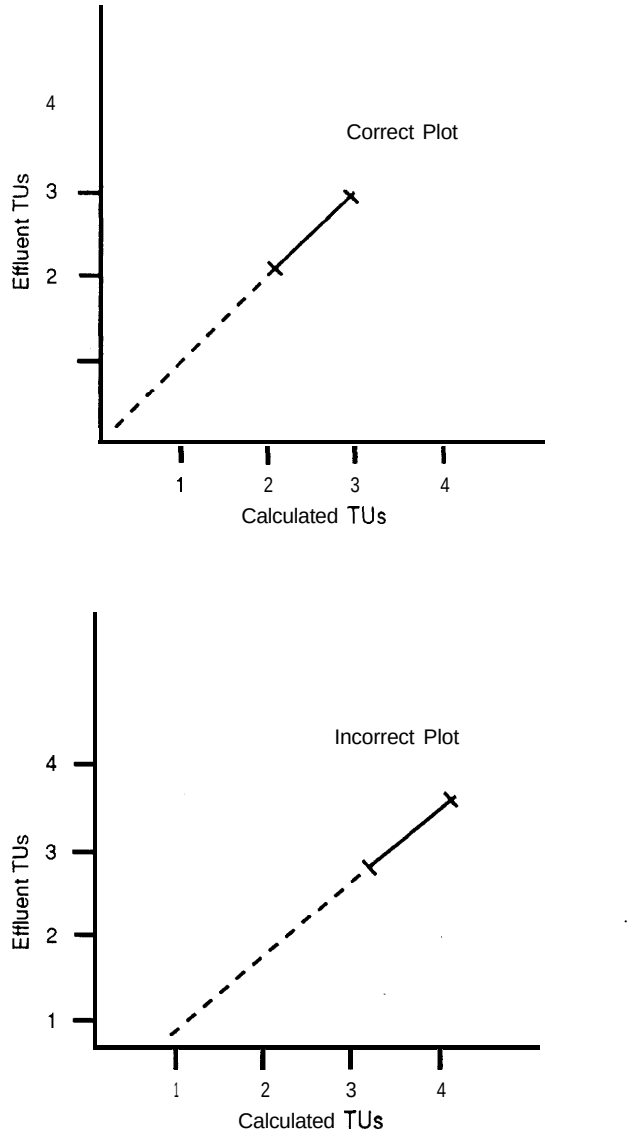

Figure 2-4. Correct (top) and incorrect (bottom) plots of toxic units (TUs) for non-additive toxicants.

incorrectly. The slopes for both plots would be 1 but a negative intercept instead of an intercept of 0 would be obtained for the incorrect plot. The more similar the TUs of each toxicant are to each other, the greater the error in the correlation will be.

2.2 Correlation Problems Caused by Matrix Effects

Correlation becomes much more difficult when the toxicants interact with the other effluent constituents in ways that change their toxicity and we refer to these changes as matrix *effects*. There are numerous matrix effects and all of them will not be discussed here; instead a framework is provided to aid in designing tests or test conditions to validly incorporate matrix effects in such a manner that useable correlation data can be obtained.

Matrix effects generally fit into one of two categories. One category is when the toxicants change form in some manner which exhibit a different toxicity. A very common example is ammonia which changes from NH_3 to NH_4^+ as pH decreases. NH_4^+ is so much less toxic than NH_3 that it is often considered nontoxic? Another example is HCN whose most toxic form is as un-dissociated HCN, a form predominating at low pH values. As pH increases the equilibrium shifts to more H^+ and CN^-. If metals are present, metal-cyanide complexes form which are often less toxic than HCN but metal-cyanide complexes might vary in toxicity depending on the metal. For example, iron-cyanide complexes are much less toxic than some of the other metal complexes. Metal-cyanide complexes might also photodecompose in sunlight releasing HCN or H^+ and CN^-, depending on pH.

A second category of matrix effects involves such physical changes as sorption or binding in some manner so as to make the toxicant unavailable to the organism. For example, non-polar organics sorb onto suspended solids, and some metals, such as copper, also sorb onto suspended solids. The presence of organic matter on suspended solids might increase the sorbtive capacity. Predictably, changes in water chemistry often change the sorption/solution equilibrium and thereby, change the portion of total toxicant that is available to the organism.

To further complicate matters, biological characteristics of the test organisms might change the availability of the same toxicant form. For example a non-polar organic sorbed on suspended solids such as bacterial cells, might be unavailable to a fish but readily available to daphnids because cells might be ingested and digested by daphnids. The uptake route then is through the digestive tract but the toxicant has entered the body none-the-less.

From the above discussion, it is obvious that one method of correlation will not be applicable for all toxicants. A temptation may be to remove the toxicant from the effluent and then use the effluent as a diluent to measure toxicity. However, because effluents are so complex and undefined, there is virtually no way to remove one or a few constituents and still be certain other characteristics have not been changed. For example, zeolite removes ammonia but it also removes some metals and non-polar organics; the C_{18} resin removes metals as well as non-polar organics; ion exchange columns remove ionized constituents, but non-polar organics also are retained by the columns. Toxicant removal procedures have utility but require very complicated simultaneous testing of the effluent and proper blanks (cf., EPA, 1992; EPA,

[2]See specific discussion in Section 3, Phase II (EPA, 1993A).

1993A) is necessary to properly interpret results (cf., Section 9 on hidden toxicants).

In Phase III, quantitative comparisons are being made between toxicity and concentrations of toxicants rather than qualitative comparisons as in Phases I and II (EPA, 1991A; EPA, 1992; EPA, 1993A). In the correlation approach, such comparisons are the essence of the technique. Therefore even small changes in form or availability might be unacceptable. This means that manipulations and changes must be minimized when effluent toxicity and toxicant concentrations are to be compared.

