THE CORRELATION OF CHROMATOGRAPHIC PARAMETERS WITH THE TOXICITY OF PETROLEUM REFINERY FINAL EFFLUENT

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CHAPTER I

INTRODUCTION

The Clean Water Act of 1972, established as one of its goals for the nation, the elimination of toxic pollutants in toxic amounts in waters of the United States. According to the 1988 National Water Quality Survey (USEPA, 1988), 45% of river miles monitored and 35% of lake acreage monitored contained elevated levels of toxic pollutants. As a way of controlling the discharge of toxic pollutants into waters of the U.S., the USEPA in 1984 implemented a policy based on water-quality parameters rather than the technology based limits previously used (USEPA, 1984). The fundamental philosophy of this policy is to determine and control toxics problems by the use of whole effluent toxicity tests involving appropriate aquatic organisms.

Approximately 25% of all wastewater released into surface waters in the U.S. consists of manufacturing and energy related discharges (Speidel et al., 1988). Because petroleum refining discharges are subject to regulation by the NPDES permitting system, in many regions, the EPA has established a set of guidelines requiring biomonitoring for effluent dischargers whose wastewater could potentially lead

to toxic effects in the receiving stream. In Oklahoma, 7-day subchronic *Ceriodaphnia dubia* and fathead minnow toxicity tests are performed to determine the degree of toxicity that effluents present to receiving waters. In order to determine the degree of toxic contamination from refinery effluents, aquatic toxicity tests are routinely being used by industry to meet permit requirements. Another toxicity test performed, but not required by regulations, is the Microtox system; this test utilizes the marine bacteria *Photobacterium phosphoreum*. These species are sensitive to the types of pollutants commonly discharged by petroleum related industries (Marchini et al., 1992; Cowgill et al., 1991).

The *Ceriodaphnia dubia* and Fathead minnow sub-chronic toxicity tests expose the organisms for 7 days and require many hours of personnel time to set up, conduct, and analyze the test results (Kszos, 1991). However, one alternative would be to develop a predictive model, a Quantitative Structure Activity Relationship (QSAR), based on chemical-physical characteristics of test compounds to screen effluents for further toxicity tests. QSARs have routinely been used to predict the toxicity of homogenous sets of chemicals. Unfortunately, in the environment, a complex mixture of chemicals is present and very little data pertaining to QSARs of unknown mixtures exists. Generally a set of biological activity parameters such as toxicity

endpoints like LC50s or EC50s are correlated with physicochemical parameters such as log P, solubility, or other electronic or steric factors. The use of Kovats gas chromatographic retention was shown to be an effective structural parameter for QSAR studies of alkylbenzenes and chlorobenzenes (Mgbeme, 1989). Many other chromatographic retention parameters have been used in correlating structural and biological activity values (Kaliszan, 1987). Another index involves the use of reverse-phase liquid chromatography retention parameters (Baker, 1979). The retention index is a parameter that describes many other parameters commonly used in QSARs. Retention indices are generally associated with an increase in molecular weight, boiling point, and the degree of hydrophobicity as contained in log P.

The proposed study involves the use of complex oil refinery effluents to test the use of structure activity relationships to predict the toxicity of unknown mixtures. One method used to reduce the complexity of complex mixtures is to fractionate or separate the sample into chemically similar and distinct components. The use of C18 silica bonded solid phase extractions allows complex samples to be fractionated based on their degree of nonpolar character (Mount and Anderson-Carnahan, 1989).

Previous research in mixture toxicity studies has examined a known mixture of compounds and assigned an index

for each chemical based on the additive toxicity displayed by the substance. Konemann (1981) has developed the Mixture Toxicity Index (MTI) as a mean of assessing the additive toxicity exhibited by a mixture of chemical compounds. The basic objective of this experiment was to develop a method for correlating the toxicity of a complex oil refinery effluent with some measure of its HPLC and Kovats Index. In order to develop the method whereby the toxicity of an unknown complex mixture may be predicted, the following null hypothesis will be tested:

1. H_0: The 7 day subchronic toxicity to Fathead minnows is not correlated with the Kovats and HPLC Index of the C18 extractable organics from a complex oil refinery effluent.
2. H_0: The 7 day subchronic toxicity to *Ceriodaphnia dubia* is not correlated with the Kovats and HPLC Index of the C18 extractable organics from a complex oil refinery effluent.
3. H_0: The acute toxicity of effluent to *Photobacterium phosphoreum* is not correlated with the Kovats Index of the C18 extractable organics from a complex oil refinery effluent.
4. H_0: The toxicity of a petroleum refinery effluent to *Photobacterium phosphoreum* is not reduced by extraction with C18 solid phase cartridges.

CHAPTER II

LITERATURE REVIEW

Contaminants in the aquatic environment may affect organisms in a number of ways; initially, the effect may be lethal or sublethal. Lethal effects are those causing the organism's death while sublethal effects are deleterious effects not immediately causing its death, but which produce some adverse behavioral, physiological, or morphological changes. Concurrently, the effect may be manifested with great severity in a short time (acute) or over a longer period of time at a continuous slow rate (chronic); chronic effects usually last from weeks to years depending on the life cycle of the organism (Rand and Petrocelli, 1985).

One of the fundamental ideas of toxicology is that structure of chemicals affects their biological activity. The importance of structure in toxicology has been recognized since the work of Meyer (1899) and Overton (1899) recognized the significance of partitioning between fat and water in producing toxic effects. The field eventually proceeded to the stage of predicting biological activity from chemical structure. In this regard, quantitative structure activity relationships (QSARs) began. A

quantitative structure activity relationship is a mathematical relationship set up to predict the biological potency of a particular set of compounds. Work by Hansch and Fujita (1963) and Hansch et al. (1963) established the importance of lipophilic character of compounds in structure activity relationships. They also suggested that steric factors and electronic distribution along with rate of penetration were parameters essential to the biological activity of chemicals. The QSAR is set up by determining the potency of a training set of compounds from a group of chemicals, for example the chlorinated alkane hydrocarbons. This part of the process involves building a model from which predictions of biological activity of other unknown members of a group may be made. One of the first requirements is that the training set of compounds be structurally similar and act by the same mode of action. Also the training set must span the space of factors that relate variation in biological activity to such factors as lipophilicity and electronic distribution. Tosato et al. (1990) examined QSARs based on statistical design to identify hazardous chemicals for additional biological testing. The importance of selecting a good training set and validation of the model was shown to be enhanced by statistical designs involving multivariate and principal component analysis.

The aquatic toxicity of many non-reactive non

electrolyte organic compounds have been shown to be consistent with a narcosis mode of action involving equilibrium partitioning into a lipid bilayer membrane. This mode of action is thought to produce physical changes as a result of movement of a chemical into the cell membrane. These compounds may be fitted to a simple linear QSAR involving only log P. However, if equilibrium is not reached between the toxicant phase and the phase at the site of biological action, a linear relationship does not exist in the QSAR. Instead, a parabolic relationship seems to hold between log P and biological activity (Hansch, et al., 1963; Lipnick, 1989)

Veith et al. (1989) investigated the toxicity of a series of acetylenic alcohols (C-C triple bond) to the fathead minnow and found that modes of action other than simple narcosis were believed to be causing excessive toxicity. Certain alcohols are thought to be altered to electrophillic aldehydes and ketones capable of reacting with nucleophilic moieties (for example -OH, -SH, $-NH_2$, -NH) present in enzymes and other important biological macromolecules. This underscores the idea that when a QSAR is developed, one must have a sound working hypothesis of the general mechanism that is causing toxicity.

Veith, et al. (1983) estimated the acute toxicity of several industrial organic chemicals to fathead minnows using a single bilinear equation for a group of alcohols

acting with the same mode of action (narcosis). There was general agreement when log P was used to predict the 96 hour LC50.

Along with the effect of length of exposure and dose, another influencing factor that must be taken into account in the environment is the impact of the combination of contaminants on organisms. Chemical mixtures complicate the toxicity to aquatic organisms by increasing the number of possible interactions. A combination of chemicals may act together as a whole to produce an additive effect; in essence, the sum of the individual toxicities equals the toxicity of the mixture. The mixture may also be less than additive (antagonistic) toxicity or there may be synergism in which the toxicity of the mixture is greater than the sum of the individual toxicities.

Albert (1987) proposed two methods whereby one could predict the toxicity of complex mixtures: the first one involves identifying the risk from individual components where data is available. The second method treats the mixture as a single toxicant and measures the change in toxicity as composition changes. However, this assumes that knowledge of the chemical composition of the mixture is available.

Past attempts have been made to predict the toxicity of mixtures of contaminants. Brown (1968) developed a method to estimate mixtures of common industrial contaminants to

rainbow trout. Based on the knowledge of water quality parameters such as pH, dissolved oxygen, and temperature, the toxicity of the mixture was calculated as the sum of the proportions of the acutely toxic concentration of each contaminant.

L.L. Marking (1977) used the concept of toxic units to determine an additive toxicity index. Each component in a binary mixture contributes to the toxicity; the contributions can be summed as follows:

$$(A_m/A_i) + (B_m/B_i) = S$$

where A and B represent individual chemicals; i and m are the toxicities of the individual chemicals and mixtures respectively; S represents the sum of the biological activity.

Values obtained for S have meaning for various ranges. An S around 1 represents additive toxicity; values greater than 1 indicate greater than additive toxicity (synergism); values less than 1 represent (antagonism) less than additive toxicity.

The determination of the toxicity of complex mixtures has included studies in aquatic toxicology. Anderson (1979) extended the study of multiple toxicity based on dose-response data and isobolograms to predict the lethal and sublethal toxicities of mixtures of heavy metals. This report found that while the toxicities of pure solutions of copper and zinc decreased with an increase in hardness,

mixtures of these metals produced virtually the same toxicity as the hardness increased.

A study by Deneer (1988) investigated the joint acute toxicity of 50 organic chemicals towards *Daphnia magna*. The model of concentration addition was used to predict the toxicity of mixtures of compounds. This method involves the use of a toxic unit; the concentration of each compound is expressed as a fraction of its lethal or effective concentration. The toxicity of the mixture is calculated by summing toxic units over all compounds. The study found that compounds present at concentrations as low as .0025 toxic units contributed to the joint toxicity of the mixture. It is apparent that a mixture of compounds acting by anesthetic action will contribute to the toxicity of the mixture even at very low concentrations. This indicates that if compounds are acting by the same mode, there is no concentration below which a contribution to overall toxicity is made.

Wherever possible, the regulation and control of complex wastes into the aquatic environment should be based on chemical analysis of the toxic components and their dilution in the receiving stream ; however, if the toxicity cannot be predicted from known components, toxicity tests using species of appropriate sensitivity are necessary. The toxicity test may serve two purposes: one to serve as a surrogate for chemical analysis when a chemical analysis

would prove difficult and secondly to serve as a surrogate for other aquatic organisms to predict the impact of the effluent on other organisms in the receiving stream (Lloyd, 1989).

Hermens et. al (1985) developed QSARs to predict the toxicity of chemicals and complex mixtures of chemicals to several aquatic species. Testing of 14 aquatic species with values were correlated with Log P estimates. The 14 day lethality to guppies of a group of anilines correlated with log P and Hammett's constant (r=.935,n=17). A group of organic halogen compounds possessing reactive toxicity correlated with log P and the reactivity toward 4-nitrobenzylpyridine, as measured by reaction rate constant (r=.956,n=15). The toxicity of the mixture appears to be consistent with the concentration addition model.

Vandermeulen has reviewed the literature about toxicity of petroleum hydrocarbons to freshwater organisms since 1979 (1987). Freshwater organisms were found to be quite variable in their toxic response to petroleum hydrocarbons. The reasons for the differences in sensitivity were attributed to life cycle stage tested, sex of the organism, feeding regime, and prior exposure history of the organism. Response may also be affected by the composition of the test material, temperature of the test, duration of the test, condition of the material, and general water quality. Differences in test methodology can significantly add to the

variability of toxicity results; the test may be static or flow-through and the test materials may be prepared by any number of ways. The toxicity of water soluble fractions of crude oil to aquatic organisms ranged from 1-10 mg/l (Vandermeulen, 1987).

