ABSTRACT

TORY L. CHAMPLIN. Use of the C18 (Octadecyl) Solid Phase Extraction Column for Wastewater Toxicity Identification and Characterization (Under the direction of DR. FRANCIS A. DIGIANO).

Biomonitoring requirements are continually being added to NPDES permits. As a result, many municipal wastewater treatment facilities have been identified as having effluent acute toxicity. To solve this problem, the Environmental Protection Agency (EPA) has developed a Toxicity Identification Evaluation (TIE) protocol. This protocol lists a set of simple procedures which are used to separate whole effluent samples into different fractions containing different classes of similar compounds. Toxic fractions are further separated and concentrated using various techniques to assist in the possible identification of certain classes of suspected toxic compounds. Once sample fractionation and toxicity testing have been thoroughly investigated, chemical-specific analyses are conducted to tentatively identify toxic constituents.

The C18 (Octadecyl) Solid Phase Extraction (SPE) Column is used in the TIE protocol to separate and concentrate moderately polar to nonpolar organic compounds from toxic effluent samples. An increasing gradient of methanol (MeOH) in water is used to elute the column; the objective is to

separate retained compounds into eight different fractions based on their polarity. The objective of conducting this research is to determine if the C18 SPE Column elution procedure is a viable technique for the identification and characterization of toxic effluents.

This study showed that the C18 column was able to remove compounds causing acute toxicity from samples collected at the Cross Creek Wastewater Treatment Plant (WWTP) in Fayetteville, NC. The 80 to 85 percent MeOH/H_2O fractions contained the most toxicity. However, laboratory tests of the procedure using known target compounds indicated that several different MeOH/H_2O fractions contained each individual compound thus showing deficiencies in resolution. Moreover, the target compounds that were selected differed widely in polarity (as indicated by the compounds octanol/water partitioning coefficient), yet this did not cause a wide separation of these compounds into specific MeOH/H_2O fractions. This research showed the C18 SPE column is capable of retaining relatively non-polar compounds as indicated by the target compound evaluation. These compounds were effectively eluted from the C18 SPE column with MeOH/H_2O, but separation was not well defined. In the situation of toxic wastewaters, where numerous non-polar organic compounds may possibly be present in WWTP's discharge, the C18 SPE column provides little information regarding the identity of the non-polar organics causing toxicity in a toxic MeOH/H_2O fraction.

TABLE OF CONTENTS

Page

LIST OF FIGURES.................................... vii

LIST OF TABLES..................................... x

ACKNOWLEDGEMENTS................................... xiii

I. INTRODUCTION...................................... 1

A. BACKGROUND..................................... 1

B. RESEARCH AND OBJECTIVES........................ 2

II. LITERATURE REVIEW................................ 4

A. C18 SPE COLUMN................................. 4

B. C18 SPE COLUMN PROCEDURE (TIE PROTOCOL)........ 11

C. TOXICITY IDENTIFICATION EVALUATION STUDIES USING THE SPE PROCEDURE........................ 18

III. EXPERIMENTAL PROCEDURE........................... 24

A. C18 SPE COLUMN PROCEDURE....................... 24

1. C18 SPE COLUMN SET-UP AND CONDITIONING..... 24

2. SAMPLE APPLICATION.......................... 28

3. ELUTION OF MeOH/H_2O FRACTIONS.............. 29

B. BIOASSAY PROCEDURE............................. 33

C. ILLUSTRATION OF PROCEDURE USED TO ANALYZE BIOASSAY DATA.................................. 34

D. THE TIME-BASED TOXICITY UNIT (TTU)............. 41

E. ILLUSTRATION OF USE OF TTU CONCEPT............. 43

F. TARGET COMPOUND SELECTION...................... 48

G. SELECTION OF WWTPs FOR TIE..................... 51

TABLE OF CONTENTS (continued)

Page

IV. EXPERIMENTAL RESULTS.................................. 56

A. BACKGROUND TOXICITY SOURCES...................... 56

1. EVALUATION OF METHANOL (MeOH).............. 56

2. EFFECT OF BIOASSAY CUP MATERIAL ON MeOH TOXICITY RESULTS........................... 57

3. TOXICITY OF MeOH/H_2O ELUENTS FROM THE C18 COLUMN................................ 59

4. TOXICITY OF DILUENT WATER.................. 61

B. EVALUATION OF TARGET COMPOUND TOXICITY......... 65

1. PHENOL (Ph)................................ 65

2. 1-METHYLNAPHTHALENE (MN).................... 68

3. NONYLPHENOL ETHOXYLATE 9 MOLE (NPE)........ 71

4. DI-N-OCTYL PHTHALATE (DOP)................. 73

5. SUMMARY OF TARGET COMPOUND EVALUATION...... 79

6. SUMMARY OF MICROTOX AND CERIODAPHNIA COMPARISONS................................ 81

C. EVALUATION OF TOXIC EFFLUENT SAMPLES........... 83

1. WESTSIDE WWTP, HIGHPOINT, NORTH CAROLINA... 83

2. CROSS CREEK WWTP, FAYETTEVILLE, NORTH CAROLINA.................................. 85

V. CONCLUSIONS AND RECOMMENDATIONS.................... 95

A. CONCLUSIONS.................................... 95

B. RECOMMENDATIONS................................ 96

REFERENCES.. 98

APPENDIX A: WASHING PROCEDURE.......................... 102

APPENDIX B: EQUIPMENT SPECIFICATIONS................... 104

TABLE OF CONTENTS (continued)

Page

APPENDIX C: REQUEST AND REPORTING FORMS.................. 105

APPENDIX D: MICROTOX TEST PROCEDURE..................... 108

APPENDIX E: LOG OCTANOL/WATER PARTITIONING COEFFICIENT ESTIMATIONS FOR TARGET COMPOUNDS.................................. 114

APPENDIX F: TIME-BASED TOXICITY UNIT DATA TABLES....... 119

LIST OF FIGURES

Figure	Description	Page
2.1	The C18 Solid Phase Extraction Column (a) A Schematic Diagram Illustrating the SPE Disposable Column and (b) Solid Phase Extraction Process	6
2.2	Chemical Structure of Octadecyl Sorbent with Trimethylchlorosilane Endcapping	8
2.3	Chemical Structure of Reversed Phase Partitioning using Solid Phase Extraction. (Sorbent: Octadecyl, Analyte: Dibutyl Phthalate)	12
2.4	Overview of Phase I Effluent Characterization Tests (6) (Note: pH(I) stands for the initial pH of the sample)	13
2.5	Schematic for Phase II Identification of the C18 Column Retained Toxicants (6)	15
3.1	Experimental Setup for Phase II C18 SPE Column Sample Application	25
3.2	Experimental Procedure for Phase II C18 SPE Column Elution Process	27
3.3	Sample Application Process	30
3.4	Column Elution Process	32
3.5	Toxicity Test Request Form	35
3.6	Timed Lethality Test Data/Worksheet	36
3.7	Percent Mortality Versus the Log of Elapsed Time for Hypothetical Samples (a) CFE0, EFE100 and EFE500 and (b) EFE750, EFE850 and EFE950	40
3.8	Composite Sample 1/16 to 1/17/89, Imaginary Creek WWTP, Example, NC (a) Post-Column Effluents	47
3.9	Schematic of Westside Wastewater Treatment Plant, Highpoint, NC	52
3.10	Schematic of Fayetteville - Cross Creek Wastewater Treatment Plant, Fayetteville, NC	54

LIST OF FIGURES (continued)

Figure	Description	Page
4.1	Evaluation of Methanol Toxicity in (a) Plastic Cups and (b) Glass Beakers	58
4.2	Evaluation of Toxicity Eluted from C18 SPE Column Using (a) Plastic Cups (Methanol From Lot 873940); (b) Glass Beakers (Methanol From Lot 873940); and (c) Plastic Cups (Methanol From Lot 884151)	60
4.3	Control Study of C18 SPE Column Using Non-Toxic Effluent Shown by (a) Post-Column Effluents; (b) Column Elutions (Conducted in Plastic Cups); and (c) Methanol Elutions (Conducted in Glass Beakers)	62
4.4	Evaluation of the Toxicity of Botany Pond Water to be Used as a Diluent Water Using (a) Post-Column Effluents and (b) Methanol Elutions	64
4.5	Ability of C18 SPE Column to Isolate and Elute Toxicity Associated with Phenol Using (a) Post-Column Effluents and (b) Methanol Elutions	66
4.6	Verification of C18 SPE Column's Inability to Retain Phenol as Demonstrated by Phase I TIE Test Procedures Using (a) Toxicity and (b) Absorbance Measurements	67
4.7	Ability of C18 SPE Column to Isolate and Elute Toxicity Associated with 1-Methylnaphthalene Using (a) Post-Column Effluents (Conducted in Glass Beakers); (b) Methanol Elutions (Conducted in Plastic Cups);and (c) Methanol Elutions (Conducted in Glass Beakers)	70
4.8	Evaluation of C18 SPE Column to Isolate and Elute Toxicity Associated with 1-Methylnaphthalene as indicated by the *Ceriodaphnia* (a) Methanol Elutions (Conducted in Plastic Cups); (b) Methanol Elutions (Conducted in Glass Beakers); and Microtox (c) Methanol Elutions	72
4.9	Ability of C18 SPE Column to Isolate and Elute Toxicity Associated with Nonyl Phenol Ethoxolate 9 Mole Using (a) Post-Column Effluents and (b) Methanol Elutions	74

LIST OF FIGURES (continued)

Figure	Description	Page
4.10	Ability of C18 SPE Column to Isolate and Elute Toxicity Associated with Di-n-Octyl Phthalate Using (a) Post-Column Effluents and (b) Methanol Elutions; and (c) Verification by Absorbance Measurements	76
4.11	Comparison Study Between the (a) Ceriodaphnia and (b) Microtox in Measuring the Methanol Elutions for Di-n-Octyl Phthalate	78
4.12	Ability of the C18 SPE Column to Isolate and Elute Toxicity Associated with Composite Effluent Sample Collected 1/16/89 to 1/17/89 at the Cross Creek WWTP, Fayetteville, NC Using (a) Post-Column Effluents and (b) Methanol Elutions	88
4.13	Ability of the C18 SPE Column to Isolate and Elute Toxicity Associated with Composite Effluent Sample Collected 1/30/89 to 1/31/89 at the Cross Creek WWTP, Fayetteville, NC Using (a) Post-Column Effluents and (b) Methanol Elutions	89
4.14	Ability of the C18 SPE Column to Isolate and Elute Toxicity Associated with Composite Effluent Sample Collected 3/21/89 to 3/20/89 at the Cross Creek WWTP, Fayetteville, NC Using (a) Post-Column Effluents and (b) Methanol Elutions	91

LIST OF TABLES

Table	Description	Page
2.1	Solvent Eluotropic Strength and Polarity	8
2.2	Applications for Reversed Phase Extraction Columns	12
2.3	Solid Phase Extraction Toxicity Test Results, Largo WWTP, Largo, Florida (1985)	20
2.4	Solid Phase Extraction Toxicity Test Results, Akron POTW, Akron, Ohio (1986)	21
2.5	Solid Phase Extraction Toxicity Test Results, Patapsco WWTP, Baltimore, Maryland (1986 to 1987)	22
3.1	Computer Generated Time Lethality Data/Worksheet	39
3.2	Example Calculation of Time based Toxicity Unit for Imaginary Creek WWTP, Example NC, Sample CFEO	44
3.3	A Comparison of ET50 and TTU Values for the C18 Column Effluent Results from the Imaginary Creek WWTP, Example, NC	45
3.4	Target Compounds, Summary of Physical and Chemical Properties	49
4.1	Methanol Control Testing	57
4.2	Summary of Target Compound Evaluation Results	80
4.3	Comparison Between the Ceriodaphnia LC50 and the Microtox EC50 for Selected Compounds	82
4.4	The Effects of Sonication on Toxic Effluent Samples Collected at Highpoint as Measured by the Ceriodaphnia	85
4.5	Summary of Cross Creek WWTP Composite Samples Fayetteville, North Carolina	92
D.1	Percent Methanol Evaluation for Figures 4.1(a) and 4.1(b)	120
D.2	Column Toxicity Evaluation for Figures 4.2(a) and 4.2(b)	121

LIST OF TABLES (continued)

Table	Description	Page
D.3	Column Toxicity Evaluation for Figure 4.2(c) ...	122
D.4	Time Lethality Toxicity Unit Data for Figure 4.3(a) ...	123
D.5	Time Lethality Toxicity Unit Data for Figure 4.3(b) ...	123
D.6	Time Lethality Toxicity Unit Data for Figure 4.4(a) ...	124
D.7	Time Lethality Toxicity Unit Data for Figure 4.4(b) ...	124
D.8	Time Lethality Toxicity Unit Data for Figure 4.5(a) ...	125
D.9	Time Lethality Toxicity Unit Data for Figure 4.5(b) ...	125
D.10	Time Lethality Toxicity Unit Data for Figures 4.6(a) and 4.6(b) ...	126
D.11	Time Lethality Toxicity Unit Data for Figure 4.7(a) ...	127
D.12	Time Lethality Toxicity Unit Data for Figure 4.7(b) ...	128
D.13	Time Lethality Toxicity Unit Data for Figure 4.7(c) ...	129
D.14	Time Lethality Toxicity Unit Data for Figures 4.8(a), 4.8(b), 4.8(c) ...	130
D.15	Time Lethality Toxicity Unit Data for Figure 4.9(a) ...	131
D.16	Time Lethality Toxicity Unit Data for Figure 4.9(b) ...	132
D.17	Time Lethality Toxicity Unit Data for Figures 4.10(a) and 4.11(a) ...	133
D.18	Time Lethality Toxicity Unit Data for Figure 4.10(b) and 4.12(a) ...	134

LIST OF TABLES (continued)

Table	Description	Page
D.19	Time Lethality Toxicity Unit Data for Figure 4.11(b)	135
D.20	Time Lethality Toxicity Unit Data for Figure 4.11(c)	136
D.21	Time Lethality Toxicity Unit Data for Figure 4.12(b)	137
D.22	Absorbance Data Collected on 10/13/88, 10/8/88 and 10/9/88 for Figure 4.12(c)	138
D.23	Timed Lethality and Microtox Data for Figures 4.13(a) and 4.13(b)	139
D.24	Timed Lethality Toxicity Unit Data for Figure 4.14(a)	140
D.25	Timed Lethality Toxicity Unit Data for Figure 4.14(b)	140
D.26	Timed Lethality Toxicity Unit Data for Figure 4.15(a)	141
D.27	Timed Lethality Toxicity Unit Data for Figure 4.15(b)	141
D.28	Timed Lethality Toxicity Unit Data for Figure 4.16(a)	142
D.29	Timed Lethality Toxicity Unit Data for Figure 4.16(b)	142

ACKNOWLEDGEMENTS

This project was funded through a grant from the Risk Reduction Engineering Laboratory of the United States Environmental Protection Agency and also through the Water Resources Research Institute at the University of North Carolina.

I. INTRODUCTION

A. Background

The Water Quality Act of 1987 established water quality-based permit limitations on all toxic pollutants. The intent of this law is to go beyond technology-based approaches that simply require installation of wastewater treatment facilities in order to protect water quality. Instead, the water quality based approach uses the combination of both whole effluent toxicity testing and chemical-specific analyses for controlling toxic pollutants. As a result, states are adding biomonitoring to their permit regulations. Toxicological data gathered in the early to mid 1980s indicated that approximately 79% of the municipal wastewater treatment facilities in the United States had effluents that were acutely lethal to aquatic life (1).

To assist permittees violating NPDES biomonitoring toxicity requirements, EPA has developed Methods for Aquatic Toxicity Identification Evaluation (TIE) protocol. This protocol lists a network of procedures which integrate analytical chemistry with acute toxicity testing. It supersedes an earlier EPA approach to toxicity elimination which focused only on identification of the 126 Priority Pollutants. The TIE protocol, on the other hand, makes no attempt initially to use a chemical specific approach to

toxicity identification. Effluent samples are separated into different fractions (based on general chemical properties), each containing different classes of similar compounds.

Many sources contribute toxicity to publicly owned treatment works (POTWs), including industrial, commercial and domestic wastes. The toxic constituents are broadly categorized into five groups by the TIE protocol based on chemical characteristics. These include oxidants, metals, volatiles, solids and organic compounds. The C18 SPE (Solid Phase Extraction) Column is used to extract and concentrate moderately polar to non-polar organic compounds from wastewater samples. A reduction in acute toxicity measured before and after sample passage indicates non-polar organics as a possible source of toxicity. Once this determination has been made, the C18 column can be used to elute and concentrate the retained organic compounds for further refined testing.

B. Research and Objectives

The main focus of this research is to investigate the use of the C18 Octadecyl Solid Phase Extraction Column in Phase II Toxicity Identification Procedures of the TIE protocol. The objectives of conducting this research are:

1. to show the differences in sensitivity between the *Ceriodaphnia* and Microtox as aquatic toxicity indicators,

2. to evaluate the C18 SPE Column elution procedure by using selected target compounds of known toxicity and polarity and examine a possible correlation between the methanol/water fraction that elutes toxicity and the log octanol/water partitioning coefficient of these target compounds

3. to test the C18 SPE Column procedure with actual effluent samples collected from POTW's in Highpoint and Fayetteville, North Carolina.

II. LITERATURE REVIEW

A. C18 SPE Column

Solid Phase Extraction (SPE) was introduced in the mid 1970's as a organics analysis preparation technique (2). Prior to this time, the traditional liquid-liquid extraction conducted in separatory funnels was the popular approach. The objective of either technique is to prepare a sample for instrumental analyses. Sample cleanup and concentration are important for gas chromatography (GC) and high pressured liquid chromatography (HPLC). The removal of impurities from a sample matrix is required when they interfere with analyte measurements or possibly shorten the life of GC or LC capillary columns. Sample concentration is essential when the measured analytes are too dilute for direct measurement.

In terms of the TIE, the SPE process was adapted to extract suspected non-polar organics from wastewater samples. Toxic organic constituents found leaving wastewater treatment facilities are typically at concentrations too low to be effectively identified by current analytical techniques. Using SPE allows for the separation as well as the concentration of these compounds so that further toxicity testing and chemical analyses may

be conducted.

The concept of the SPE test recommended by EPA in its TIE protocol is similar to low pressure liquid chromatography. A small, disposable SPE column can contain a variety of possible sorbents. Figure 2.1a shows the schematic diagram of a typical column (3). The polypropylene reservoir can vary in size ranging from 1 to 6 ml in capacity. The columns are prepacked by the manufacturer (J.T. Baker) with 100, 200, 500 or 1000 mg of sorbent sandwiched between two, 20-micron polyethylene frits.

Figure 2.1b illustrates the process of solid phase extraction (3). The first step is to condition the column with an appropriate solvent (e.g., methanol, hexane or chloroform). This activates the functional groups of the sorbent. In addition, a small volume of sample is applied and wasted to remove any portion of remaining solvent. The sample is forced through the column either by aspiration or positive pressure. Sample application is followed by column washing. Impurities or possible interferences retained by the column can be selectively removed by an appropriate solvent leaving the analytes remaining. The last step in the process is to elute the purified analytes from the column. This can be accomplished by the selection of a solvent strong enough to displace the analytes from the sorbent.

Two of the major SPE categories are normal and reversed

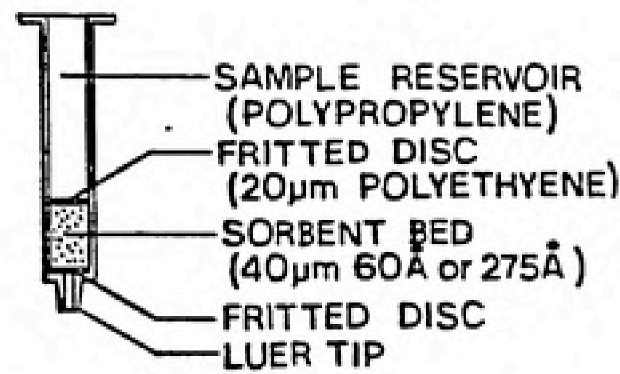


Figure 2.1a

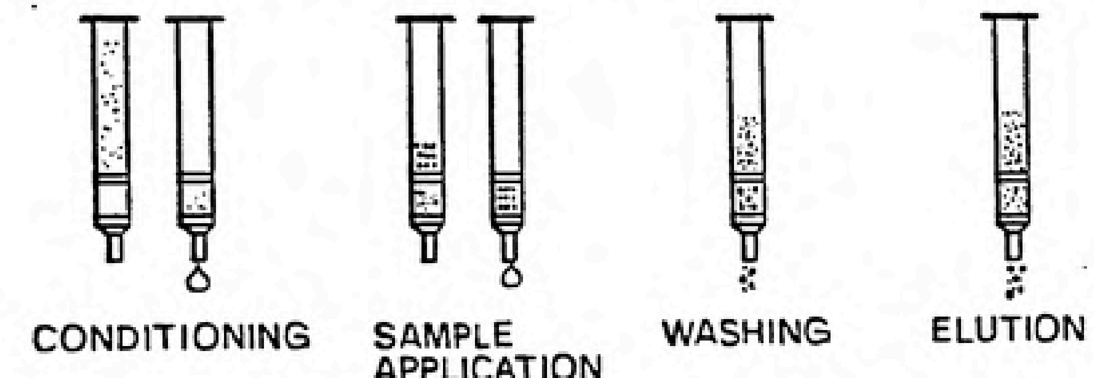


Figure 2.1b

Figure 2.1 The C18 Solid Phase Extraction Column (a) A Schematic Diagram Illustrating the SPE Disposable Column and (b) Solid Phase Extraction Process.

phase chromatography. Normal phase chromatography (NPC) refers to a system where the sorbent is more polar than the mobile phase or sample solution whereas reversed phase chromatography (RPC) refers to the opposite. Silica, kieselguhr, alumina and Florisil (activated magnesium silicate) are commonly-used, normal phase adsorbents in the separation of polar compounds from relatively nonpolar solvents (4). Reversed phase chromatography came about from the synthesis of bonded sorbents in the late 1960's. The free silanol groups of silica were treated with mono-, di-, tri-halo or alkoxy silyl derivatives to form siloxanes. The original intent of bonded silica was to create a non-polar adsorbent; however polar bonded phases were also developed.

The eluotropic strength of a solvent used in SPE procedures is represented by E^o. The eluotropic series shown in Table 2.1 is an arrangement of a group of solvents in order of decreasing strength for elution of analytes from pure silica and was determined experimentally. Other eluotropic series have been developed and are available in the literature for other sorbents.

Table 2.1 lists some commonly used chromatographic solvents according to their E^o and p' (polarity index) for silica. Both of these indices are important to consider when designing an extraction process (2). The polarity index ranks chromatographic solvents according to their solvating ability for a variety of test solutes. This index was developed to assist analysts using liquid-liquid

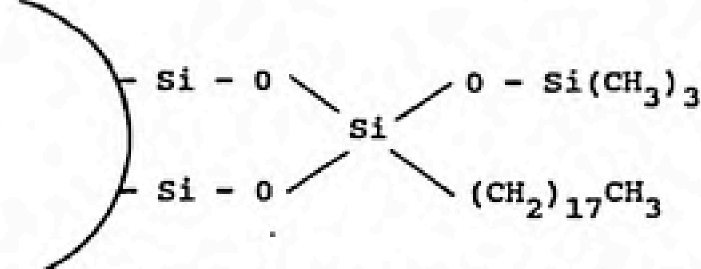


Figure 2.2 Chemical Structure of Octadecyl Sorbent with Trimethylchlorosilane Endcapping.

Table 2.1
Solvent Eluotropic Strength and Polarity (2)

Solvent	E^{o}*	p'**
Acetic Acid, Glacial	>0.73	6.2
Water	>0.73	10.2
Methanol	0.73	6.6
Z-Propanol	0.63	4.3
Pyridine	0.55	5.3
Isobutyl Alcohol	0.54	3.0
Acetonitrile	0.50	6.2
Ethyl Acetate	0.45	4.3
Acetone	0.43	5.4
Methyl Ethyl Ketone	0.39	4.5
Tetrahydrofuran	0.35	4.2
Methylene Chloride	0.32	3.4
Chloroform	0.31	4.4
Tert-Butyl Methyl Ether	0.29	-
Ether, Anhydrous	0.29	2.9
Benzene	0.27	3.0
Toluene	0.22	2.4
Carbon Tetrachloride	0.14	1.6
Cyclohexane	0.03	0.0
Pentane	0.00	0.0
n-Hexane	0.00	0.06
n-Heptane	0.00	0.2
Hexanes	0.00	0.06

* E^{o} = eluotropic strength, eluting solvent strength on silica.

** p' = polarity index, measure of solvent's ability to interact as a proton donor, proton acceptor or dipole.

extraction (conducted typically in separatory funnels).

The eluotropic series was developed to aid in liquid-solid chromatography (SPE). For the series listed in Table 2.1, the sorbent is pure silica and the chromatographic phase is normal (NPC). As can be seen, the eluotropic strength and the polarity index do not always correspond. This is why it is important to include both indices when designing an extraction process. For example, isobutyl alcohol (E^{o} = 0.54) has a higher eluotropic strength than acetonitrile (E^{o} = 0.50), indicating a higher degree of solvating strength on pure silica. However, the corresponding polarity index shows isobutyl alcohol (p'= 3.00) having a lower degree of polarity than acetonitrile (p'= 6.20). In the case of NPC/SPE, acetonitrile would be considered to have a higher degree of solvating strength for elution of analytes from pure silica even though isobutyl alcohol has a higher polarity.

The C18 SPE Column used in the TIE protocol is composed of a porous silica sorbent which has been treated with a single layer of octadecyl groups (5). This non-polar, bonded-phase attracts non-polar compounds and metal chelates (which are soluble in hexane and chloroform) strongly from an aqueous sample. The stationary phase (sorbent) is less polar than the mobile phase (sample); this is RPC.

Figure 2.2 shows the structure of the octadecyl sorbent with the addition of the trimethylchlorosilane endcapping. Octadecyl substituted siloxanes are capable of extracting

nonpolar to slightly polar analytes from solvents having E^o (eluotropic strength of adsorption on pure silica) values greater than approximately 0.6 (Table 2.1). The analytes are eluted from the column with solvents having lower E^o values (2).

The column elution procedure as outlined in Phase II Toxicity Identification Procedures of the TIE protocol designates methanol ($E^o = 0.73$) as the eluting solvent (5). However, methanol (MeOH) is one of the weaker solvents to use in reversed phase chromatography (RPC) where wastewater ($E^o > 0.73$) is the mobile phase and the sorbant (less polar) is the stationary phase. The objective would be to choose an eluting solvent that has an E^o much less than that of wastewater. Therefore, a far better solvent than MeOH would be hexane ($E^o = 0.0$); this would also facilitate GC/MS analyses. Unfortunately, hexane is not miscible in water nor is it nontoxic to the test organisms. Miscibility and a non-toxic response are essential to verification of toxicity in the column elution procedure. MeOH ($E^o = 0.73$), on the other hand, meets these two important criteria. However, MeOH is not a very good solvent to use to GC/MS analysis, because it will shorten the life of capillary columns (5). Nevertheless MeOH is used because of its low toxicity and ability to elute compounds from a C18 column.

The intermolecular interactions between the analyte molecules and the octadecyl functional groups on the sorbent create the separation mechanisms which allow for the

extraction process to work. The intermolecular forces which play a key role in the separation process are: ionic interactions, hydrogen bonding, dipole-dipole, dipole-induced dipole and dispersion forces (induced dipole-induced dipole) (2). Figure 2.3 illustrates the diester dibutyl phthalate as it is partitioned onto the C18 bonded phase.

The octadecyl bonded phase is considered the most versatile in terms of retaining a wide range of different compounds as compared with the other RPC sorbents that are available. Various applications are listed in Table 2.2. It is also considered to have the highest degree of retention for non-polar analytes. Under certain circumstances the interactions between some analytes and the sorbent may be too significant to be disrupted by the eluting solvent. A less polar solvent or a change in the bonded phase to a shorter alkyl chain (octyl, butyl or methyl) could solve this problem. However, changing the solvent or sorbent must not cause toxicity or other interferences.

B. C18 SPE Column Procedure (TIE Protocol)

A detailed description of the C18 SPE Column procedure is given in the EPA Guidance Documents describing Phase I (6) and Phase II (5). Figure 2.4 is an overview of the Phase I Toxicity Characterization Procedures (6). The reference to Day 1 and Day 2 identifies the time at which toxicity testing is conducted on the specified aliquots.

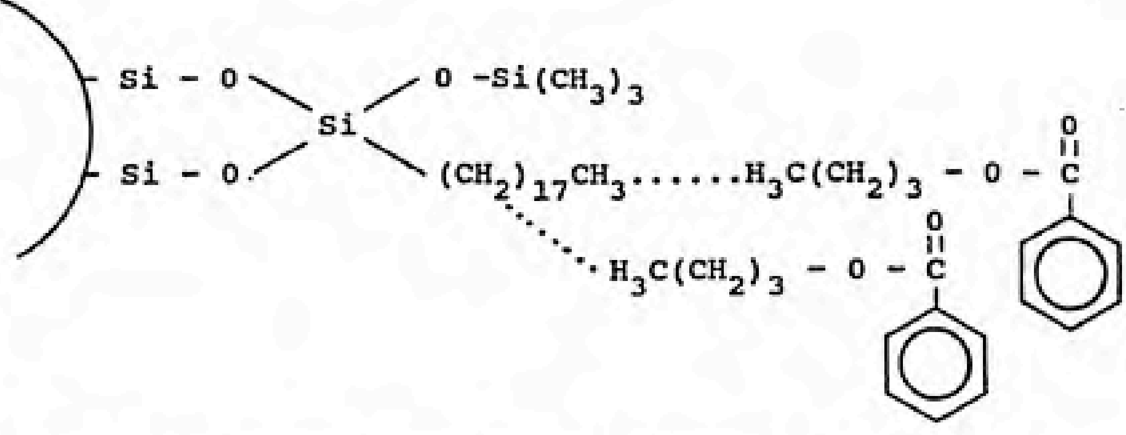


Figure 2.3 Chemical Structure of Reversed Phase Partitioning Using Solid Phase Extraction. (Sorbent: Octadecyl, Analyte: Dibutyl Phthalate)

Table 2.2
Applications for Reversed Phase Extraction Columns (2)

Sorbent	Application
Octadecyl (C_{18})	Abused Drugs, Acetaminophen, Amines, Analgesics, Anthraquinones, Antiarrthmics, Anticonvulsants, Antiepileptics, Antibiotics, Aromatics, Barbiturates, Benzodiazepines, Caffeine, Cannabis, Carbohydrate Carboxylic Acid, Carotenoids, Cholesterol Esters, Dye Intermediates, Essential Oils, Ethchlorvynol, Ethosuximide, Fatty Acids, Food Preservatives, Fungicides, Hydrocarbons, Hypnotics, Lidocaine, Lipids, Oil Soluble Vitamines, Phenols, Phthalate Esters, Priority Pollutants, (Pesticides, PNA's, RAH, PCB's), Sedatives, Steroids, Sulfonamides, Surfactants, Tetracyclines, Theophylline, Tricyclic Antidepressants, Triglycerides, Valproic Acid.
Octyl (C_8)	Priority Pollutants (Pesticides, PNA's, PAH's, PCB's) and other compounds adsorbed too tightly to Octadecyl (C18).
Phenyl (C_6H_5)	Offers less retention of hydrophobic compounds.