Solvent extraction, so commonly used for organic analyses, is likely to extract biologically unavailable organics as well as soluble forms. The total measured concentration may be larger than the true exposure concentration. Use of the C_8 SPE column also is not free from problems as the C_{18} SPE column is a finer filter than the glass fiber filters commonly used for pre-column filtration. Therefore solids are likely to be physically retained on the upper part of the column. When the column is eluted with methanol, the methanol extracts toxicant(s) from the solids (which might not be biologically available) as well as elutes the C_{18} sorbent itself. For Phases I and II, this might be unimportant, but for the Phase III correlation step where careful quantitative comparison is necessary, the effect might be unacceptable. Such problems probably reach a maximum when working with samples such as highly organic sediment pore water (with high organic characteristics) where much of the chemical might be biologically unavailable.

The central problem for either type of matrix effect is the difficulty of analytically measuring the biologically available portion of the specific toxic form. A correlation for a POTW effluent where for nickel was suspected of causing the toxicity is shown in Figure 2-5. During Phase I, the acute toxicity was removed with EDTA additions, and in Phase II the nickel was measured at toxic concentrations to C. *dubia.* The toxicity correlated very well with total nickel concentration (r^2 = 0.89 and a slope of 1 .17) and it appeared that only nickel seems to be involved. But the intercept of -12.34 is quite different from the expected zero. Such an intercept would be expected if there were a relatively fixed amount of nickel which was not biologically available in all samples. In this example, because all other confirmation data corroborated nickel as the toxicant, a constant concentration of nontoxic nickel was thought to provide the explanation for the unexpected intercept value. However, there is no obvious reason to think that the quantity, or even the percentage of total toxicant, is the same across samples for other toxicants, or for nickel in other matrices.

For the effluent samples that lose their toxicity in a short time, the nontoxic effluent can be used for the suspect toxicant(s) tests as a diluent in parallel tests using a standard dilution water to elucidate matrix effects on toxicity. Toxicity test results with quite different toxicity would reflect matrix effects. If toxicity is persistent, developing two separate correlations using pure chemical additions on two different effluent samples, each with substantially different toxicant concentrations, might be useful. If the toxicity test results indicate that the biologically unavailable portion changes with measured concentrations, the slope should be different than one. This approach requires careful work and the investigator must consider incorporating equilibrium time experiments (cf., EPA, 1993A).

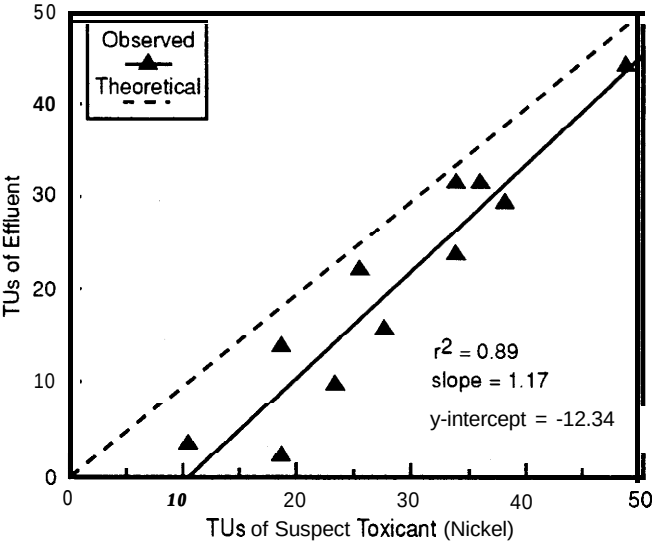

Figure 2-5. Correlation of toxic units (TUs) for a POTW effluent and the suspect toxicant, nickel.

Metals can be especially difficult toxicants to implicate using correlation because the toxicity of metals is typically very matrix dependent. When the knowledge of these characteristics is extensive for a chemical, as it is with ammonia (see Phase II), testing can be tailored to the chemical and a very powerful correlation obtained. The large amount of available information on ammonia does not exist for most metals. In these instances, the logic pattern should to be reversed where the approach has to become: if x *is the toxicant, what are the matrix effects?.* These can be found by pure chemical testing combined with Phases I or II manipulations. Once an adequate understanding of matrix effects is obtained, the information can be used to answer the question: Is the *effluent toxicant behavior consistent with the matrix effects for the suspect toxicant?*

Matrix effects will have varying impacts on toxicant behavior that also depends on the effluent effect concentration. For effluents which have effect concentrations in the <10% range, the test solutions will more closely resemble the diluent water matrix than the effluent. If the effluent has effect concentrations in the 50% to 100% range, the matrix effects of the test solution will most likely resemble those of the effluent, not of the dilution water. Since effluent TUs are calculated from

responses occurring in the dilution near the effect concentration, the matrix characteristics of that concentration are of the most concern for correlation. Thus the importance of the effluent matrix effects diminishes as the toxicity of the effluent is greater (i.e., matrix at effect level is more like dilution water).

One can safely say that the difficulty of simulating the matrix effects with a simulated effluent is quite large so that the choice is clearly to use the actual effluent when possible. An important reason for this choice is that so few matrix effects have been studied extensively, and beyond pH and hardness little data exists. Even then the interrelationship between pH, alkalinity and hardness were often ignored.

The above discussion does not provide all of the options on how to handle matrix effects. However, it should provide convincing evidence that more than the correlation step alone is necessary to provide adequate confirmation!

In summary, the TIE research experience has revealed two major areas of potential problems in using the correlation approach. The lack of additivity for toxicants found in effluents requires careful analysis when calculating TUs for regression purposes. Secondly, when there are matrix effects, correlation becomes difficult because the effluent matrix might change from sample to sample and because there are no analyses specific for the toxic forms. For such effluents, other confirmation techniques should be used more extensively to better support the overall confirmatory efforts.

Section 3
Symptom Approach

Different chemicals may produce similar or very different symptoms in a test species. Probably no symptom of intoxication is unique to only one chemical. Therefore, while similar symptoms observed between two samples means the toxicant(s) could be the same or different, different symptoms means the toxicant(s) is definitely different, or there are multiple toxicants in the two samples. By observing the symptoms displayed by the test organisms in the effluent and comparing them to the symptoms displayed by test organisms exposed to the suspect toxicants, failure to display the same symptoms means the suspect toxicant(s) is probably not the true one or the only one.