Upon examination it seems that the most sensitive development stages of organisms to crude oil occur in the larval and juvenile stages of the life cycle. Much of a crude oil's toxicity has been attributed to its aromatic, especially its di and triaromatic content. As expected, the amount of oil required to elicit a chronic sublethal response is generally much lower than typical acute responses. The effect may be seen in developmental, physiological, behavioral, and pathological responses.

Petroleum refinery effluents are usually complex mixtures of aliphatic and aromatic hydrocarbons and heteroatom substituted aromatic compounds (USEPA, 1978). Depending on the nature and concentration of components in an effluent, either acute or chronic effects on aquatic organisms may result.

Pickering and Henderson (1966) examined the acute toxicity of some petrochemicals to the fathead minnow. They proposed the use of long term studies to determine sublethal effects in order to derive water quality requirements for aquatic life.

Industrial wastewater similar to oil refinery effluents

has been characterized for acute toxicity to aquatic organisms. The acute toxicity of coal conversion effluents to *Daphnia magna* found that cresols and phenols were the most acutely toxic substances in the wastewater and that their toxicities were additive in character. The 48-hour LC50 of the untreated effluent was 1.06 ml/l while the LC50 of the treated effluent was 111.6 ml/l. (Parkhurst et al., 1979).

Schultz et al. (1978) studied contaminants from aqueous coal-conversion effluents and found that of the compounds tested, toxicity increased and solubility decreased with increasing alkyl substitution. Another study by Schultz et al. (1980) examined some nitrogenous compounds present in coal conversion effluents and found that toxicity increased with increasing partition coefficient, boiling point, and molecular weight.

Generally, acute toxic effects of oil refinery effluents are significantly reduced after biological treatment. Field studies have suggested there is a low potential for long-term biological effects based on chronic lab tests; impacts appear to be limited to the mixing zone (Stubblefield and Maki, 1984). However, some refractory compounds escape treatment and are passed into the receiving streams. Once the effluent reaches the streams, deleterious effects on resident organisms and the overall structure of the river ecosystem may take place. Barclay et

al. (1980) investigated the effect of an oil refinery effluent discharge on the diversity of macrobenthos in a small Texas river. Species diversity was impacted at sampling sites below refinery discharges when compared to controls. Smith (1987) correlated the diversity of macrobenthos in an Oklahoma stream receiving refinery effluent with concentration of non-polar organics.

Although the acutely lethal toxicity of petroleum refinery effluents appears to be significantly reduced following treatment, chronic and sublethal effects and effects at the ecosystem level may be revealed by further studies.

Reece and Burks (1985) performed *Daphnia magna* acute toxicity tests on the methylene chloride extractable fraction of petroleum refinery wastewater. The research concluded that the compounds causing most of the acute toxicity of the petroleum refinery wastewaters tested were the steam volatile base neutral aromatic fractions. The work identified eleven polycyclic aromatic hydrocarbons in the steam volatile base-neutral aromatic fraction. Many compounds remained unidentified although they appeared to be similar to the previously identified compounds and modified by addition of oxygen, nitrogen, and sulfur. The compounds were believed to be acting in an additive or synergistically effect because these compounds typically are not acutely toxic at the low concentrations found in the samples.

Identification of compounds previously identified in petroleum refinery effluents indicates that polynuclear aromatic hydrocarbons (PNAs) and various aromatic alkyl substituted compounds make up a significant portion of the content of the effluent (USEPA, 1978). While many compounds of this type are typically not acutely toxic they have the potential to accumulate in aquatic organisms and produce deleterious sublethal effects.

Southworth et al. (1978) studied the bioaccumulation potential of some polynuclear aromatic hydrocarbons in *Daphnia pulex*. Regression of the 24 hour concentration factor vs log P yields the expression:

$$\log C.F. = .7520 \log P - .4362, \quad R^2 = .85.$$

The bioaccumulation potential of the PAH increased approximately by a factor of 10 with each additional ring structure added to the compound.

Trucco, Engelhardt, and Stacey (1983) studied the toxicity, accumulation, and clearance of benzene, naphthalene, phenanthrene, benzo(a)anthracene, and benzo(a)pyrene to *Daphina pulex*. Toxicity, as measured by the 96-h LC50, increased in the order just mentioned and ranged from 15 mg/l for benzene to 5 μg/l for benzo(a)pyrene.

Borey and Parcell (1980) studied the toxicity of carbon treated refinery effluent to the estuarine minnow *Cyprinodon variegatus* exposed over a period of thirty days. Results

from tests on half strength effluent showed that activated carbon treated effluent ET50's (time necessary to affect 50% of the test organisms) were significantly higher than conventional treated effluent. Tests with full strength effluent over longer time periods resulted in greater mortality due to upsets in the operation of the plant. Overall mortality was too low to detect a consistent difference between effluents treated in a conventional manner and those treated with activated carbon.

Barclay and Harrel (1985) investigated the effect of waste water discharges, including a petroleum refinery, on the community structure of macrobenthos in two streams in southeastern Texas. Species diversity was significantly lower at sites below refinery effluent as calculated by Shannons diversity index (1.03 compared to 4.20) compared to relatively clean sampling stations. The coefficient of similarity between the refinery site and a clean site was .122, while the coefficient between two clean sites was .688. The streams were characterized by generally poor water quality as indicated by physicochemical and macrobenthic parameters.

Rowe et al. (1983a) examined the sublethal effects of a treated oil refinery effluent to American flagfish (*Jordanella floridae*). Fish were exposed for their entire life cycle at four concentrations up to a maximum concentration of 28% effluent. Second generation fry were

then separated and exposed for another 30 days to the refinery effluent. There were no significant differences among the groups mortality. There was significant difference in wet weight after 92 days for males at 9% effluent and .92% for females. Days of spawning, frequency of spawning, and number of eggs per spawning were significantly affected at 28% effluent. Survival in the second generation was not greatly affected at any effluent concentration. There were spinal abnormalities and erosion of the fins especially at higher concentrations. The 9% effluent concentration was considered as the threshold for chronic sublethal effects on flagfish. Overall there were significant sublethal effects at the highest effluent concentration and minimal effects at all other concentrations. The effects of area, side of effluent entry, and concentration were all non significant. Considering this, the authors concluded that avoidance behavior is not likely to be affected in the field by well treated refinery effluents.

Another report by Rowe et al. (1983b) investigated the sublethal effects of refinery effluent on growth of rainbow trout. Four concentrations of effluent were used up to a maximum of 30%. Random samples were taken and growth and weights were measured at 0, 11, 22, 33, and 44 days.

At the end of 44 days, fish weight in the 30% effluent was not statistically different than control. Specific

growth rates were statistically different from control for 10 and 30% effluent. Results for fish length followed the same pattern of response. The threshold concentration for fish growth based on this experiment was estimated to be between 3 and 10% effluent.

Westlake et al. (1983a) examined the sublethal effects of petroleum refinery effluent on avoidance behavior and other locomotor responses of rainbow trout. Fish were placed in flow-through observation tanks containing concentrations of refinery effluent. Both respiratory rate and number of coughs /minute increased with increasing concentration of effluent. These measures were relatively insensitive compared to other responses; the estimated threshold for respiratory rate was 71% and 35% for coughing.

Westlake et al. (1983c) investigated the sublethal response of petroleum refinery effluent on survival and reproduction of *Daphnia pulex* and found them to be more sensitive to effluents than flagfish and rainbow trout. Chronic lethality thresholds were estimated at 6.4% and threshold of 5% inhibition in reproduction was estimated as 0.52%.

An in vitro enzyme inhibition assay using inhibition of glucose-6-phosphate dehydrogenase (G6PDH) was evaluated by Rutherford et al. (1979) on a simulated petroleum effluent. The research also used the in vitro enzyme inhibition procedure for toxicity screening of petroleum refinery

effluents. Tests were conducted on artificial refinery mixtures (ARM) and actual refinery effluents using inhibition of glucose-6-phosphate dehydrogenase as the test response. G6PDH was inhibited by 18% by raw refinery effluent; inhibition was reduced to 12% following activated sludge treatment. The effects on the ARM indicated that a combination of antagonistic, additive, and synergistic effects was taking place.

Boese et al. (1982) investigated the effect of two petroleum refinery wastewaters on gill Na,K-ATPase in the Pacific Staghorn Sculpin (*Leptocottus armatus*). Results indicated that one refinery effluent low in total extractable organic material produced statistically significant differences in enzyme activity at 20% effluent. The refinery effluent with high total extractable organics produced significant differences in enzyme activity at effluent concentrations of 2 percent. Petroleum refinery wastewaters thus have the potential to affect the ability of estuarine organisms to maintain osmoregulatory ability.

Not only does the possibility exist for organisms to be directly affected by contaminants in the water column, but also those organisms living in the sediment may be impacted. Knap and Williams (1981) have investigated the fate of petroleum hydrocarbons from a petroleum refinery effluent in an estuarine system. Adsorption of hydrocarbons onto estuarine sediment appears to be the major sink; about 70%

of the hydrocarbons in an experimental system could be found in the sediment.

Peterson et al. (1986) studied defensive, teritorial (agonistic) behavior of male orangespotted sunfish (*Lepomis humilis*) when exposed to treated petroleum refinery wastewaters. There was a significant decrease in the number of agonistic behavior displays (approaches, fin erections, chases, bites, and avoidance). If such behavior was significantly altered in the environment by refinery wastewaters, the potential exists for deleterious effects on the behavioral responses of this species.

A cell culture assay for determining the quality of oil refinery effluents has been previously described (Richardson, 1977). There was significant inhibition of cell growth at 22 and 45% effluents tested. The test was also found to be more sensitive than *Daphnia* when subjected to m-cresol as a reference toxicant.

As part of a research program studying the possibility of using bacteria for conducting toxicity tests, the Microtox analyzer was developed. Microtox utilizes the luminescent bacteria *Photobacterium phosphoreum* as the test organism. This bacterium is native to marine environments and emits light as a part of its normal metabolic processes. The emission of light is the basis for the test; if bacteria are subjected to a toxicant, metabolic processes will be inhibited and the amount of light produced will be reduced.

Consequently, the reduction in light is directly proportional to the toxicity of a substance. The information provided by Microtox allows for the calculation of an EC50, the amount of toxicant needed to produce a 50% reduction in light output. Microtox has been applied in numerous environmental contamination settings. Among the more common situations are contaminated groundwater, hazardous waste sites, toxicity of pure compounds, and toxicity monitoring of complex wastewater effluents (Bulich, 1984; Symons and Sims, 1988).

The Microtox toxicity test system has been used in QSAR studies of both pure compounds and complex mixtures. One area of research that has been examined frequently is the correlation of Microtox with predictions of toxicity to higher organisms like *Daphnia* and fathead minnows. Curtis et al. (1982) investigated the relationship between Microtox EC50 and fathead minnow LC50 values for 68 organic chemicals. Good correlation was found when the chemicals were divided into homologous groups. It could then become possible to predict the toxicity of a chemical to fish based on the results obtained with Microtox taking into account the variability of toxicity values. Indorato et al. (1983) also studied Microtox as a possible screen for toxicity to other fish as well as fathead minnows. The toxicity of 39 industrial effluents to *Photobacterium phosphoreum* and *Daphnia magna* agreed for 86% of the toxic wastewaters

(Vasseur, et al., 1984a). Microtox was found to produce fairly replicable results that corresponded with results from standard toxicity tests when analyzed with 15 test chemicals (De Zwart, 1983).

Microtox is frequently used in typical QSARs of a homologous series of compounds. A study to determine the reliability of using bacterial assays found that the EC50 for Microtox correlated with the LC50 for fathead minnows of a series of alcohols with R^2 of .96 (Curtis et al., 1982).