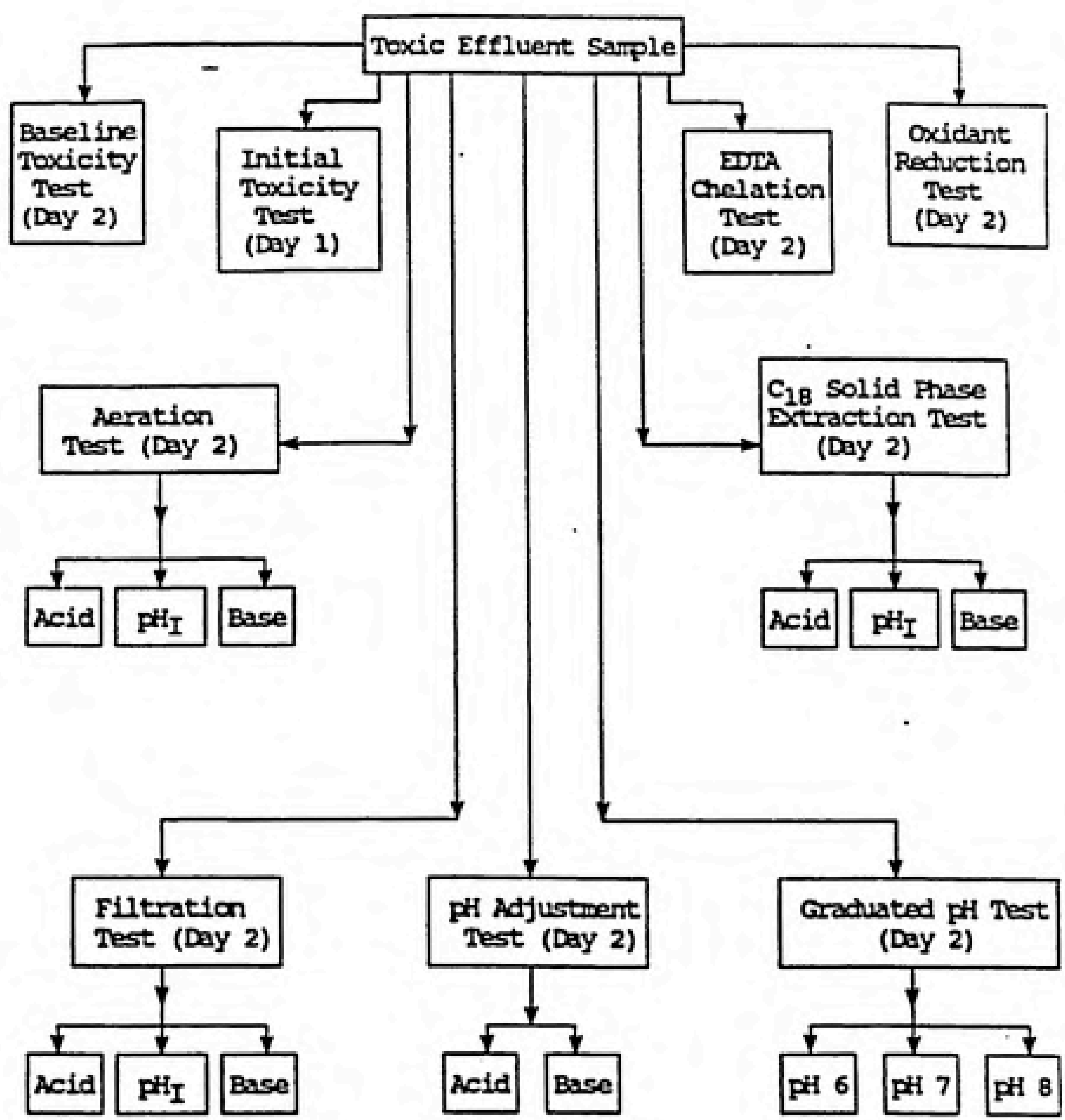
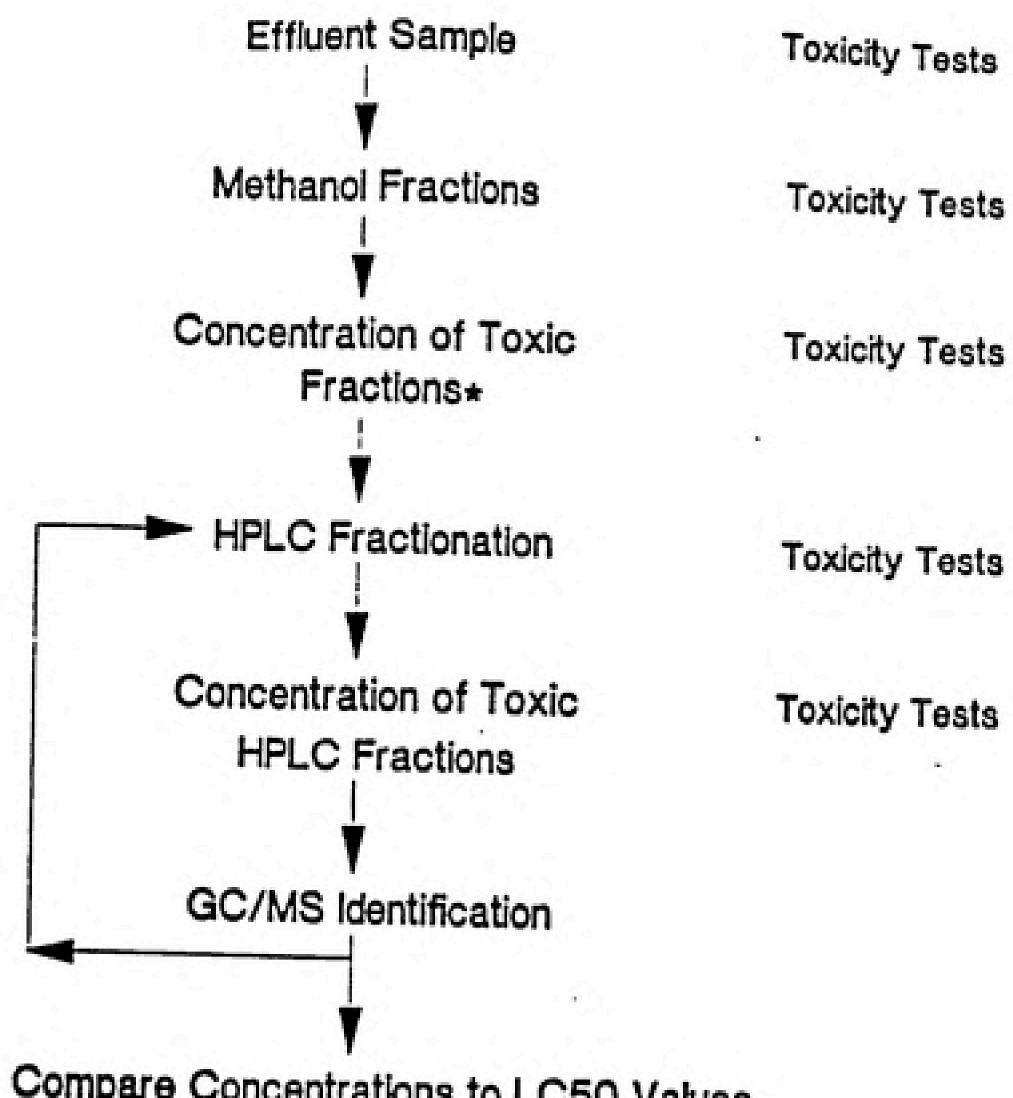


Figure 2.4 Overview of Phase I Effluent Characterization Tests (6) (Note: pH(I) stands for the initial pH of the sample)

Day 1 is considered the day when the effluent sample arrives in the laboratory. On this day the manipulative procedures are conducted and the different aliquots are generated. Day 2 is the initiation of toxicity testing on the fractions generated from the previous day. Initial and baseline toxicity tests are conducted to identify and to insure the continual presence of toxicity. The baseline test is considered the norm for the toxicity measurements taken of the aliquots.

As shown in Figure 2.4, the bioassays for the C18 Solid Phase Extraction Test are conducted on Day 2. Three different aliquots are passed through three conditioned columns. Two of the aliquots are adjusted to pH 3 (to remove uncharged organic acids) and 9 (to remove uncharged organic bases); the uncharged form of organic acids and bases is less polar and thus able to be retained by the C18 column. The pH of all of the aliquots generated by Phase I testing are readjusted back to the initial pH (pHI) of the sample before toxicity tests are conducted. Bioassays are performed on aliquots taken before and after the column. If a noticeable reduction in toxicity is indicated by the test, then Phase II efforts will focus on the C18 column elution procedure. However, if the test indicates no reduction in toxicity, no elution is needed.

The identification of the nonpolar organic toxicants is accomplished in Phase II Toxicity Identification Procedures of the TIE protocol. Figure 2.5 shows the general process



* In rare cases GC/MS can be useful here.

Figure 2.5 Schematic for Phase II Identification of C18 Column Retained Toxicants (6)

used to identify the toxic analytes. In this phase, the C18 column is used to extract, concentrate and elute the toxic analytes from the effluent sample. A large portion of sample (about 1 liter) is passed through the column. The sample pH may be adjusted depending on the results from Phase I. Obviously, the pH that caused the largest decrease in toxicity would be used in this procedure. As with Phase I C18 column tests, the toxicity of the aliquots before and after passage through the column verify the retention of toxic compounds and insure the capacity of the column to remove toxicants has not been reached.

A column that contains toxicity causing compounds is eluted sequentially with small volumes of 25, 50, 75, 80, 85, 90, 95 and 100 percent MeOH/H_2O mixtures. The increasing solvent gradient is used to separate and remove the retained analytes in terms of their decreasing polarity and solubility. These MeOH/H_2O fractions are then tested for toxicity. Those which show significant toxicity are combined together, diluted one to ten with high purity water and passed through a smaller C18 column. Another elution is performed, in this instance using a small volume of 100 percent MeOH. The purpose of this step is to concentrate the analytes even further and eliminate water from the mixture. The eluted fraction is tested for toxicity to insure that the toxic analytes are still present. It also allows for recovery estimations to be determined of the toxic analytes by comparing toxicity values of the eluent

and the whole effluent sample. A recovery of 100% is not crucial at this point, but significant toxicity should be present for the analysis to continue.

The concentrated eluent is injected into a reversed phase, HPLC column. In the initial stages of testing, the C18 packing used in the SPE column is also used in the HPLC column; these differ only in particle size with that used in HPLC being much smaller. As more is known about the toxic analytes, other HPLC packed columns may be utilized to achieve better resolution.

As with the SPE column, the HPLC column is eluted with a concentration gradient of MeOH and water. The elution gradient begins at 30 percent MeOH/H_2O and continues to 100 percent MeOH. Twenty-five fractions are collected and tested for toxicity. The toxic fractions are concentrated again through a small C18 SPE column. This step, as before, concentrates the analytes and eliminates the presence of water. Judgment is required to determine if the toxic fractions should be concentrated separately or combined. This is crucial when considering the cost of GC/MS analyses. For example, if three successive fractions are determined to be toxic, the probability that the same toxicant is present in all three is fairly reasonable (5). These three fractions could be combined reducing the work load. The concentrated fractions are tested for toxicity. This verifies the presence of toxicity and allows for recovery estimations to be made.

The final step in the identification of the toxic compounds is GC/MS analyses. The concentrated fractions generated from the HPLC stage of the procedure are injected onto a capillary column. MeOH, as mentioned before, is not a typical solvent used for GC analysis. However, MeOH is required for toxicity verification. The life of a capillary column will be shortened by the injection of MeOH, and routine GC/MS QA/QC procedures are necessary to monitor column performance. Once the mass spectral data have been generated, peak detection and integrated algorithms can be used to reduce the data (5). A library search is conducted to identify all detectable peaks. A list of identified compounds is assembled and confirmed using various techniques outlined in Phase II and Phase III of the TIE protocol. If the spectral analysis is cluttered by the presence of numerous compounds, then the HPLC fractionation technique may be modified to attain higher resolution.

C. Toxicity Identification Evaluation Studies Using the SPE Procedure

As of this writing, no TIE result oriented studies have been published in the Journal of the Water Pollution Control Federation (JWPCF) or in Environmental Toxicology and Chemistry (SETAC). Many papers have been presented on the TIE topic at annual professional meetings (WPCF and SETAC), but as of yet non of these have been published in their respective journals. The only source of information found

is published by EPA (7, 8, 9). However, many of these documents are still in draft form and are not for public release. Additionally, EPA has not published any data to show the development of the C18 SPE Column procedure. This lack of supportive information has made it difficult to understand EPA's rationale for the procedures developed.

The EPA sponsored studies using the C18 column procedure were conducted at the Largo WWTP in Largo, Florida (7), the Akron POTW in Akron, Ohio (8) and the Patapsco WWTP in Baltimore, Maryland (9). All three indicated a significant reduction in toxicity when the effluent samples were passed through a C18 SPE column. Non-polar organic compounds would therefore appear to have been the major toxic constituents in the effluent streams of all three treatment facilities.

The limited results received from the study conducted at the Largo WWTP (7) are summarized in Table 2.3.

Table 2.3

Solid Phase Extraction Toxicity Test Results, Largo WWTP, Largo, Florida (1985)

	Percent $MeOH/H_2O$							
Date/Type	25	50	75	80	85	90	95	100
Oct.24,1985/C				-	-	-	-	T
Oct.24,1985/C	-	-		*				
Dec.16,1985/C				T	*	T	T	T

* denotes toxic peak
- denotes not tested
C = composite sample
T = toxicity found in that fraction

Samples collected on October 24 were used to establish if the C18 column was capable of extracting and eluting toxic analytes from the effluent stream. A toxicity peak was found at 80 percent $MeOH/H_2O$ (see second entry in Table 2.3). The third sample, collected approximately on December 16, 1985, showed that the toxicity peak had shifted to the 85 percent MeOH fraction. The 85 and 90 percent $MeOH/H_2O$ fractions of this sample were then taken to dryness and reconstituted with dilution water to check for toxicity; both showed complete mortality of Ceriodaphnia within 24 hr.

The results from the Largo WWTP suggested that the toxicants, in addition to being relatively non-polar, are also relatively non-volatile (7). The shift in toxicity peak suggested by the data in Table 2.3 may indicate variability in the sources of toxicity. However, these data are too limited to make firm conclusions.

Table 2.4 is a summary of the column elution results

obtained from testing the effluent from the Akron POTW in Akron, Ohio (8).

Table 2.4

Solid Phase Extraction Toxicity Test Results, Akron POTW, Akron, Ohio (1986)

Date/Type	Percent $MeOH/H_2O$ 25	50	75	80	85	90	95	100
Jan 13,1986/C	-	-			T			T
Jan 14,1986/C	-	-						T
Jan 15,1986/C	-	-			T			
Feb 8,1986/C					T			
Feb 9,1986/C					T			
Mar 28,1986/C	-	-			T			
Mar 29,1986/C	-	-						
Mar 30,1986/C	-	-			T			
Apr 22,1986/1G	-	-			T			T
/2G	-	-			T			T
/3G	-	-			T			T
/4G	-	-			T			T
Jul 10,1986/2G				T	T		T	
Aug 6,1986/G					T			T
Aug 14,1986/C					T			
Aug 18,1986/C					T			
Aug 26,1986/C					T			

- denotes not tested
C = composite, G = grab sample
T = toxicity found in that fraction

The 85 percent $MeOH/H_2O$ fraction contained most of the toxicity. However, toxicity was also found in the 100 percent MeOH fraction. Because the toxicity was found in the higher percent $MeOH/H_2O$ fractions, the toxic compounds were considered relatively non-polar. Additionally, the appearance of toxicity in the 100 percent $MeOH/H_2O$ fraction was only observed when mortality of the <u>Ceriodaphnia</u> ensued

rapidly in the 85 percent MeOH/H_2O fraction (8).

The C18 column was also tested at the Patapsco WWTP in Baltimore, Maryland (9). Many industrial sources contribute to the influent stream of this facility. Acute toxicity using Ceriodaphnia is observed on a continual basis. Table 2.5 shows the column elution results from testing the primary and secondary effluents (9).

Table 2.5

Solid Phase Extraction Toxicity Test Results
Patapsco WWTP, Baltimore, Maryland (1986-1987)

	Percent MeOH/H_2O							
Date/Origin	25	50	75	80	85	90	95	100
Jul 9,1986/2nd Eff	t	t	t	t	T	*	T	t
Jul 23,1986/Pri Eff	t	t	t	T	T	*	T	t
Jul 23,1986/2nd Eff	t	t	t	T	T	*	T	t
Dec 10,1986/2nd Eff	t	t	T	T	*	T	t	t
Jan 6,1987/2nd Eff	t	t	T	T	*	T	t	t
Jan 8,1987/2nd Eff			t	T	*			

* denotes toxic peak
T = significant toxicity found in that fraction
t = slight toxicity found in that fraction
Primary Effluent (Pri Eff)
Secondary Effluent (2nd Eff)

Toxicity was present over a wide range of MeOH/H_2O fractions. This would indicate that the effluent stream contains a complex variety of non-polar organic compounds. The shift in the toxic peak from 90 percent to the 85 percent MeOH/H_2O fraction was suggested to be seasonal; however no explanation for this change in peak values was

offered.

An attempt was made during the Patapsco study to identify the toxic constituents found in each $MeOH/H_2O$ fraction found to contain toxicity. The GC/MS analyses did not show the presence of suspected toxic compounds (9). In fact, the majority of the chromatographic peaks could not be identified and for the few which could, they did not appear to be the cause of toxicity.

The operational and managerial viewpoint of the Toxicity Reduction Evaluation conducted at the Patapsco WWTP was recently presented (10). The column elution procedure was criticized for not yielding meaningful results. The procedure was found to produce poor spectral analysis through GC/MS. This was claimed to be caused by too many compounds being present in the influent or effluent of a large municipal wastewater treatment plant. The results obtained with the C18 column fractionation technique were noted to be similar to those at the Akron WWTP in Akron, Ohio. Yet, these treatment facilities have different industrial sources. Therefore, it was difficult to explain why samples from two plants showed toxicity in the same 85 percent $MeOH/H_2O$ fraction. The procedure was recommended for use as a research tool but not as a practical technique for wastewater treatment plant personnel.

III. EXPERIMENTAL PROCEDURE

A. C18 SPE Column Procedure

A.1 C18 SPE Column Set-up and Conditioning

The C18 SPE column procedure was followed exactly as presented in the EPA Phase I Toxicity Characterization Procedures Guidance Document (6). This procedure, however, deals only with passage of samples through the C18 SPE Column and subsequent measurements of toxicity after passage. While this research was underway, EPA published (November 1988) its Phase II Toxicity Identification Procedures in draft form (5). This aspect of the procedure deals with elution of $MeOH/H_2O$ fractions and measurements of their toxicity. Unfortunately, much of the elution work had already been done for this study when the EPA document became available. The elution procedure was developed from the limited information found in specific extracts sent by EPA from the City of Largo evaluation (11) and Las Vegas report (12). The procedures used closely resembled those adopted by EPA in the draft report of Phase II; differences between them will be addressed.

The experimental setup for conducting the column elutions is shown in Figure 3.1. It consists of a sample reservoir (1 liter volume), needle valve, C18 SPE Column and

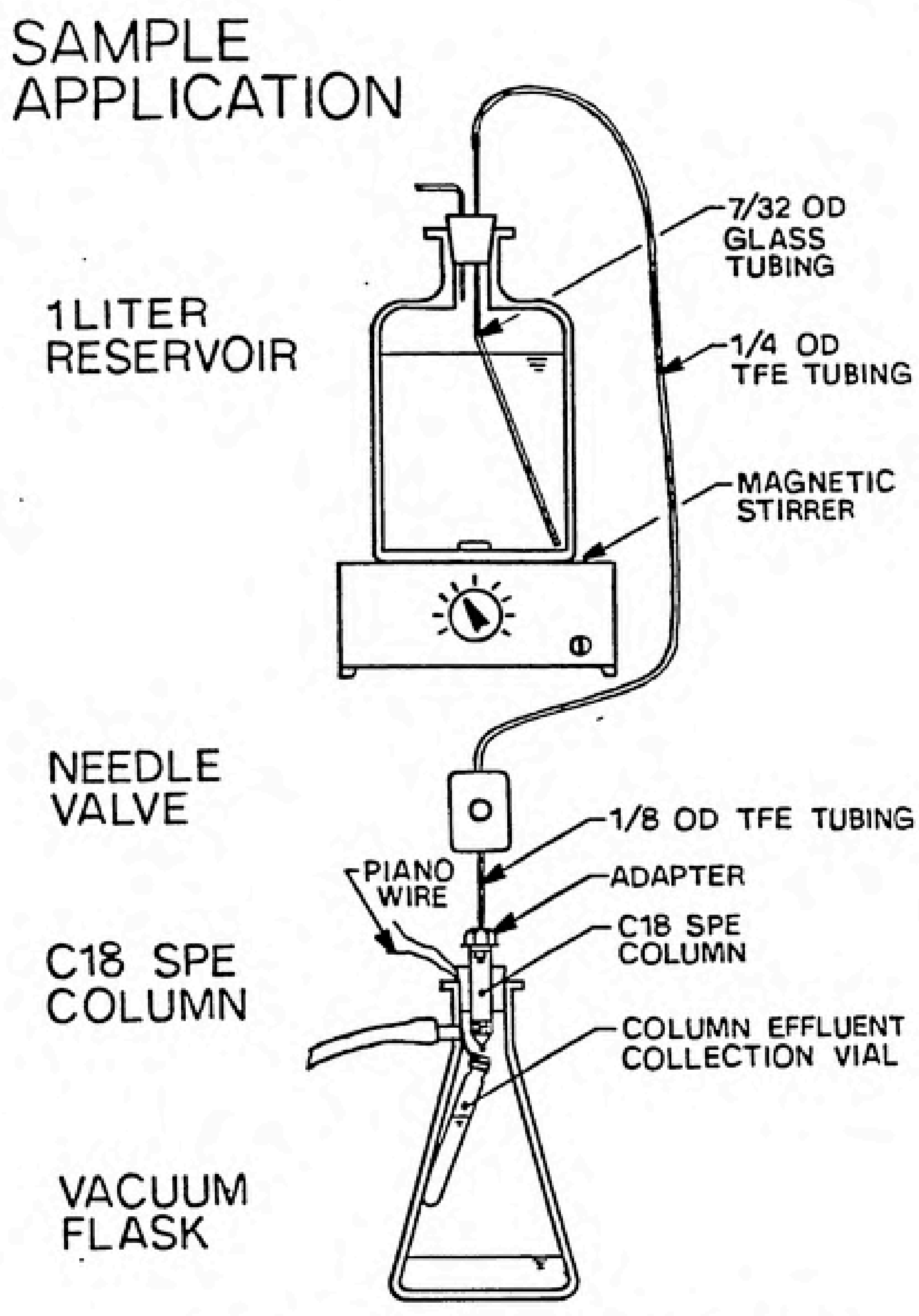


Figure 3.1 Experimental Setup for Phase II C18 SPE Column Sample Application.

vacuum flask. A rubber stopper held the glass tubing that was used to extract the sample and vent the interior space of the reservoir bottle. The extraction tube was bent to allow easy access to the last remaining portions of sample. A needle valve controlled the flowrate of the column. During the design of this setup it was discovered that the stopper used on the discharge tube of a standard 250 ml filtration apparatus was ideal for holding the C18 SPE column. Additionally, a looped piece of piano wire was inserted into the vacuum flask on the outer edge of the rubber stopper to hold the sample collection vials and to allow easier control of the vacuum process through the continual bleeding of the pressure.

The column was first conditioned with MeOH. The column manufacturer (J.T.Baker) suggests 10 ml of MeOH followed by a 10 ml rinse with high purity water (3); however, the EPA Phase II Draft Document recommends increasing this to 25 ml (5). The manufacturer's procedure was used here.

After conditioning, 3 ml of each MeOH/H_2O elution was passed through and collected in analytically clean vials. These fractions served as controls to determine if the interaction between MeOH and the column sorbent was causing toxic interferences.

The MeOH/H_2O elution process is shown in Figure 3.2. The 5 ml syringe, containing 3 ml of the MeOH/H_2O mixture and 2 ml of headspace, was inserted into the column adaptor. The syringe was carefully depressed to allow the mixture to

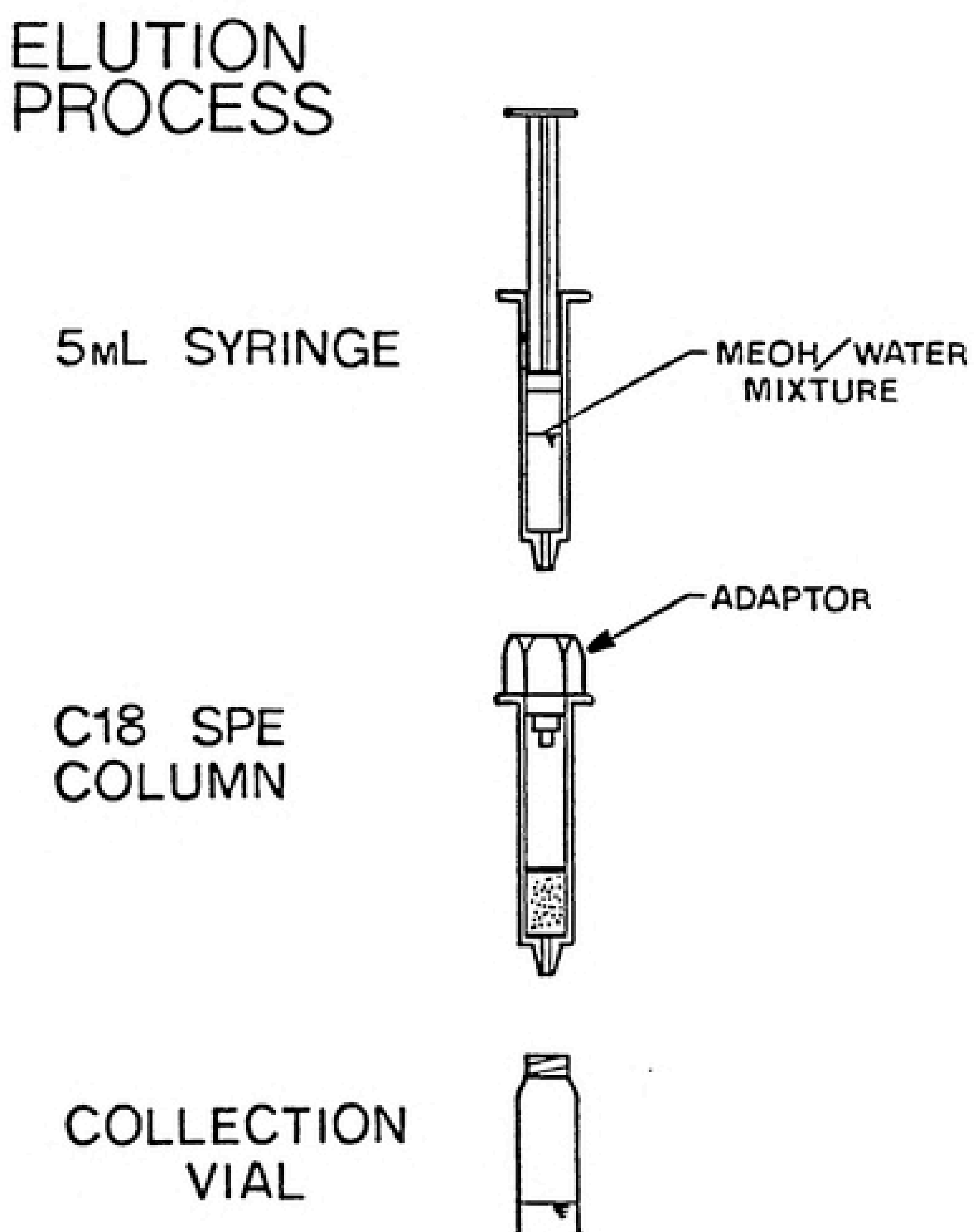


Figure 3.2 Experimental Procedure for Phase II C18 SPE Column Elution Process.

flow through the column at a rate of 5 ml/min or less. The 2 ml headspace was required to expel the remaining fluid from the packing. Each elution was collected in a separate vial and labelled for toxicity testing. At this point, EPA suggests the column should be allowed to dry between each 3 ml elution volume and a maximum flow rate of 4 ml/min should not be exceeded (6). The column was not extensively dried beyond the 2 ml headspace required to flush-out the remaining MeOH/H_2O mixture. A maximum flow rate of 5 ml/min was used as prescribed by the manufacturer (3). After the column elution blanks were passed, the column was reconditioned with 10 ml of MeOH followed by 10 ml of high purity water.

A.2 Sample Application

Each sample was processed by first filling the reservoir bottle with one liter of sample. The conditioned column was inserted in the rubber stopper and placed in the top of the vacuum flask, Figure 3.1. The sample feeding line (1/8 inch OD, Teflon TFE tubing) was inserted into the adaptor of the column. Air must be removed from this line; otherwise, the column will become partially dry. The packing must not be allowed to dry between conditioning and sample application. The system was primed by first inserting the feeding line into the adaptor of a so-called, "waste column". This column was inserted into another rubber stopper and placed in the vacuum flask. The needle valve was opened and the

vacuum lever was turned on. Once the sample had reached the end of the tube (1/8 inch OD, Teflon TFE tubing), the needle valve was used to stop the flow, the feeding line was placed back into the adaptor of the conditioned column and the sample was processed. The flowrate was established by opening the needle valve and carefully adjusting the vacuum; both the needle valve and vacuum can be used to achieve finer adjustment of the flowrate once processing has begun.

Post-column effluent samples were taken after 100, 500, 750, 850 and 950 ml have passed through the column as shown in Figure 3.3. These were subjected to toxicity testing. The EPA Phase II Draft Document suggests collecting post-column effluent samples after 25, 500 and 950 ml have passed (5). However, additional samples were collected to obtain a better indication of whether sorptive capacity of the column was exceeded. Post-column effluent samples were taken by carefully removing the stopper containing the column from the vacuum flask (Figure 3.1), wrapping the piano wire around the threads of the collection vial, placing the vial under the luer tip, and inserting the vial and column back into the flask. No sample concentration was done following passage through the column.

A.3 Elution of MeOH/H_2O Fractions

Once the sample was passed through the column, the MeOH/H_2O elutions were repeated in the same manner as described for the controls (Figure 3.2). A set of eight, 5

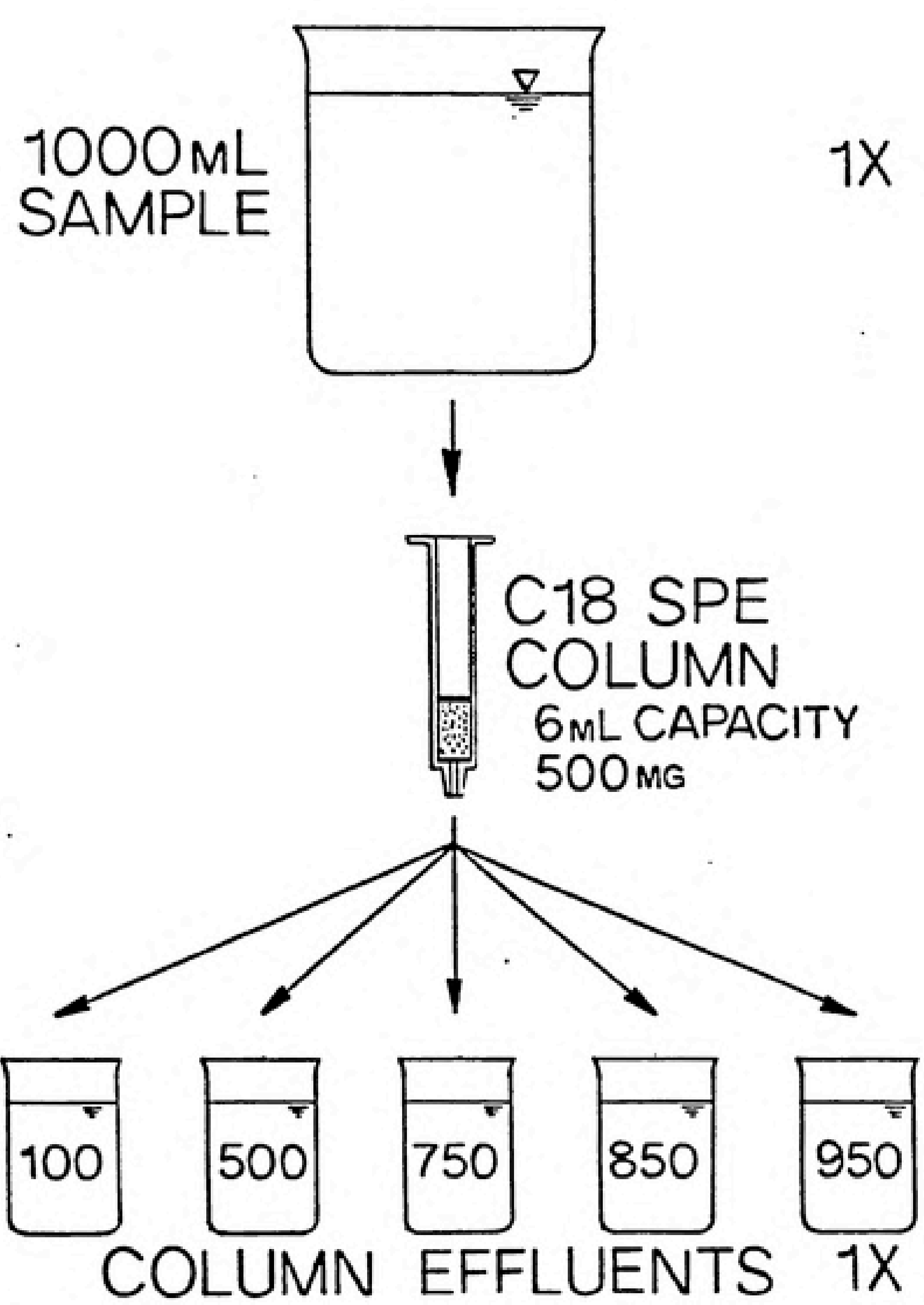


Figure 3.3 Sample Application Process.

ml disposable syringes, each containing 3 ml of the required percent MeOH/H_2O mixtures (25, 50, 75, 80, 85, 90, 95, 100%) with the addition of a 2 ml of headspace were inserted in ascending order of percent MeOH/H_2O.