Behavior of most test species is difficult to put into words so that a clear image of behavior is obtained. Behavioral and morphological changes of 30-d old fathead minnows (*Pimephales promelas*) were used as diagnostic endpoints in 96 h flow-through single chemical tests. Organic chemicals of various modes of action were tested and video recordings were used to monitor the behavioral response (Drummond et al., 1986; Drummond and Russom, 1990). Substances within a single chemical classification did not necessarily cause the same type of response (Drummond and Russom, 1990). Therefore, it is difficult to predict chemical classification using behavioral monitoring alone.

This type of behavioral monitoring data does not exist for the cladocerans or the newly hatched fathead minnows or other species that are most frequently used in the TIE process. However, noting various symptoms is useful in the TIE. This is done by simply exposing the test species to the suspect toxicant(s) and observing how they react. By the time confirmation is initiated, toxicity tests with the suspect toxicants will have been conducted using pure compounds and symptoms may have been observed. It is important to note the symptoms observed during all testing because such characteristics can be very helpful in confirmatory work.

The intensity of exposure concentrations might change the symptoms observed with the suspect toxicant in the effluent. Therefore, it is important to compare symptoms at concentrations that require about the same period of onset. This can be done by comparing symptoms at exposure concentrations that have similar TUs. In this way both the unknown (sample) and the known toxicants (pure compound) can be set at the same toxicity level.

Observations of the organisms should not be delayed until the normal length of the test has elapsed. With some toxicants, the test organisms will show distinctive symptoms soon after the exposure begins, whereas later, symptoms are often more generalized and less helpful. For some other toxicants, a sequence of different symptom types are displayed by the test organism over the exposure period and the sequence may be more definitive for a given chemical than the individual symptoms. In few cases will the symptoms be unique enough to specifically identify the toxicant, but symptoms different from those caused by the pure suspect toxicant are convincing evidence that the suspect toxicant is not the true or only one.

A second caution is needed regarding mixtures of toxicants. Mixtures of toxicants can produce symptoms in test animals different from the symptoms of the individual toxicants comprising the mixture. When more than one toxicant is involved, the investigator must not only include all the toxicants, but include them in the same ratio as measured in the effluent. Often the toxicant of the mixture at the highest concentration relative to its effect concentration will cause most of the symptoms. As for single toxicants, the mixture concentration causing the same endpoint in a similar exposure period should be compared. Spiking effluent with the suspect toxicants and comparing the results of the spiked effluent sample and the unspiked effluent sample toxicity tests, both near their effect concentrations, is a good approach to take (Section 5).

Symptoms caused by the toxicant(s) might be quite different among different species of organisms; therefore the use of two or more species provides increased definitiveness of the observations. For both species, the researcher must compare symptoms at concentrations that are equitoxic. The greater the difference in sensitivity, the more important this becomes, The chemical concentration is unimportant; the important consideration is that equitoxic concentrations are compared.

Suppose, for example, species A and B have LC50 values for a suspect toxicant of 1 and 80 mg/l. Then concentrations of 2 and 160 mg/l may be used to compare symptoms of species A and B, respectively. If the onset of symptoms is rapid, then perhaps 1.25 and 100 mg/l (1.25xLC50) should be tried. Since symptoms vary with the exposure intensity, using various multiples of the LC50 (i.e., 0.5, 1, 2x) can add additional confirmation data, if the same set of symptoms are seen in both series. If more than one toxicant is involved, and the ratio of the two species' LC50 values for toxicant A is markedly different than for toxicant B, C, D, ... then the definitiveness of using symptoms is even greater.

For acute toxicity, time-to-mortality at equitoxic concentrations can be used as a symptom type of test. Some chemicals cause mortality quickly and some cause mortality slowly. If for two effluent samples, toxicity is expressed quickly for one and for the other very slowly, the toxicants are probably not the same.

In chronic testing, use of symptoms is also applicable. For example, adult mortality, number of young/female, death of young at birth, growth retardation, abortion, or time to onset of symptoms, all can also be monitored and such observations may be useful. The shape of the dose response curve may also be a determinant in assisting in confirmation. Some chemicals show an all or none type of response (diazinon) while others (i.e.. NaCl) display a relatively flat concentration-response slope for chronic toxicity.

Section 4
Species Sensitivity Approach

The effect concentrations can be compared for the effluent of concern and the suspect toxicants, using species of different sensitivities. If the suspect toxicant(s) is the true one(s), the effect levels of effluent samples with different toxicity to one species will have the same ratio as for a second species of different sensitivity. Also the ratio for each species should be the same as for known concentrations of the pure toxicant. The same ratio of effect values for two species implies the same toxicant in both samples of effluent. Obtaining the same effluent toxicity ratio among various effluent samples for each species as is obtained by exposure to comparable concentrations of known toxicants, implies that the suspect toxicants are the actual ones present. However, if other effluent characteristics affect toxicity and if they vary, the ratios could also be affected.

The common notion that goldfish are resistant to most toxicants and trout are sensitive to most toxicants is not readily substantiated (AQUIRE, 1992). Many species are more sensitive to certain groups of toxicants than trout. Of course, there are generalizations that can be made. For example, sunfish (Centrarchids), frequently are much more resistant to metals than goldfish, minnows, and daphnids (AQUIRE, 1992). Daphnids tend to be more resistant to chlorinated hydrocarbon insecticides than many fish species and more sensitive to organophosphate insecticides (AQUIRE, 1992). These differences must always be verified for the suspect toxicants; generalities can only be used as an initial guide to species selection. Sensitivity differences of 1 O-i 00x may occur in some chemical groups and not in others. If several toxicants are involved, interpreting the results and designing the ancillary experiments is more difficult. If successful, the power of the result for multiple toxicants is much greater than for a single toxicant. The difference in sensitivity between *Ceriodaphnia* and fathead minnows has, on several occasions, revealed either a change in the suspect toxicants present in a series of effluent samples, or the presence of other toxicants in addition to those suspected.