Many research papers have been published establishing a correlation between Microtox toxicity and the fathead minnow and *Ceriodaphnia* bioassays (Blum and Speece, 1989; Munnkitrick, 1991). Some research has found that Microtox has a sensitivity comparable to the traditional bioassay procedures. This research has suggested using Microtox as a screen for compounds or effluents to determine if further tests with other species is necessary (Vasseur et al., 1984b).

The use of Microtox as a screen for aquatic toxicity of complex effluents offers some important advantages. First, the test is relatively simple to perform and requires much less time than the traditional fish bioassays. Second, bacteria are important members at the bottom of the ecological food chain; data about contaminants impact at this trophic level may be helpful in understanding their impacts at the ecosystem level of response (Liu, 1984).

Microtox has been applied to the prediction of the toxicity of mixtures of shale oil components. A multiple linear regression model was used in predicting the toxicity of several mixtures; many mixtures were found to be synergistic using the Mixture Toxicity Index of Konemann (Warne et al., 1989). Mixtures such as complex effluents may be amenable to routine screening for toxic effects by Microtox because it seems to reproduce the acute and chronic effects seen with higher organisms (Pols, 1989).

The effectiveness of industrial treatment processes was analyzed using fish and Microtox (Casseri et al., 1983). The research found that Microtox correlated with 48-hour *Daphnia* and 96-hour fathead minnow data. The researchers concluded Microtox can be used in evaluating the effectiveness of chemical, biological, and adsorption processes employed in treating complex industrial wastewaters.

Eisman et al. (1991) describes the use of the Microtox Toxicity Test System in determining petroleum hydrocarbon toxicity of petroleum fuels and fuel components water soluble fractions, and soil leachates likely to contaminate the subsurface environment. The fuels tested were diesel, unleaded gas, JP4, and JP5 aviation fuel. Individual compounds chosen for testing were taken from groups of alkanes, cycloalkanes, alkenes, and alkylbenzenes. The toxicity of water soluble fractions was found to be comparable to samples run through soil leachate procedures.

Also the EC50 values obtained at different time intervals did not significantly differ indicating that volatilization did not drastically alter the test results.

Because many wastewater discharges have the potential to cause not only acute but also chronic effects on higher aquatic organisms, a relatively quick toxicity test that reveals the presence of chronically toxic contaminants has become necessary. Norberg and Mount (1985) have shown the utility of using the 7-day static renewal subchronic toxicity test on *Pimephales promelas* when conducting tests to estimate the chronic toxicity of effluents.

A short term method for estimating the chronic toxicity of effluents to *Ceriodaphnia dubia* has provided a compliment to fish toxicity studies (Mount and Norberg, 1984).

In an effort to develop simple and reproducible retention parameters and to make identification of unknown substances more reliable, Kovats (1958) introduced a method of calculating an index whereby the retention is determined relative to a series of homologous n-hydrocarbons. Mathematically the relationship can be expressed as follows:

$$I=100*\frac{logT_i-logT_n}{logT_{n+1}-logT_n}+100n$$

where T represents adjusted retention time, subscript i refers to the compound of interest and n represents the

carbon number of the n-alkane emerging just before the compound of interest, and n+1 represents the standard alkane emerging immediately after the compound of interest.

Because environmental samples routinely consist of complex mixtures of organic compounds, chromatographers have developed methods to improve the resolution of such complex mixtures using temperature programmed chromatographs. This involves starting the chromatogram off at a relatively low temperature and increasing the temperature at a constant rate. Lee and Vassilaros (1979) used this principle to separate a complex mixture of polycyclic aromatic hydrocarbons and calculate a retention index based on 2,3,4, and 5 ring PAH standards.

Another chromatographic index, developed by Baker et al. (1979), uses a retention index based on the relative retention of a homologous series of C_3 - C_{23} 2-keto alkane standards. This index has been applied to systems utilizing high performance liquid chromatography. The retention index of phenacetin was found to be relatively constant as percent composition of the mobile phase changed (Baker and Ma, 1979). Correlation between biological activity of some propranolol analogues and barbiturates and the HPLC index based on 2-ketoalkanes was carried out by Baker et al. (1979). The retention index of some barbiturates correlated with their ability to inhibit division of *Arbacia* eggs based on the model as follows:

$$- \log C = aX + bX^2 + C$$

$$(r = .959 \; F = 80.9 \; N = 9)$$

Baker, et al. (1979) investigated the antiarrhythmic activity of propanolol derivatives to rabbit hearts correlated with the HPLC retention index in the same study (r= .866 F = 13.5 N = 12).

Henry, et al. (1979) investigated the correlation of the chromatographic retention volume (V_R) used in HPLC to minimum inhibitory concentration of sulfonamides in vitro against *E. coli* on log V_R. Higher correlations were usually produced using reversed phase C18 pellicular column. Using a simple linear model gave a fairly good correlation (r = .805, s = .45, n = 11). Correlation using a parabolic model was more significant (r = .963, s = .22, n = 11).

Davydov (1982) found a relationship between toxicity and HPLC retention volumes of 17 cardiac glycosides.

The Kovats and HPLC index are associated with physical properties of compounds; the molecular weight, boiling point, solubility, and logP are related to chromatographic indices. Previous QSARs involving physical-chemical properties have correlated with the aquatic toxicity displayed by a homogenous set of compounds. An experiment set up to measure the indices of a complex petroleum refinery will be evaluated for its correlation with toxicity to Microtox, *Ceriodaphnia dubia* and *Pimephales promelas*.

CHAPTER III

MATERIALS AND METHODS

Water samples from the raw wastewater effluent of two Oklahoma petroleum refineries were collected on a monthly basis. Samples were obtained over a period of 24 hours proportional to the flow of the effluent. Samples were transported to the lab in polyethylene cube containers for a series of subsequent toxicity tests and chemical analysis (Fig.1). Water samples were used for 7-day subchronic toxicity to Fathead minnows and 7-day subchronic toxicity to *Ceriodaphnia dubia*. Aliquots were utilized for toxicity evaluations with the luminescent bacteria *Photobacterium phosphoreum*. The complete test produced an EC50 value describing the amount of effluent causing a 50% reduction in light output by the bacteria.

Chemical oxygen demand and ammonia levels were monitored according to APHA Standard Methods (1989).

Solid phase extraction (SPE), the use of bonded silica particles for adsorbing compounds from water, was used to extract compounds from the effluent sample. The functional group bound to the silica often contains significant nonpolar character for adsorbing nonpolar compounds from the water sample. The type of SPE used in this experiment was

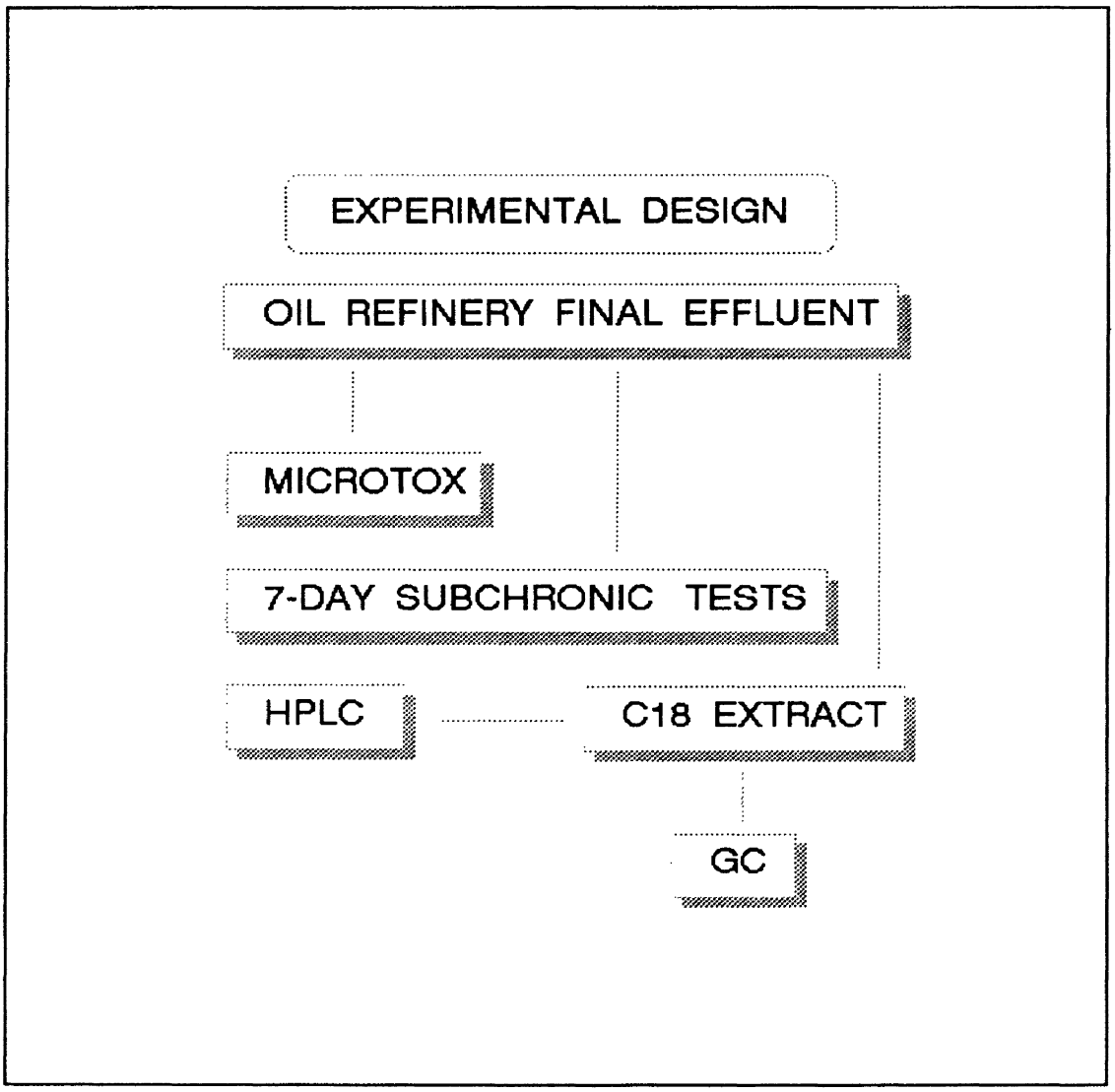

Figure 1. Basic experimental design for data collection of chromatographic and toxicity values.

C18 (Octadecylsilane). C18 solid phase extractions have been used in a variety of situations to remove organic chemicals from water (Junk et. al, 1988; Wells et al., 1987). Durhan et al. (1990) found that, on average, C18 SPE removed 60% of the toxicity to Ceriodaphnia. Extractions were carried out with C18 bonded silica extraction cartridges using a setup similar to EPA Method 525 for analysis of drinking water. The 6-ml high capacity cartridges contain 1000 mg of C18 bonded to 40-μm silica (J.T. Baker, Phillipsburg, NJ). The sorbent was activated with 20 ml of high purity methanol and allowed to remain in the cartridge for 5 minutes before removal. Ten ml of filtered distilled deionized water was then passed through the cartridge. One liter of effluent sample to which 5 ml methanol was added to keep the column conditioned was passed through the column at a rate of 10 ml per minute. The sorbent was dried under vacuum for 5 minutes. The sorbent was eluted with 4 ml of pesticide grade methylene chloride (Burdick & Jackson, Muskegon, Michigan) into a 15-ml extraction tube. The extract was dried by passage through a column of anhydrous $NaSO_4$ and concentrated to 500 μl in isooctane by introducing N_2 gas into the tube.

Gas chromatographic data were collected on a Tracor 565 gas chromatograph with a flame ionization detector and He carrier gas utilizing a 15 m DB608, (J&W Scientific, Folsom CA) 0.53 mm i.d., .83 μm film thickness bonded phase column.

Injection volumes of 1 μl were subject to the following temperature program: Initial temperature of 50°C held for 5 minutes to 250°C at 10°C/min with a 5-min hold at 250°C. The detector and injection port temperature was 250°C.