Figure 3.4 shows the extent of sample concentration caused by the elution procedure and the subsequent dilution required for toxicity testing. By using 3 ml of each eluent and 1 liter of sample, the concentration factor was 333X. From each elution, 150 uL was diluted with 10 ml of dilution water (a dilution of 66.7X) and tested for toxicity. Thus, the final concentration of the compounds to which the test organisms were exposed was 5X that of the original sample. However, this assumes that the elution process was 100 percent efficient and that each toxic compound had eluted into one particular fraction. Neither one of these assumptions can be achieved experimentally. Therefore, the expected concentration of the eluted compounds should be less than 5X. It should also be noted that the final percent MeOH at the point of toxicity testing varies between 0.375 to 1.5 percent for this elution series; keeping the percent MeOH less than 1.5 was important so as to eliminate MeOH toxicity.

Post-column effluents and MeOH/H_2O elutions from the column procedure were stored overnight in a refrigerator at $4^{o}C$ and toxicity tests are conducted the next day. The glassware and tubing used during this process must be thoroughly cleaned (see Appendix A for details) before the

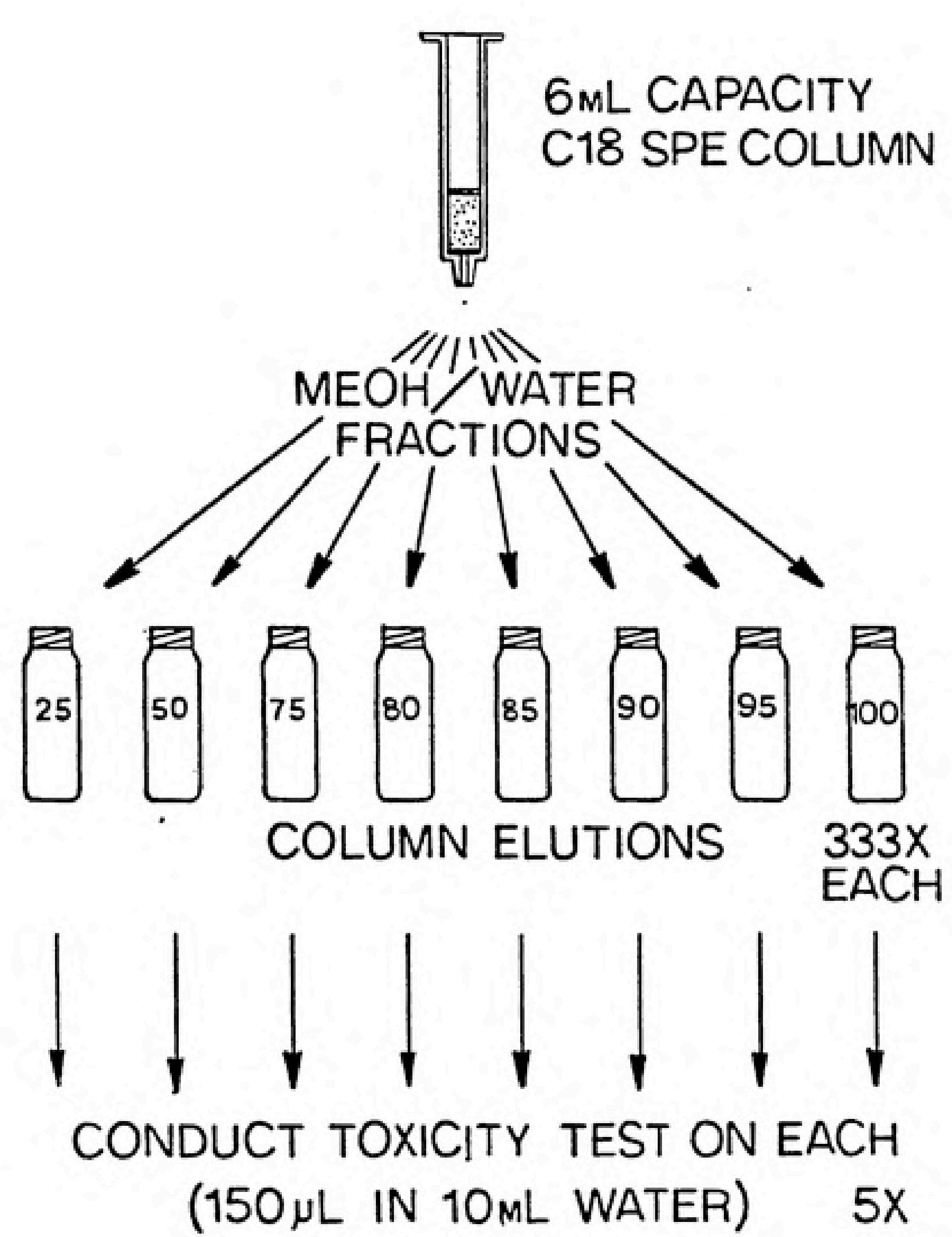


Figure 3.4 Column Elution Process.

next sample can be tested. A list of equipment and materials used in the column elution procedure is given in Appendix B.

B. Bioassay Procedure

Two types of bioassays were used to measure acute toxicity during this research: a bioassay based on the Ceriodaphnia C. dubia species and the Microtox (Microbics Corp.) assay procedure. The Ceriodaphnia can be described as a small crustacean found typically in most surface water sources in the United States. It is part of the Cladocera Order under the Phylum Arthropoda (13). EPA has selected this organism for testing based on many factors such as sensitivity, availability and cost (14). The Ceriodaphnia are preferred over the more well-known Daphnia magna and Daphnia pulex because they are smaller in size, have shorter generation times and are more sensitive. Typically, Ceriodaphnia can produce 3 to 4 broods per week under optimal laboratory conditions (15). A large number of neonates are required to test for toxicity as outlined by the TIE protocol, and the Ceriodaphnia are amenable to this need.

The Microtox System uses a strain of marine photoluminescent microorganisms that resemble photo-bacterium phosphoreum (16). These organisms emit light as a by-product of respiration (17). If something interferes or inhibits respiration, a reduction or elimination of light

output is observed. The Microtox instrument has a photomultiplier which is sensitive to this light emission. A decrease in light output after the bacteria have been exposed to a sample gives an indication of toxicity; the effect is proportional to the amount of toxicity present.

C. Illustration of Procedure Used to Analyze Bioassay Data

The following hypothetical example is given to illustrate the process of conducting a Ceriodaphnia toxicity test. Although, these data are not real, the results of this research gave most of the patterns included here for discussion. The process begins with the Toxicity Request Form (Appendix C) shown in Figure 3.5. A composite sample was taken at the Imaginary Creek WWTP in Example, North Carolina, between the dates of 1/16/89 to 1/17/89. The sample was considered toxic by baseline tests taken on 1/18/89. The C18 SPE Column elutions were conducted on 1/23/89. The Toxicity Request Form was filled out for the post-column effluents (slanted lettering style) and given to the bioassay laboratory. This informs the bioassay laboratory personnel of the in-coming samples and allows them to prepare for testing the following day. This usually entails the isolation of adult test organisms, labeling sample cups, filling out the top portions of the data recording form (Figure 3.6) and preparing the dilution water.

The samples were prepared for toxicity testing the next

TOXICITY TEST REQUEST FORM
Ceriodapnia dubia

NAME: T. CHAMPLIN DATE: 1/23/89

LOG #	SAMPLE DATE	SAMPLE I.D.	LOCATION	TREATMENT DATE	TREATMENT TIME
CH1.0	1/16	CFEO	RF 12	1/23	AM
CH1.1	1/16	EFE100	RF 12	1/23	AM
CH1.2	1/16	EFE500	RF 12	1/23	AM
CH1.3	1/16	EFE750	RF 12	1/23	AM
CH1.4	1/16	EFE850	RF 12	1/23	AM
CH1.5	1/16	EFE950	RF 12	1/23	AM

TYPE OF TEST:
- X TIMED LETHALITY (10 mL; no dilutions)
- __ TIMED LETHALITY (10 mL; w/ dilutions)
- __ ACUTE STATIC (50 mL; 24 & 48 HR LC50)
- __ 7-DAY MINI CHRONIC (15 mL; reproductive)
- __ OTHER (______________________)

SPECIAL INSTRUCTIONS: NONE

RESULTS:

	LOG #	CH1.0	CH1.1	CH1.2	CH1.3	CH1.4	CH1.5		
_A	ET50=	1.4	>48	>48	8.0	8.0	3.2		
_B	LC50=								
_C	CHRC=								

(PASS/FAIL)

REQUEST RECEIVED BY: MJM
DATE: 1/23/89
TEST DATES: 1/24/89 to 1/26/89

COMMENTS:

Figure 3.5 Toxicity Test Request Form

TIMED LETHALITY TEST DATA / WORKSHEET
Ceriodaphnia dubia

SAMPLE I.D.: Imaginary Creek WWTP , 1/16/89 to 1/17/89 Composite NAME: T.Champlin

CONTROL/DILUTION WATER SOURCE: Botany Pond/ Fish Tank LOT #: 15
INITIAL:
Filter: GF/C Temp: ____ pH: ____ D.O.: SAT Alk: ____ Hardness: ____ Cond: ____

INITIAL DATE: 1/24/89 TIME: 0900 ANALYST: MJM/TLC
FINISH DATE: 1/26/89 TIME: 0900 ANALYST: MJM

TEST VOL: 10mls # ORGANISMS/CUP 5 DILUTIONS: none ADULTS ISOLATED AT: 1355 1/23

SAMPLE		CFE 0		EFE 100		EFE 500		EFE 750		EFE 850		EFE 950	
REPLICATE		A	B	A	B	A	B	A	B	A	B	A	B
# ORGANISMS		5	5	5	5	5	5	5	5	5	5	5	5
TIME:	NAME												
0930	MJM	OK	1d	OK	OK	OK	OK	OK	OK	OK	1d	OK	OK
1000	MJM	2d 2tw	2d 3tw	OK	OK	OK	OK	OK	OK	1d	1d	1d	OK
1100	MJM	3d 2tw	3d 2tw	OK	OK	OK	OK	OK	OK	1d 1tw	2d 1tw	2d	1d 1tw
1300	MJM	5d	5d	OK	OK	OK	OK	1d 1tw	1d 2tw	2d 1tw	2d 1tw	3d 1tw	3d
1700	TLC	–	–	OK	OK	OK	OK	2d 1tw	3d	3d 2tw	2d 2tw	4d	3d 1tw
0900	MJM	–	–	1d	1tw	1tw	OK	3d 1tw	3d 1tw	5d	4d	5d	5d
0900	MJM	–	–	2d 1tw	1d 1tw	1d 2tw	1d 2tw	5d	5d	–	5d	–	–
Temp (C)													
pH		7.8	7.7					7.5	7.7	7.6	7.5	7.5	7.8
DO (mg/L)		8.1	8.0					8.0	8.2	8.1	8.0	8.2	8.1

COMMENTS:

Figure 3.6 Timed Lethality Test Data/Worksheet

day by removing them from the refrigerator and warming them to 25°C in a water bath. The sample vials were then removed and poured into the testing cups. Replicated cups were rinsed first with a small portion of sample and then 10 ml of sample were poured into each cup. Replicates were used to ensure against the invalidation of the test from the accidental loss of a cup. The Timed Lethality Test was performed using five neonates, born from the isolated adult population from the previous day which were transferred to each test cup. The initiation of the toxicity test began once the neonates were transferred. Samples are not diluted in the Timed Lethality Test; rather, mortality readings are taken typically at 0.5, 1.0, 2.0, 4.0, 8.0, 24.0 and 48.0 hr past the initial transfer.

Figure 3.6 shows the mortality readings as they were obtained for this test. Death was reported by the lower case letter "d" and twitching by "tw". Additionally, "er" was used to indicate erratic characteristics shown by the organisms behavior. A complete description of the terms used to describe the behavior of aquatic life can be found in IERL-RTP Procedures (18). After the 48 hr reading, dissolved oxygen (DO) and pH measurements were conducted on the test cups showing significant mortality. This insured that DO and pH were within acceptable limits for *Ceriodaphnia* survival and thereby eliminating artifactual mortality.

The mortality results were stored on Lotus 1-2-3 work

sheets which can be used to generate tables such as shown in Table 3.1. The results of the replicated cups were combined and reported in the cellular blocks on this form. ET50 and TTU values were determined and reported at the bottom of each cell. The ET50 value refers to the elapsed time required for 50 percent mortality to occur. It is determined from a mortality versus log of elapsed time plot as illustrated in Figure 3.7. Mathematically this is the same as a linear interpolation based on a logarithmic scale. The Time-based Toxicity Unit (TTU) was developed in this research and is explained in Section III.D. Its purpose was to provide additional information regarding the progression of toxicity over time.

The Standard Microtox procedure was followed exactly as given in the Microbics Manual (17). This procedure required the bacterial reagent to be exposed to a sample dilution series of 50, 25, 12.5 and 6.25 percent of the original sample. Light output measurements were taken of the reagent before the sample was introduced. As soon as the readings were reported on the strip chart recorder, the bacteria were exposed to the sample dilution series. Light output readings were taken at 5 minutes and 15 minutes to record any reduction in light output. Once testing was finished, the data recorded on the strip chart was reduced using a computer program developed by the Microbics Corporation or by following the instructions in the Microtox manual (19). The final results were reported in terms of an EC50 value.

Table 3.1 Computer Generated Time Lethality Data/Worksheet

TIMED LETHALITY DATA / WORKSHEET
TESTS STARTED ON 1/24/89
(NO MORTALITY IN THE CONTROLS)

IMAGINARY CREEK WWTP, EXAMPLE, NC
COMPOSITE SAMPLE 1/16/89 TO 1/17/89
FRACTIONATION SPE C-18 COLUMN

BIOASSAYS CONDUCTED IN
PLASTIC CUPS
****

TIME HOURS	LAPSED TIME	LOG TIME	SAMPLE 01-16-CFE0 NUMBER ORGANISMS	NUMBER DEAD	PERCENT MORTALITY	SAMPLE 01-16-EFE100 NUMBER ORGANISMS	NUMBER DEAD	PERCENT MORTALITY	SAMPLE 01-16-EFE500 NUMBER ORGANISMS	NUMBER DEAD	PERCENT MORTALITY
900	0		10	0	0.0%	10	0	0.0%	10	0	0.0%
930	0.50	-0.301	10	1	10.0%	10	0	0.0%	10	0	0.0%
1000	1.00	0.000	10	4	40.0%	10	0	0.0%	10	0	0.0%
1100	2.00	0.301	10	6	60.0%	10	0	0.0%	10	0	0.0%
1300	4.00	0.602	10	10	100.0%	10	0	0.0%	10	0	0.0%
1700	8.00	0.903	10	10	100.0%	10	0	0.0%	10	0	0.0%
900	24.00	1.380	10	10	100.0%	10	1	10.0%	10	0	0.0%
900	48.00	1.681	10	10	100.0%	10	3	30.0%	10	2	20.0%
			ET50 = 1.4		TTU = 46.5%	ET50 > 48		TTU = 1.7%	ET50 > 48		TTU = 0.8%

TIME HOURS	LAPSED TIME	LOG TIME	SAMPLE 01-16-EFE750 NUMBER ORGANISMS	NUMBER DEAD	PERCENT MORTALITY	SAMPLE 01-16-EFE850 NUMBER ORGANISMS	NUMBER DEAD	PERCENT MORTALITY	SAMPLE 01-16-EFE950 NUMBER ORGANISMS	NUMBER DEAD	PERCENT MORTALITY
900	0		10	0	0.0%	10	0	0.0%	10	0	0.0%
930	0.50	-0.301	10	0	0.0%	10	1	10.0%	10	0	0.0%
1000	1.00	0.000	10	0	0.0%	10	2	20.0%	10	1	10.0%
1100	2.00	0.301	10	0	0.0%	10	3	30.0%	10	3	30.0%
1300	4.00	0.602	10	2	20.0%	10	4	40.0%	10	6	60.0%
1700	8.00	0.903	10	5	50.0%	10	5	50.0%	10	7	70.0%
900	24.00	1.380	10	6	60.0%	10	9	90.0%	10	10	100.0%
900	48.00	1.681	10	10	100.0%	10	10	100.0%	10	10	100.0%
			ET50 = 8.0		TTU = 12.2%	ET50 = 8.0		TTU = 28.2%	ET50 = 3.2		TTU = 25.6%

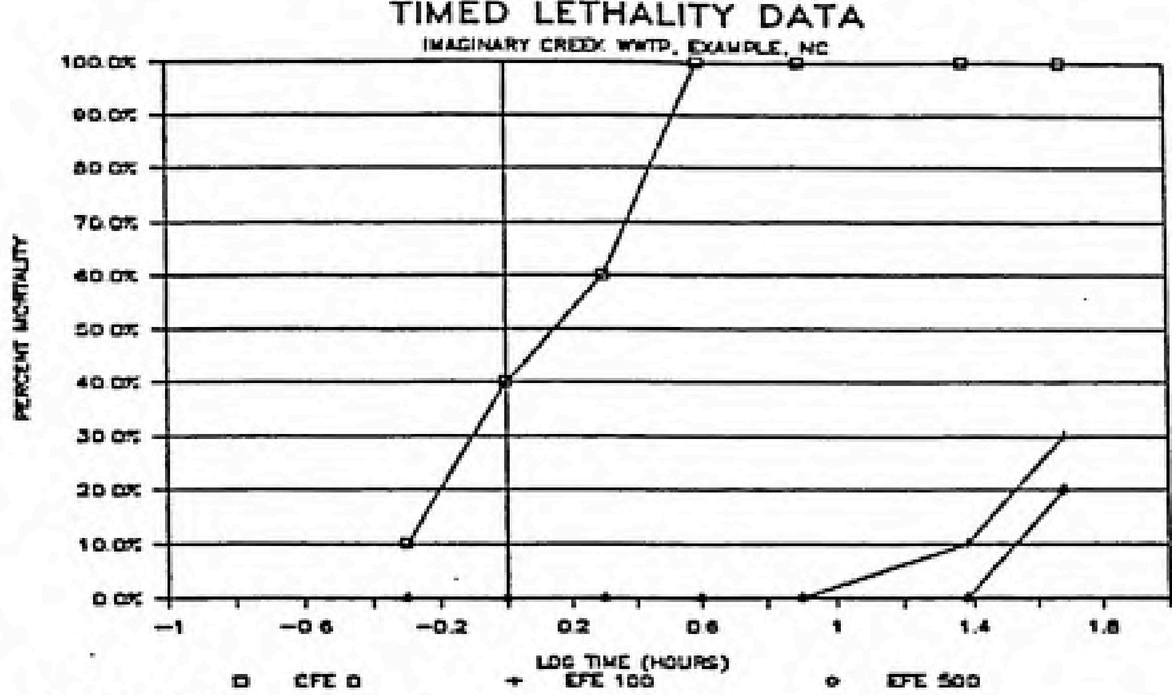


Figure 3.7a

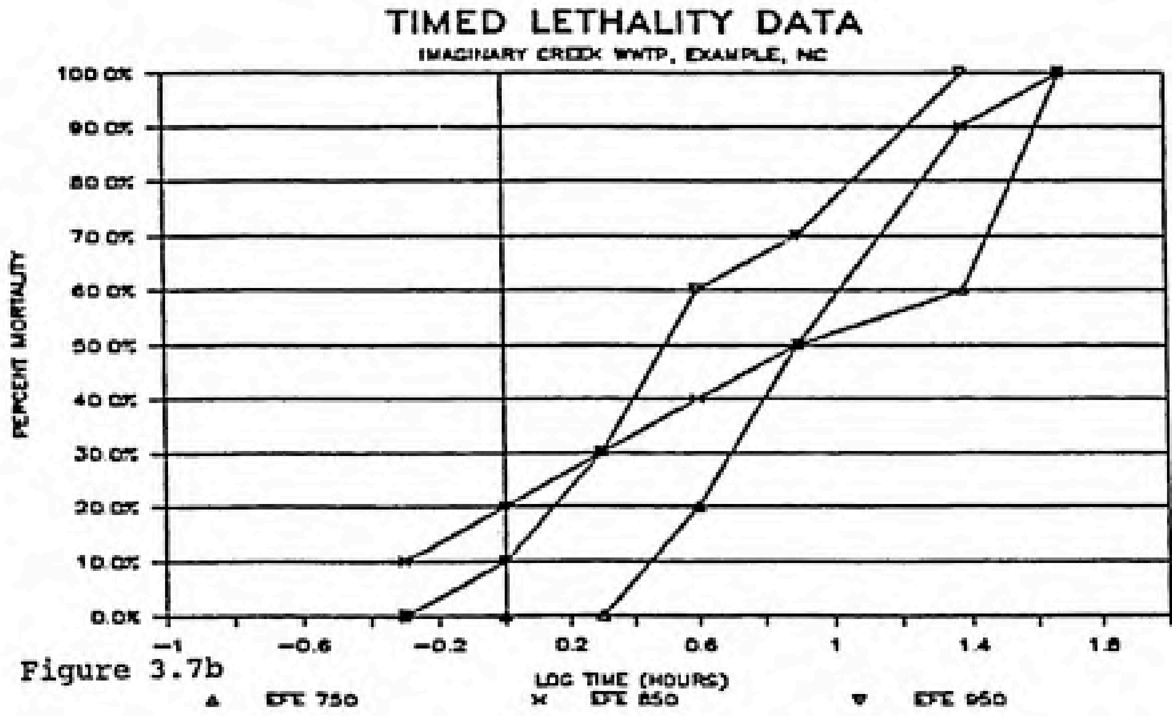


Figure 3.7b

Figure 3.7 Percent Mortality Versus the Log of Elapsed Time for Hypothetical Samples (a) CFE0, EFE100 and EFE500 and (b) EFE750, EFE850 and EFE950.

This value represents the effective concentration at which 50 percent of the light output capability of the bacteria has been reduced. A quick-reference guide for the Microtox procedure is given in Appendix D of this report.

D. The Time-based Toxicity Unit (TTU)

The results of toxicity testing can be expressed in Toxic Units (TU) to obtain a direct relationship between the reported values of mortality and toxicity. The TU value is a measure of the strength of a chemical expressed as a fraction or proportion of its lethal threshold concentration (20). The strength may be calculated as shown in the following equation:

$$TU = \frac{\text{concentration of toxic compound}}{\text{lethal threshold concentration of toxic compound}}$$

A TU value greater than 1.0 would represent mortality of more than 50 percent of the test organisms, while a value less than 1.0 would indicate less than 50 percent mortality. A TU value of exactly 1.0 would indicate lethality of 50 percent of the organisms. Higher values are assigned to higher degrees of toxicity establishing a direct relationship.

A TU was developed from the timed lethality (ET50) data to facilitate comparisons of samples before and after C18 column passage and to provide better data interpretation. ET50 values, or the time required to observe 50 percent

mortality of the test organisms, establishes an inverse relationship between the degree of toxicity and the reported value. For example an ET50 value of 2 hr is considered to be significantly more toxic than an ET50 value of 8 hr. An inverse relationship is awkward to use when trying to illustrate graphically the reductions in toxicity due to sample treatments in the TIE protocol (Figure 2.4). In addition, the ET50 provides no information on the progression of toxicity with time. The following Time-based Toxicity Unit (TTU) was developed to overcome these disadvantages:

$$TTU = \frac{\sum \frac{M_t}{\log(t+1)}}{\sum \frac{M_{max}}{\log(t+1)}} \times 100\% \qquad \text{Eq.(1)}$$

where M_t represents the number of mortalities observed at time t, and M_{max} refers to the maximum number of mortalities which could possibly be observed at time t. The value of M_{max} is the total number of test organisms. A TTU of 100 percent would represent the observance of complete mortality at the first reading (i.e., $M_t = M_{max}$) and subsequently all other readings. A TTU value of zero percent would indicate the observance of no mortality over the duration of the test.

The TTU expression (Eq. 1) establishes a direct relationship between the intensity of toxicity and the

reporting of toxicity. This unit is not unique; other schemes are possible. It was created primarily to present the results of the ET50 tests in graphical form so that the larger the value of the plotting point, the greater the toxicity. The reciprocal of elapsed time provides a weighting scheme which places emphasis on the initial readings. That is, mortalities observed in the early stages of testing indicate more significant toxicity than those occurring later. A logarithmic time scale allows for the weighting to be expressed over two orders of magnitude to accommodate observations taken from 1 to 48 hr; an arithmetic time scale would have placed too little significance on mortality observed at 24 and 48. Use of log(t+1) rather than log(t) avoids taking the log of a number less than or equal to one, which would cause a negative or zero value to be calculated at the 0.5 and 1.0 hr readings, respectively.

E. Illustration of Use of TTU Concept

The hypothetical mortality data of one sample, given in Table 3.1, are expressed in terms of TTU values in Table 3.2.

Table 3.2

Example Calculation of Time-based Toxicity Unit for Imaginary Creek WWTP,Example, NC, Sample CFEO

Elapsed Time(t)	$\frac{1}{\log(t+1)}$	Observed Mortality M_t	$\frac{M_t}{\log(t+1)}$	$\frac{M_{max}}{\log(t+1)}$
0.5	5.6	1	5.6	56.0
1.0	3.3	4	13.2	33.0
2.0	2.1	6	12.6	21.0
4.0	1.4	10	14.0	14.0
8.0	1.0	10	10.0	10.0
24.0	0.7	10	7.0	7.0
48.0	0.6	10	6.0	6.0
		TOTALS:	68.4	147.0

$$TTU = \frac{\sum \frac{M_t}{\log(t+1)}}{\sum \frac{M_{max}}{\log(t+1)}} \times 100\% = \frac{68.4}{147.0} \times 100\%$$

$$TTU = 46.5\%$$

The TTUs for the other samples were calculated in a similar manner and presented along with their corresponding ET50 values in the lower portion of Table 3.1.

Table 3.3 compares the ET50 and TTU values for the C18 post-column effluent samples and illustrates the advantages of the TTU.

Table 3.3

A comparison of ET50 and TTU Values for the C18 Column Effluent Results from the Imaginary Creek WWTP,Example,NC

Sample Identification	ET50(hr)	TTU(%)
CFEO	1.4	46.5
EFE100	>48	1.7
EFE500	>48	0.8
EFE750	8.0	12.2
EFE850	8.0	28.2
EFE950	3.2	25.6

Samples EFE100 and 500 both have ET50 values greater than 48 hr, yet the data shown in Table 3.1 reveal that mortality was actually observed in both of these samples. In contrast, toxicity values can be assigned to these samples using the TTU approach even though mortality was less than 50 percent after 48 hr. These TTU values (1.7 and 0.8) are small, however, because mortality observed in the later stages of testing is not very important on this toxicity scale. Thus, the weighting scheme devised for time at which mortality occurs provides additional information about the expression of toxicity in the sample.

The TTU approach can also distinguish between toxicity in two samples with the same ET50 but different time progressions of toxicity. For example, Table 3.3 shows that samples EFE750 and 850 have identical ET50 values (8 hr). However, their corresponding TTU values are significantly different. Figure 3.7b shows that while there two samples

reach 50 percent mortality in 8 hr, the time progression of mortality is different; i.e., mortality begins two readings earlier for the EFE850 sample. This would imply that sample EFE850 should be more toxic because mortality was observed sooner in the test. The weighting scheme of the TTU assigns more significance to death in the earlier stages of testing and, therefore, would assign a higher TTU value to EFE850.

The TTU can also result in comparisons of toxicity between two samples that are opposite to those based on ET50. This is illustrated by samples EFE850 and 950. The ET50 values for EFE850 and EFE950 are 8 and 3.2 hr, respectively. However, the corresponding TTU values indicate the reverse ordering. Figure 3.7b shows that the progression of mortality begins earlier and is faster in the initial stages of testing for sample EFE850. It is not until the 2 hr reading that more mortality occurs in sample EFE950. Thus, the time-weighting factor again assigns more importance to mortality occurring in the initial stages of testing and produces a higher TTU for EFE850 even though 50 percent mortality occurs earlier in the EFE950 sample.

Figure 3.8 is a graphic representation of the TTU values obtained from the data of the hypothetical example. The ability of the C18 column to retain the toxic compounds is seen by comparing toxicity of the sample before passing it through the column (BEFORE COLUMN) and samples collected

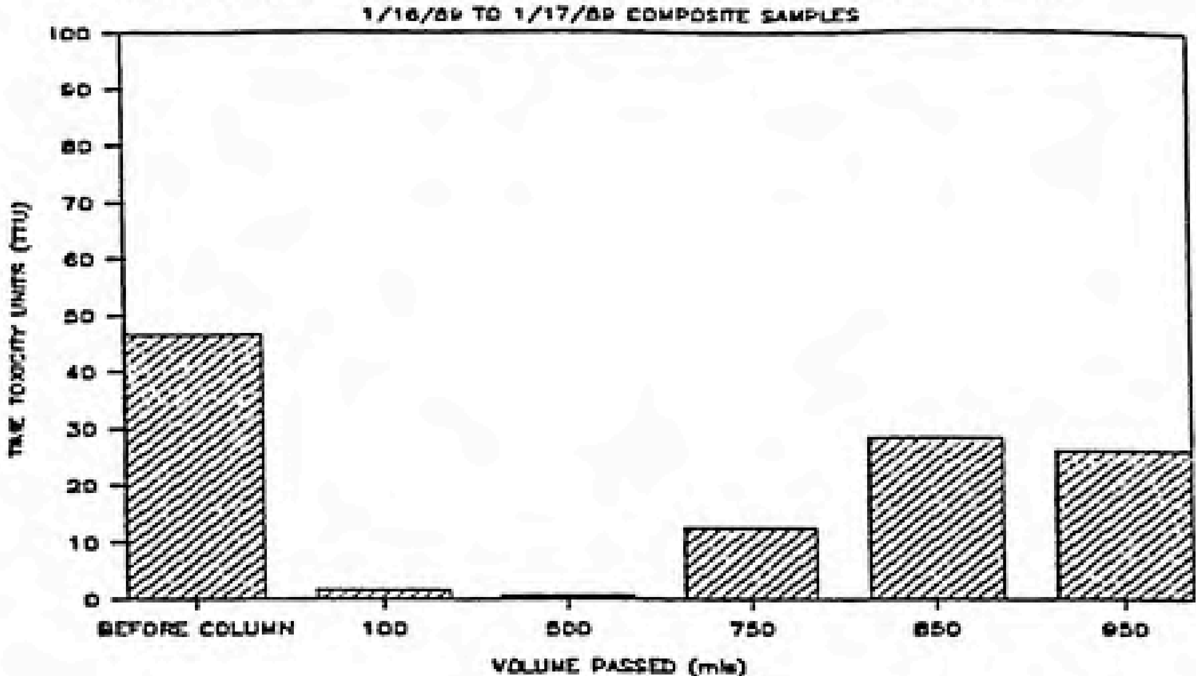


Figure 3.8 Composite Sample 1/16 to 1/17/89, Imaginary Creek WWTP, Example, NC (a) Post-Column Effluents.

after passage. The initial volume passed has no toxicity remaining. However, toxicity increases as more sample is passed thus indicating that the sorptive capacity of the C18 column has been exceeded. Reducing the volume passed from one liter to 500 ml would most likely alleviate this problem.

Bar graphs of the TTU values will always be presented using a 0 to 100 percent scale as shown in Figure 3.8. This gives the proper qualitative perspective of toxicity from sample to sample and for the various percent $MeOH/H_2O$ fractions. The intent of such bar graphs is to illustrate the efficiency of the column in retaining toxic compounds

and in segregating them into different fractions.