Comparison of sensitivity among species has another very important use. Some species may evidence toxicity from an effluent constituent that the TIE test species did not. If this happens, then the above comparison will be confused, but at least there will be a warning that the suspect toxicant may not be the cause of toxicity. In order to determine what is happening, the investigator should step back to Phase II, and possibly step back to Phase I to characterize the additional toxicant and then identify the toxicant using the new species. A second Phase III effort might be necessary for this toxicant and species. It is important not to assume that the resident species have the same sensitivity as the TIE test species. Especially for freshwater discharges into saltwater this concern is critical when a saltwater organism triggered the TIE, because at present the techniques and procedures described in Phases I and II are most likely to be done using freshwater organisms especially since the effluent is freshwater. If the concern is for marine organisms and their protection cannot be assumed (cf., Section 8, Phase I; EPA, 1991A), confirmation must be conducted with marine organisms.

In chronic testing, chemical and physical conditions might differ more among tests on different species because food must be provided during the test period and different foods are used for each species. For example, the final pH of fathead minnow 7-d tests might be lower than in acute fathead minnow tests and both are likely to be lower than in *Ceriodaphnia* chronic tests due to greater respiration rates for fish than cladocerans and food in fish tests. If the investigation was to confirm ammonia toxicity, this pH difference could result in confusing results by showing the *Ceriodaphnia* to be more sensitive than the fathead minnows when the reverse should be true (cf., EPA, 1993A; Phase II). The above example illustrates reasons to maintain careful quality control in Phase III work.

Section 5
Spiking Approach

In spiking experiments, the concentration of the suspect toxicant(s) is increased in the effluent sample and then toxicity is measured to see whether toxicity is increased in proportion to the increase in concentration. While not conclusive, if toxicity increases proportionally to an increase in concentration, considerable confidence is gained about the true toxicant(Two principles form the basis for this added confidence. To get a proportional increase in toxicity from the addition of the suspect toxicant when it is in fact not the true toxicant, both the true and suspect toxicants would to have 1) very similar toxicity and 2) to be strictly additive. The probability of both of these coinciding by chance is small.

Removing the suspect toxicants from the effluent without removing other constituents or in some way altering the effluent is usually not possible. The inability to do this makes the task of establishing the true toxicity of the suspect toxicants in the effluent difficult. For many toxicants, effluent characteristics, such as TOC, suspended solids, or hardness, affect the toxicity of a given concentration, Some characteristics, such as hardness, can be duplicated in a dilution water, but certainly not TOC or suspended solids because there are many types of TOC and suspended solids, and generic measurements do not distinguish among the different types. For example, effluent TOC occurs as both dissolved and suspended solids. In POTW effluents, the source of the TOC is likely to be largely from biological sources, both plant and animal (e.g., bacteria) and bacteria are likely to make up a large component of suspended solids. If there have been recent storms, oily materials from stormwater runoff might be high. Simulating TOCs from such variable sources is next to impossible because TOC is not solely the result of man-made organic chemicals. For suspended solids, shape, porosity, surface-to-volume ratio, charge and organic content (all or any), will impact sorption characteristics. None of these qualities are measured by the standard methods for measuring suspended solids nor can they be reproduced in a simulated effluent.

In a simple system, such as reconstituted soft water, it is reasonable to expect that for most chemicals a doubling of the chemical concentration will double the toxicity, at least in the effect concentration range. If the solubility of the toxicant is being approached or there are effects from water characteristics such as suspended solids, then the toxicity might not double or conceivably could more than double. For example, if a chemical with a large n-octanol/water partition coefficient (log P) is largely sorbed on solids, doubling the total concentration might more than double the toxicity because the added chemical might remain in solution. Another important issue is that equilibrium might not be established during the entire test period and is probably unlikely to occur before the test organisms are added. For example, in our TIE research, we found various surfactants sorb to solids and can be removed by filtration (Ankley et al., 1990). In these experiments, however, filtration failed to remove surfactants immediately after they were spiked in an effluent but surfactants were removed after a few days equilibrium time. Other chemicals are likely to show similar behavior in regard to equilibrium time.

If several toxicants are involved, then their interaction (additivity, independent action, synergism) must be measured or otherwise included in the confirmation process (cf., Section 2). Since ratios might be as important as concentration, the best way to spike when multiple toxicants are involved is to increase each toxicant by the same number of TUs (e.g., by doubling each). In this way the ratios of the toxicities remain constant.

The fact that two or more toxicants fail to show additivity is useful evidence in confirmation. Interpreting spiking data might require a very high level of competence in both toxicology and chemistry; otherwise the data could be very misleading. Using more than one species of differing sensitivity is effective in adding confidence to the results. When matrix effects are complicated, other types of spiking can be done to reduce the effects of the effluent matrix characteristics. If a method exists for removing the toxicants from the effluent, such as the C_{18} SPE procedures (EPA, 1993A), the extracts or methanol fractions can be spiked with pure chemicals in addition to spiking effluent, using the same principles as described for effluents. The advantage in this approach is that matrix characteristics such as suspended solids and TOC will be absent or much reduced and will not affect spiking experiments as much. The disadvantage is that proof that the extracts or fractions contain the true toxicants must be generated. Some approaches for doing

this are given in Section 6. The use of the spiking approach is especially applicable to fractions from the C_{18} SPE column or the high performance liquid chromatography (HPLC) column used for the isolation of non-polar organics. In these procedures, the constituents are separated from much of the TOC, suspended solids and hardness, so that spiked additions might be strictly additive where they might not be in the effluent. Suggestions and precautions about ratios and all other previously discussed concerns apply here too. In addition, concerns about the methanol percentages in the toxicity tests, the amount of SPE or HPLC eluate required for the toxicity tests and the issue of toxicity enhancement by methanol must be considered in order to generate the appropriate toxicity data. Spiking the methanol fractions with suspect toxicants, however, does not provide the same confidence about the cause of toxicity in the effluent as spiking the effluent directly. The mass balance approach described in Section 6 could be coupled with spiking the effluent with a portion of the fractions to make the data more relevant to whole effluent toxicity.