Samples for high performance liquid chromatography were adsorbed onto C18 columns in the manner described for GC analysis. 2 ml of pesticide grade methanol (E M Science, Gibbstown, NJ) was used to elute the column. The extract volume was reduced to 1 ml by bubbling N_2 gas into the extraction tube. The extract was filtered with a .45 μm polyvinylidene difluoride (PVDF) membrane filter (Gelman Sciences, Ann Arbor, Michigan) prior to addition of 2-ketoalkanes and internal standard.

The experiment was run using two Waters 501 pumps, a variable wavelength detector set at 278 nm, and Maxima Chromatography Data Analysis program. 10 μl of sample were injected onto a C18 reversed phase 10 μm pellicular column 30 cm long x 4.9 mm i.d. (Phenomonex, Torrance, CA). The solvent gradient was initially 70:30 water:methanol linearly increasing to 100% methanol over a 30 minute period. The solvent was held at 100% methanol for 15 minutes. The column equilibrated to original conditions over 15 min. Flow rate was 1 ml/min.

Toxicity tests with *Photobacterium phosphoreum* were conducted according to the Microbics operating manual (Microbics, Carlsbad, CA) with some modifications to the

100% assay. Briefly, effluent samples were osmotically adjusted to 2% NaCl and cooled to the test temperature of 15°C. Each concentration of effluent plus the control was run in quadruplicate. A Model 500 Microtox (Microbics, Carlsbad, CA) toxicity analyzer was used to monitor luminescence of *Photobacterium phosphoreum* after 5 and 15 minutes. The procedure allowed for the estimation of the EC50 (the concentration of effluent that resulted in a 50% reduction in bioluminescence).

The EC50 estimates were obtained by converting the percent inhibition of light emitted at each effluent concentration into a τ value defined as the ratio of light lost to the amount of light remaining (Johnson, 1974). Values of τ were calculated as a function of percent effluent; the EC50 was determined as the point where τ equals 1. The 95% confidence limits were calculated through the use of a program provided with the Microtox software.

Fathead minnow 7-day subchronic toxicity tests were conducted according to the latest EPA method (USEPA, 1989). Briefly, fathead minnows less than 24 hours old were exposed to effluent concentrations diluted with a synthetic mineral water made up in the lab. The dilutions were renewed with fresh effluent daily. Ten neonates (organisms less than 24 hours old) each were placed in 4 bowls for each effluent concentration tested. Organisms were counted and the dead removed. At the end of seven days, the surviving organisms

were dried and weighed on an analytical balance to determine the difference in growth between controls and treatment samples.

Ceriodaphnia 7-day subchronic toxicity tests were conducted according to the latest EPA method (USEPA, 1989). To summarize, *Ceriodaphnia dubia* neonates were exposed to effluent concentrations diluted with a synthetic mineral water made up in the lab. One neonate was placed in each of 10 cups for each effluent concentration. The number of organisms surviving and the number of young produced were counted daily. The test was terminated after 3 broods of neonates were produced.

Calculation of Kovats Index

Kovats index is a gas chromatographic index that compares the retention time of unknown compounds to the retention of a series of standard n-alkanes. Because petroleum refinery effluents are such complex mixtures, another method became necessary to describe its Kovats Index.

In order to calculate the index, the median point of the total area of the chromatogram was determined. The retention time at this point was used to calculate the Kovats Index according to the following equation:

$$I=100*\frac{T_i-T_n}{T_{n+1}-T_n}+100n$$

where T_i is the retention time of the median point in the chromatogram, T_n is the retention time of the n-alkane standard eluting just before the median point, T_{n+1} is the retention time of the n-alkane standard eluting just after the median point; n represents the carbon number of the n-alkane eluting prior to the median point.

Statistical Methods

LC50s for the fathead minnow and *Ceriodaphnia* survival were calculated by the Trimmed Spearman-Karber method (Hamilton et al., 1977). An IC50 is the concentration of effluent required to reduce a non-quantal effect by 50 percent; calculations were performed with the computer program BOOTSTRAP. In order to determine the concentration of effluent causing no statistically significant difference from control response, the NOEC was calculated using the TOXSTAT (Version 3.2) program. The procedure tests the difference between control response means and treatment means employing Dunnett's test (Dunnett, 1955).

Chromatographic and toxicity data were correlated using Pearson's r value. Determination of quality of correlations was carried out with the use of SYSTAT v.5.01

CHAPTER IV

RESULTS

Chemical Analysis

Chemical characterization of effluents

Refinery effluents characterized by HPLC and capillary gas chromatography in all cases showed a fingerprint of petroleum contamination; the fingerprint can be described as an unresolved complex mixture (UCM). In studies of discharges of hydrocarbons from municipal wastewater, Hoffman and Quinn (1987) and Van Vleet and Quinn (1977) described a UCM produced from capillary gas chromatograms. Extracts of the C18 SPE refinery wastewater samples performed during this study exhibited similar chromatographic complexity. Chromatographic data was obtained on both GC and HPLC (Appendix A). The mixture consisted of chemically similar molecules having boiling point and polarities alike enough to be unresolvable by the columns used in this study (i.e., HPLC chromatograms of refineries A and B are shown in Figures 2 and 3 respectively and a representative gas chromatogram is shown in Appendix A). These chromatograms clearly show the UCM present in both refinery effluents. An analysis of the amounts bracketed by

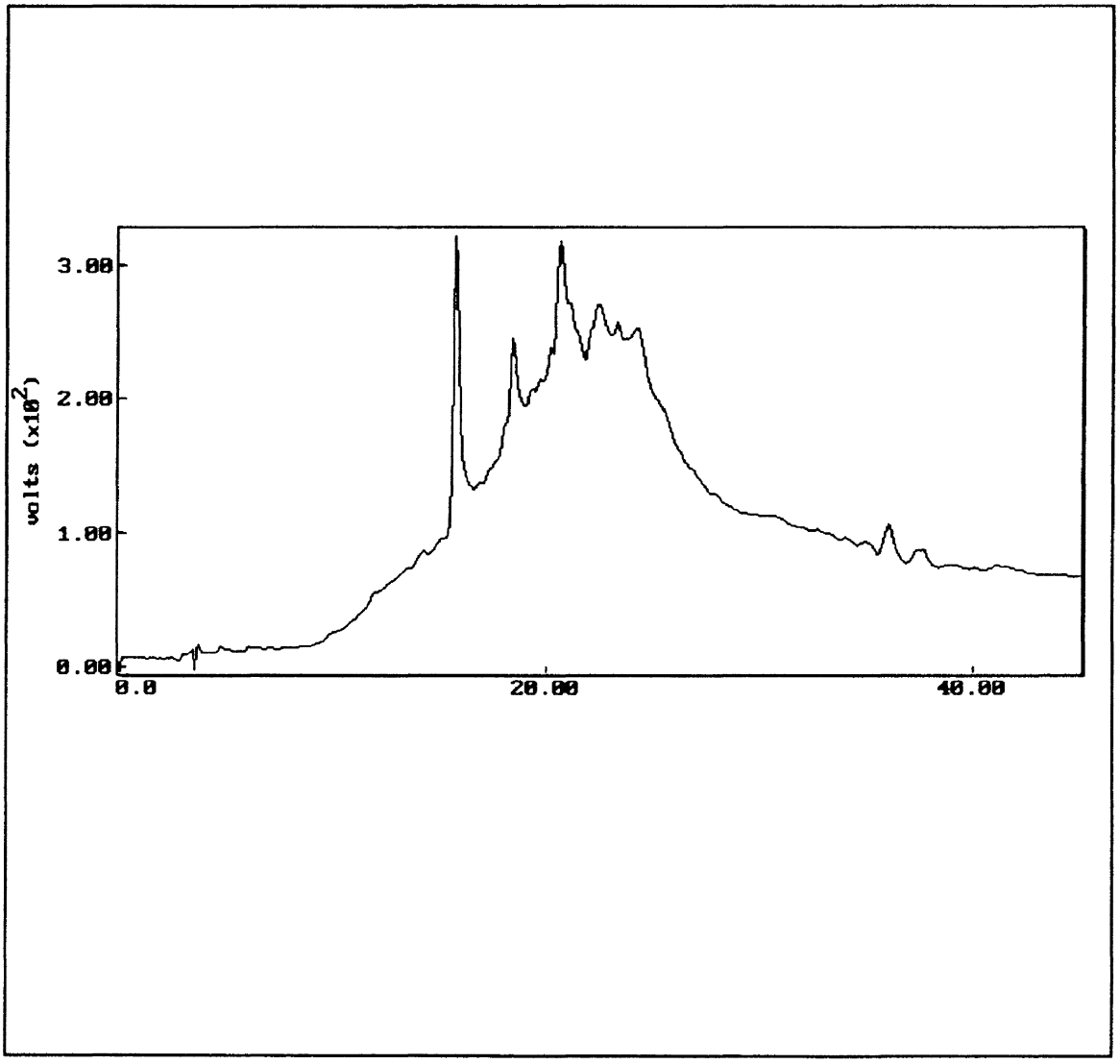

Figure 2. HPLC chromatogram of a refinery A final effluent adsorbed onto C18.

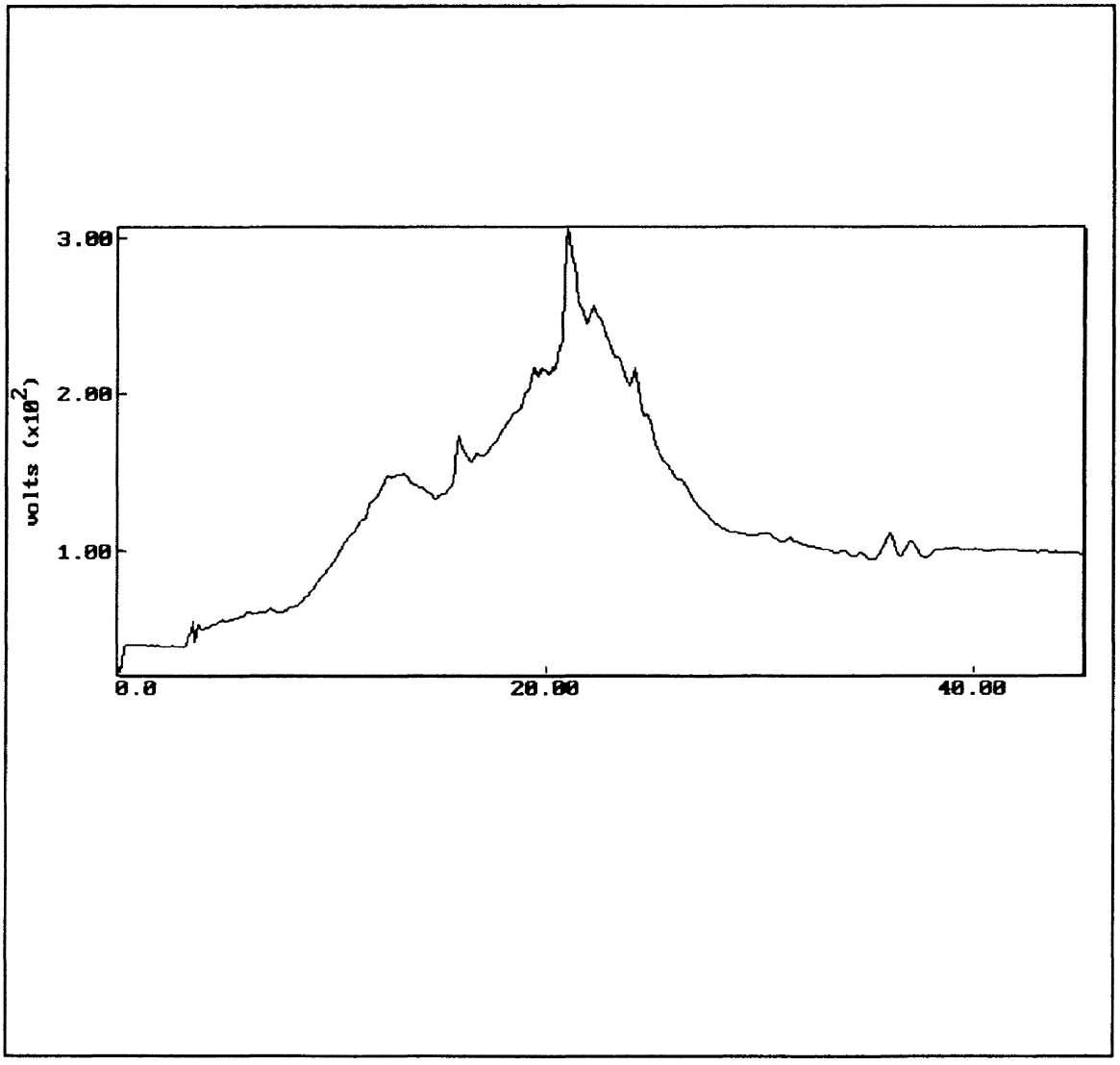

Figure 3. HPLC chromatogram of refinery B final effluent adsorbed onto C18.