F. Target Compound Selection

Phenol (Ph), 1-methylnaphthalene (MN), nonyl phenol ethoxylate 9 mole (NPE) and di-n-octyl phthlate (DOP) were selected to determine if the C18 SPE Column provides an accurate means of detecting their presence. The selection criteria were:

1. wide representation of log octanol/water partitioning coefficients,
2. acute toxicity to Ceriodaphnia at reasonably attainable concentrations and
3. a concern at Highpoint Westside WWTP in North Carolina, a case study in this research, or at POTWs in general.

Table 3.4 is a summary of the physical and chemical properties of these four compounds.

The octanol/water partition coefficient (Kow) is the ratio of a chemical's concentration in the octanol phase to its concentration in the aqueous phase in a two-phase system. Ph and DOP were selected because they gave a very wide range of log Kow values (1.46 to 9.2). Such a wide range was necessary to determine if a direct relationship exists between increasing log Kow (i.e.,increasing non-polar nature) and elution in increasing percent MeOH/H_2O fraction. These compounds were selected to represent the ends of the partitioning coefficient scale. Ph is acutely toxic to Ceriodaphnia at a lethal concentration in which 50 percent (LC50) mortality is observed after a 48 hr period of 4.3

Table 3.4
Target Compounds
Summary of Physical and Chemical Properties

	Phenol (Ph)	1-Methylnaphthalene (MN)	Nonylphenol Ethoxylate 9 mole (NPE)	Di-n-octyl Phthalate (DOP)
Chemical Formula	C_6H_6O	$C_{10}H_7CH_3$	$C_{33}H_{60}O_{10}$	$C_{24}H_{38}O_4$
Chemical Structure	OH	CH_3	$O(CH_2CH_2O)_9H$; R; R = $-CH(CH_3)-CH_2-CH(CH_3)-CH_2-CH(CH_3)-CH_3$ (printed as R-CH-CH2-CH-CH2-CH-CH3 with CH3 under each CH)	$C(=O)-O-C_8H_{17}$; $C(=O)-O-C_8H_{17}$
Molecular Weight	94.11 gm/mole (26)	142.2 gm/mole (22)	616.4 gm/mole (25)	391.0 gm/mole (26)
Log Octanol/Water (Observed) Partition Coefficient	1.46 (26)	3.9 (24)	--------	9.2 (26)
Log Octanol/Water Partition Coefficient (Estimated)	1.46 (27)	3.84 (27)	7.8 (28)	9.53 (27)
Density	1.071 gm/cm3 (26)	1.0202 gm/cm3 (22)	1.057 gm/cm3 (25)	0.982 gm/cm3 (26)
Solubility	93,000 mg/L @ 25 C (26)	--------	--------	3 mg/L @ 25 C (26)
Vapor Pressure	0.5293 torr (26)	--------	--------	< 0.2 torr @ 150 C (26)
pKa	10.02 (26)	--------	--------	--------
LC50 Value	4.3 mg/L @ 24 C (21)	1.42 mg/L (21)	5.5 mg/L (23)	0.32 mg/L (**)

(**) NOEC Reproduction of Daphnia magna (21)

mg/L. Ph has also been identified as one of the compounds discharged from the Highpoint Westside WWTP (21). There were no lethal concentrations values found for DOP. However, a no-effect concentration value (NOEC) was located for the reproduction of Daphnia magna at 0.32 mg/L. DOP has been identified in the influent stream of the Highpoint Westside WWTP (21).

MN was selected because its log Kow is 3.9, thus being between Ph and DOP (24). MN is acutely toxic to Ceriodaphnia (LC50 value of 1.42 mg/L), and it has been identified in both the influent and effluent of the Highpoint Westside WWTP.

NPE is an all purpose detergent and wetting agent used for its good dispersing and emulsifying qualities (25). This compound was chosen because it is presently being used by industries at Highpoint, is acutely toxic to Ceriodaphnia (LC50 = 5.5 mg/L), and generally represents an important class of pollutants (surfactants) in municipal wastewater treatment. The log Kow is suspected to be greater than the reported value for MN. However, no value was found in the standardly used compendium of log Kow (26, 27, 28). Therefore , only an estimate could be made. The value of 7.8 reported in the summary table was determined using a linear regressed equation which relates log Kow with density values (28). This value should be viewed as a crude estimate. Additionally, a value of 15.9 was determined from a linear relationship between log Kow and molecular weight

(28). Structural fragment addition led to a log Kow value of -7.35 (27). There was no measured value found in the literature. The calculations used to determine log Kow estimations for all of the target compounds are shown in Appendix E.

G. Selection of WWTPs for TIE

Secondary effluents from the Westside WWTP in Highpoint, North Carolina and the Cross Creek WWTP in Fayetteville, North Carolina were tested using the procedures outlined in Phase I (Toxicity Characterization Procedures) of the TIE protocol. Additionally, Phase II (Toxicity Identification Procedures) C18 column elutions were conducted on samples which indicated a reduction in toxicity after passage through the C18 Column.

A flow diagram of the Westside WWTP at Highpoint is given in Figure 3.9. Biological treatment consists of a trickling filter and activated sludge system operating in series. The detention time in activated sludge treatment is about 14 hr, providing for excellent nitrification. The design flow rate is 6.2 MGD. Before discharging into Rich Fork Creek, the effluent is passed through a tertiary filter. About 15 industries, including metal platers and finishers, oil manufacturing, textiles, organic chemical manufacturing, and drum cleaning, discharge their wastewater to this plant; these comprise about 12% of the flow. The effluent stream from the treatment facility is approximately

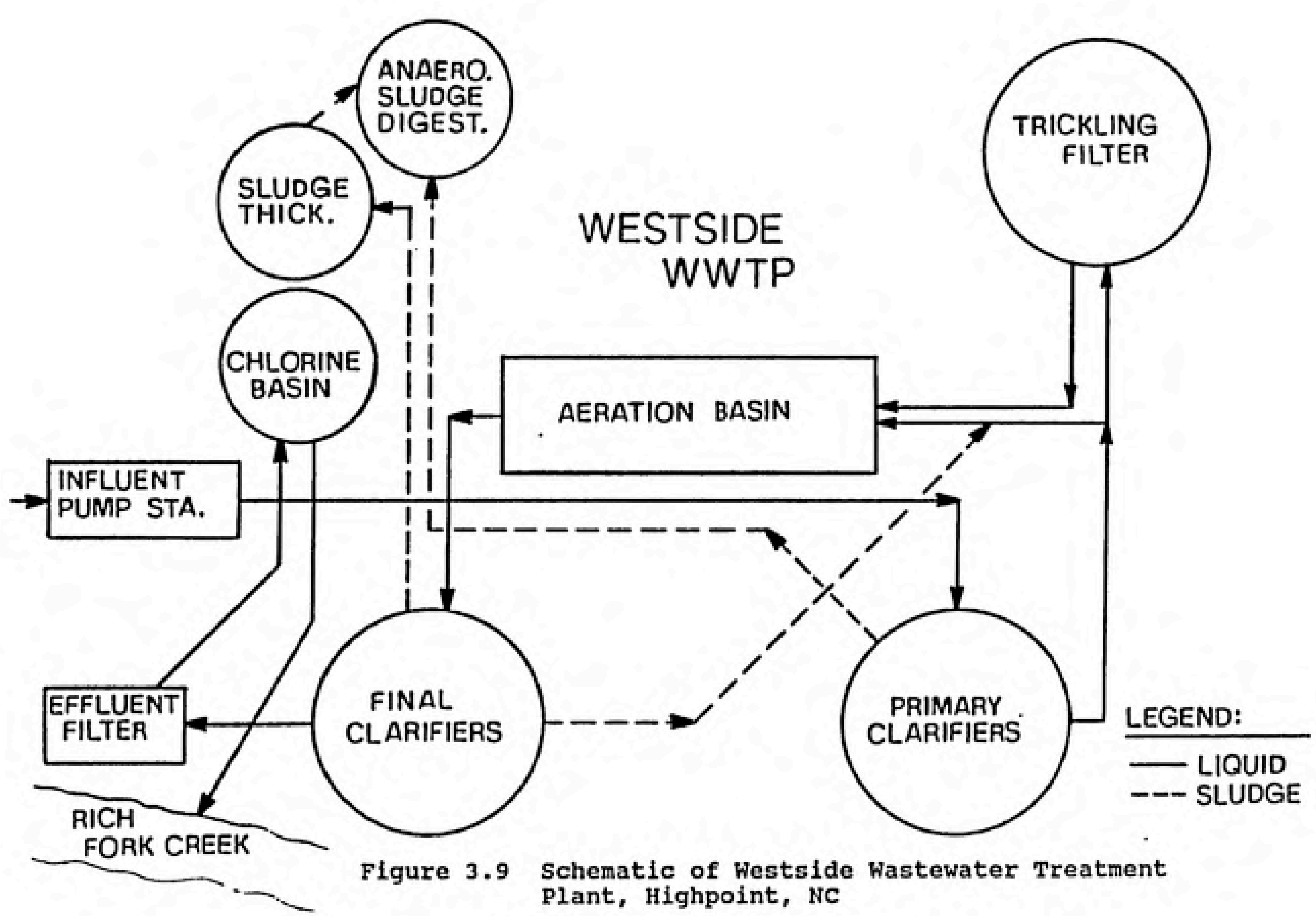


Figure 3.9 Schematic of Westside Wastewater Treatment Plant, Highpoint, NC

95 percent of the total stream flow expected during a 7Q10 low flow period. Thus, the State of North Carolina has imposed strict biomonitoring requirements. A more complete description of the treatment facility and operational characteristics is given by Storm, DiGiano, and Christman (21).

A flow diagram for the Fayetteville - Cross Creek WWTP is given in Figure 3.10. The treatment processes consist of a pure oxygen activated sludge process, clarifiers and post instream chlorination in series (29). There is no primary clarification before aeration and the tertiary filters as well as the sludge drying beds are not in use. The detention time in the activated sludge process is only 2 hr which means that no nitrification occurs. Fayetteville, Mills and the Town of Hope are the major domestic sources contributing wastewater to this facility. Approximately 10 percent of the facility's total design flow (16 MGD) is considered industrial waste by volume. Typical industrial sources contributing wastes to the facility are organic chemical manufacturing, textiles, metal platters and finishers, oil manufacturing and a large tire manufacturer. The instream waste concentration (IWC) is 3.58 percent based on the 7Q low flow period for the Cape Fear River (29). Because the IWC is very low, the effluent is diluted sufficiently to allow the Cross Creek WWTP to pass the State of North Carolina biomonitoring requirements for acute toxicity. However, this plant still fails to meet limits

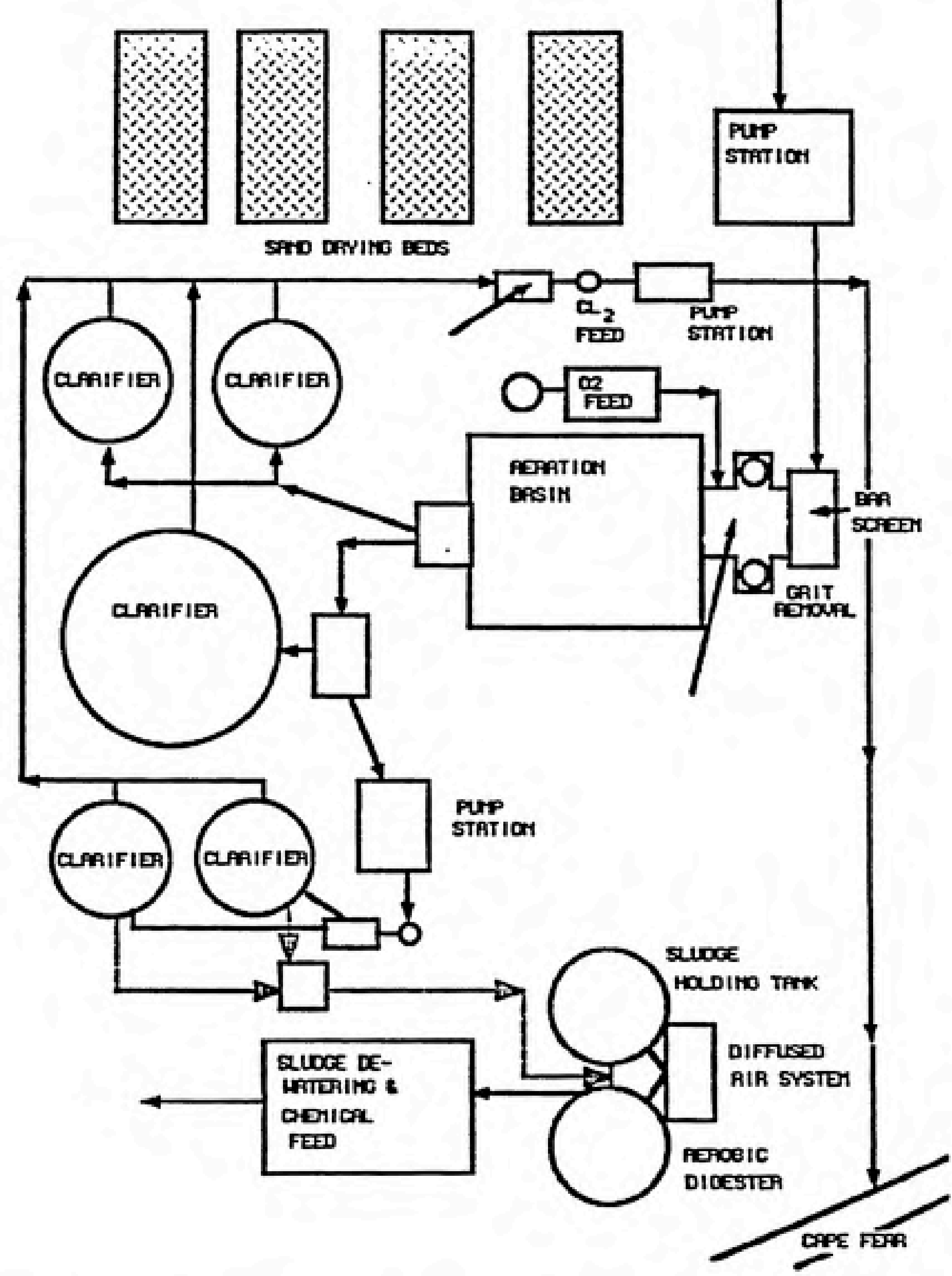


Figure 3.10 Schematic of Fayetteville - Cross Creek Wastewater Treatment Plant, Fayetteville, NC

est for chronic toxicity.

IV. EXPERIMENTAL RESULTS

A. Background Toxicity Sources

A.1 Evaluation of Methanol (MeOH)

While MeOH was chosen by EPA to be the appropriate solvent for the C18 SPE column elutions, it is known to cause acute toxicity to Ceriodaphnia at fairly low concentrations. In the January 1987 draft of Phase I, Toxicity Characterization Procedures (14) and subsequently in the City of Largo Evaluation (11) and in the Las Vegas Report (12), 2.0 percent MeOH was reported as the concentration at which no mortality was observed over a 48 hr duration. Before proceeding with this study, it was deemed important to confirm that dilution of MeOH/water fractions to 2 percent MeOH would not cause background toxicity.

Early results suggested that 2 percent MeOH was not toxic. However, continual blank testing through the target compound evaluation indicated sporadic toxicity. The LC50 of MeOH was repeatedly tested during this research as illustrated in Table 4.1. The results confirm the suspicion of significant toxicity at about 2 percent.

Table 4.1
Methanol Control Testing

Date	LC50 VALUE(30)	95% Confidence
4/27/88	2.9%	2.6 to 3.3
4/27/88	2.9%	2.6 to 3.3
4/27/88	2.9%	2.6 to 3.3
4/27/88	2.4%	2.1 to 2.7
7/19/88	2.0%	1.5 to 2.5
7/22/88	2.0%	1.9 to 2.0

Personal contact with Dr. Mount of U.S./EPA Duluth Laboratory (31) confirmed these results; MeOH contamination by either the manufacturer's distillation process or laboratory use were suggested as causes. Dr. Mount recommended decreasing the MeOH percentage to 1.5 to avoid such problems.

Even after adopting 1.5 percent MeOH, some mortality was noted within a 48 hr period. This was accounted for as "background toxicity" and subtracted from total toxicity of the sample through use of the Time-based Toxicity Unit (TTU).

A.2 Effect of Bioassay Cup Material on MeOH Toxicity Results

The results of MeOH bioassays using plastic and glass cups are presented in Figures 4.1a and b, respectively. The raw data are given in Table D.1 of Appendix F. Figure 4.1a

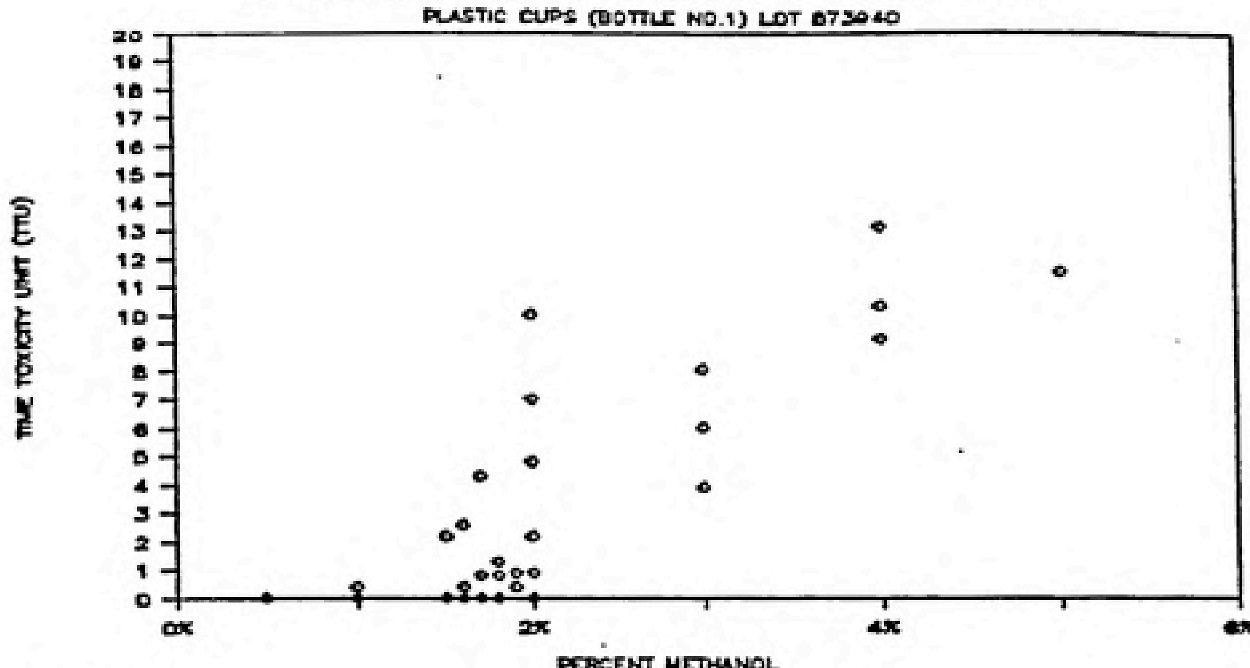


Figure 4.1a

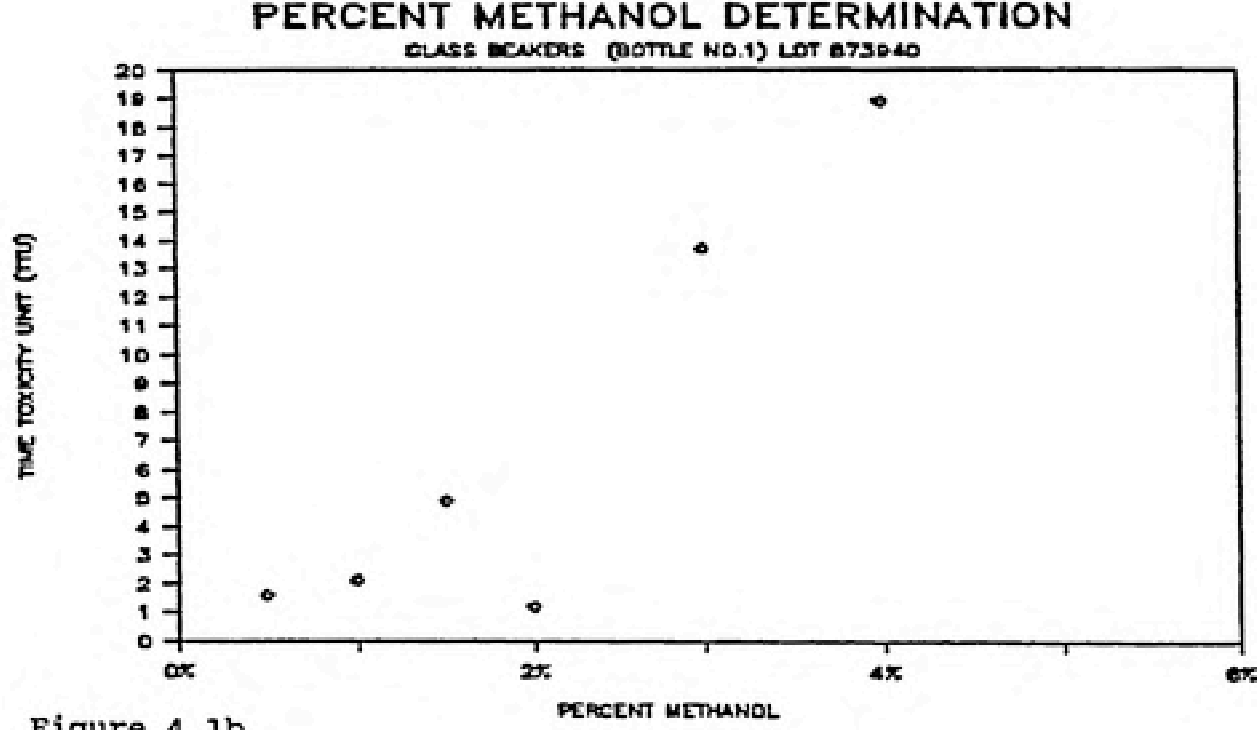


Figure 4.1b

Figure 4.1 Evaluation of Methanol Toxicity in (a) Plastic Cups and (b) Glass Beakers

shows mortality occurred between 1.5 to 2.0 percent MeOH whereas Figure 4.1b shows mortality at even lower percent MeOH (it should be noted that bioassays were conducted in 10 ml glass beaker, as opposed to a 30 ml plastic cups). These results suggested that the Ceriodaphnia experienced a higher degree of sensitivity to the MeOH when the test was conducted in glass. No reason was determined for this result.

A.3 Toxicity of MeOH/H_2O Eluents from the C18 Column

The toxicity of MeOH/H_2O eluents from the C18 column were tested as outlined by the procedures given in section IIIA. These MeOH/H_2O solutions served as controls to check the possibility of MeOH reacting with the C18 sorbent to elute compounds that may cause toxicity to Ceriodaphnia. As mentioned in the C18 SPE procedure, the MeOH/H_2O solutions (25, 50, 75, 80, 85, 90, 95, 100 % MeOH/H_2O) were introduced to the column prior to the sample in each experiment. Figures 4.2a, b and c illustrate the toxicity of these eluents (after required dilution) using plastic (Figure 4.1a and c) and glass (Figure 4.2b) bioassay cups. The raw data are given in Table D.2 and Table D.3 of Appendix F. All three figures show that mortality existed at 1.5% MeOH. Figure 4.2 shows that more mortality was observed using glass rather than plastic cups which is consistent with results of MeOH control testing presented in Figures 4.1a and b.

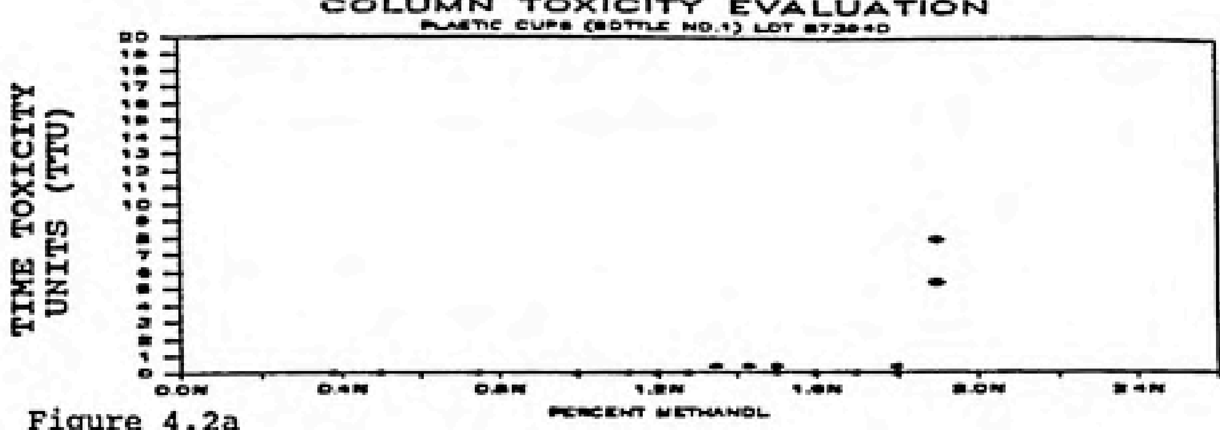


Figure 4.2a

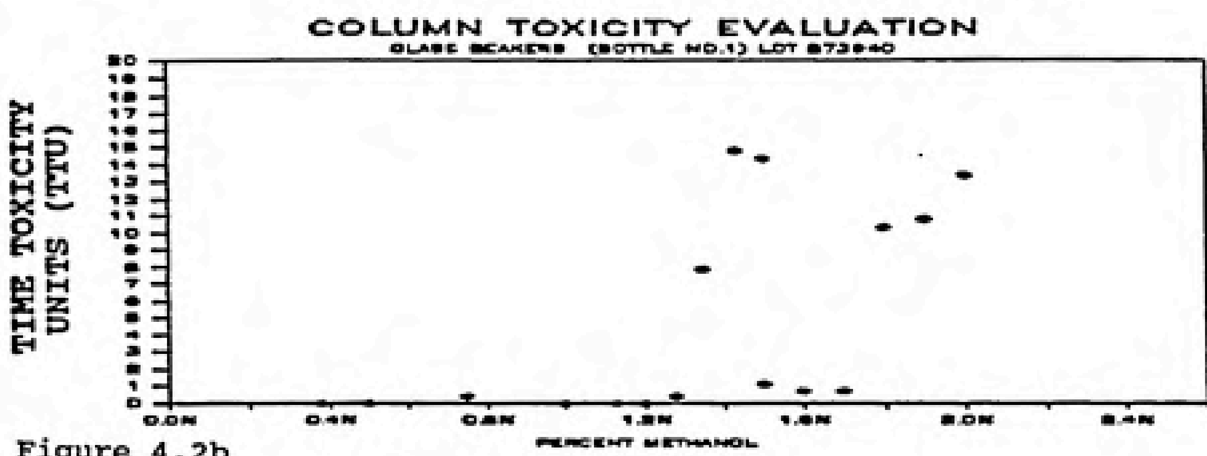


Figure 4.2b

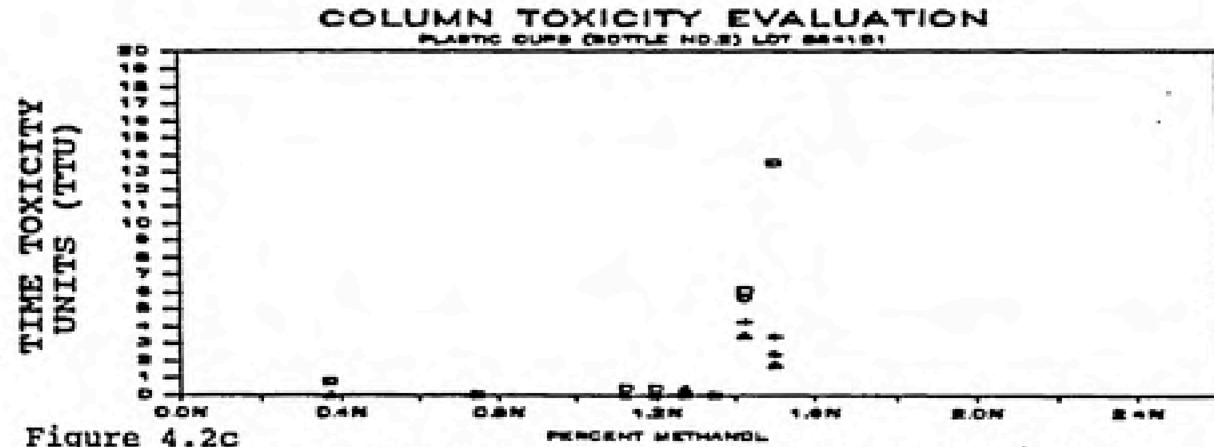


Figure 4.2c

Figure 4.2 Evaluation of Toxicity Eluted from C18 SPE Column Using (a) Plastic Cups (Methanol From Lot 873940); (b) Glass Beakers (Methanol From Lot 873940); and (c) Plastic Cups (Methanol From Lot 884151).

A.4 Toxicity of Diluent Water

Because the target compounds were added to diluent water, a control study was needed to insure that the diluent was not causing toxicity either in the samples passed through the column or in the elution of the column with $MeOH/H_2O$. The two diluents used in the evaluation of the target compounds were "non-toxic" effluent from the Westside WWTP at Highpoint, North Carolina and natural water from Botany Pond in Chapel Hill, North Carolina. The phrase "non-toxic" effluent refers to wastewater samples that were determined not to cause mortality of the *Ceriodaphnia* after a 48 hr period.