For chronic testing spiking a portion of the methanol fractions, such as C_{18} SPE methanol fractions into dilution water to mimic the effluent, requires some special considerations as discussed in the chronic Phase I (EPA, 1992) and the new Phase II (EPA, 1993A). For any test species, the effects of the methanol at the effluent spiking concentration for the test species must either be essentially non-existent or clearly established so that proper interpretation is applied. The use of spiking for chronic toxicants of the methanol fractions is not as easy as the spiking for acute toxicants due to the limitations in the quantity of methanol that would be added with each fraction for the toxicity test. If the chronic toxicity effect level is around or <25% effluent and the highest fraction tested is 4x higher than the chronic effect level, add-back tests can be conducted similar to the acute add-backs but the quantity of methanol required for the testing and analysis must be considered (cf., Section 2; EPA, 1993A). As discussed in Phase II, once a suspect toxicant has been tentatively identified, the steps of confirmation should be started although sample volumes of methanol eluates might limit the amount of testing (see Phase II, Section 2; EPA, 1993A) with chronically toxic samples. Spiking of appropriate levels for chronic toxicity for single chemicals (or mixtures) is limited as sublethal data are not as plentiful as acute data. The acute toxicity of some chemicals might be altered by methanol (i.e., surfactants). The possibility that this is occurring must be checked and a correction applied if warranted. Spiking fractions also has applicability for hidden toxicants; refer to Section 9 for further details.

Spiking can also be done effectively when the suspect toxicant(s) of concern can be removed. However, since other toxicants might also be removed, the data must be carefully interpreted. Ammonia is a good example (cf., Phase II; EPA, 1993A) to use with this technique where one toxicant can be removed. Ammonia can be removed from the effluent by passing samples over the zeolite resin, after which the concentration can be restored in the post-zeolite effluent by the addition of ammonia. If toxicity is also restored, then it is likely that there is sufficient ammonia to cause the toxicity observed. However, it cannot be concluded from these data alone, that ammonia is the cause of toxicity because the zeolite can also remove substances other than ammonia. Another substance which is non-additive with ammonia yet present at a lesser or the same number of TUs could cause the initial effluent toxicity but not be discernable by this removal technique. This is an example of a hidden toxicant (see Section 9). For acute toxicity, zinc could behave exactly this way because it is non-additive with ammonia yet zinc is also removed by zeolite. Using other ammonia removal methods, such as high pH stripping, followed by spiking to the initial ammonia concentration will enhance confidence that a hidden toxicant is not present. Other examples involving the C_{18} SPE column and various ion exchange resins would be approached and interpreted similarly.

Section 6
Mass Balance Approach

This approach is applicable only to those situations in which the toxicant(s) can be removed from the effluent and recovered in subsequent manipulation steps. The objective is to account for all toxicity to assure that small amounts of toxicity are not being lost. This concern is partly covered by the correlation approach (Section 2); however, a totally different toxicant present at a small concentration could appear as experimental variability in the correlation and go unnoticed.

The mass balance concept is best described by illustration for acutely toxic effluents and the C_{18} SPE fractions. As described in Phase II (Section 2.2.7; EPA, 1993A) for acutely toxic effluents, the effluent has been passed over a C_{18} SPE column which is then eluted with the methanol/water fractions. After the toxicity tests on the individual fractions are completed, add-back tests can be initiated to determine whether all of the toxicity in the original sample was accounted for in the SPE fractions. For this step, there are three separate tests (with dilutions and replicates to calculate effect endpoints) that must be conducted which consist of the all-fraction test, the toxic-fraction test, and the nontoxic-fraction test. Assuming a complete recovery of all non-polar organics from the SPE column, this should yield a solution of non-polar organic compounds equal to the original sample concentrations, In the mass balance approach, these add-back tests are conducted using an aliquot of the effluent that has passed through the C_{18} SPE column (post-SPE column nontoxic effluent) or an aliquot of dilution water. Each toxic fraction is added back to the post-SPE column effluent, so that each is present at original effluent concentrations (i.e., lx effluent concentration). For example for acutely toxic effluents, the toxic-fraction test solution is prepared using methanol concentrations as described in Phase II (i.e., Section 2.2.7; EPA, 1993A) and for each fraction where toxicity was observed in the fraction toxicity test, 30 µl of each is added to the same 10 ml of nontoxic post-C_{18} SPE column effluent (or dilution water). A portion of each of the remaining fractions where toxicity was not demonstrated are now added to a second post-SPE column aliquot at effluent concentrations for the nontoxic-fraction test. Finally portions of all the fractions (e.g., n= 8 for acutely toxic effluents) are added to a third post-SPE column aliquot at effluent concentrations for the all-fraction test. If all the toxicity is exhibited in the toxic-fraction test, then the all-fraction test results and the toxic-fraction test results should be the same as in the unaltered effluent. Results from the nontoxic-fraction test should indicate that no toxicity is present. This mass balance (or add-back) approach allows the researcher to ascertain whether or not the toxicity in the toxic-fraction test equals the effluent toxicity. Small amounts of toxicity can be undetectable in the toxic-fractions when tested separately or the toxicant(s) might not have been eluted from the C_{18} SPE columns. Unless mass balance experiments are conducted, such loss of toxicity might not be detected. In the effluent example discussed in Section 2, the toxicity was contained usually in the 75%, 80%, and 85% fractions and occasionally in the 70% fraction! The r^2-value, slope, and intercept were all close to the expected values if two toxicants (diazinon and CVP) were causing the effluent toxicity (Figure 2-3). However, in Table 6-l the results of mass balance tests indicate that toxicity from the all-fraction test was greater than the toxicity of the toxic-fraction test. While this difference is small, it did seem to be real and was attributed to a small amount of another toxicant in the 70% fraction. In 11 of 12 samples, the results from the all-fraction tests indicate there was greater toxicity than was found in the toxic-fraction tests. On the few occasions when the 70% fraction was toxic, it did not contain any of the three suspect toxicants. Without the mass balance data, consistent presence of the additional toxicant would not have been discovered.

At the stage where the toxic-fractions have been identified, the test of the fractions in a mass-balance test is highly desirable. For chronic toxicity testing, the amount of eluate available might be limited following the fraction toxicity tests. Using eluate for the add-back tests might be a trade-off between tracking toxicity and having sufficient eluate to concentrate for further analysis. This limits the add-back tests broad applicability for chronic toxicity TI Es unless the effluent is toxic enough that at 4x the chronic effect level, the methanol concentrations do not exceed

[3]During development of the non-polar organic procedures, various elution profiles were used that included the 70% methanol/water fraction.