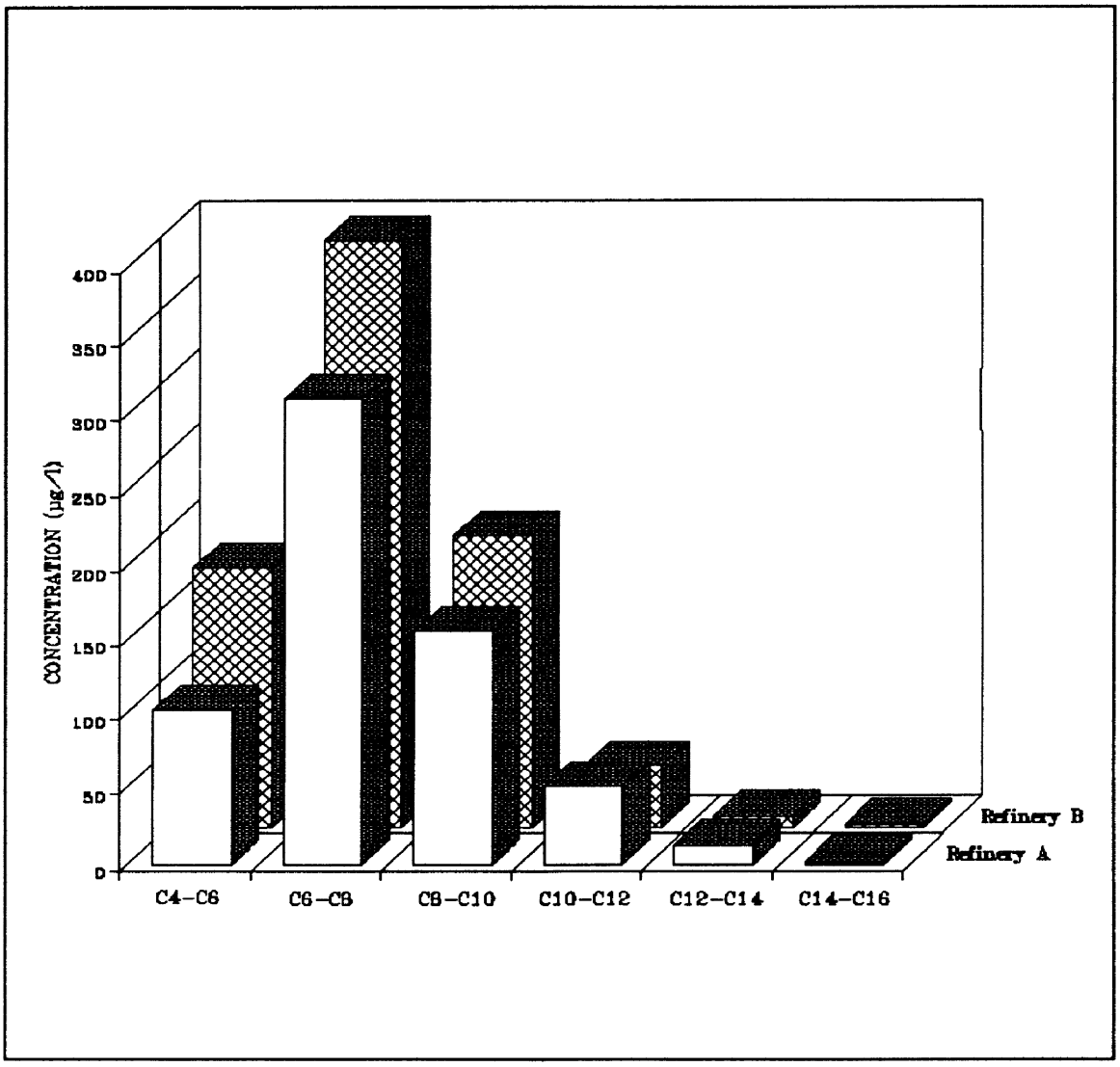

Figure 4. Amounts bracketed by HPLC standards for Refinery A and B final effluents.

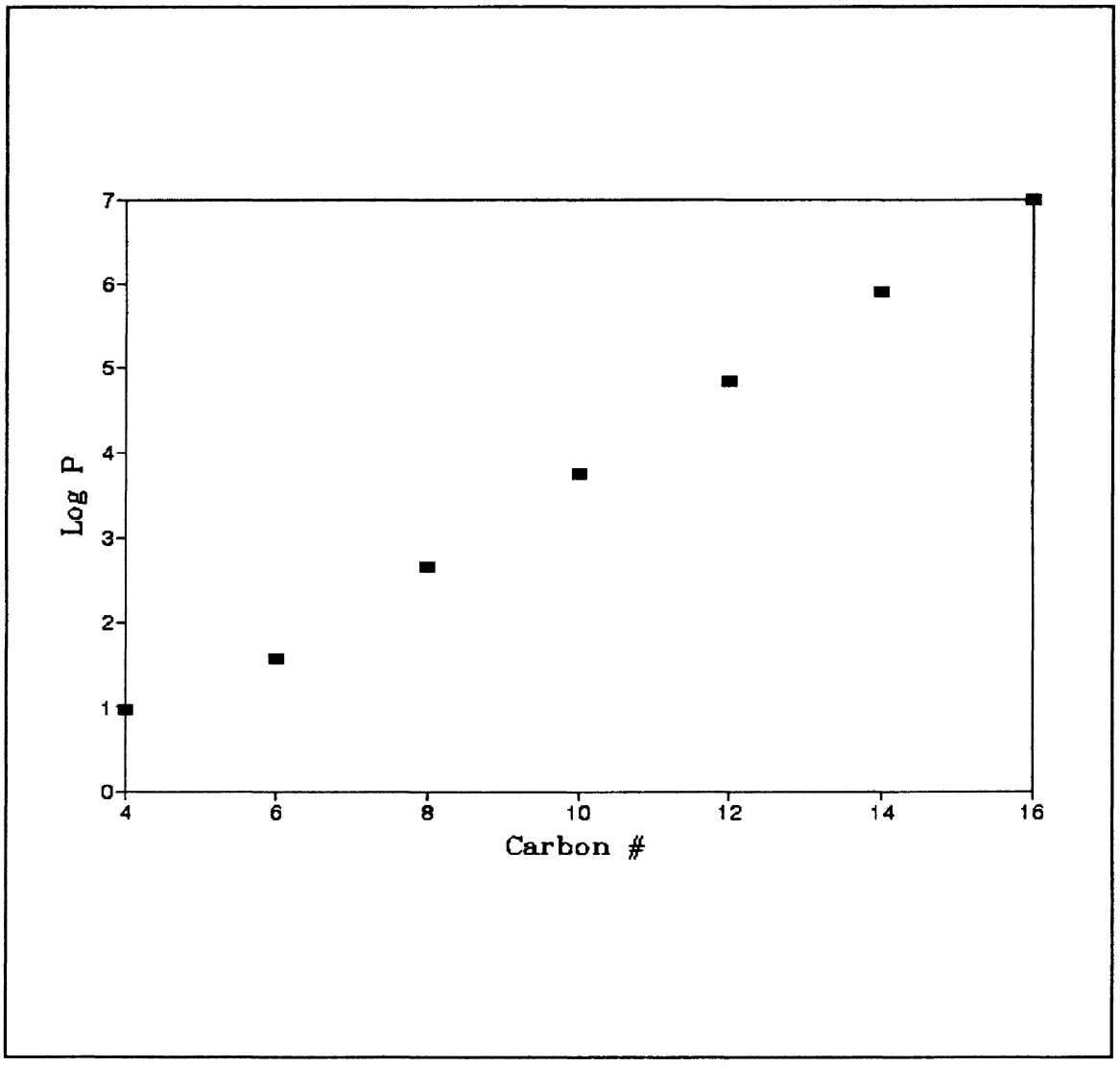

Figure 5. Log P for the 2-ketoalkane standards as a function of number of carbon atoms in the molecule.

the standards showed some differences. In general, refinery B had more material in the first three groups while refinery A had more material in the last three groups (Figure 4). The HPLC standards used in this experiment ranged from C4 to C16 2-ketoalkanes; log P for these standards increased as the carbon number increased indicating greater lipophilic character as the number of carbon atoms substituted increases (Figure 5).

Standard chemical analysis were also performed on both refinery final effluents. Results of the tests show ,in general, both effluents had similar water quality parameters; one notable exception was the higher total NH3 and COD values of Refinery B (Appendix B). This may be due to the quality of biotreatment obtained before the final effluent is discharged.

C18 Extraction Of Refinery Effluents

Results from C18 extraction of both refinery effluents indicated that toxicity to *Photobacterium phosphoreum* was significantly reduced. No observed effect concentrations (NOEC) to *Photobacterium phosphoreum* for each refinery tested resulting from the extractions are shown in Table I. Refinery A NOECs were reduced on average by 74.8% upon adsorption of sample onto C18. The difference between before and after NOEC's was significant at $p<.001$ (paired t-test,$t=9.1$,$n=12$). Refinery B NOEC's were reduced on average by 73.1% upon adsorption onto C18. The difference between before and after NOEC's was significant at $p<.001$ (paired t-test,$t=16.2$,$n=6$). Observations of the cartridges after extracting one liter, showed the appearance of a dark material adsorbed onto most of the volume of the cartridge. Apparently, most of the nonpolar compounds were being adsorbed onto the column and as a result of this action, the toxicity to *Photobacterium phosphoreum* was largely reduced. The removal of most of the toxicity by C18 extraction provides good evidence that nonpolar organics were causing most of the toxicity to *Photobacterium phosphoreum*.

Table I

EFFECTS OF C18 SOLID PHASE EXTRACTION ON THE NO OBSERVED EFFECT CONCENTRATION OF LUMINESCENCE OF *PHOTOBACTERIUM PHOSPHOREUM* EXPOSED TO FINAL EFFLUENTS FROM TWO REFINERIES

SAMPLE	NOEC (% Effluent) Before C18	AfterC18
A8591	11	98
A8591t	5.6	98
A8791t	5.6	98
A8791	2.8	98
A8991t	2.8	98
A8991	5.6	98
B82091	27	98
B82291	40	98
B82491	27	98
A12092	11	98
A12292	11	65
A12492	11	98
B12892	18	91
B13092	18	91
B2192	5.6	98
A21092	91	91
A21292	40	91
A21492	27	91

Both Figure 6 and 7 show gamma as a function of effluent concentration for refineries A and B respectively. Gamma is simply the ratio of amount of light lost to the amount of light remaining. High values of gamma indicate greater toxicity than low values. The figures show that in general, the trend was the same but the magnitude of effect differed for some effluents. As can be seen in both figures, the adsorption of nonpolar organic compounds onto C18 significantly reduces toxicity for both types of effluents. The results of this experiment seem to concur with the work by Johnson (1990) in which C18 reduced acute toxicity of petroleum refinery process waters and final effluent to *Ceriodaphnia dubia* and *Pimephales promelas*.