Figure 4.3 illustrates the results of passing "non-toxic" effluent through a C18 column and subsequently eluting it with various $MeOH/H_2O$ fractions. No toxicity was found in this diluent sample before introduction to the column (see BEFORE COLUMN in Figure 4.3a). The slight amount of toxicity found in the post-column effluent sample taken after 850 ml of the diluent had passed may have been caused by contamination of the collection vial or retention of the MeOH used during the conditioning of the column; this is not considered a serious problem. The toxicity of $MeOH/H_2O$ fractions eluted from the column after passage of the Westside WWTP diluent is presented in Figures 4.3b (plastic cups) and 4.3c (glass beakers). The 95 and 100 percent $MeOH/H_2O$ fractions were slightly toxic, a result

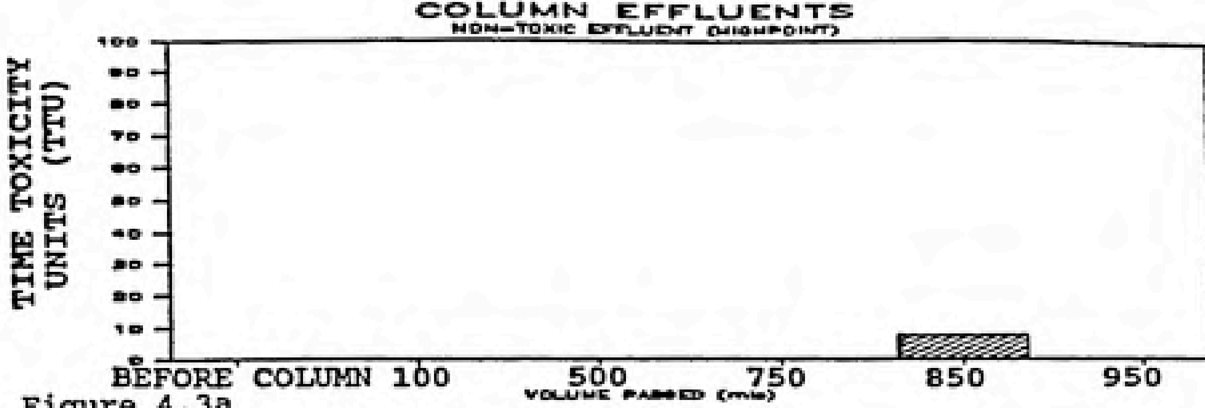


Figure 4.3a

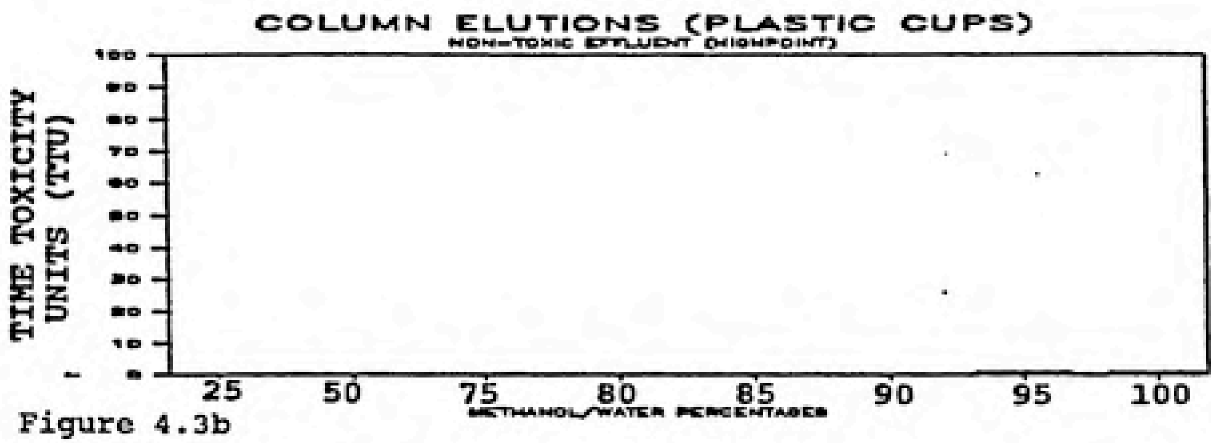


Figure 4.3b

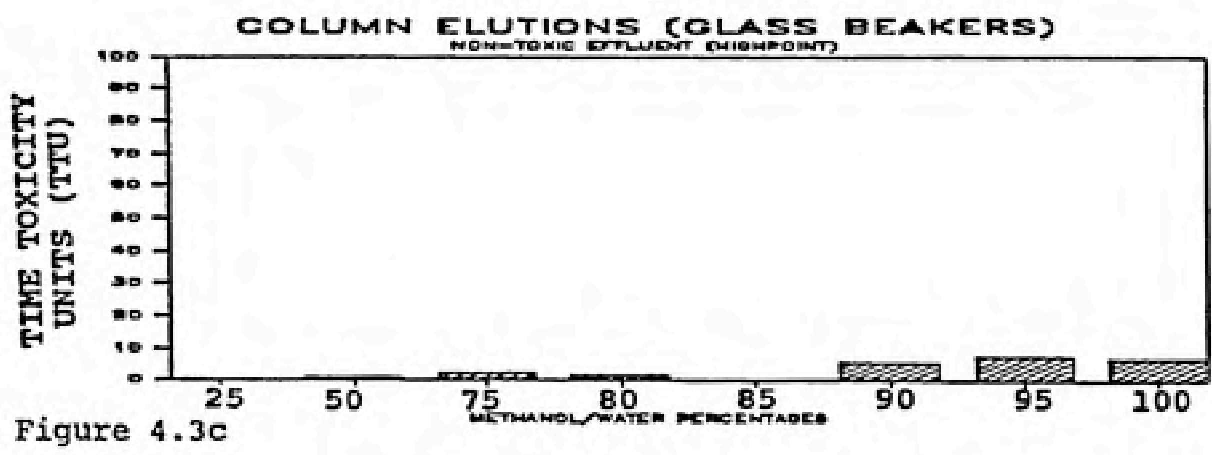


Figure 4.3c

Figure 4.3 Control Study of C18 SPE Column Using Non-Toxic Effluent Shown by (a) Post-Column Effluents; (b) Column Elutions (Conducted in Plastic Cups); and (c) Methanol Elutions (Conducted in Glass Beakers).

typically associated with the higher percent MeOH/H_2O fractions. The final percent MeOH for conducting the bioassays from these fractions were 1.43 and 1.5, respectively. These are close to the percentage where toxicity due to MeOH itself is found.

A comparison of Figure 4.3b and c shows that more toxicity was eluted when glass rather than plastic cups were used for the bioassay; this is again consistent with earlier control experiments. The small toxicity peak at 75 percent MeOH in Figure 4.3 (c) is also of interest. This may be due to the concentration of sublethal compounds; a result which in theory is possible and is noted by EPA in their Phase II Toxicity Characterization Procedures (5). Chemical specific analyses were not conducted to verify this possibility.

The toxicity of the other diluent used - Botany Pond water - is given by Figure 4.4. These results can be compared directly to those in Figure 4.3 for "non-toxic" Westside WWTP effluent as the diluent. No toxicity was found before passage through the column (see BEFORE COLUMN in Figure 4.4a) and very little was observed in samples collected after passage (see 500 and 850 ml post-column effluent samples in Figure 4.4b). The only toxicity noted during MeOH/H_2O elution was found in the 95 and 100 percent MeOH/H_2O fractions (Figure 4.4b). As noted before, this artifactual toxicity appears unavoidable and was probably due to MeOH rather than diluent toxicity. It is subtracted from the toxicity measured for the target compounds are

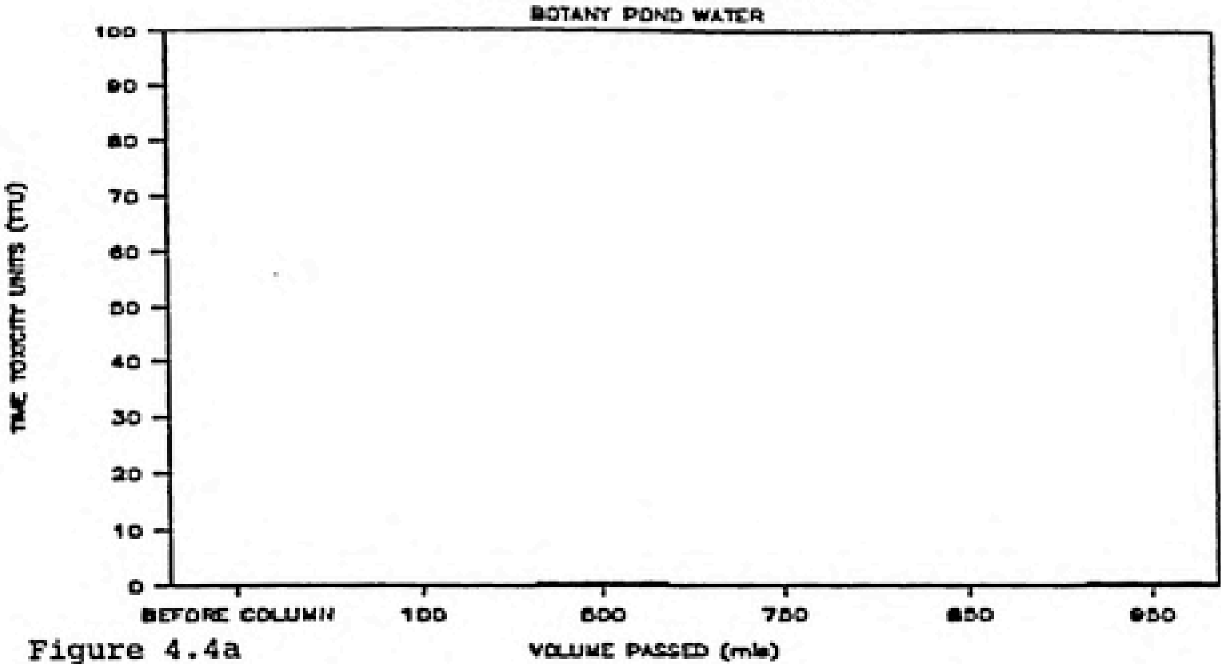


Figure 4.4a

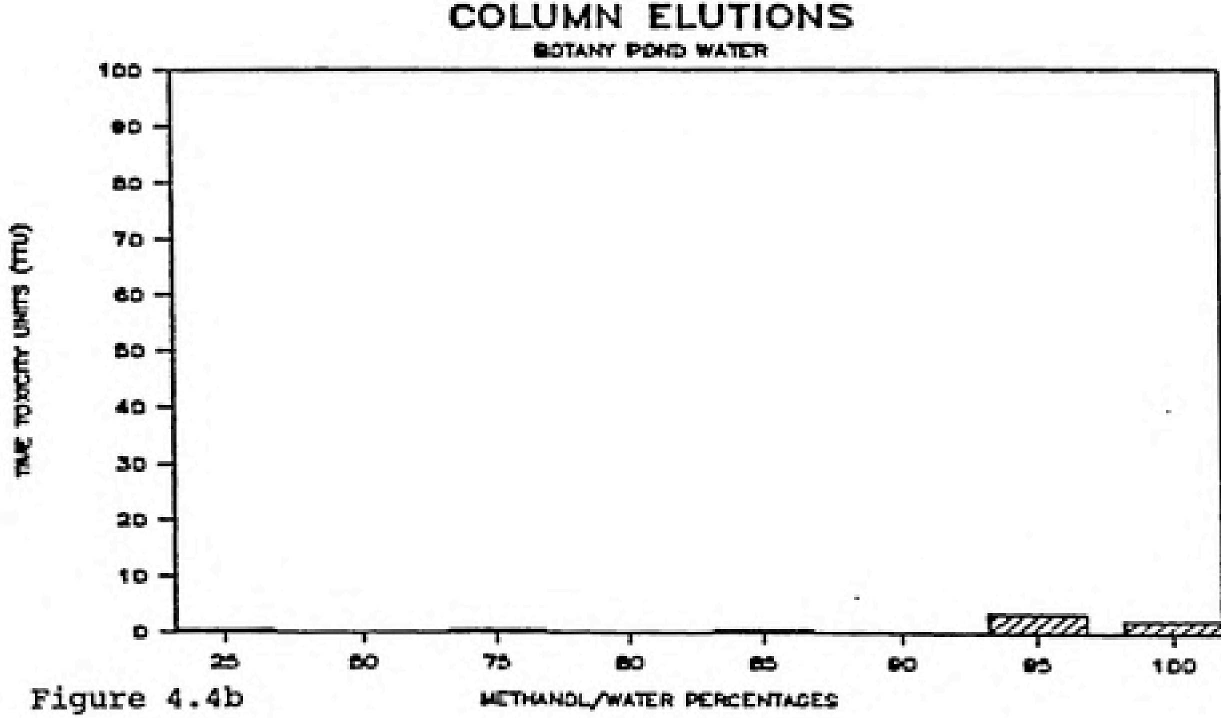


Figure 4.4b

Figure 4.4 Evaluation of the Toxicity of Botany Pond Water to be Used as a Diluent Water Using (a) Post-Column Effluents and (b) Methanol Elutions.

tested.

B. Evaluation of Target Compound Toxicity

B.1 Phenol (Ph)
Concentration Tested: 43 mg/L

Figure 4.5a shows the results of passing a Ph solution (43 mg/L) prepared with Botany Pond water, through a C18 column. Ph was not retained by the C18 column. If it was retained, toxicity present before passage would have been removed by the column. Instead, all post-column effluent samples contained toxicity equal to that of the feed to the column. The absence of toxicity in the eluted fractions (Figure 4.5b) further reinforces this point. The Ph solution had a measured pH of 7.8, approximately two log units lower than the reported pKa for Ph of 10.02 (25) or 9.9 (32). Thus, Ph was at least 99% in its uncharged form, which theoretically should favor removal by non-polar interactions between Ph and the sorbent.

The Phase I Toxicity Characterization Procedures for the C18 column included adjusting sample pH to 3 and 9 before passage (see Section IIB). However, no retention of Ph toxicity occurred at either pH value (Figure 4.6a). Further confirmation of lack of Ph retention was obtained by absorbance measurements (268 nm) of the solution, before and after the column (Figure 4.6b). Figure 4.6b does show a slight decrease in absorbance at pH 3 after 25 ml have passed; however, this was not supported by corresponding

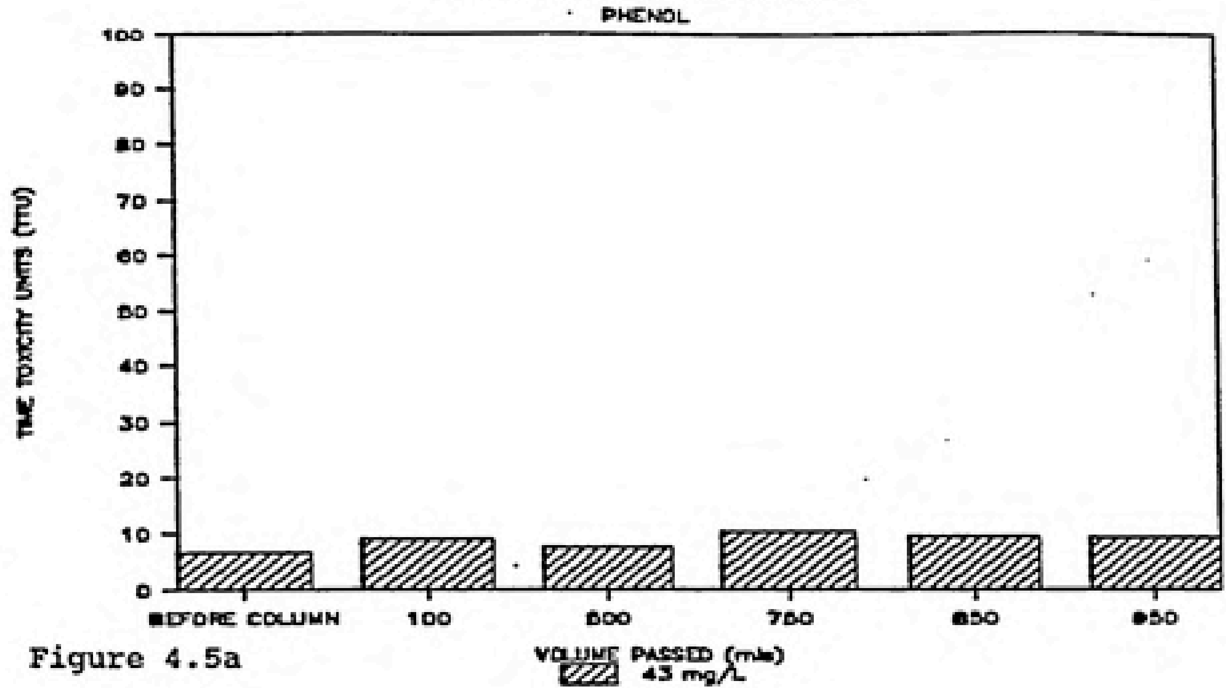


Figure 4.5a

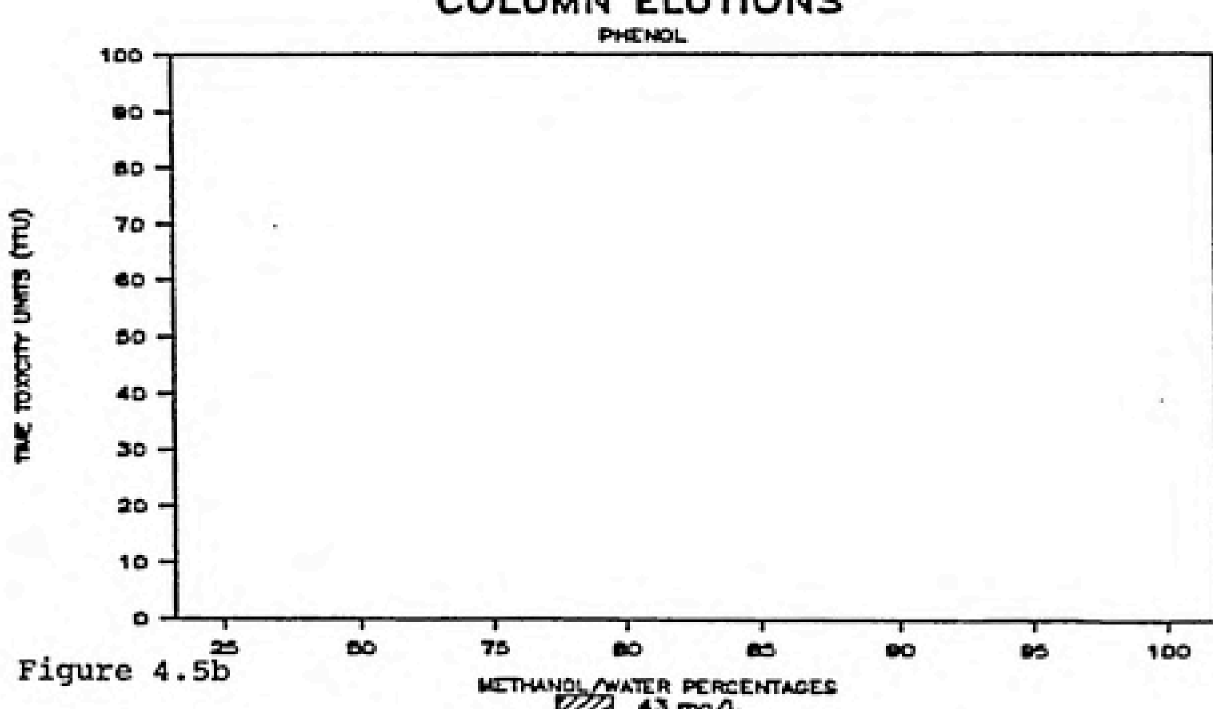


Figure 4.5b

Figure 4.5 Ability of C18 SPE Column to Isolate and Elute Toxicity Associated with Phenol Using (a) Post-Column Effluents and (b) Methanol Elutions.

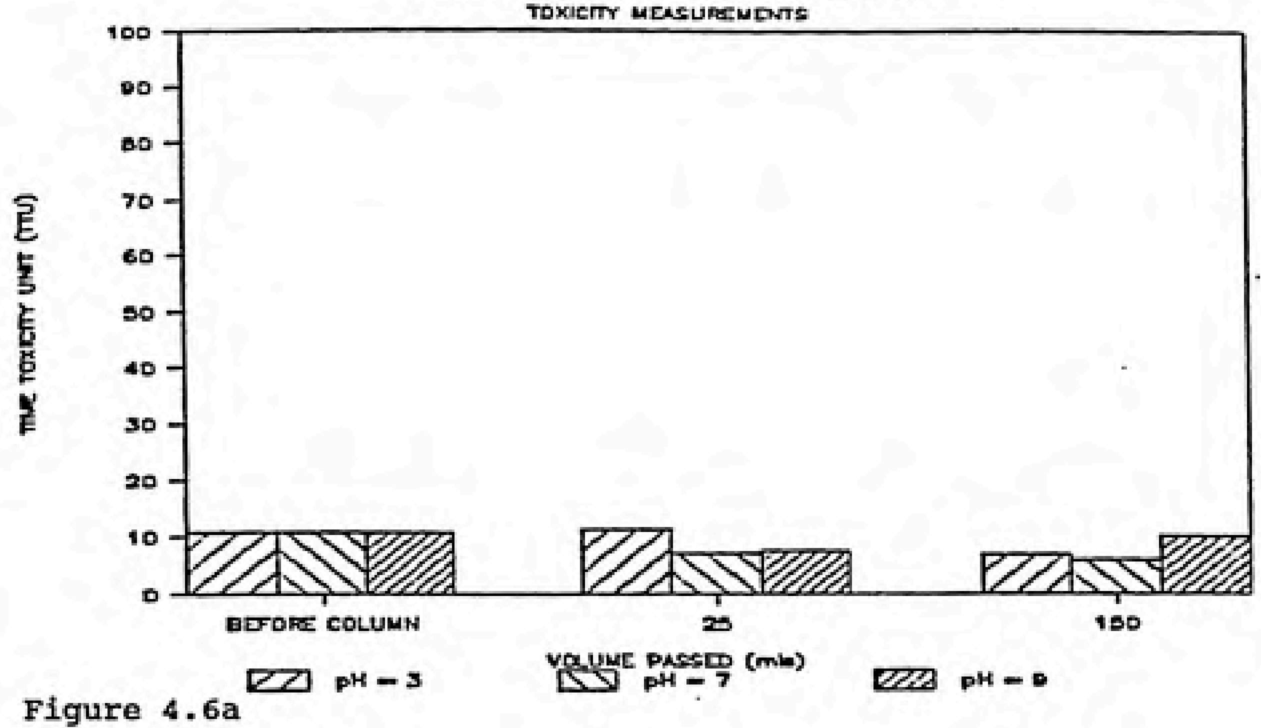


Figure 4.6a

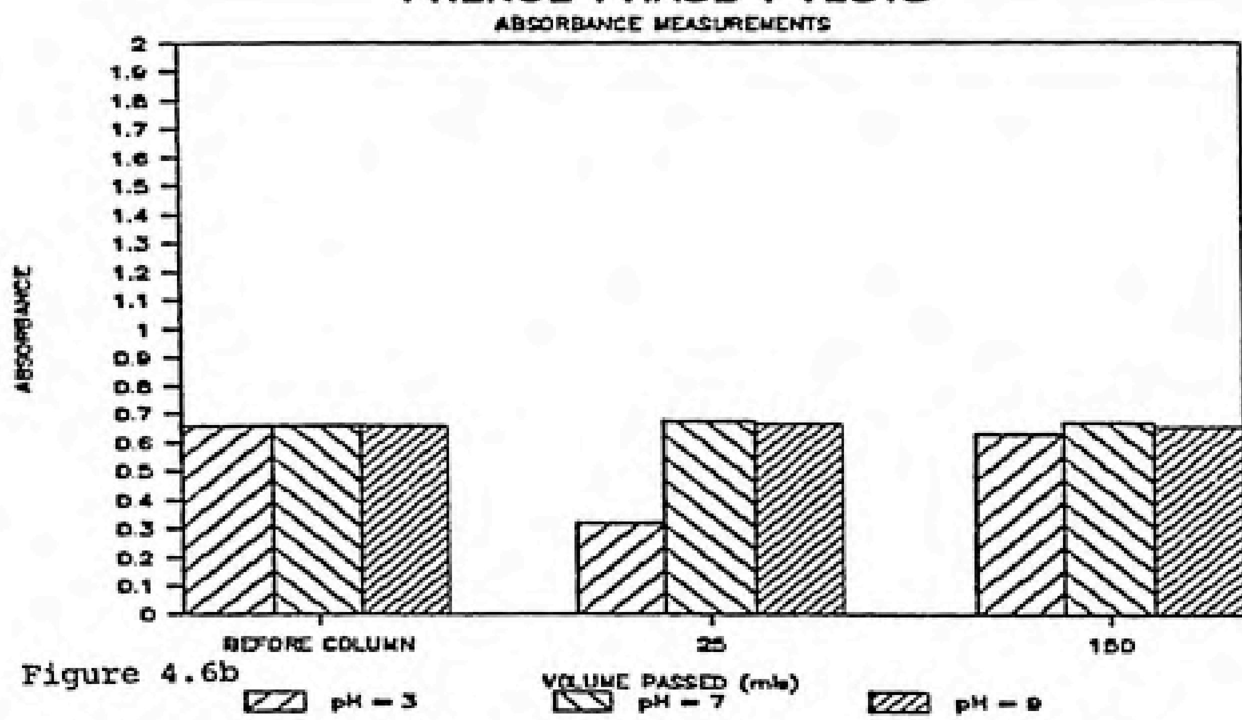


Figure 4.6b

Figure 4.6 Verification of C18 SPE Column's Inability to Retain Phenol as Demonstrated by Phase I TIE Test Procedures Using (a) Toxicity and (b) Absorbance Measurements.

toxicity measurements (Figure 4.6a). The C18 column would appear to be unable to retain Ph from the spiked solution.

Two alternative explanations are possible for failure of the C18 SPE Column to retain Ph. The first centers around the low Kow (log Kow of 1.46), and high water solubility (93,000 mg/L at 25°C (26)) of Ph. Both indicate relatively high polarity and suggest that Ph should not be retained by a non-polar sorbent. The second explanation involves the conditions used for operating the C18 SPE Column. The flow rate (5 ml/min) and volume (1 Liter) of sample used by SPE to concentrate organic toxicants from dilute aqueous samples is considerably greater than the flowrate (0.02 ml/min) and volume (microliters) used in HPLC column work. This may explain why C18 HPLC Columns are able to retain Ph, while C18 SPE Columns are not. At the volume and flowrate used for SPE, Ph may be retained momentarily and then subsequently eluted back into the aqueous mobile phase.

B.2 1-Methylnaphthalene (MN)
Concentrations Tested: 2.9, 7.1, 35.5 mg/L

Because MN was the first target compound to be tested, the toxicity artifact caused by 2.0 percent MeOH had not been realized before experiments began. It was therefore necessary to modify the procedure outlined in the preceding section (Section IIIA.3) to maintain a maximum of 1.5 percent MeOH in the bioassay. Instead of conducting another series of C18 column tests (based on 1.5% MeOH) and

generating new elutions, 150 ul of each MeOH/H_2O eluent (based on the original 2.0% MeOH) was diluted with 10 ml of dilution water. This modification insured a maximum of 1.5 percent MeOH and a concentration factor of 3.75X at the point of bioassay testing. The only significant change from the original procedure is a concentration factor of 3.75X instead of the usual 5X. The results indicate no appreciable loss in toxicity even though the concentration factor had been decreased.

The C18 column test was performed using three different MN concentrations (2.9, 7.1, 35.5 mg/L). Figure 4.7a shows an increase in toxicity with increasing concentration as expected (see BEFORE COLUMN results), and no significant breakthrough of toxicity with passage of up to 1L of sample, even at the highest feed concentration. MeOH/H_2O elutions, using plastic (Figure 4.7b) and glass (Figure 4.7c) both showed that the 80 percent MeOH/H_2O fraction contained most of the toxicity, regardless of the MN concentration introduced to the column. The recovery of toxicity from these three different feed concentrations of MN is also consistent, i.e., the TTU value for the 80 percent MeOH/H_2O fraction is highest for the highest feed concentration. As noted before, the column elutions conducted in plastic cups seem to attenuate toxicity to _Ceriodaphnia_ when compared to results using glass. MN was the only target compound tested in plastic and glass; the bioassays for the other compounds were conducted in plastic cups as outlined by the standard

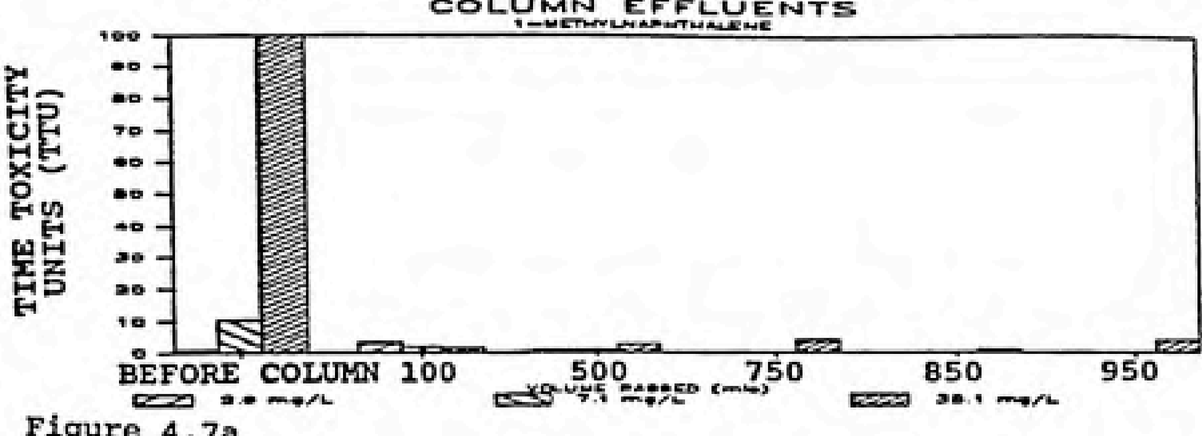


Figure 4.7a

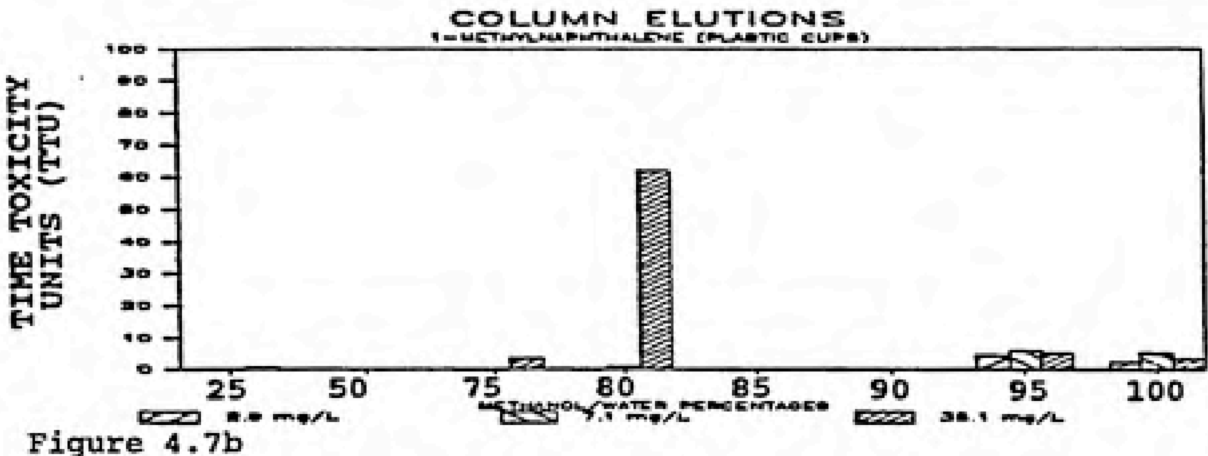


Figure 4.7b

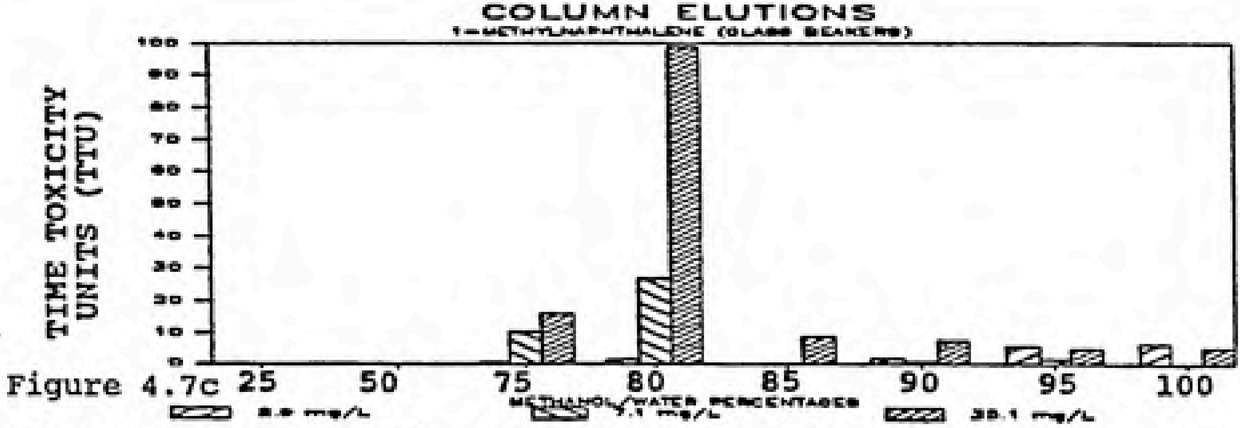


Figure 4.7c

Figure 4.7 Ability of C18 SPE Column to Isolate and Elute Toxicity Associated with 1-Methylnaphthalene Using (a) Post-Column Effluents (Conducted in Glass Beakers); (b) Methanol Elutions (Conducted in Plastic Cups);and (c) Methanol Elutions (Conducted in Glass Beakers).

procedure (section IIIC).

A comparison of _Ceriodaphnia_ and Microtox bioassay of column elutions is presented in Figure 4.8. The feed concentration of MN selected for this study was 7.1 mg/L, the second highest concentration presented in Figure 4.7. When the _Ceriodaphnia_ test was conducted in plastic cups (Figure 4.8a), no toxicity was recovered in the 80 percent $MeOH/H_2O$ fraction. A similar result was repeated at this same MN feed concentration (Figure 4.8b). Toxicity in the higher $MeOH/H_2O$ fractions (90 and 95%) was due either to the additive effects between MeOH and MN or due to MeOH alone. However, the Microtox bioassay procedure was capable of showing the 80 percent toxicity peak (Figure 4.8c) that had not been observed using the standard _Ceriodaphnia_ test (Figure 4.8a) conducted in plastic cups. Therefore, these results of MN suggest Microtox to be more sensitive than the _Ceriodaphnia_ bioassay procedure when plastic cups were used. Comparing results in Figure 4.8a and Figure 4.8b again confirmed that glass increases the sensitivity of the _Ceriodaphnia_ bioassay. Comparison of Figure 4.8b and c therefore , shows a similar response of the two bioassay procedures in recovery of toxicity in the 80 percent $MeOH/H_2O$ fraction.