Table 6-1. Comparison of Eff luent Toxicity and Toxicity Measured in Effluent Fraction Add-back Tests

Sample	Toxic Units (TUs)		
	Effluent	All-fractions	Toxic-fractions
12/03/87	1.18	1.64	1.43
01/12/88	2.00	2.94	3.13
01/13/88	1.93	2.86	2.53
02/03/88-I[a]	cl .oo	1.15	<1 .oo
02/03/88-II	2.00	1.75	1.64
03/03/88-I[a]	1.15	1.06	<1.00
03/03/88-II	1.33	1.52	1.13
03/23/88-I	3.70	3.03	2.86
03/23/88-II	2.86	2.86	2.44
04/28/88	2.27	1.72	1.64
05/17/88	2.27	2.04	2.00
05/17/88	2.27	1.67	1.59
Mean	2.13	2.18	2.00

[a]Values excluded from mean calculations due to less than values.

the organisms tolerance. For chronically toxic samples, the all-fraction add-back test with C. *dubia* is not possible due to high methanol concentrations in test cups unless chronic toxicity is below 25% and add-backs are done using 25% effluent as the high test concentration (cf., Phase II; EPA, 1993A). The data from the individual methanol/water tests may be summed; however this approach must be considered more tentative than add-back tests (see below).

A deficiency in the above approach to mass balance is that there can be some toxicity in the post-SPE column effluent which has not been removed by the C_{18} SPE but which is not present in concentrations high enough to detect. The above mass balance approach alone will not identify this. However, if the add-back tests described above are repeated using a standard dilution water, residual toxicity in the post-SPE column effluent should cause the toxic-fraction test and all-fraction test to show more toxicity when added to the post-SPE column effluent than when added to dilution water. A confounding effect of this approach is that if the toxicity is changed by matrix effects (suspended solids or TOC), then the toxicity will be different in the clean water test. Matrix effects can be discerned, in part, by a third spiking experiment where a portion of all of the fractions and a portion of each toxic-fraction test are spiked into whole filtered effluent (which has not passed through the C_{18} SPE column). If the addback tests in dilution water indicates greater toxicity than the addback tests with the post-SPE column effluent, and the same type of addback test experiment with filtered effluent (i.e., 1 µm filter) indicate that the fractions are exactly additive, then matrix effects are indicated.

Some post-SPE column effluent samples develop fungal or bacterial growth or perhaps a precipitate forms after the effluent passes through the column. For the fungal type of growth, this is thought to occur when some methanol bleeds into the effluent as it passes through the column and more rinsing will not eliminate this problem. Some effluents consistently develop this type of growth in the post-column effluent while others exhibit this pattern in only an occasional sample. To alleviate this problem, conditioning the column with acetonitrile has helped (cf., the acute Phase I (EPA, 1991A) and chronic Phase I (EPA, 1992) for details). When methanol fractions are spiked into the effluent this problem might or might not be enhanced; we have found this to be an effluent-specific occurrence.

Caution is warranted in situations where toxicity is contained in more than one SPE fraction. The researcher should not necessarily expect the toxicity expressed by each individual fraction that is tested separately to add up to the total effluent toxicity. First, toxicants may not be additive and second, some toxicity which cannot be detected in individual fractions may add to the whole toxicity. For example, any one C_{18} SPE fraction may not show toxicity but may contain some of the toxicant that is in the adjacent toxic-fraction. In this case, the toxicity of the toxic-fraction test would be less than expected. If this happens in more than one pair of fractions, the sum of the toxicity from the toxic-fraction test will be less than the effluent toxicity or all-fraction test. These concerns are especially important when several toxicants are involved and one or more occur in more than one fraction.

For effluents where the C_{18} SPE column is not used, but where the toxicants can be removed from the sample, the same objectives should be achievable, but the methods will be different. For example, if an effluent appears to contain a volatile toxicant, the mass balance could be done on the trap and on the purged sample. Since we have not yet done mass balance on samples such as these we have no experience from which to offer additional guidance or advice.

Some of the mass balance process begins in Phase II, and there is a subtle difference in the purpose of mass balances in Phases II and III. In Phase II, usually only a few samples are used and mass balances are necessary to determine the need for more identification in those few samples. The mass balance is useful in early stages of Phase II as well before toxicants are identified at all, because it allows the investigator to decide if the toxicants present at 2x or 4x whole effluent concentrations are also expressing toxicity at lower concentrations.

In Phase III as many samples are tested, the mass balance approach can provide information over time with many samples whether or not the suspect toxicants consistently account for all or the majority of the toxicity. As illustrated above, the power of the mass

balance approach to detect small degrees of toxicity is better than for the correlation approach.

When a portion of the toxicant is not biologically available and therefore does not contribute to toxicity, care must be taken to assure that removal of the toxicant from the sample does not remove biologically non-available portions. An example of this situation may be the alternative solvent extraction procedures which may remove a bound toxicant(s) sorbed on suspended solids with the solvent and is now toxic, yet it was not toxic in the unaltered sample.

Section 7
Deletion Approach

In some situations, particularly for industrial discharges, keeping the suspect toxicants out of the waste stream influent or effluent for short periods of time and also conducting toxicity tests on the wastewater simultaneously may be practical. When this approach can be used, it offers the most convincing evidence obtainable that the suspect toxicants are the true ones. Care must be taken however, that other substances are not deleted or that some characteristic such as pH does not change also. If a researcher can be certain that all changes are known, then this approach is definitive. Changes in the toxicants with time are as much of a concern here as in any other approach. These can be handled by the approaches outlined in earlier sections and the deletion approach need not be done repeatedly; however, if it were practical to do so, it would certainly be effective. If some samples do not contain one or more suspect toxicants, these effluent samples can be used to the advantage in confirmation in much the same way as intentional deletions described in this section can be used to confirm toxicity.

Section 8
Additional Approaches

This section mentions only a few of many steps that can be used to further confirm the cause of toxicity. The steps mentioned are mostly those that we have used and found helpful and practical.