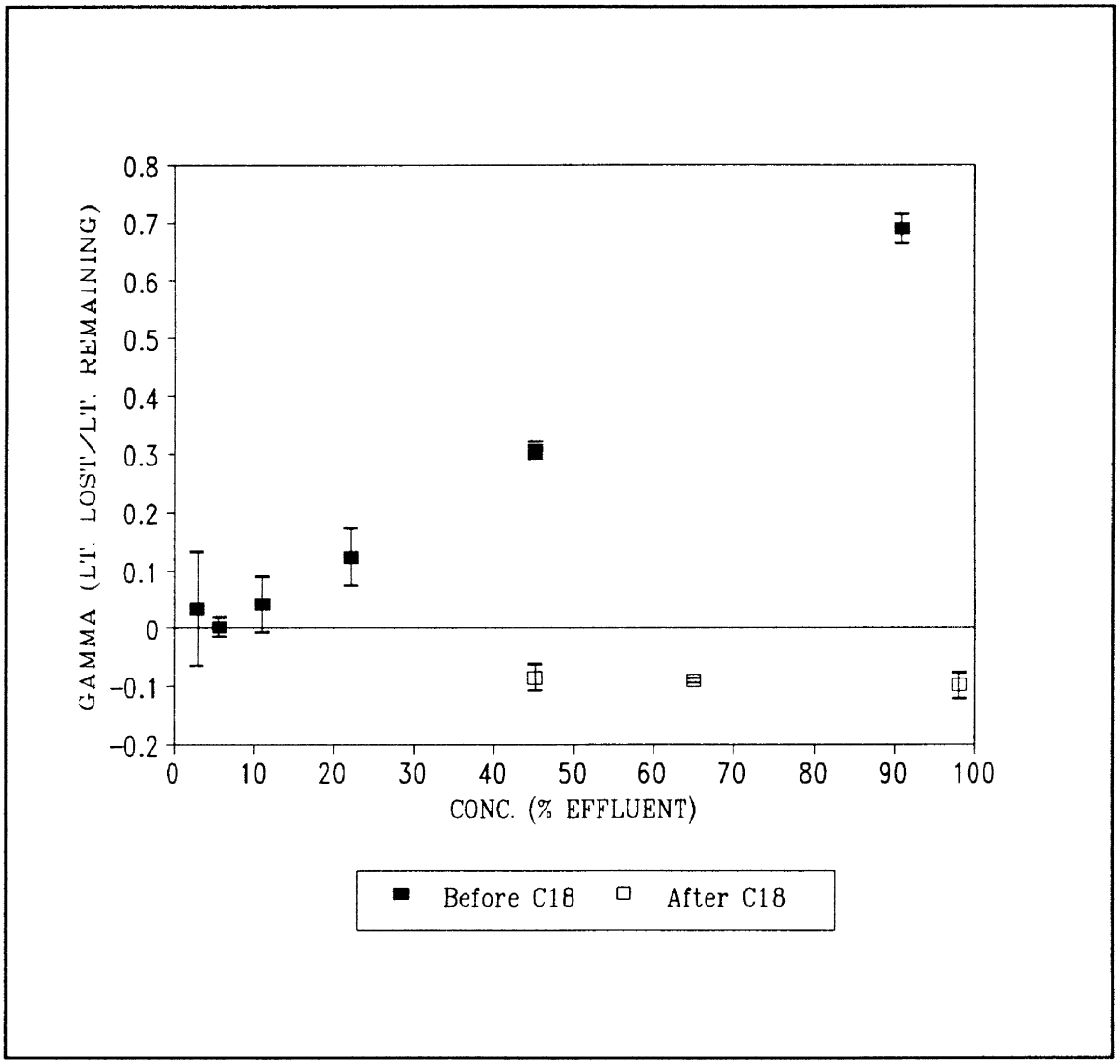

Figure 6. The effect of C18 extraction on *Photobacterium phosphoreum* light output exposed to refinery A wastewater.

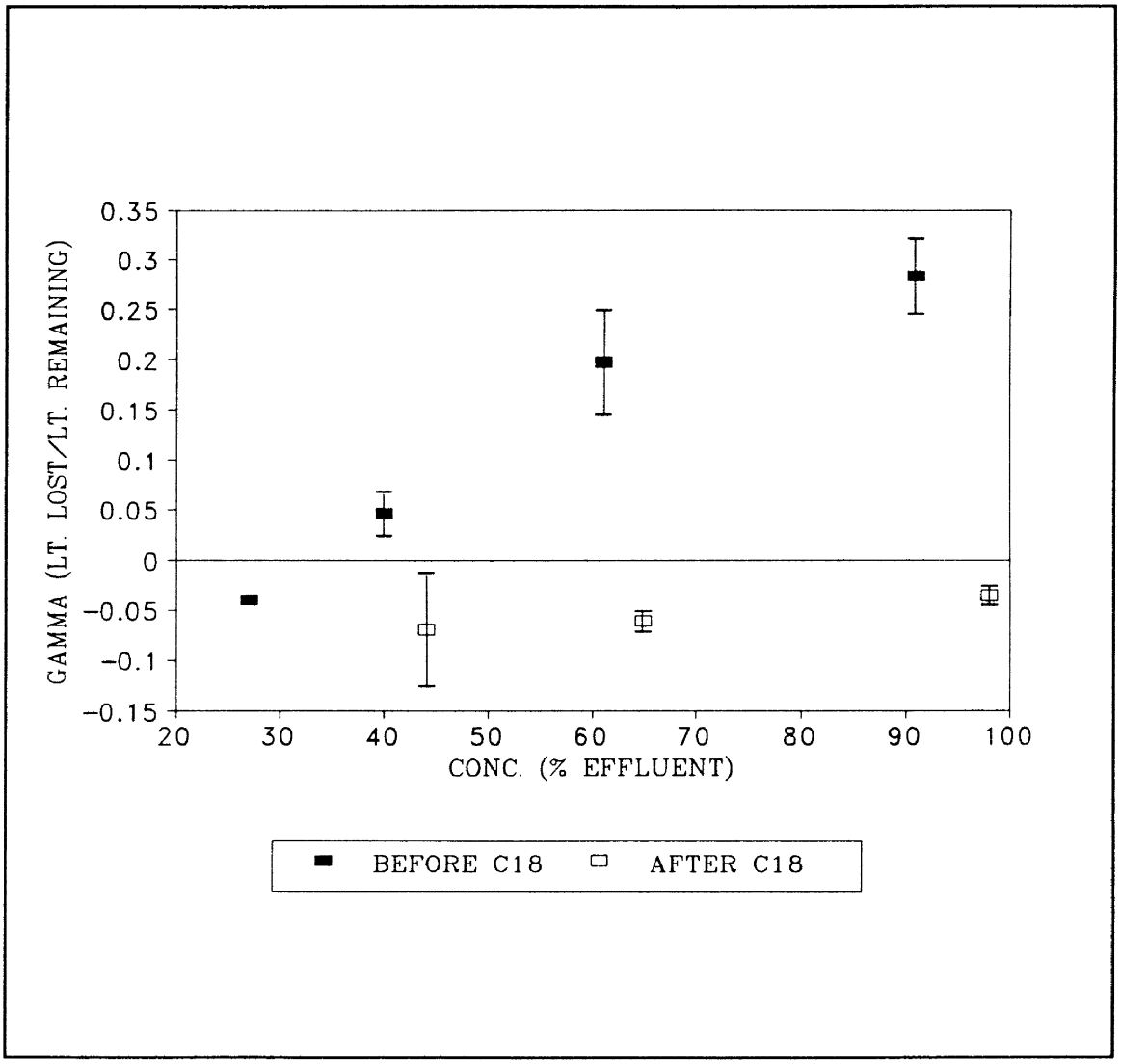

Figure 7. The effect of C18 extraction on *Photobacterium phosphoreum* light output exposed to refinery B wastewater.

Bioassays

Microtox and HPLC results

The results of Microtox toxicity test values for refinery A and refinery B are shown in Tables II and III respectively. There was very little difference between the response shown by *Photobacterium phosphoreum* to the two refinery final effluents. The NOEC response of Refinery A ranged from 2.8 to 91 percent. For Refinery B the Noec response ranged from 2.8 to 40 percent. A comparison of toxicity results shows that there was no great difference between NOEC or IC25 values for either Refinery A or Refinery B. For Refinery A, the concentration at which light output was reduced by 25% correlated with the amount of material bracketed by standards having between 14 and 16 carbons atoms (Appendix C). Scatterplots of toxicity to *Photobacterium phosphoreum* as a function of amount of material bracketed by HPLC standards for Refinery A are shown in figures 8 through 11. The NOEC of light output by the bacteria correlated with the amount of material bracketed by standards having 6-8 and 8-10 carbon atoms. For Refinery B, the concentration at which light output was reduced by 25% correlated with the amount of material bracketed by standards having 6-8,8-10,10-12, and 12-14 carbon atoms (Appendix C). Scatterplots of toxicity values obtained from Refinery B as a function of

TABLE II

EFFECT OF REFINERY A FINAL EFFLUENT ON
PHOTBACTERIUM PHOSPHOREUM
LIGHT OUTPUT*

Sample	NOEC	IC25
A8591t	5.6	54.5
A8791t	5.6	40.2
A8791	2.8	48.1
A8991t	2.8	37.2
A8991	5.6	52.5
A91691	27	70.3
A91891	5.6	55.1
A92091	27	77.6
A101491	27	62.1
A101691	27	75.8
A101891	27	73.3
A12092	11	49.1
A12292	11	48.3
A12492	11	71.9
A21092	91	52.5
A21292	40	77.2
A21492	27	56.3

* - All values given as percent effluent.

TABLE III
EFFECT OF REFINERY B FINAL EFFLUENT ON
PHOTOBACTERIUM PHOSPHOREUM
LIGHT OUTPUT*

Sample	NOEC	IC25
B82091	27	85.5
B82291	40	86.6
B82491	27	78.4
B92491	19	80.2
B92691	29	82.5
B92891	19	65.8
B102291	18	63.8
B102491	27	68.4
B102691	27	62.3
B111391	22	51.6
B111891	22	47.5
B111991	22	63.4
B112191	22	60.5
B112391	11	36.4
B121091	2.8	41.3
B121291	11	35.6
B121491	11	47.9
B121791	11	54.8
B12892	18	27.1
B13092	18	41.0
B2192	5.6	47.4
B22792	2.8	12.1
B22992	5.6	32.2
B3392	11	24.3
B32492	11	60.4
B32692	23	51.5
B32892	23	79.2

* - All values given as percent effluent.

amount of material bracketed by HPLC standards are shown in figures 12 through 14. These figures show that as the amount of material increases, the toxicity value is lower. The NOEC of light output by the bacteria correlated with the amount of material bracketed by standards having 6-8, 10-12, and 12-14 carbon atoms. Evidently, molecules having anywhere from approximately 6 to 14 carbon atoms are passing through membranes of the bacteria and altering the metabolic processes that leads to bioluminescence.

Results for correlations with HPLC Indices were not as significant as the results based on amounts bracketed by standards. For Refinery A, a marginally strong positive correlation was found between indices of 12-14 and 14-16 carbon atoms and the EC25 of the effluent (Appendix C). This contrasts with the negative correlation with indices of 6-8 carbon atoms and the IC25 of the effluent. There may be some opposing toxic effect occurring within the effluent when described by HPLC Indices.

Microtox and GC Indices

Chromatography of 11 refinery final effluents produced the unresolved complex mixture typical of petroleum contamination. There were very few significant correlations with both amounts bracketed by n-alkane standards and GC indices (Appendix C). Two significant correlations between amounts around C18-C20 with toxicity have positive signs.

These compounds molecular weight and hydrophobic character may be high enough to prevent their passage into the *Photobacterium phosphoreum* membrane.

Correlations between GC Indices and toxicity values were again very few in number; However, indices bracketed by C16 and C18 were significant with IC25 and NOEC values.