B.3 Nonylphenol Ethoxylate 9 Mole (NPE)
Concentrations Tested: 11, 27.5, 55 mg/L

The feed concentrations of NPE through the C18 column were 11, 27.5, 55 mg/L. Figure 4.9a demonstrates that all

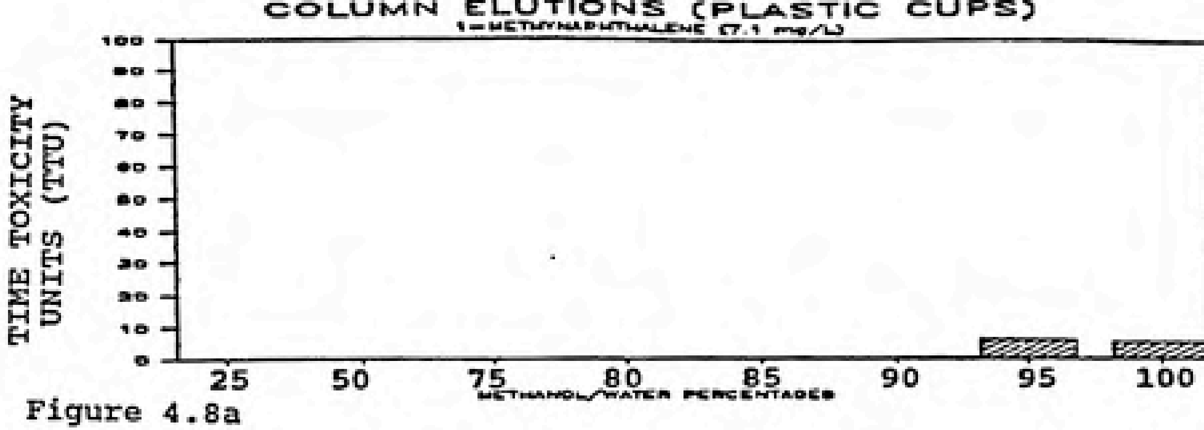


Figure 4.8a

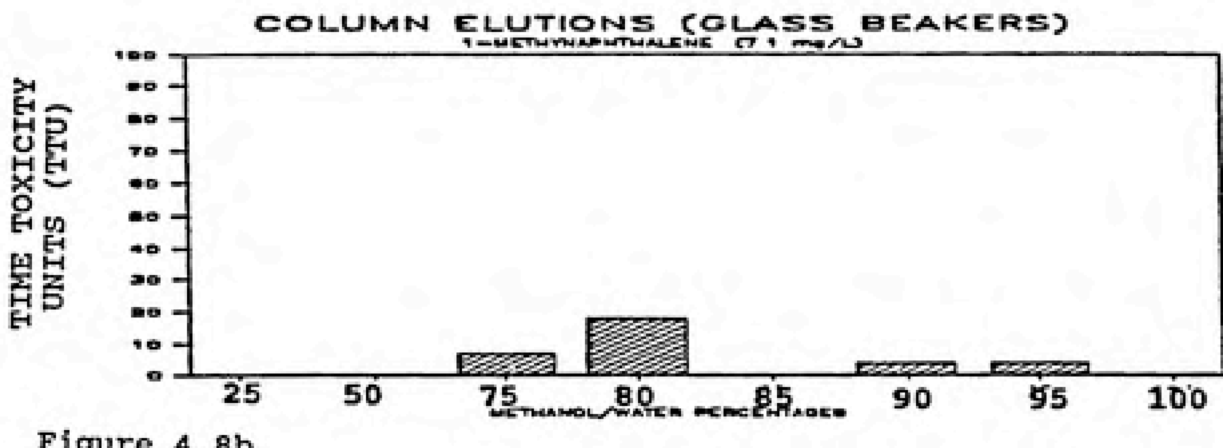


Figure 4.8b

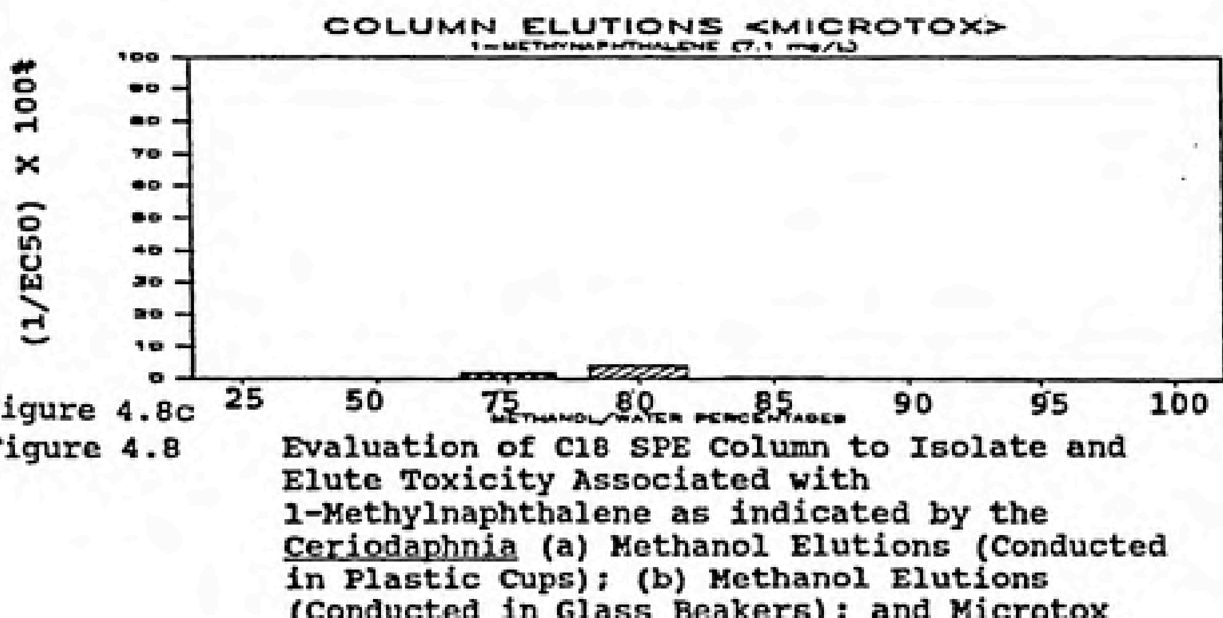


Figure 4.8c

Figure 4.8 Evaluation of C18 SPE Column to Isolate and Elute Toxicity Associated with 1-Methylnaphthalene as indicated by the Ceriodaphnia (a) Methanol Elutions (Conducted in Plastic Cups); (b) Methanol Elutions (Conducted in Glass Beakers); and Microtox (c) Methanol Elutions.

three solutions had significant initial toxicity (see BEFORE COLUMN results) which was in all cases retained completely by the C18 sorbent. Figure 4.9b shows that feed concentration produced some variability in the location of MeOH/H_2O fraction that eluted most of the toxicity. At the lowest concentration (11 mg/L), the 85 percent MeOH/H_2O fraction was only slightly more toxic than the 80 percent MeOH/H_2O fraction whereas the highest concentration (55 mg/L), it was the reverse ordering. The important point (shown by Figure 4.9b) is that the elution procedure of the C18 column was unable to isolate cleanly the toxicity due to a single known compound into one MeOH/H_2O fraction. Instead, toxicity peaked in the 80 to 85 percent MeOH/H_2O fractions.

B.4 Di-n-octyl Phthalate (DOP)
Concentrations Tested: 164, 491, 1473 mg/L

Toxicity testing of DOP indicated that a higher concentration was required to produce significant mortality than was originally expected; the 48 hr LC50 was determined in-house to be 90 mg/L. Despite having to use very high concentrations (far greater than the solubility of DOP in water of 3 mg/L at 25°C) as feed to the C18 column and risk the possibility of exhaustion of sorptive capacity, testing was continued because DOP is representative of very non-polar compounds (log Kow > 9) in contrast to Ph and MN.

Early breakthrough of DOP occurred in the C18 column effluent. As shown by Figure 4.10a, the TTU values of the

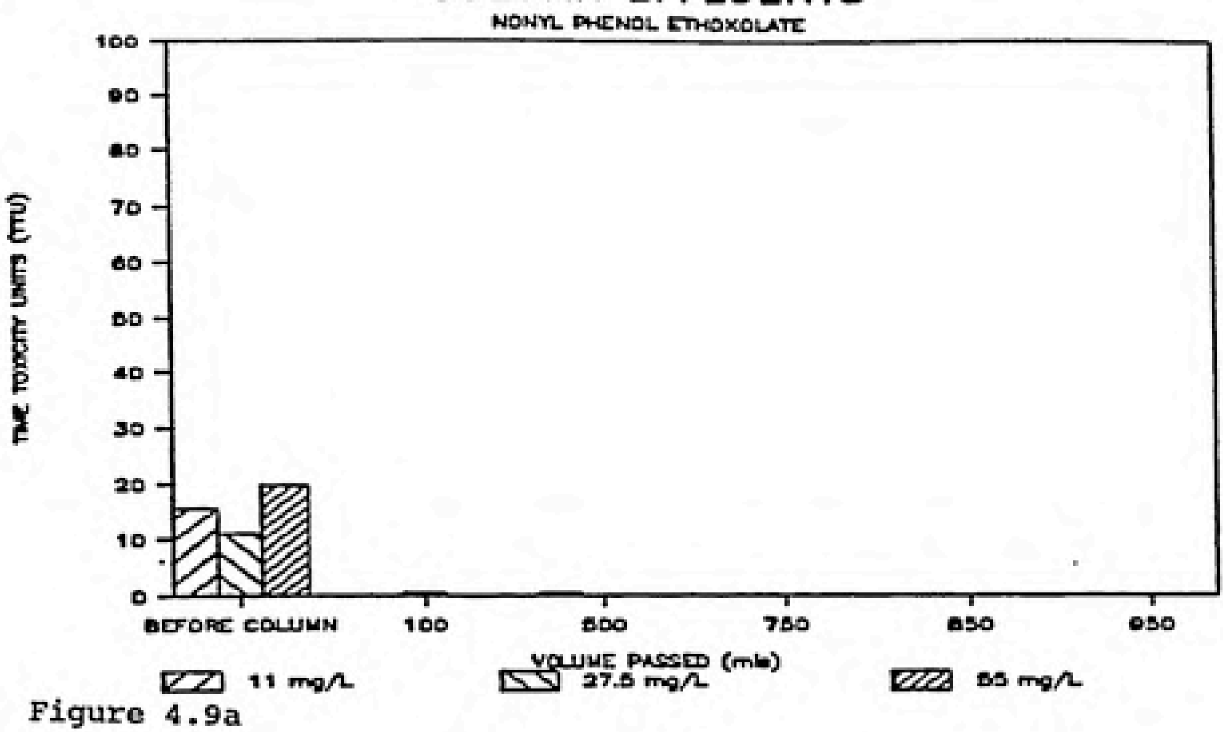


Figure 4.9a

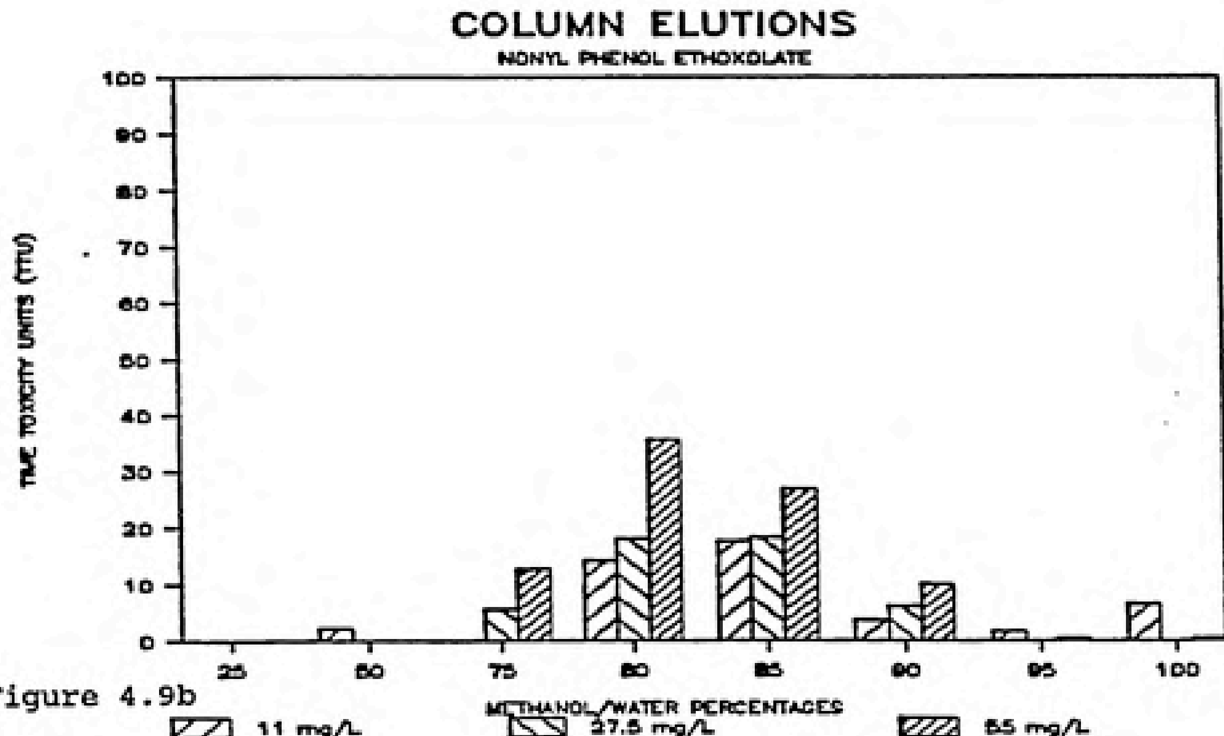


Figure 4.9b

Figure 4.9 Ability of C18 SPE Column to Isolate and Elute Toxicity Associated with Nonyl Phenol Ethoxolate 9 Mole Using (a) Post-Column Effluents and (b) Methanol Elutions.

post-column effluent samples are similar to those of the feed. The feed solutions of DOP were probably not true solutions and this may have affected the TTU pattern observed in the effluent samples. The lowest concentration (164 mg/L) was milky white whereas the other two concentrations (491 and 1473 mg/L) appeared to contain minute droplets in suspension; these latter solutions were composed of two distinct, liquid phases. This problem is obviously due to the DOP concentrations required for a lethal response being far in excess of the solubility of DOP (3 mg/L at 25°C) in water. The concentrations of DOP used for testing would be unlikely in the discharge of municipal WWTP. However, industrial sources may on occasion discharge this compound into the collection system of POTWs in the concentration range tested here; these tests should indicate possible results of industrial effluent fractionation.

Despite breakthrough of the C18 column by DOP, it was still possible to examine the MeOH/H_2O elution results to determine which fraction(s) contained the most toxicity. Figure 4.10b shows extensive smearing of toxicity into fractions ranging from 75 to 95 percent MeOH/H_2O.

A comparison of elution toxicity (Figure 4.10b) and presence of DOP in each fraction, as measured by absorbance at 420 nm (Figure 4.10c) is shown. Absorbance was used here rather than concentration because MeOH had a pronounced effect on the slope of standard concentration versus absorbance curve. Therefore the presence of DOP in

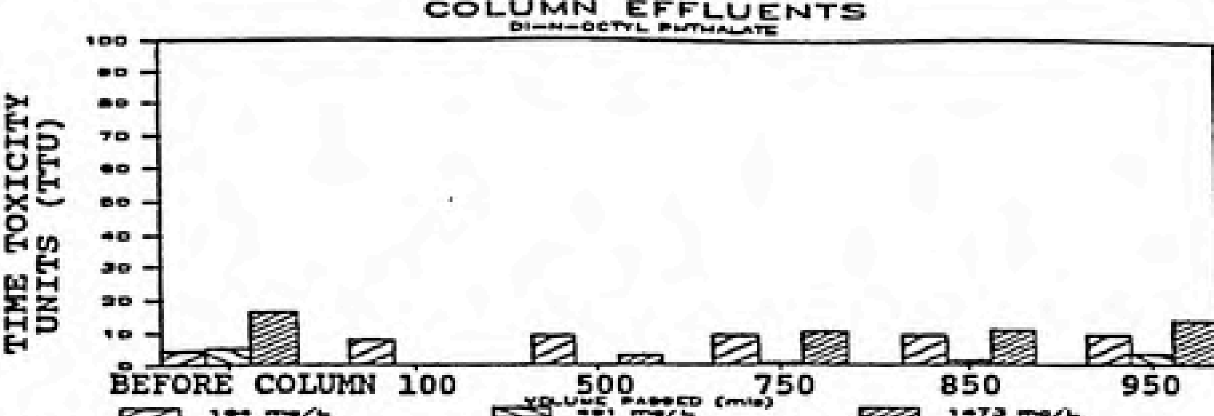


Figure 4.10a

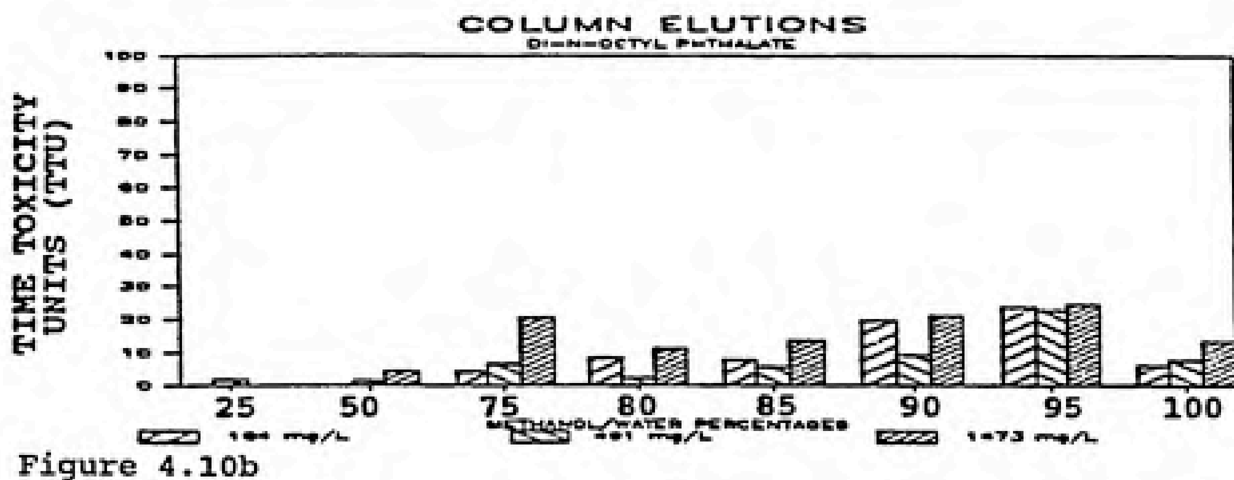


Figure 4.10b

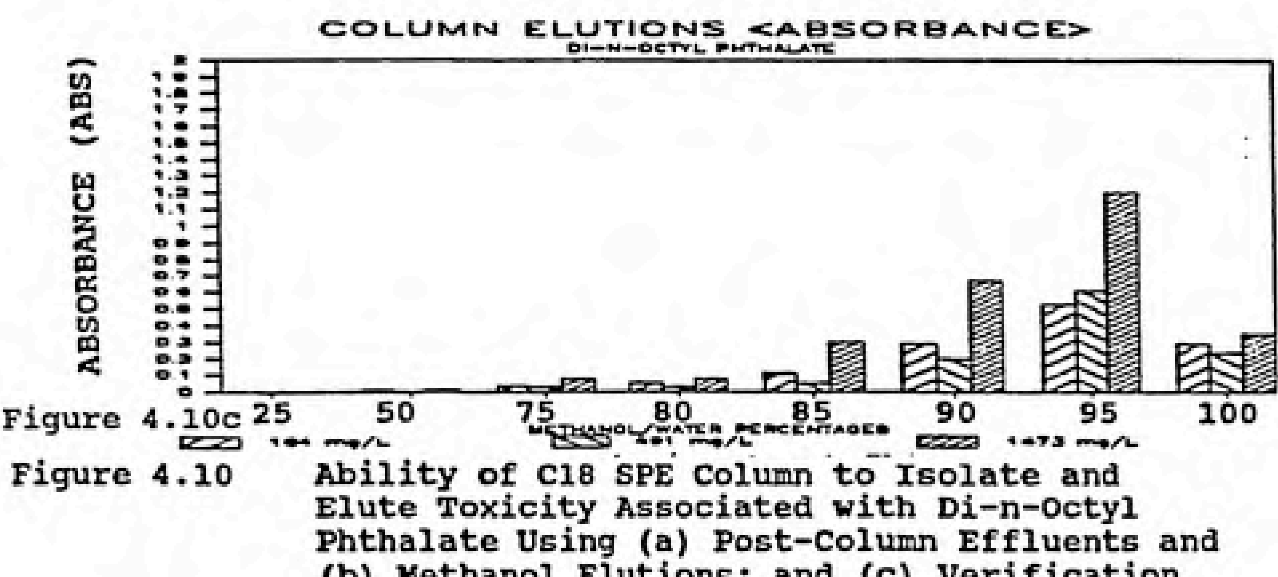


Figure 4.10c

Figure 4.10 Ability of C18 SPE Column to Isolate and Elute Toxicity Associated with Di-n-Octyl Phthalate Using (a) Post-Column Effluents and (b) Methanol Elutions; and (c) Verification by Absorbance Measurements.

different $MeOH/H_2O$ fractions cannot be determined precisely owing to varying degrees of MeOH interferences. A rough correlation was observed between toxicity and absorbance in each $MeOH/H_2O$ elution. This gives some support to the idea that the toxicity expressed by each eluted fraction is, in fact, due to the presence of DOP in that fraction and possibly indicates a correlation between toxicity and concentration; such a correlation is one of the objectives considered in EPA's Phase III Toxicity Confirmation Procedures.

Finally, the Microtox bioassay procedure was also used on the $MeOH/H_2O$ elutions of the C18 column receiving the highest DOP concentration (1473 mg/L). The results are compared to those using Ceriodaphnia bioassay in Figure 4.11. The Microtox bioassay was unable to measure toxicity in the 95 percent $MeOH/H_2O$ fraction (Figure 4.11b) which the Ceriodaphnia had shown to be the most toxic fraction (Figure 4.11a). If Microtox had been the only bioassay procedure conducted, the 95 percent toxicity peak would not have been identified. The Microtox procedure was repeated three times on the 90, 95 and 100 percent $MeOH/H_2O$ fractions to determine if experimental error could be responsible. However in all cases, no noticeable toxicity was found. Additionally, the standard color correction test recommended by Microbics (19) for colored samples was conducted, but it did not change the results.

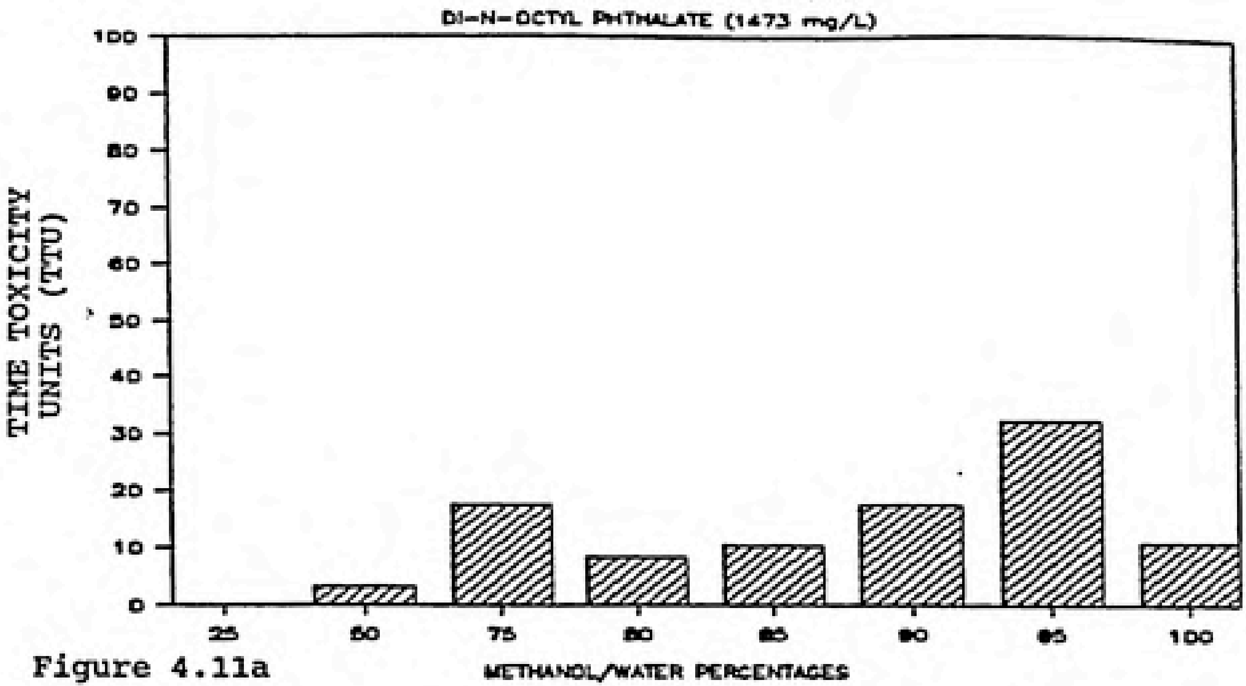


Figure 4.11a

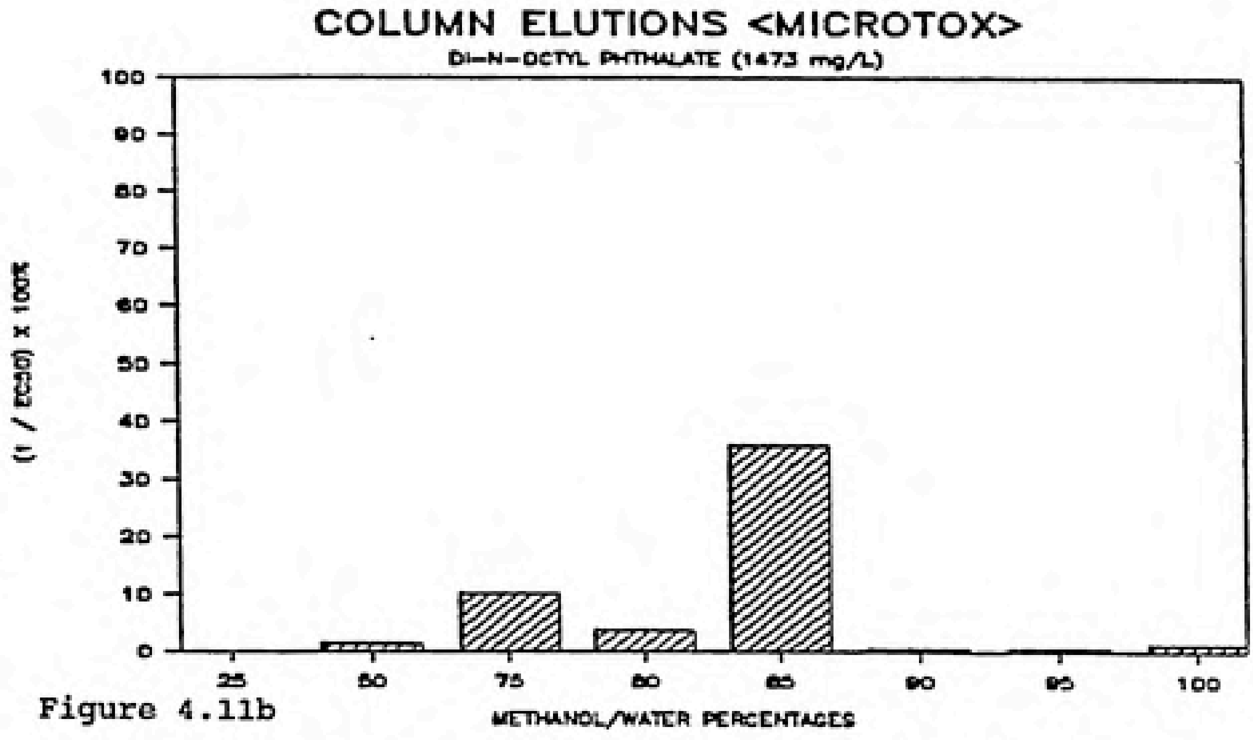


Figure 4.11b

Figure 4.11 Comparison Study Between the (a) <u>Ceriodaphnia</u> and (b) Microtox in Measuring the Methanol Elutions for Di-n-Octyl Phthalate.

B.5 Summary of Target Compound Evaluation.

Table 4.2 summarizes the results from the target compound evaluation. This table shows the comparison of log Kow values and the percent MeOH/H_2O fraction for which toxicity peaked for the different target compounds tested. Theoretically, a correlation between increasing MeOH/H_2O percentages and increasing log Kow values should exist. A compound with a low log Kow, if retained by the column, should be removed in a lower percent MeOH/H_2O fraction due to its relatively high polarity and hydrophilic nature. As the log Kow values increase, a decrease in polarity or increase in non-polarity is indicated and compounds become increasingly hydrophobic. These compounds would require a higher percentage of MeOH/H_2O before eluting from the column sorbent.

Although only four target compounds were evaluated, there is some broad indication of a relationship between log Kow and percent MeOH/H_2O (Table 4.2). However, the separation of these compounds is not well defined or established in any particular fraction. In the Patapsco study (conducted in Baltimore, Maryland), the class of compounds present in the 85 to 90 percent MeOH/H_2O fractions was determined to have a range of log Kow values between 3.6 and 9 (9). In this study, all three target compounds (MN, NPE, DOP) retained by the C18 column produced toxicity in the 75 to 90 percent MeOH/H_2O fractions. Experimentally, it would seem reasonable that mixtures of these compounds at

Table 4.2
Summary of Target Compound Evaluation
Results

Target Compound	log Kow Observed	log Kow Estimated	MeOH/water Fraction to Peak Toxicity Ceriodaphnia	MeOH/water Fraction to Peak Toxicity Microtox
Phenol (Ph)	1.46 (26)	1.46 (27)	Not Retained	Not Tested
1-Methylnaphthalene (MN)	3.9 (24)	3.84 (27)	80%	80%
Nonylphenol Ethoxylate 9 mole (NPE)	--------	7.8 (28)	80% to 85%	Not Detected
Di-n-Octyl Phthalate (DOP)	9.2 (26)	9.53 (27)	95%	85%

varying concentrations could elicit toxicity peaks ranging between 75 and 95 percent MeOH/H_2O. This possibly verifies the wide range of log Kow values found in the Patapsco fractions, and also shows the column's inability to separate classes of compounds (as defined by a broad range of log Kows values tested here) into more distinct fractions.

B.6 Summary of Microtox and Ceriodaphnia Comparisons

Table 4.3 shows the comparison between the Ceriodaphnia and Microtox assay procedure based on LC50 and EC50 values, respectively. As indicated by Table 4.3, Microtox is less sensitive than the standard Ceriodaphnia bioassay test (conducted in plastic cups). These results were expected due to the physiological differences and contact-response time between Microtox (marine bacterium) and the Ceriodaphnia (a small crustacean). The Ceriodaphnia are capable of digesting soluble as well as insoluble forms of compounds while the marine bacterium is restricted to the soluble form. Toxicants associated with colloidal particles in water can be digested by the Ceriodaphnia causing a toxic response while not effecting the marine bacteria. This difference in nutrient uptake between these two test organisms is sufficient to cause a difference in sensitivity.