The pH is one of the most important effluent characteristics that changes toxicity. The pH of POTW effluents, sediment pore or elutriate waters, and ambient waters will almost always rise when they are exposed to air, especially in the small test volumes used in TIE work. Commonly, pH in an effluent sample at 25°C will rise from 7.1-7.3 to 8.3-8.5 during a 24 h period. That pH change is enough to increase ammonia toxicity (based on total ammonia) about three fold. Such pH changes can destroy work for some purposes, but by regulating these pH changes, the pH fluctuations can be used to great advantage for other purposes.

Phase II (EPA, 1993A) describes the use of pH change to identify ammonia toxicity. The toxicity of some metals, hydrogen cyanide and hydrogen sulfide among others, is altered by pH change. Other characteristics, such as hardness, can also be varied to see if the changes in toxicity follow a predictable pattern. The toxicity of some metals could be approached in this way. Not all equilibria are as rapid as the ammonia equilibrium, so the amount of time for equilibria to occur should be controlled and standardized (cf., Phase II; EPA, 1993A). Various time . periods may have to elapse before the expected changes occur and this may differ with each effluent. With the improved methods of pH control described in the Phase I documents (EPA, 1991; EPA, 1992), much more use can be made of pH manipulation.

Often chemicals in effluent samples may not be biologically available, and if they are not, then they are not likely to cause toxicity. They may be made biologically available through some manipulation in Phase I and subsequently identified in Phase II. Through confirmation, the toxicity due to such a toxicant will become apparent when the correlation indicates a poor fit (cf., Section 2). For many toxicants, biological availability can be demonstrated by measuring body uptake. If the constituent of concern enters the body from the effluent, it is certainly biologically available. Exposure to pure compounds may be necessary to establish which particular organ should be evaluated for the toxicant. In acute metal exposures using fish, most metals concentrate first in the gills while non-polar organics concentrate in fatty tissues such as the liver. When a chemical is metabolized by the organism, a residue measurement for that compound is not a valid measure of the lethal body burden because it is unknown whether the metabolite is more of less toxic than the parent compound. If the suspect toxicant has a known mode of action, such as the acetylcholinesterase inhibition produced by organophosphate pesticides, this exposure effect can be measured to assess if toxic effects conform with the predicted effect. The use of enzyme blockers such as piperonyl butoxide (PBO) is also an aid in confirming toxicity caused by specific classes of toxicants (cf., Phase II; EPA, 1993A).

As additional steps are needed for confirming the cause of toxicity, combinations of various Phase I and Phase II procedures should always be used whenever practical. When several results are combined and all results are indicating the same type of toxicant, the data are more conclusive than when only one procedure yields predicted results.

Total dissolved solids (TDS) are a common problem in certain areas of the country and for certain industries. TDS will not cause toxicity from osmotic stress (this can easily be shown because their toxicity is not related to osmotic pressure) but rather TDS acts as a set of specific toxicants. For toxicity caused by TDS, the ratios and concentrations of the major cations and anions can be measured analytically. A similar mix of these major ions can be added to a dilution water to see if the expected toxicity is present. By testing various mixtures, the researcher can ascertain which of the TDS components contribute most to the toxicity.

Section 9
Hidden Toxicants

In the previous section, references were made to the problem of hidden toxicants. Essentially there are two situations which may produce the problem of hidden toxicants. The first situation occurs when disparate ratios of TUs of two toxicants are present in the effluent sample. Since the effect concentration is measured by diluting the effluent, when disparate ratios occur, the TUs of the toxicant present in fewer TUs in 100% effluent are so low at the effect diluent, that its contribution if any, is not measurable. This problem exists whether the toxicants are additive or non-additive. This situation generally will not be encountered in effluents that have very slight toxicity (i.e., effect concentration 75% to 100%) because little or no dilution is required to achieve the effect concentration. For those toxicants present in disparate ratios in effluents with marginal toxicity, the chemical present at the low levels may be nontoxic even in 100% effluent.

The second situation where hidden toxicant(s) occurs is when the toxicants are non-additive or partially additive in the effluent sample. These toxicants may occur at approximately equal TUs or at disparate ratios of TUs, as long as those present at lesser TUs are present at 1 TU in the 100% effluent (cf., discussion of performing correlation on these types of toxicants, contained in Section 2).

If confirmation is being conducted for both acute and chronic toxicity or if acute toxicity is being used as a surrogate for chronic toxicity, the acute to chronic ratio must also be considered. For example, consider an effluent with toxicants A and B for which the acute-to-chronic ratios are 3 and 12, respectively and the TUs for acute toxicity are 2 and 1 in an effluent sample for A and B, respectively. By definition, 1 acute TU (TU,) for toxicant A equals 3 chronic TUs (TU_c) and for B, 1 TU, = 12 TU,. In this example, the acute toxicity of the effluent will be determined by A and the chronic toxicity will be determined by B. If in another situation, the acute-to-chronic ratios for two compounds were similar, then one of the toxicants would determine the effect concentration for both acute and chronic toxicity. These examples illustrate the importance of acute-to-chronic ratios for non-additive toxicants. Acute-to-chronic ratios have special importance for additive toxicants when acute toxicity is being used as a surrogate measure for chronic toxicity. If acute toxicity is being used as a surrogate it must be demonstrated that the cause of the acute toxicity is the same as the chronic toxicity. When acute toxicity is used as a surrogate for chronic toxicity in Phases I and II, interpretation of the results can easily be biased and these considerations are important.

When a toxicant can be removed from the effluent and recovered, the identification of the presence of a hidden toxicant is more readily known. For example, the use of the C_{18} SPE column may remove hidden toxicants. The toxicant(s) is recovered in the eluate a&measured both analytically and toxicologically. This type of hidden toxicant may be observed if ammonia is present at concentrations that could cause toxicity. For example, in an effluent sample ammonia is present at 3 TUs. Ammonia will not be removed by the C_{18} SPE column and yet an additional 1.5 TU of a non-polar organic toxicant is evident when the C_{18} SPE eluate test is conducted. If the discharger applied remedial treatment they would be able to remove the ammonia toxicity yet the effluent would still be toxic. The same concept of hidden toxicants can be found when toxicants are removed by sublation which is followed by recovery and concentration of toxicity (cf., Phase I; EPA, 1991 A; EPA, 1992). For example, sublation can separate some surfactants, resin or fatty acids, and polymers from such constituents as metals and ammonia. Hydrogen sulfide can be removed by a purge and trap method, thereby separating it from other effluent constituents.