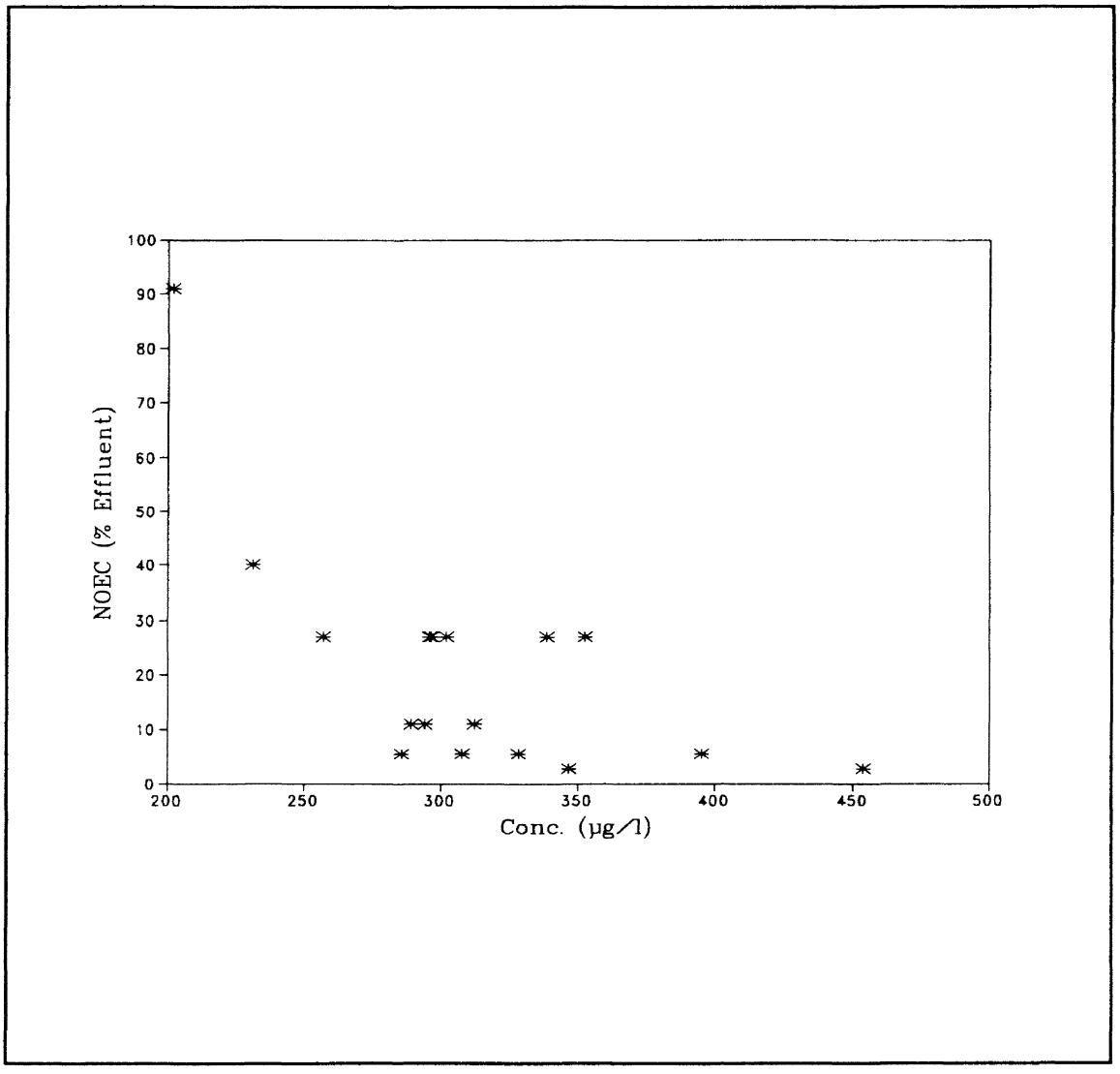

Figure 8. NOEC of *Photobacterium phosphoreum* for Refinery A as a function of material bracketed by HPLC standards having 6 and 8 carbon atoms.

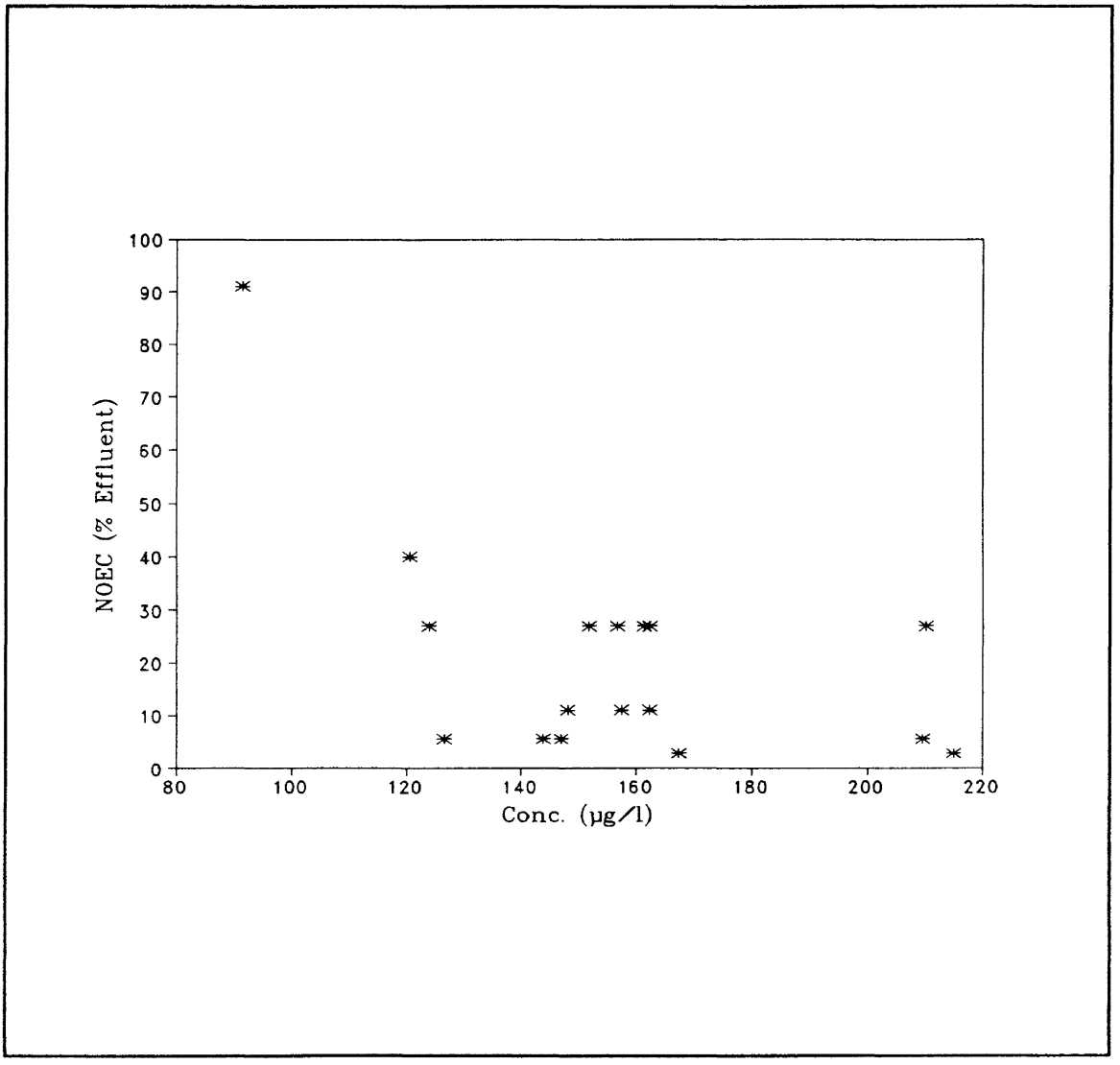

Figure 9. NOEC of *Photobacterium phosphoreum* for Refinery A as a function of amount of material having 8 and 10 carbon atoms.

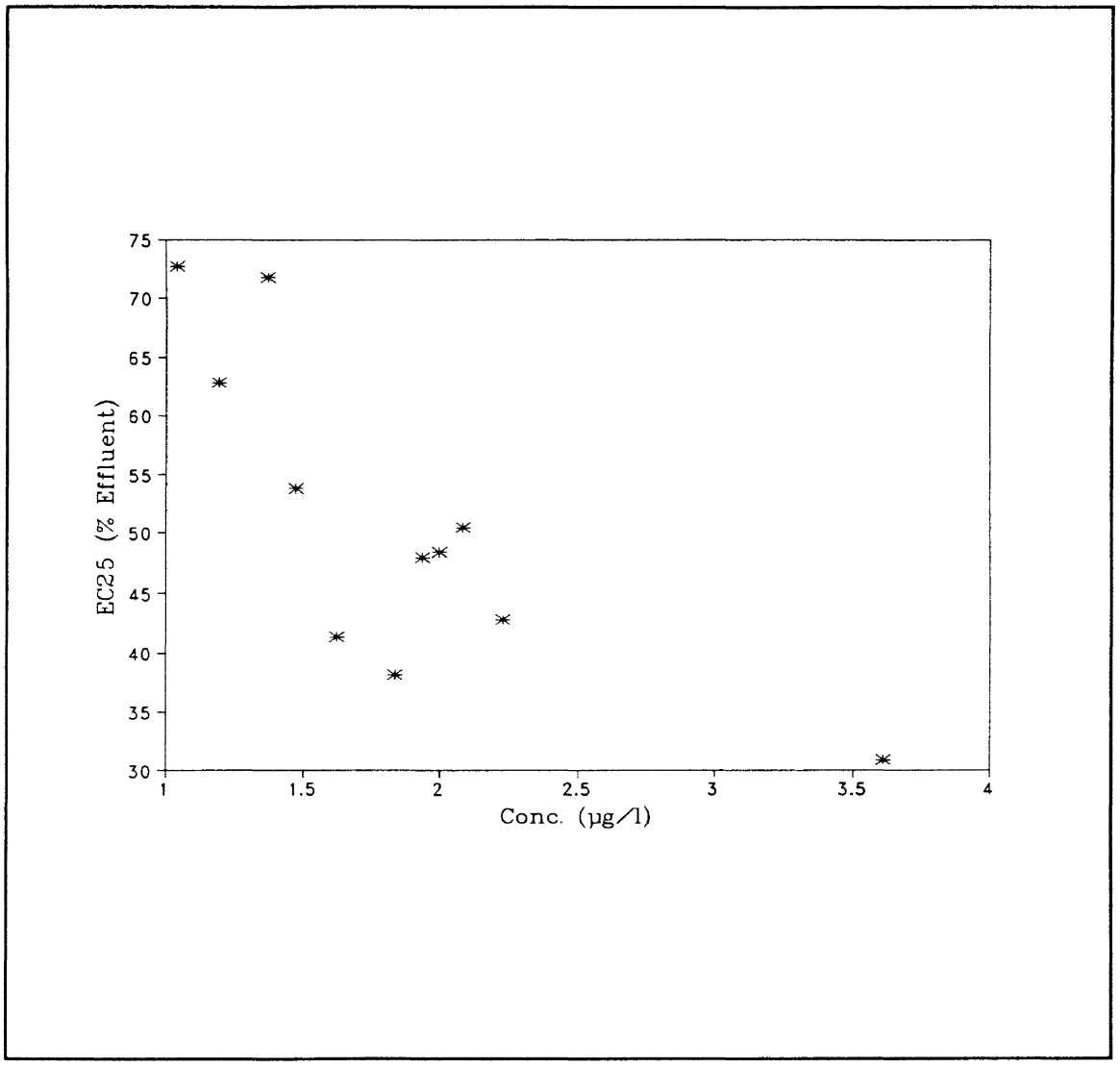

Figure 10. EC25 of *Photobacterium phosphoreum* for Refinery A as a function of amount of material bracketed by HPLC standards having 14 and 16 carbon atoms.