The advantage of the Microtox bioassay procedure is a fast response time. Wastewater samples can be determined to

Table 4.3
Comparison Between
the Ceriodaphnia LC50 and the Microtox EC50
for Selected Compounds

Chemical Analyzed	Median Lethal Concentration (LC50) Ceriodaphnia (30)	Median Effective Concentration (EC50) Microtox (19)
Phenol (Ph)	LC50 = 10.3 mg/L 95% Confidence (7.7 to 14.2)	EC50 = 41.5 mg/L 95% Confidence (18.6 to 92.8) R = 0.99093
1-Methylnaphthalene (MN)	LC50 = 4.7 mg/L 95% Confidence (3.5 to 6.3)	EC50 = 8.4 mg/L 95% Confidence (1.1 to 63.2) R = 0.95366
Nonylphenol Ethoxylate 9 mole (NPE)	LC50 = 6.7 mg/L 95% Confidence (5.2 to 8.7)	EC50 > 275 mg/L
Di-n-Octyl Phthalate (DOP)	LC50 = 89.7 mg/L 95% Confidence (74.1 to 108.7)	EC50 = 262 mg/L 95% Confidence (153 to 448) R = 0.97527
Methanol (MeOH)	LC50 = 2.0% 95% Confidence (1.9 to 2.0)	EC50 = 3.1% 95% Confidence (2.5 to 4.0) R = 0.99869

be toxic within typically 20 minutes as opposed to the 48 hr required by the standard Ceriodaphnia test. This so-called "quick response" is obviously not without a loss sensitivity (see Table 4.3). However, the cost of conducting the TIE procedures and setting up the bioassays makes this "quick response" a possible savings in time and money. Microtox can be effectively used to screen toxic from non-toxic samples. Samples that are determined to be toxic by Microtox are typically very toxic to the Ceriodaphnia and this determination within 20 minutes allows for samples to be processed even sooner than if the baseline toxicity test (24 hr) had been determined by the Ceriodaphnia. This "quick response" is crucial when dealing with samples that show toxicity degrading rapidly over time.

C. Evaluation of Toxic Effluent Samples

C.1 Westside WWTP, Highpoint, North Carolina

Two toxic, composite effluent samples (ET50 = 21 hr and LC50 < 10%) were obtained from the Westside WWTP in Highpoint, NC. Both samples, dated 3/7/88 and 3/8/88, were passed through C18 columns. The columns were found to be completely ineffective in terms of reducing toxicity or retaining any toxic organics. This was subsequently confirmed by the absence of toxicity in the column elutions. However, Phase I characterization tests (see Figure 2.4 for overview) indicated a significant reduction in toxicity with

the addition of varying concentrations of EDTA (33). This would imply metals toxicity and explains why toxicity was not removed by the C18 column. Further laboratory evidence from Highpoint indicated a higher than normal concentration of nickel measured during this acute event (an influent nickel concentration of 2.4 mg/L and an effluent nickel concentration of 0.5 mg/L).

Besides the standard Ceriodaphnia toxicity test, the samples were tested with Microtox. No toxicity was found for both samples, yet the samples were definitely toxic to the Ceriodaphnia. John B. Razza, a sales representative from the Microbics Corporation, was consulted to explain these contradictory results. He suggested the sample should be sonicated prior to bioassay testing as a technique to release the metal ions associated with colloidal surfaces. Both samples were sonicated at three different frequencies for a total of 15 minutes, and then subsequently tested for toxicity using the Ceriodaphnia and Microtox. Again, no toxicity was detected by the Microtox instrument. However, an increase in toxicity with sonication was observed using the Ceriodaphnia procedure as is shown in Table 4.4; this is consistent with the idea that metals toxicity could increase due to the presence of free rather than bound forms.

Table 4.4

The Effects of Sonication on ET50
of Effluent Samples Collected at Highpoint
as Measured by the *Ceriodaphnia*

Date Samples Collected	ET50 Pre-sonication	ET50 Post-sonication
3/7/88	21.4 hr	18.8 hr
3/8/88	31.1 hr	21.4 hr

The Microtox instrument was successful in measuring the standard toxicant copper used in the *Ceriodaphnia* laboratory. An EC50 value equivalent to 156.3 ug/L was determined with a 95 percent confidence interval between 141.9 to 172.2 and a correlation coefficient of 0.99998. Thus, Microtox can detect metals toxicity, but the test organism (marine bacterium) is apparently not as sensitive as the *Ceriodaphnia*.

C.2 Cross Creek WWTP, Fayetteville, North Carolina

The first composite sample (collected from 1/16/89 to 1/17/89) obtained from Fayetteville was acutely toxic to the *Ceriodaphnia* (ET50 = 3 hr, LC50 = 57%). Phase I of Toxicity Characterization Procedures (see Figure 2.4 for outline) were conducted the following day (1/18/89), and the bioassays were setup on the day after (1/19/89). The results from this first round of testing showed almost the complete elimination of toxicity after the sample had been

passed through the C18 SPE Column. None of the other Phase I Toxicity Characterization Procedures gave as significant of a reduction in toxicity (for detailed results consult M. Frey (34))

The possible importance of combined chlorine toxicity was also noted in the first sample. This was implied by a decrease in toxicity observed during sample storage. The day the composite was collected (1/17/89), a so-called "pre-initial" toxicity test (not stipulated by Phase I Toxicity Characterization Procedures) was conducted resulting in an ET50 of 3 hr; this would be considered Day 0 by the Phase I Toxicity Characterization Procedures (Figure 2.4). The next day (Day 1) the initial toxicity test was conducted resulting in an ET50 of 4.3 hr. By Day 2, the actual day for conducting Phase I toxicity tests, the baseline of the effluent sample had increased to an ET50 value of 13 hr. Thus, sample storage alone reduced toxicity and could imply that a portion of toxicity was caused by the presence of combined chlorine (because this WWTP does not nitrify, the chlorine added is most likely reacting with ammonia immediately). However, this should not rule out the possibility of volatile organic compounds; they too could exhibit the same reduction observed due to sample storage (for detailed results consult M. Frey (34)).

On 1/23/89, the C18 SPE Column elution procedure was conducted. A 500 ml portion of toxic effluent was passed through a new C18 column and subsequently eluted. Figure

4.12a shows the complete retention of the toxic compounds present in the feed to the column (see BEFORE COLUMN). When the column was eluted with the MeOH/H_2O mixtures, the majority of the toxic material was released in the 80 to 85 percent fractions as indicated by Figure 4.12b.

The second sample received from Fayetteville was composited between 1/30/89 and 1/31/89. This sample was also acutely toxic, having an ET50 of 3 hr and a LC50 of 71%. Phase I Toxicity Characterization Procedures (for detailed results consult M. Frey (34)) were conducted on the following day (2/1/89) and the bioassays were setup on the day after (2/2/89). As found for the previous sample, a significant reduction in toxicity occurred from Day 0 to Day 2 (suggesting toxicity due to combined chlorine), and the C18 column showed complete elimination of toxicity (suggested toxicity due to non-polar organics). On Day 2, 2/2/89, one liter of sample was passed through a new C18 column and subsequently eluted (Figure 4.13). Figure 4.15b indicates that the pattern of toxicity eluted with MeOH/H_2O fractions was different than found for the first Fayetteville sample (Figure 4.12b) That is, the majority of the toxicity eluted into the 80 to 85 percent MeOH/H_2O fractions for the first sample as compared with the 75 to 80 percent MeOH/H_2O fractions for the second sample. This shift may be an indication that the toxic organic composition changed between sample dates, a span of about 15 days. However, chemical specific analyses have not been

COLUMN EFFLUENTS FOR CROSSCREEK WWTP

1/16/89 TO 1/17/89 COMPOSITE SAMPLES

TIME TOXICITY UNITS (TTU)

100
90
80
70
60
50
40
30
20
10
0

BEFORE COLUMN
50
250
450

VOLUME PASSED (mls)

Figure 4.12a

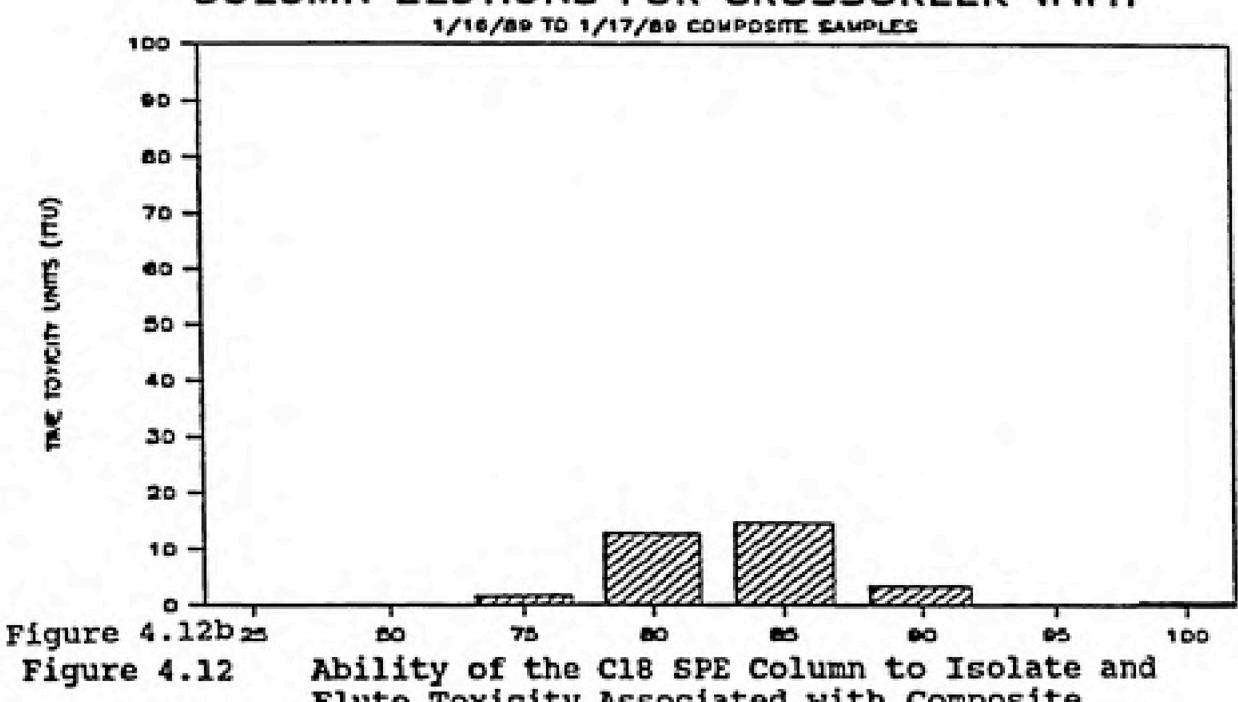


Figure 4.12b

Figure 4.12 Ability of the C18 SPE Column to Isolate and Elute Toxicity Associated with Composite Effluent Sample Collected 1/16/89 to 1/17/89 at the Cross Creek WWTP, Fayetteville, NC Using (a) Post-Column Effluents and (b) Methanol Elutions.

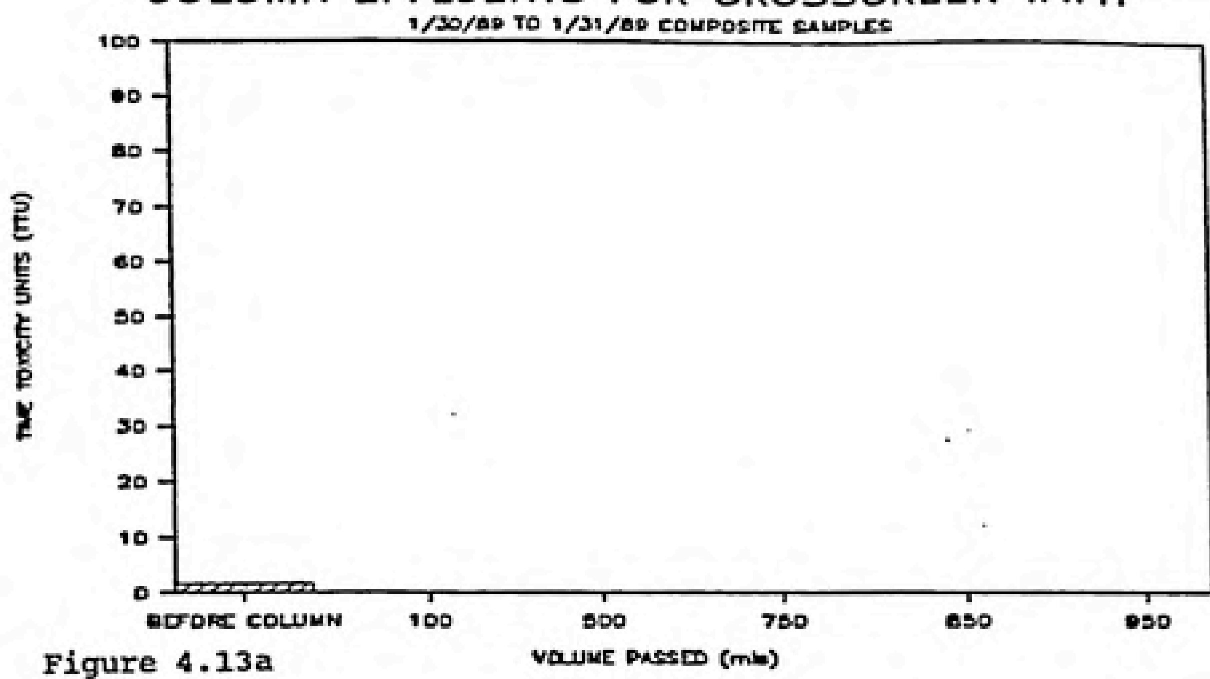


Figure 4.13a

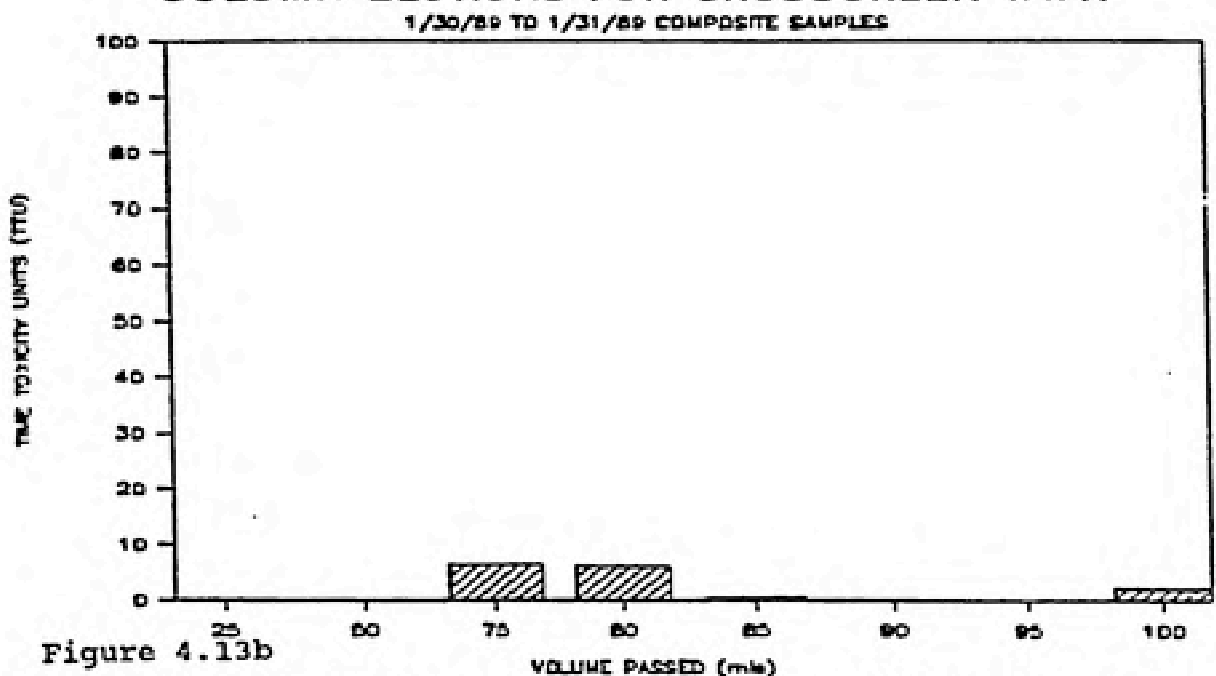


Figure 4.13b

Figure 4.13 Ability of the C18 SPE Column to Isolate and Elute Toxicity Associated with Composite Effluent Sample Collected 1/30/89 to 1/31/89 at the Cross Creek WWTP, Fayetteville, NC Using (a) Post-Column Effluents and (b) Methanol Elutions.

conducted on the fractions to determine this possibility.

The third Fayetteville sample was composited between 3/20/89 and 3/21/89. Again, acute toxicity was observed (ET50 = 23 hr, LC50 = 63%). Phase I Toxicity Characterization Procedures (for detailed results consult M. Frey (34)) were conducted on the following day (3/22/89) and the bioassays were setup on the day after (3/23/89). As with the previous two Fayetteville samples, the C18 column retained the toxic compounds completely. Aeration at a high pH was the only other Phase I Toxicity Characterization Procedure that showed the complete elimination of acute toxicity (34). On 3/23/89, one liter of sample was passed through a new C18 column and subsequently eluted (Figure 4.14). The toxicity elution profile (Figure 4.14b), resembled that of the first sample (Figure 4.12b) more closely than that of the second (Figure 4.13b). The 75 to 90 percent MeOH/H_2O fractions eluted the majority of toxicity compounds.

A fourth Fayetteville sample was composited between 2/20/89 and 2/21/89. This sample also gave acute toxicity (ET50 = 20 hr, LC50 = 63%). The Phase I Toxicity Characterization Procedures (for detailed results consult M. Frey (34)) were conducted. As found before, the C18 column was able to retain toxicity; however, time did not permit an elution of the column with MeOH/H_2O to identify toxic fraction.

Table 4.5 is a summary of the C18 column work conducted

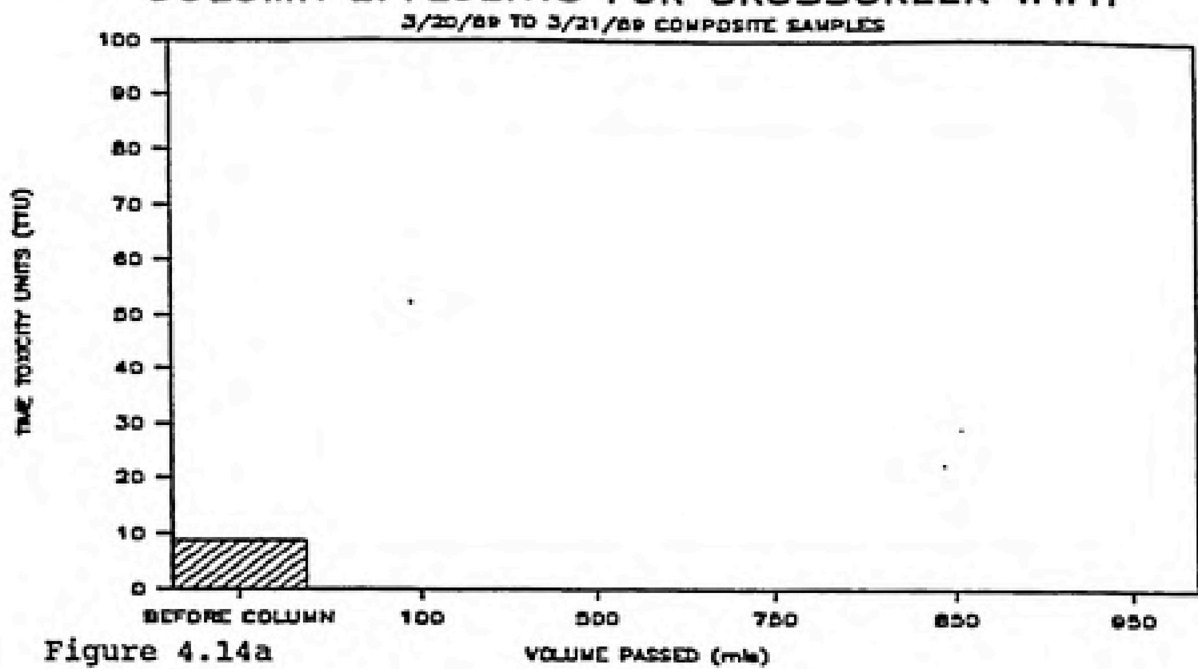


Figure 4.14a

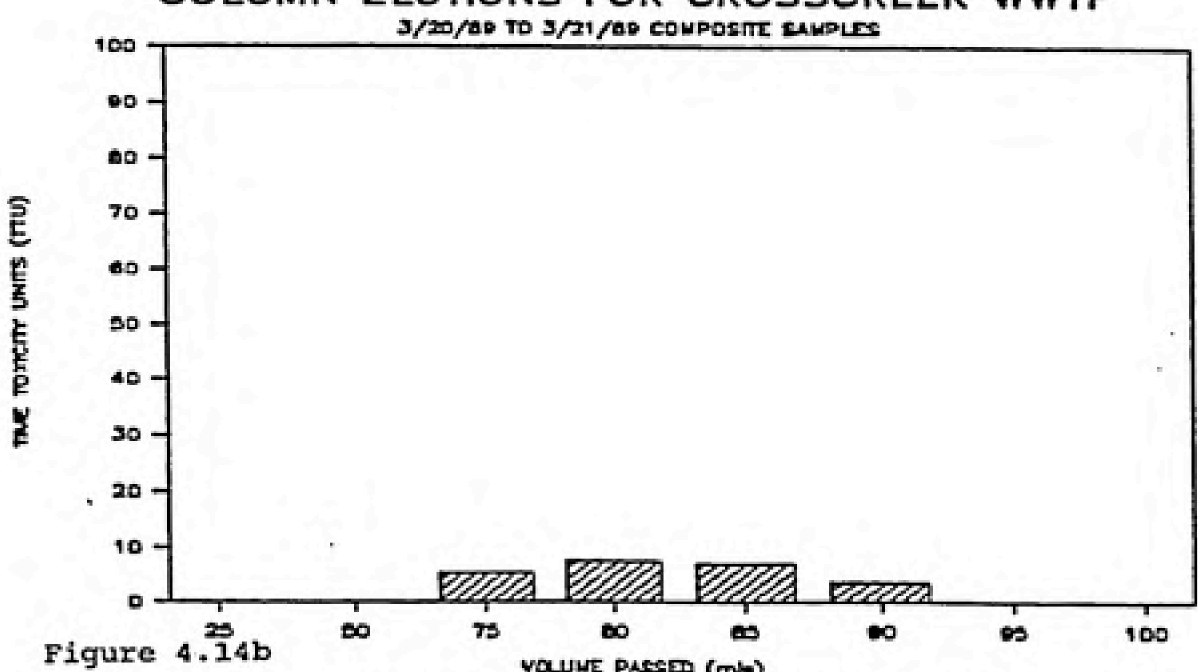


Figure 4.14b

Figure 4.14 Ability of the C18 SPE Column to Isolate and Elute Toxicity Associated with Composite Effluent Sample Collected 3/21/89 to 3/20/89 at the Cross Creek WWTP, Fayetteville, NC Using (a) Post-Column Effluents and (b) Methanol Elutions.

Table 4.5
Summary of Cross Creek WWTP Composite Samples
Fayetteville, North Carolina

Sample Number	Composite Sample Collection Date	ET50 (hr)			C18 SPE Column Results	
		Day 0	Day 1	Day 2	Toxicity Removed (Yes/No)	MeOH/water Fraction Containing Most Toxicity (%)
1	1/16/89 to 1/17/89	3	4.3	13	Yes	80 to 85
2	1/30/89 to 1/31/89	2.8	20	48	Yes	75 to 80
3	2/20/89 to 2/21/89	20	16	13	Yes	Not Determined
4	3/20/89 to 3/21/89	23	24	30	Yes	75 to 90

on the four Fayetteville - Cross Creek WWTP composite samples. Because toxicity was eliminated after the passage of these samples through the C18 SPE Column (see Post-Column Effluent results in Figures 4.12a, 4.13a, 4.14a), moderately polar to non-polar organic compounds were indicated as a source of toxicity. The shift in toxicity peaks from 80 to 85 percent MeOH/H_2O fractions for the first sample (1/16/89 to 1/17/89) to the 75 to 80 percent MeOH/H_2O fractions for the second (1/30/89 to 1/31/89) might be an indication that the organic composition changed between sample dates, a span of 15 days. However, due to the unreliable nature of the C18 SPE Column elution procedure and the fact that the baseline toxicity for the second sample was minimal (ET50 = 48 hr), the shift in toxicity peaks may not be significant. Chemical specific analyses were not conducted to determine a change in organic composition occurred between sample dates.

Further sample collection and testing will be required to determine if moderately polar to non-polar organics are a source of the continual acute toxicity. Because of in-stream dilution, the Cross Creek WWTP is not in violation of acute toxicity but rather, chronic toxicity. Thus, the question remains as to whether results of a TIE aimed at finding the cause(s) of acute toxicity can be extrapolated to chronic toxicity. One possible follow-up would be to identify these compounds retained by the C18 SPE Column using GC/MS procedures as described in the EPA Phase II Toxicity Identification Procedures (5). The importance of

these compounds in producing chronic toxicity could then be assessed through literature or laboratory study.

VI. CONCLUSIONS AND RECOMMENDATIONS

A. Conclusions

1. The comparison study conducted between the *Ceriodaphnia* and Microtox as aquatic toxicity indicators showed that the *Ceriodaphnia* were more sensitive to the target compounds tested (Table 4.3).

2. The target compound results (see section IV.B) showed that the C18 SPE Column was capable of retaining and eluting moderately polar to non-polar organic compounds from diluent water. A very rough correlation was obtained between the major percent $MeOH/H_2O$ fraction which elutes a known compound (as indicated by toxicity) and the log Kow value of the compound (see Table 4.5). The C18 SPE Column was not able to produce a sharp elution of each target compound into one $MeOH/H_2O$ fraction and this limited the data interpretation.

3. Passage of the composite effluent samples collected at the Cross Creek WWTP in Fayetteville, North Carolina through the C18 SPE Column produced a significant reduction in toxicity (as indicted by Figures 4.12a, 4.13a and 4.14a). Furthermore, toxicity was successfully eluted from the C18

column into the 75 to 90 percent $MeOH/H_2O$ fractions (as indicated by Figures 4.12b, 4.13b and 4.14b). Both of these results would indicate moderately polar to non-polar organic compounds as a source of toxicity in the effluent of the Cross Creek WWTP.

B. Recommendations

1. The Microtox bioassay procedure should be utilized as a sample screening device. Typically, a sample that is determined to be toxic by Microtox is very toxic to the Ceriodaphnia and combined with the fact that toxicity is determined within 20 minutes makes the Microtox bioassay procedure a viable system in TIE studies.

2. More target compounds should be tested to define the achievable resolution of the C18 SPE Column and to determine if a correlation exists between the percent $MeOH/H_2O$ fraction in which the compound is recovered and the log Kow of the compound.

3. Further sample collection, toxicity testing and TIE work will be required at the Cross Creek WWTP to determine if non-polar organics are a consistent source of acute toxicity. Because of in-stream dilution, the Cross Creek WWTP is not in violation of acute toxicity but rather, chronic toxicity. Thus, the question remains as to whether results of a TIE aimed at finding the cause(s) of acute toxicity can be extrapolated to chronic toxicity. One

possible follow-up would be to identify these compounds retained by the C18 SPE Column using GC/MS procedures as described in the EPA Phase II Toxicity Identification Procedures (5). The importance of these compounds in producing chronic toxicity could then be assessed through literature or laboratory study.

REFERENCES:

1. Versteeg, D. J. and Woltering, D. M., "A Chemical Fractionation Technique for Evaluating the Aquatic Toxicity of Municipal Wastewater Treatment Plant Effluents," Presented at CESIO Second World Surfactant Congress, Paris, France, May 24-27, 1988.

2. Zief, M. and Kiser, R., "Solid Phase Extraction for Sample Preparation," J. T. Baker, Inc., Phillipsburg, NJ.

3. Baker - Disposable SPE Column Instructions, Handout shipped with columns, Literature #8005 to 8006, J.T. Baker, Inc., Phillipsburg, NJ.

4. Baker - 10 SPE Applications Guide, Vol. I (1982), Vol. II (1984), J. T. Baker, Inc., Phillipsburg, NJ.

5. Mount, D. I. and Anderson-Carnahan, L., Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures, Draft, EPA/600/3-88/035. Nov. 1988.

6. Mount, D. I. and Anderson-Carnahan, L., Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures, EPA 600/3-88/034.

7. EPA/Atlanta, "Toxicity Evaluation, City of Largo WWTP, NPDES Permit No. FL0026603," U.S. Environmental Protection Agency, Region IV, Atlanta, GA.

8. Steen, A. E., "Biomonitoring to Achieve Control of Toxic Effluents," Cuyahoga River and Akron WWTP Evaluation, Akron, Ohio, Sept. 1987, EPA/625/8-87/013.

9. Botts, J. A., Braswell, J. W., and Sullivan, E. C., "Toxicity Reduction Evaluation at the Patapsco Waste Water Treatment Plant," Engineering Science, Fairfax, VA, Cooperative Agreement No. CR812790-01, Water Research Division, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, OH, EPA/600/2-88/034.

10. Slattery, G. H., "Case Study - Operations & Managements Viewpoint, Lessons Learned - Patapsco WWTP," Balitmore, MD, Presented at VWPCA May 1-3, 1988.

11. U.S. Environmental Protection Agency, "Toxicity Evaluation for the City of Largo WWTP, NPDES Permit No. FL0026603," Region IV, Atlanta, GA, 1985.

12. Mount, D. T., "Las Vegas Report," Selected pages sent by Anderson-Carnahan, L., Environmental Protection Agency, Jan. 1988.

13. Standard Methods for the Examination of Water and Wastewater, APHA, AWWA, WPCF, 15th Edition, Washington, D.C., 1980.

14. Anderson-Carnahan, L. and Mount, D. I., Methods for Toxicity Reduction Evaluations Phase I Toxicity Characterization Procedures Draft - January 1987, EPA/600/1987.

15. Berner, D. B., "Taxonomy of Ceriodaphnia (Crustacea: Cladocera) in U.S. Environmental Protection Agency Cultures," Biological Methods Branch, Environmental Monitoring and Support Laboratory, Cincinnati, OH, 1985.

16. Kurz, G. E., Leslie, N., Henderson, R. J., "A Rapid Industrial Waste Screening Method," Presented at the 39th Purdue Industrial Waste Conference, May 9, 1984.

17. Microtox, "How to Run a Standard Microtox (TM) Test," Microbics Corporation, Part No. 555880, Release 2, Microbics Corporation, 1988.

18. Brusick, D. J. and Young, R. R., "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests," Industrial Environmental Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC, EPA 600/8-81/024.

19. Microtox, "How to Reduce Microtox Test Data," Microbics Corporation, Draft 18, Oct. 1987.

20. Rand, G. M. and Petrcelli, S. R. Fundamentals of Aquatic Toxicology, Hemisphere Publishing Corporation, Washington, 1985.

21. Storm, J. F., "Tentative Identification of Organic Compounds in the Influent and Effluent of the Highpoint Westside Wastewater Treatment Plant and Implications for Aquatic Toxicity." Master's Report, University of North Carolina at Chapel Hill, Environmental Sciences and Engineering Department, School of Public Health, 1987.

22. Eastman Kodak Co., Catalog & Price List-Kodak Laboratory Chemicals, Catalog No. 52, Laboratory and Research Products Division, Rochester, NY, July 1985.

23. Frazier, W. D., "Analytical Chemist," Central Laboratory Services, City of High Point, High Point, NC.

24. Goodman, N., "Evaluation of Methodology for Analysis of Organic Contaminants in Soil," Thesis Report, University of North Carolina at Chapel Hill, Environmental Sciences and Engineering Dept., School of Public Health, 1985.

25. Union Carbide Corporation, "Tergitol NP Nonionic Surfactants, Union Carbide Ethylene Oxide Derivatives," Booklet, Union Carbide Corporation, 1980-1981.

26. Callahan, M.A., Slimak, M. W., Gabel, N. W., May, I.P., Fowler, C. F., Freed, J. R., Jennings, P., Durfee, R. L., Whitmore, F. C., Maestri, B., Mabey, W. R., Holt, B. R., and Gould, C., "Water-related Environmental Fate of 129 Priority Pollutants," Vol I and II, U.S. Environmental Protection Agency, Washington, D.C., EPA/440/4-79/029a and EPA-440/4-79/029b.

27. Lyman, W. J.,Reehl, W. F., Rosenblatt, D. H., Handbook of Chemical Property Estimation Methods, McGraw-Hill: New York, 1982, p 1-1 to 1-38.

28. Mailhot, H. and Peters, R. H., "Empirical Relationships Between the 1-Octanol/Water Partition Coefficient and Nine Physiochemical Properties," Environ. Sci. Technol., 22: 12: 1479-1488, (1988).

29. North Carolina Department of Natural Resources and Community Development, Water Quality Section, "Fayetteville-Cross Creek WWTP Toxicity Examination NPDES NO. 0023957," Oct. 1986.

30. Hamilton, M. A., Russo, R. C., and Thurston, R. V., "Trimmed Spearman-Karber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays," Environ. Sci. Technol. 11(7) 714-719 1977, Correction 12(4) 417 (1978).