Specific blockers of toxicity such as EDTA for metals and PBO for organophosphates are also useful in establishing the cause of toxicity. The more specific the blocker, the more definitive are the results, However, present knowledge does not allow us to be certain that compounds such as EDTA do not also affect the toxicity of other chemicals. Use of two specific blockers such as EDTA and sodium thiosulfate for copper, allows more definitive conclusions (cf., Phase I; EPA, 1992).

Manipulating characteristics such as pH is useful but can easily mislead thinking. For example, if the effluent has ammonia toxicity, the toxicity due to ammonia should disappear if the pH is lowered appropriately. These results do not allow a conclusion that there are no hidden

toxicants. If, however, the pH is lowered so as to eliminate ammonia toxicity but the effluent toxicity exists or even increases, then the likelihood of a hidden toxicant is high. Unfortunately a complication to this rationale is that the toxicity expressed at the lower pH may be totally artifactual due to mechanisms of pH adjustments.

The best approach to find hidden toxicants is to first use, those methods that alter the effluent the least, can remove and recover removed hidden toxicants, and are most specific for a few toxicants. This advice is most applicable where the effort is to try to find out if some specified type of toxicant is a hidden one, e.g., is there a non-polar organic as a hidden toxicant.

If, however, the search is for any type of hidden toxicant then every conceivable technique should be used that would help to distinguish a hidden toxicant from the suspect toxicant(Hidden toxicants are very hard to find when ammonia is the primary toxicant. Various tests used to identify ammonia as the toxicant, i.e., use of the zeolite resin, graduated pH tests and air-stripping (EPA, 1993A), all have a reasonable probability of changing the toxicity of many other potential toxicants. For instance, it is known that zeolite removes some non-polar organics and metals. Air-stripping (at pH 11) could also remove or destroy many other chemicals as it often must be done for a extended period of time to achieve good ammonia removal. The graduated pH test results might also implicate a metal as a toxicant (EPA, 1993A). If these tests were conducted in Phase II (EPA, 1993A) and the results consistently indicated ammonia toxicity, these data indicate that there are no hidden toxicants. The required characteristics for a hidden toxicant to behave exactly as ammonia are very specific and obtaining results like those described above for a toxicant other than ammonia is unlikely.

If the hidden toxicant is additive with the suspect toxicant but occurs in a disparate ratio, the confirmation effort must first emphasize confirming the cause of toxicity (or remove the toxicity) of the primary toxicant. Then toxicity from the hidden toxicant should be measurable. The probability a hidden toxicant that has additive toxicity will not express its toxicity using several Phase I or Phase II techniques is less than the probability that a non-additive toxicant will express its toxicity using several of the same techniques.

If the remedial action for a primary toxicant is specific and easy, such as a product substitution, the search for hidden toxicants perhaps should be done after the remedial action has reduced or eliminated the primary toxicant from the effluent. The remedial action (especially if it is treatment) may also eliminate the hidden toxicant(What must be avoided if at all possible, is to carry out expensive remedial action only to find that the effluent is still toxic.

The problem of hidden toxicants is a major reason a researcher should not accept the presence of toxic concentrations of suspect toxicant as sufficient confirmation (cf., Section 1). The presence of biologically unavailable forms (cf., Section 8) is a compelling reason not to do so.

A thorough confirmation is resources well spent in most instances. Non-additivity and disparate ratios complicated by non-availability occur too frequently to bypass confirmation. Seasonal changes or changes without a pattern, in effluent toxicants are further reasons to perform the confirmation over a period of time to assure that the entire suite of toxicants has been found.

Section 10
Conclusions

Often the most laborious and difficult part of the TIE is developing data to adequately establish the cause of toxicity. In our experience, frequently the suspect cause of toxicity is found without difficulty but developing a convincing case to prove that the suspect cause is the true toxicant is the challenge.

Especially for POTW plants, this confirmation phase must be performed over a considerable period of time to be certain that the cause of toxicity is not changing. TIEs on POTWs and some industrial categories are not likely to be a one time event but will have to be repeated as long as the inputs to the plant change. Our current wastewater treatment plants were not designed to remove specific chemicals, so there is no reason to expect that they will remove everything which they receive. Especially where the control over the influent is not complete, as is the case with POTW plants, a solid case must be developed to assure that the cause of toxicity is not changing.

Section 11
When the Treatability Approach Has Been Used

As discussed in Phase I, two main approaches may be used to remove a toxicity problem--toxicant identification and source control or treatability. Phases I and II involve the first approach while treatability procedures accompanied by toxicity testing are used in the second (EPA, 19898; EPA 1989C).

In the second approach, treatment methods are varied to determine which will remove toxicity without identifying the specific toxicants. The treatability approach requires as much confirmation as the toxicant identification approach. Since the treatability approach should remove toxicity, the confirmation procedures are somewhat different.

Repeat samples should be tested to ensure that toxicity has been successfully removed. This should be done over a sufficient length of time to assure that the range of conditions are included during the confirmation phase. Such events as seasonal changes, production changes, storms, and intermittent operations all should be included during the confirmation phase. Toxicity should be consistently removed or appropriately reduced, as required. Either acute or chronic toxicity removal can be confirmed this way.

One must be absolutely sure that the toxicity to resident species has been successfully removed. As has been pointed out in Phases I and II, the effluent constituents producing toxicity to one species may not be the same for other species. Toxicity by a given treatment method may remove all toxicity for one species but not for another. The species of concern must be tested in the effluent from the treatment method selected. If chronic toxicity is the concern, this testing may be more difficult because chronic testing methods may not be available for resident species. In selected cases, symptoms may be substituted for the usual endpoints of chronic tests but their use would be case specific.

Section *12*
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