31. Mount, D. T., Personal Communication, U.S. EPA Environmental Research Laboratory, Office of Research and Development, Duluth, MN, Aug. 1988.

32. Snoeyink, V. L., Jenkins, D., Water Chemistry, John Wiley & Sons Inc., New York, p.91.

33. DiGiano, F. A.,"Case History: Toxicity Reduction Evaluation at Highpoint, North Carolina", Presented at VWPCA, Inc, Wastewater Toxics Management Seminar, Oct. 19-20 (1988).

34. Frey, M. V.,"Evaluation of the Causative Toxicant Characterization Tests of EPA's Toxicity Identification Evaluation." Master's Report, University of North Carolina at Chapel Hill, Environmental Sciences and Engineering, School of Public Health (1989).

35. Beckman, "Abbreviated Assay Procedure (Duplicate Determination for Beckman's Microtox (TM) Analyzer," Beckman Instruments, Inc., Microbics Operations, Carlsbad, CA (1982).

36. Dolan, J. W., Chapter 17 in Snyder, L. and Kirkland,J., Introduction to Modern Liquid Chromatography, John Wiley & Sons, New York, NY (1979).

37. Mount, D. I., "Effluent Toxicity Reduction Strategies and Fractionation Techniques," (Phase One and Two). Aquatic Toxicity Testing Workshop for Consulting Laboratories, Charlotte, NC, North Carolina/South Carolina and U.S. Environmental Protection Agency, Sept. 8, 1988.

38. North Carolina Division of Environmental Management, "Toxicological Examination of Highpoint Westside Wastewater Treatment Facility NPDES No. NC0024228," Technical Services Unit, Aquatic Toxicology Group, (Jan. 1984).

39. Zweig, G. and Sherma, J., Handbook of Chromatography, Vol. II, pp.191-254, CRC Press, Cleveland, OH (1972).

APPENDIX A

Washing Procedure

In the Phase I Toxicity Characterization Procedures outlined in draft form in January of 1987, standard laboratory practices were established to insure and to protect the quality of the data generated from conducting a Toxicity Reduction Evaluation. Under this quality assurance program, the washing procedure was outlined. "All glassware used in toxicity testing should be washed with detergent, and sequentially rinsed in 10% nitric acid (to remove trace metals), hexane, acetone (to remove trace organics), and finally high quality water." Glassware used for the first time should be soaked for three days in 10% nitric acid to remove any possibility of contaminated metals. (14)

The sequencing of washes as previously outlined was restructured due to the fact that the acetone was originally shipped in a metal container. The 10% nitric acid wash was moved from its leading position to the end of the washing train to assure any metals introduced by the acetone would be removed by the acid wash. A brief outline is listed illustrating the washing procedure which was followed during toxicity testing.

Washing Procedure:

1. Detergent wash
2. Rinse 3 times in tap water
3. Rinse once in hexane
4. Rinse 3 times in tap water
5. Rinse once in acetone
6. Rinse 3 times in tap water
7. Rinse 3 times in distilled water
8. Rinse and/or store in 10% nitric acid for 3 days
9. Rinse 3 times in distilled water
10. Rinse 3 times in high quality water
11. Prior to use rinse again in high quality water.

APPENDIX B

Equipment Specifications

The following is a list of the necessary materials required to setup three SPE column processing units as described in the C18 SPE Column Procedure.

1. Box of C18 columns and adapters
2. 30 X 50ml test tubes with PTFE rubber caps
3. 3 X 1 liter reservoir bottles
4. 3 TFE needle valves with 1/8" NPT thread
5. 3 PFA male connectors for 1/8"OD
6. 3 PFA male connectors for 1/4"OD
7. 3 X 1-liter vacuum flasks
8. 6 customized rubber stoppers
9. 12 FT of 1/8"OD TFE tubing
10. 12 FT of 1/4"OD TFE tubing
11. Disposable syringes 5ml & 10ml (boxes)
12. 10 X 125ml polypropylene wide mouth bottles
13. HPLC grade methanol (1 liter)
14. House vacuum or suitable pump
15. 3 magnetic stirrers with stir bars
16. 26 FT of vacuum hose and vacuum piping
17. 2 X 100ml graduated cylinders
18. 1 utility box (4 X 5 X 8 inches)
19. 6 FT of Piano wire
20. 48 X 5ml vials with PTFE rubber caps
21. 2 microliter syringes
22. 10 plastic centrifuge tubes
23. 4 FT glass tubing 7/32 OD

APPENDIX C

Request and Reporting Forms

LOG #:__________

BIOASSAY REQUEST FORM
Ceriodapnia dubia

NAME:________________________________ DATE:__________

SAMPLE I.D.:________________________ LOCATION:__________

SAMPLE DATE:__________ TREATMENT DATE____________ TIME:___

TYPE OF TEST:
- __ TIMED LETHALITY (10 mL; no dilutions)
- __ TIMED LETHALITY (10 mL; w/ dilutions)
- __ ACUTE STATIC (100 mL; 24 & 48 HR LC50)
- __ 7-DAY MINI CHRONIC (15 mL; reproductive)
- __ OTHER (__________________________________)

SPECIAL INSTRUCTIONS: ____________________________________

____

____

____

RESULTS:
- _A ET50= ____
- _B LC50= ____
- _C CHRONIC= PASS / FAIL _______________________

____

____

____

REQUEST RECEIVED BY:___________________________
DATE:_________________
TEST DATES:___________________ to _______________________

COMMENTS:

TIMED LETHALITY TEST DATA / WORKSHEET
Ceriodaphnia dubia

SAMPLE I.D.:______________________________ NAME:____________

CONTROL/DILUTION WATER SOURCE:____________________ LOT #:__________
INITIAL:
Filter:_____ Temp:_____ pH:_____ D.O.:_____ Alk:_____ Hardness:_____ Cond:_____

INITIAL DATE:______________ TIME:______________ ANALYST:______________
FINISH DATE:______________ TIME:______________ ANALYST:______________

TEST VOL:________ # ORGANISMS/CUP______ DILUTIONS:_______ ADULTS ISOLATED AT:_______

SAMPLE REPLICATE # ORGANISMS	A	B										
TIME: NAME												
Temp (C)												
pH												
DO (mg/L)												

COMMENTS:

APPENDIX D

Microtox Test Procedure

The following procedure was developed as a quick reference guide for verifying the readiness and assisting in the operation of the Microtox Unit. This procedure combines the structural format of the Abbreviated Assay Procedure for Duplicate Determinations (35) and the additional instructions outlined in the Microtox Manual: How to Run a Standard Microtox Test (17). The purpose of including this guide is to illustrate the experimental procedure which was followed and to assist anyone that may use the instrument in the future.

Analyzer Readiness Guide

1. INSTRUMENT INSPECTION

* Insure that the Microtox unit is plugged into a powered outlet and has been operating for at least 15 minutes before attempting this procedure. Ideally the instrument should be left operating on a continual basis during periods of frequent testing.

* Visually inspect the instrument and surrounding area for possible obstructions which may block the wells or ventilation ports.

2. VERIFYING TEMPERATURE SETTINGS

* Precooling well should be at 5 degrees C

* Turret and incubating wells should be maintained at 15 degrees C but may vary between 12 C to 25 C.

* If the temperature of the turret or incubating wells is unsatisfactory it may be adjusted using the Temp Set control. <Unlock> the Temp Set control and Twiddle the dial until it reads 1.5 or the LED display indicates a temperature in the proper range, then <lock>.

3. INSTRUMENT CALIBRATION

* Select the X-10 Sensitivity Range by depressing the X-10 button.

* <Unlock> the Span control and then turn it clockwise to its maximum setting. <lock>

* Depress the HV or High Voltage Switch to the On position. The photomultiplier is now energized and should be allowed to stabilize before continuing.

* With the turret closed and no reagent, the LED display should read 0 0 0. If this is not the case the instrument will need to be zeroed. To zero the photomultiplier output <unlock> the Zero control and twiddle the dial until 0 0 0 reading is obtained then <lock> the control. This adjustment should only be done with X-10 Sensitivity range and the Span control turned to its maximum setting.

4. CALIBRATION CHECK

* <Unlock> the Span control and turn it counterclockwise to a setting of two, and then very carefully turn the control clockwise until the large outside ring is set to four and the inside dial is set to zero <lock>.

* Verify that the turret is closed, Span control is at 4.0 and Sensitivity range is set in the X-10 mode.

* Depress the CAL Check button and look at the LED display. The display should indicate a value between 80 to 120. If this value is not obtained consult the Microtox Manual page 12. Release the CAL Check button.

Microtox Assay Procedure

1. ANALYZER PREPARATION

* Verify analyzer readiness (previous procedure)

* Remove storage cuvettes from wells and replace them with new clean cuvettes.

* Add 1.0 mls of Reconstitution Solution to cuvette in the precooling well.

* Add 1.0 mls of Microtox Diluent to wells A1 through A4

* Add 0.5 mls of Microtox Diluent to wells C1 through C5

2. SAMPLE PREPARATION

* Visually inspect the sample to determine if the light absorbance correction procedure will be necessary

* Place 2.5 mls of sample into well A5

* Adjust NaCl concentration of the sample by adding 0.25 mls of Microtox Osmotic Adjustment Solution to well A5. Mix this well five times with pipettor.

* Transfer 1 ml from well A5 to A4 and mix five times with pipettor.

* Transfer 1 ml from well A4 to A3 and mix five times with pipettor.

* Transfer 1 ml from well A3 to A2 and mix five times with pipettor

* Wait five minutes for temperature equilibrium.

3. REAGENT PREPARATION (Read Before Attempting)

* Opening the reagent bottle
  - Note hissing sound due to vacuum packing. Disregard reagent if no sound is heard.
  - Seat the reagent pellet into the bottom of the vial.

* Reconstituting reagent
  - Take the cuvette of Reconstitution Solution from the precooling well and place the reagent bottle right under the lip of the cuvette.
  - "Suddenly" dump the Reconstitution Solution into the bottle

- Swirl the bottle three to four times fast.
- Pour the contents back into the cuvette and place the cuvette back into the precooling well.
- Aspirate twenty times with the 250 ul pipettor and start recorder chart drive.

* Transfer 10 ul of reconstituted reagent to wells C1 through C5.

* Flick each cuvette five times with finger.

* Depress X1 Sensitivity range.

* Place the cuvette from well C1 into the turret and close.

* Adjust the DPM reading to approximately 0 9 0 by using the Span control. <unlock> adjust <lock>

* Verify reagent equilibrium by watching the chart recorder. (Approximately a 15 minute wait)

4. ASSAY PROCEDURE

* Take initial reagent readings cycling the cuvettes in the following order:
  C1, C2, C3, C4, C5
  Reset Span if any of the cuvettes read over 100 and cycle cuvettes again.

* Immediately transfer 500 ul from well A1 to C1 and mix by aspirating and dispensing 5 times with the pipettor

* Using the procedure previously described, make the following dilution transfers

  500 ul From A2 to C2
  500 ul From A3 to C3
  500 ul From A4 to C4
  500 ul From A5 to C5

  Aspirate and dispense 5 times after each transfer

* Five minutes after the last I (0) light level was taken as indicated by the chart recorder, cycle the cuvettes in the following order to obtain the I (5) light levels:
  C1, C2, C3, C4, C5

* If the test indicates a stepwise regression then toxicity exists, and the results can be tabulated and reduced.

* Fifteen minutes after the last I (0) light level was taken, recycle the cuvettes in the following order to obtain I (15) light levels:
  C1, C2, C3, C4, C5

* If the test indicates a stepwise regression then toxicity exists, and the results can be tabulated and reduced.

* It is possible to repeat the cycling of the cuvettes for light level readings up to 30 minutes or more beyond the last I (0) reading if deemed necessary.

APPENDIX E

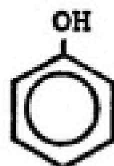
Log Octanol/Water Partitioning Coefficient Estimations for Target Compounds

---

Phenol (Ph)

Chemical Formula: C_6H_6O

Chemical Structure:



Calculation:

<u>Leo's Fragment Constant Method (27)</u>

$$f_{C_6H_5} = 1(1.90) = 1.90$$

$$+\ f^{o}_{OH} = 1(-0.44) = -0.44$$

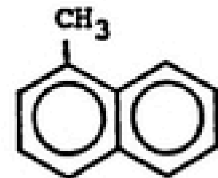
$$\log K_{OW} = 1.46$$

$$(\text{Observed } \log K_{OW} = 1.46)$$

1 - Methylnaphthalene (MN)

Chemical Formula: $C_{10}H_7CH_3$

Chemical Structure:



Calculation:

Leo's Fragment Constant Method (27)

$$
\begin{aligned}
7f^{o}_{\underline{CH}} &= 7(0.355) &&= 2.48 \\
+ f^{o}_{\underline{C}}o &= 1(0.255) &&= 0.26 \\
+ f^{o}_{\underline{C}}\ast &= 1(0.44) &&= 0.44 \\
- f^{o}_{H} &= -1(0.23) &&= -0.23 \\
+ f^{o}_{CH_3} &= 0.89 &&= 0.89 \\
\hline
\log K_{ow} &&&= 3.84 \\
(\text{Observed } \log K_{ow} &&&= 3.9)
\end{aligned}
$$

Nonyl phenol ethoxylate 9 Mole (NPE)

Chemical Formula: $C_{33}H_{60}O_{10}$

Chemical Structure:

$R-C_6H_4-O(CH_2CH_2O)_9H$

$R = -CH(CH_3)-CH_2-CH(CH_3)-CH_2-CH(CH_3)-CH_3$

Calculations:

Leo's Fragment Constant Method (27)

$$f_{C_6H_5} = 1(1.90) = 1.90$$
$$+ 9f_{CH_3} = 9(0.89) = 8.01$$
$$- 9f_H = - 9(0.23) = - 2.07$$
$$+ 8f_O = 9(-1.82) = - 14.56$$
$$+ f_{OH} = 1(-1.64) = - 1.64$$
$$+ f_O^O = 1(-0.61) = - 0.61$$
$$- f_H^O = 1(-0.23) = - 0.23$$
$$+ f_C^O = 1(0.20) = 0.20$$
$$+ 3f_H = 3(0.23) = 0.69$$
$$+ 2f_C = 2(0.20) = 0.40$$
$$+ 6f_{CH_3} = 6(0.89) = 5.34$$
$$- 2f_H = - 2(0.23) = - 0.46$$

Nonyl phenol ethoxylate 9 Mole (NPE) continued

$$+ (37-1)F_b = 36(-0.12) = -4.32$$

$$\log K_{ow} = -7.35$$

(Observed log Kow not available)

Mailhot and Peters' Empirical Relationship Method (28)

Nonyl phenol ethoxylate (NPE):

Molecular Weight = 616.4 gm/mol
Density = 1.057 gm/cm^3

This method is based on the relationship between log Kow and different physiochemical properties for the alkane family.

Molecular Weight (M):

Linear Equation: $\log K_{ow} = 1.12 + 0.024M$

$(n = 17, r^2 = 0.728, MSE = 0.156)$

Calculation: $\log K_{ow} = 1.12 + 0.024(616.4 \text{ gm/mol})$

$\log K_{ow} = 15.9$

(no observed value)

Density (D):

Linear Equation: $\log K_{ow} = -4.26 + 11.4D$

$(n = 17, r^2 = 0.701, MSE = 0.172)$

Calculation: $\log K_{ow} = -4.26 + 11.4(1.057 \text{ gm/cm}^3)$

$\log K_{ow} = 7.8$

(no observed value)

Di - n - octyl phthalate (DOP)

Chemical Formula: $C_{24}H_{38}O_4$

Chemical Structure:

$$C_6H_4(COOC_8H_{17})_2$$

Calculation:

Leo's Fragment Constant Method (27)

$$
\begin{aligned}
f_{C_6H_5} &= 1(1.90) &&= 1.90 \\
- f^{o}_{H} &= -1(0.23) &&= -0.23 \\
+ f^{o}_{CO_2} &= 2(-0.56) &&= -1.12 \\
+ 2(8)f_{CH_3} &= 2[8(0.89)] &&= 14.24 \\
+ 2(7)f_{H} &= 2[-7(0.23)] &&= -3.22 \\
+ (18-1)F_b &= 17(-0.12) &&= -2.04 \\
\hline
\log K_{ow} & &&= 9.53
\end{aligned}
$$

(Observed $\log K_{ow}$ = 9.2)

APPENDIX F

Time Based Toxicity Unit Data Tables

TABLE D.1
PERCENT METHANOL EVALUATION
FOR FIGURES 4.1(A) AND 4.1(B)

BOTTLE NO. 1 (4 LITER)
LOT NO. 873940
OPENED 3/15/88

PERCENT METHANOL	SAMPLES TIME BASED TOXICITY UNIT (TTU) — BIOASSAY CONDUCTED IN PLASTIC CUPS							GLASS BEAKERS
	3/16	4/27	4/27	4/27	7/22	7/22	7/22	7/19/88
0.5		0.0	0.0	0.0	0.0	0.0	0.0	1.6
1.0		0.0	0.0	0.0	0.4	0.0	0.0	2.1
1.5		0.0	0.0	0.0	0.0	2.2	0.0	4.9
1.6					0.0	2.6	0.4	
1.7					0.0	4.3	0.8	
1.8					0.0	1.3	0.8	
1.9					0.4	0.9	0.9	
2.0	4.8	0.0	0.0	10.0	7.0	0.9	2.2	
3.0		3.9	6.0	8.0				1.2
4.0		10.3	9.1	13.1				13.7
5.0	11.5							18.9

TABLE D.2
COLUMN TOXICITY EVALUATION
FOR FIGURES 4.2(A) AND 4.2(B)

BOTTLE NO. 1 (4 LITER)
LOT NO. 873940
OPENED 3/15/88

PERCENT METHANOL	SAMPLES TIME BASED TOXICITY UNIT (TTU)				
	PLASTIC CUPS			GLASS BEAKERS	
	3/22	7/20	8/4	7/19	8/5
0.38			0.0		0.0
0.50	0.0	0.0		0.0	
0.75			0.0		0.4
1.00	0.0	0.0		0.0	
1.13			0.0		0.0
1.20			0.0		0.0
1.28			0.0		0.4
1.35			0.4		7.8
1.43			0.4		14.8
1.50	0.0	0.0	0.4	1.1	14.3
1.60	0.0	0.0		0.7	
1.70	0.0	0.0		0.7	
1.80	0.0	0.4		10.3	
1.90	5.3	7.9		10.8	
2.00	0.0	7.5		13.3	

TABLE D.3
COLUMN TOXICITY EVALUATION
FOR FIGURE 4.2(C)

BOTTLE NO. 2 (1 LITER)
LOT NO. 884151
OPENED 8/16/88

PERCENT METHANOL	SAMPLES TIME BASED TOXICITY UNIT (TTU) BIOASSAY CONDUCTED IN PLASTIC CUPS			
	9/22	9/23	9/27	9/28
0.38	0.8	0.0	0.0	0.0
0.75	0.0	0.0	0.0	0.0
1.13	0.4	0.0	0.0	0.0
1.20	0.4	0.0	0.0	0.0
1.28	0.0	0.0	0.0	0.4
1.35	0.0	0.0	0.0	0.0
4.43	6.1	4.3	5.7	3.5
1.50	13.5	3.4	2.4	1.8

TABLE D.4

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.3(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 8/10/88

NON-TOXIC EFFLUENT FROM HIGHPOINT NC
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)
BEFORE COLUMN	0.0
100	0.0
500	0.0
750	0.0
850	7.3
950	0.0

TABLE D.5

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.3(B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 8/10/88

NON-TOXIC EFFLUENT FROM HIGHPOINT NC
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)	
	PLASTIC	GLASS
25	0.0	0.0
50	0.0	0.8
75	0.0	2.0
80	0.0	1.2
85	0.0	0.0
90	0.0	5.7
95	0.8	7.7
100	0.9	6.9

TABLE D.6

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.4(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 10/5/88

CONTROL WATER FROM BOTANY POND
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)
BEFORE COLUMN	0.0
100	0.0
500	0.4
750	0.0
850	0.0
950	0.4

TABLE D.7

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.4(B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 10/5/88

CONTROL WATER FROM BOTANY POND
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)
25	0.4
50	0.0
75	0.4
80	0.0
85	0.4
90	0.0
95	3.3
100	2.2

TABLE D.8

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.5(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 9/22/88

PHENOL
(10 X LC50 = 4.3 mg/L)
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)
BEFORE COLUMN	6.5
100	9.0
500	7.4
750	10.2
850	9.5
950	9.2

TABLE D.9

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.5(B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 9/22/88

PHENOL
(10 X LC50 = 4.3 mg/L)
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)
25	0.0 (-0.4)
50	0.0
75	0.0 (-0.4)
80	0.0
85	0.0 (-0.4)
90	0.0
95	0.1
100	0.0 (-0.1)

TABLE D.10

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURES 4.6(A) AND 4.6(B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 9/22/88

PHENOL
(10 X LC50 = 4.3 mg/L)
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)			ABSORBANCE DATA (ABS)		
	pH = 3	pH = 7	pH = 9	pH = 3	pH = 7	pH = 9
BEFORE COLUMN	10.7	10.7	10.7	0.66	0.66	0.66
25	11.1	7.0	7.6	0.322	0.672	0.644
150	6.9	5.9	10.0	0.628	0.667	0.653

TABLE D.11

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.7(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 8/5/88,7/13/88,7/6/88

1-METHYLNAPHTHALENE
LC50 = 1.393 ul/L
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN GLASS BEAKERS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)		
	2.8 ul/L 2 X LC50	7.0 ul/L 5 X LC50	34.8 ul/L 25 X LC50
BEFORE COLUMN	0.8	10.4	100.0
100	2.9	1.6	1.2
500	0.4	0.4	2.7
750	0.0	0.0	4.0
850	0.0	0.0	0.8
950	0.0	0.0	3.9

TABLE D.12

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.7(B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 8/5/88,7/13/88,8/9/88

1-METHYLNAPHTHALENE
LC50 = 1.393 ul/L
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)		
	2.8 ul/L 2 X LC50	7.0 ul/L 5 X LC50	34.8 ul/L 25 X LC50
25	0.0	0.0	0.4
50	0.0	0.0	0.0
75	0.0	0.0	3.5
80	0.0	0.8	61.8
85	0.0	0.0	0.0
90	0.0	0.0	0.0
95	4.2	5.8	5.2
100	2.3	5.3	3.4

TABLE D.13

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.7(C)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 8/5/88,7/13/88,7/6/88

1-METHYLNAPHTHALENE
LC50 = 1.393 ul/L
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN GLASS BEAKERS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)		
	2.8 ul/L 2 X LC50	7.0 ul/L 5 X LC50	34.8 ul/L 25 X LC50
25	0.0	0.0	0.0
50	0.0 (-0.8)	0.0 (-0.8)	0.0 (-0.8)
75	0.3	10.3	15.7
80	1.5	26.6	98.8
85	0.0	0.0	8.7
90	1.8	1.0	7.4
95	5.6	1.6	4.8
100	6.4	0.0 (-2.0)	5.2

TABLE D.14

TIME LETHALITY AND MICROTOX DATA
FOR FIGURES 4.8(A), 4.8(B) AND 4.8(C)

FRACTIONATION SPE C-18 COLUMN

1-METHYNAPHTHALENE
(5 X LC50=1.393ul/l)
1.5% METHANOL EXTRACTION

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	MICROTOX TOXICITY UNIT (1 / EC50) X 100%	CERIODAPHNIA DUBIA TOXICITY (1 / ET50) X 100%	
		PLASTIC	GLASS
25	0.0	0.0	0.0
50	0.0	0.0	0.0
75	1.7	0.0	6.8
80	3.7	0.0	17.8
85	0.6	0.0	0.0
90	0.0	0.0	3.7
95	0.0	6.0	3.9
100	0.0	5.0	0.0

TABLE D.15

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.9(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 9/26/88,9/27/88,9/29/88

NONYL PHENOL ETHOXYLATE
LC50 = 5.5 mg/l
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)		
	11 mg/L 2 X LC50	27.5 mg/L 5 X LC50	55 mg/L 10 X LC50
BEFORE COLUMN	15.6	10.9	19.6
100	0.0	0.4	0.0
500	0.4	0.0	0.0
750	0.0	0.0	0.0
850	0.0	0.0	0.0
950	0.0	0.0	0.0

TABLE D.16

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.9(B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 9/26/88,9/27/88,9/29/88

NONYL PHENOL ETHOXYLATE
LC50 = 5.5 mg/l
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)		
	11 mg/l 2 X LC50	27.5 mg/l 5 X LC50	55 mg/l 10 X LC50
25	0.0 (-0.4)	0.0 (-0.4)	0.0 (-0.4)
50	2.1	0.0	0.0
75	0.0 (-0.4)	5.7	12.7
80	14.2	18.0	35.7
85	17.6	18.3	26.7
90	3.6	6.0	9.9
95	1.6	0.0 (-1.1)	0.5
100	6.5	0.0 (-0.1)	0.4

TABLE D.17

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURES 4.10(A) AND 4.11(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 10/11/88,10/6/88,10/7/88

DI-N-OCTYL PHTHALATE
LC50 = 89.7 mg/l
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)		
	164 mg/L 1.8 X LC50	491 mg/L 5.5 X LC50	1473 mg/L 16.4 X LC50
BEFORE COLUMN	4.2	5.1	16.0
100	7.8	0.0	0.0
500	8.8	0.0	3.0
750	8.8	0.8	10.2
850	8.8	1.2	10.7
950	8.8	3.6	13.4

TABLE D.18

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.10(B) AND 4.12(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 10/11/88,10/6/88,10/7/88

DI-N-OCTYL PHTHALATE
LC50 = 89.7 mg/l
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)		
	164 mg/L 1.8 X LC50	491 mg/l 5.5 X LC50	1473 mg/L 16.4 X LC50
25	0.0 (-0.4)	1.6	0.0 (-0.4)
50	0.0	1.1	4.2
75	4.3	6.6	20.2
80	8.1	2.3	11.1
85	7.5	5.6	13.7
90	19.3	9.0	20.9
95	23.7	22.5	24.6
100	6.1	7.8	13.1

TABLE D.19

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.11 (B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 10/11/88,10/6/88,10/7/88

DI-N-OCTYL PHTHALATE
LC50 = 89.7 mg/l
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	(1 / ET50) X 100%		
	164 mg/L 1.8 X LC50	491 mg/L 5.5 X LC50	1473 mg/L 16.4 X LC50
BEFORE COLUMN	2.6	3.4	11.1
100	5.7	0.0	0.0
500	6.1	0.0	2.7
750	6.1	0.0	8.3
850	6.1	0.0	6.9
950	6.1	2.6	10.0

TABLE D.20

ABSORBANCE DATA COLLECTED ON 10/13/88,10/8/88,10/9/88
FOR FIGURE 4.11(C)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 10/11/88,10/6/88,10/7/88

DI-N-OCTYL PHTHALATE
LC50 = 89.7 mg/l
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS
ABSORBANCE READ AT THE END OF THE BIOASSAY TEST

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	ABSORBANCE (ABS)		
	164 mg/L 1.8 X LC50	491 mg/L 5.5 X LC50	1473 mg/L 16.4 X LC50
BEFORE COLUMN	0.080	0.287	0.209
100	0.330	0.044	0.004
500	0.550	0.060	0.011
750	0.560	0.060	0.055
850	0.560	0.064	0.089
950	0.570	0.072	0.194

TABLE D.21

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.12(B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 10/11/88,10/6/88,10/7/88

DI-N-OCTYL PHTHALATE
LC50 = 89.7 mg/l
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	(1 / ET50) X 100%		
	164 mg/L 1.8 X LC50	491 mg/l 5.5 X LC50	1473 mg/L 16.4 X LC50
25	0.00	0.00	0.00
50	0.00	0.00	3.07
75	2.74	4.78	17.54
80	5.46	2.08	8.33
85	5.59	4.17	10.20
90	11.63	6.21	17.54
95	30.30	25.00	32.26
100	5.88	6.85	10.75

TABLE D.22

ABSORBANCE DATA COLLECTED ON 10/13/88,10/8/88,10/9/88
FOR FIGURE 4.12(C)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 10/11/88,10/6/88,10/7/88

DI-N-OCTYL PHTHALATE
LC50 = 89.7 mg/l
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS
ABSORBANCE READ AT THE END OF THE BIOASSAY TEST

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	ABSORBANCE (ABS)		
	164 mg/L 1.8 X LC50	491 mg/L 5.5 X LC50	1473 mg/L 16.4 X LC50
25	0.004	0.004	0.003
50	0.006	0.001	0.010
75	0.034	0.022	0.080
80	0.053	0.028	0.075
85	0.110	0.054	0.300
90	0.289	0.200	0.667
95	0.522	0.603	1.199
100	0.285	0.238	0.346

TABLE D.23

TIME LETHALITY AND MICROTOX DATA
FOR FIGURES 4.13(A) AND 4.13(B)

FRACTIONATION SPE C-18 COLUMN

DI-N-OCTYL PHTHALATE
CONCENTRATION = 1473 mg/L
1.5% METHANOL EXTRACTION

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	MICROTOX TOXICITY UNIT (1 / EC50) X 100%	CERIODAPHNIA DUBIA TOXICITY DATA (1 / ET50) X 100%
25	0.0	0.0
50	1.4	3.1
75	10.2	17.5
80	3.8	8.3
85	35.7	10.2
90	0.4	17.5
95	0.6	32.3
100	1.3	10.8

TABLE D.24

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.14(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 1/24/89

FAYETTEVILLE CROSSCREEK WWTP MINUS C18T SERIES BLANKS
SAMPLE TAKEN BEFORE CHLORINATION
COMPOSITE SAMPLE 1/16/89 TO 1/17/89
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)
BEFORE COLUMN	11.3
50	0.0
250	0.0
450	0.0

TABLE D.25

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.14(B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 1/24/89

FAYETTEVILLE CROSSCREEK WWTP MINUS C18T SERIES BLANKS
SAMPLE TAKEN BEFORE CHLORINATION
COMPOSITE SAMPLE 1/16/89 TO 1/17/89
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)
25	0.0
50	0.0
75	1.6
80	12.7
85	14.5
90	3.5
95	0.0 (-0.3)
100	0.7

TABLE D.26

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.15(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 2/3/89

FAYETTEVILLE CROSSCREEK WWTP MINUS C18T SERIES BLANKS
SAMPLE TAKEN BEFORE CHLORINATION
COMPOSITE SAMPLE 1/30/89 TO 1/31/89
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)
BEFORE COLUMN	1.7
100	0.0
500	0.0
750	0.0
850	0.0
950	0.0

TABLE D.27

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.15(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 2/3/89

FAYETTEVILLE CROSSCREEK WWTP MINUS C18T SERIES BLANKS
SAMPLE TAKEN BEFORE CHLORINATION
COMPOSITE SAMPLE 1/30/89 TO 1/31/89
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)
25	0.3
50	0.0 (-0.5)
75	6.4
80	6.2
85	0.5
90	0.0 (-1.2)
95	0.0
100	2.1

TABLE D.28

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.16(A)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 3/24/89

FAYETTEVILLE CROSSCREEK WWTP MINUS C18T SERIES BLANKS
SAMPLE TAKEN BEFORE CHLORINATION
COMPOSITE SAMPLE 3/20/89 TO 3/21/89
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

VOLUME OF SAMPLE PASSED THROUGH C-18 COLUMN (mls)	TIME BASED TOXICITY UNIT (TTU)
BEFORE COLUMN	8.8
100	0.0
500	0.0
750	0.0
850	0.0
950	0.0

TABLE D.29

TIME LETHALITY TOXICITY UNIT DATA
FOR FIGURE 4.16(B)

FRACTIONATION SPE C-18 COLUMN
BIOASSAY STARTED ON 3/24/89

FAYETTEVILLE CROSSCREEK WWTP MINUS C18T SERIES BLANKS
SAMPLE TAKEN BEFORE CHLORINATION
COMPOSITE SAMPLE 3/20/89 TO 3/21/89
1.5% METHANOL EXTRACTION
BIOASSAY CONDUCTED IN PLASTIC CUPS

SPE C-18 COLUMN EXTRACTION PERCENT METHANOL / WATER ELUTION	TIME BASED TOXICITY UNIT (TTU)
25	0.0 (-4.6)
50	0.0
75	5.4
80	7.6
85	7.0
90	3.7
95	0.0 (-1.4)
100	0.0 (-2.7)