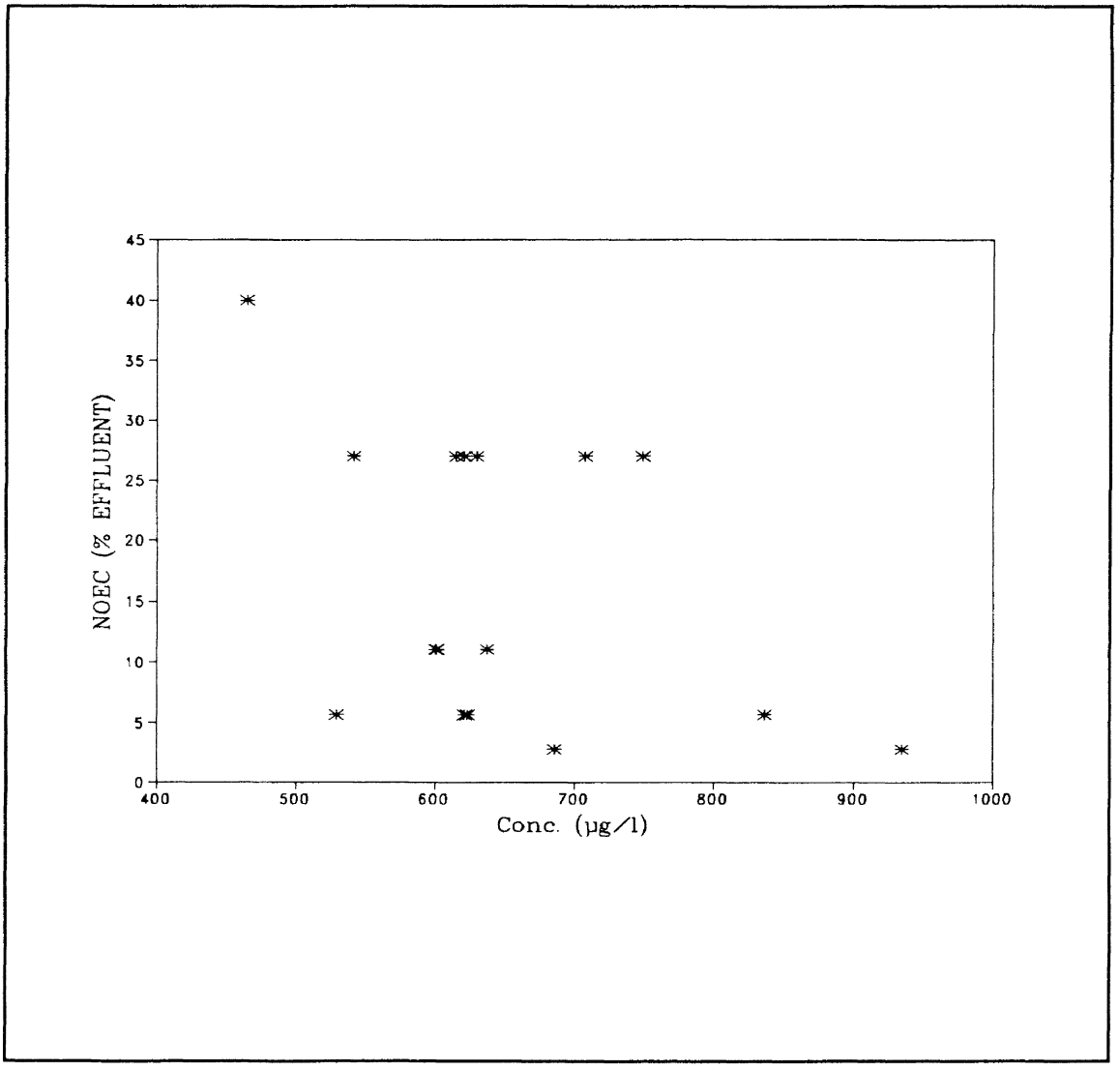

Figure 11. NOEC of *Photobacterium phosphoreum* for Refinery A as a function of amount of material bracketed by HPLC standards having 4 and 16 carbon atoms.

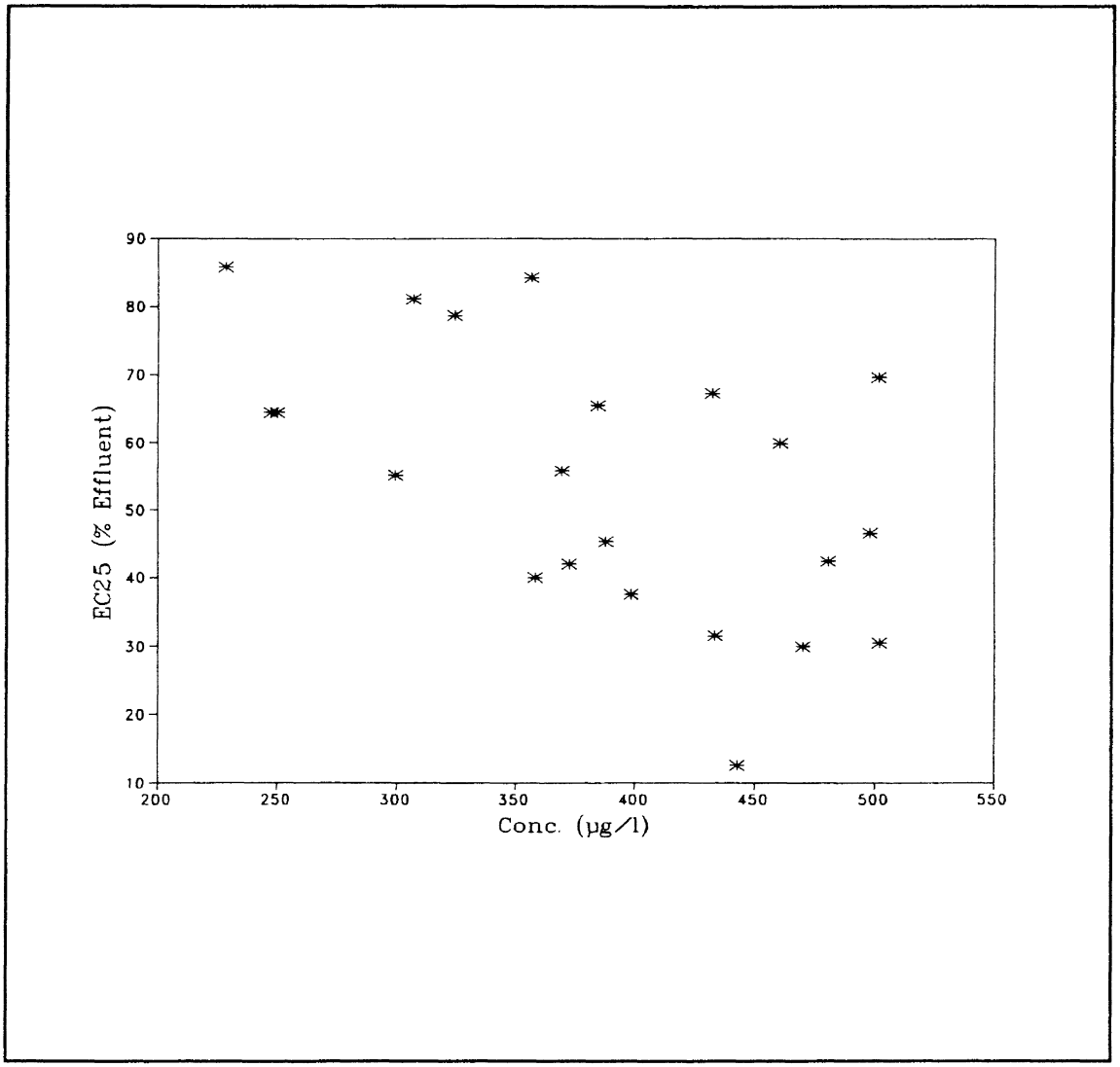

Figure 12. EC25 of *Photobacterium phosphoreum* for Refinery B as a function of amount of material bracketed by HPLC standards of 14 and 16 carbon atoms.

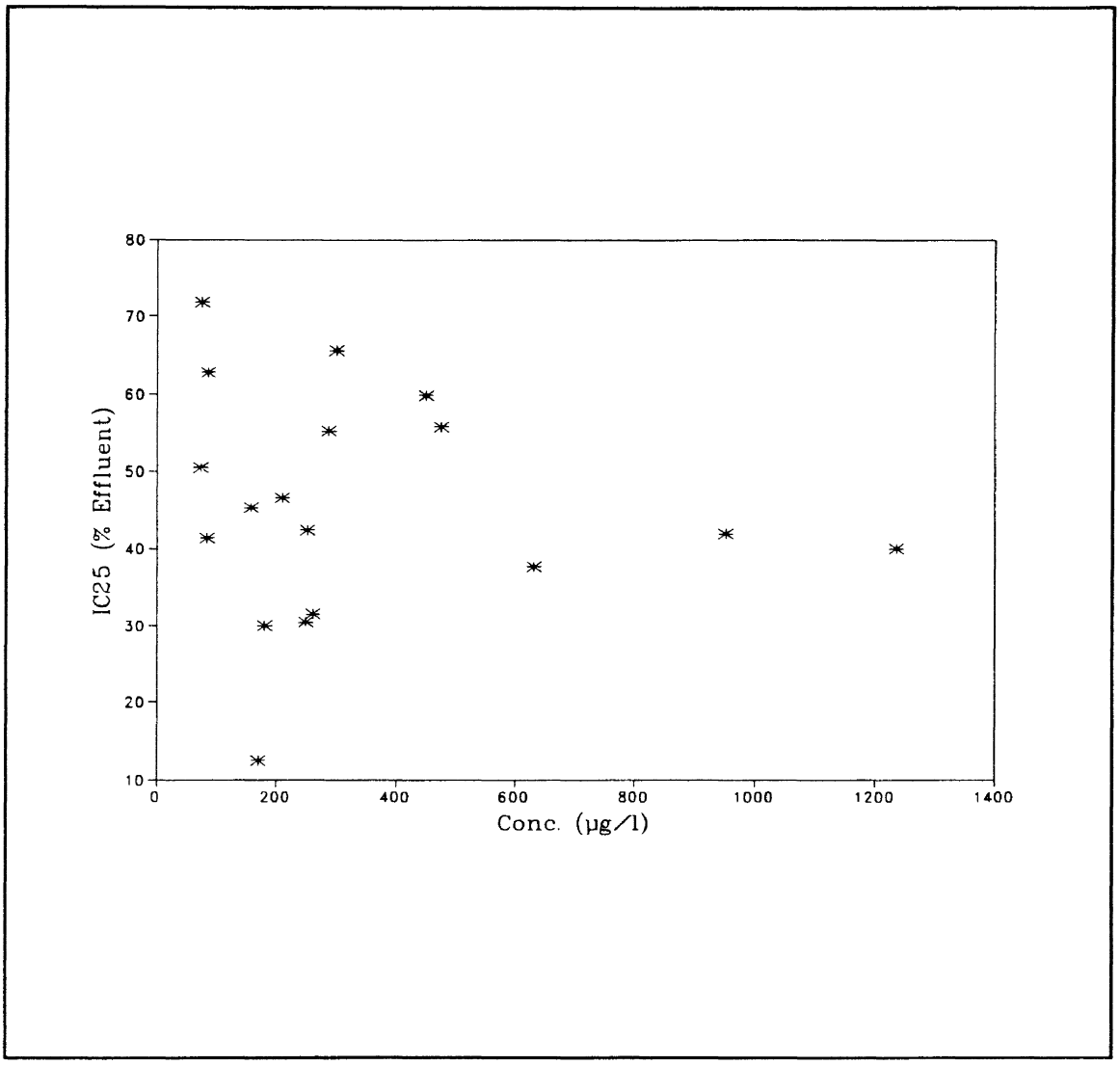

Figure 13. IC25 of *Photobacterium phosphoreum* for Refinery B as a function of amount of material bracketed by HPLC standards of 14 and 16 carbon atoms.

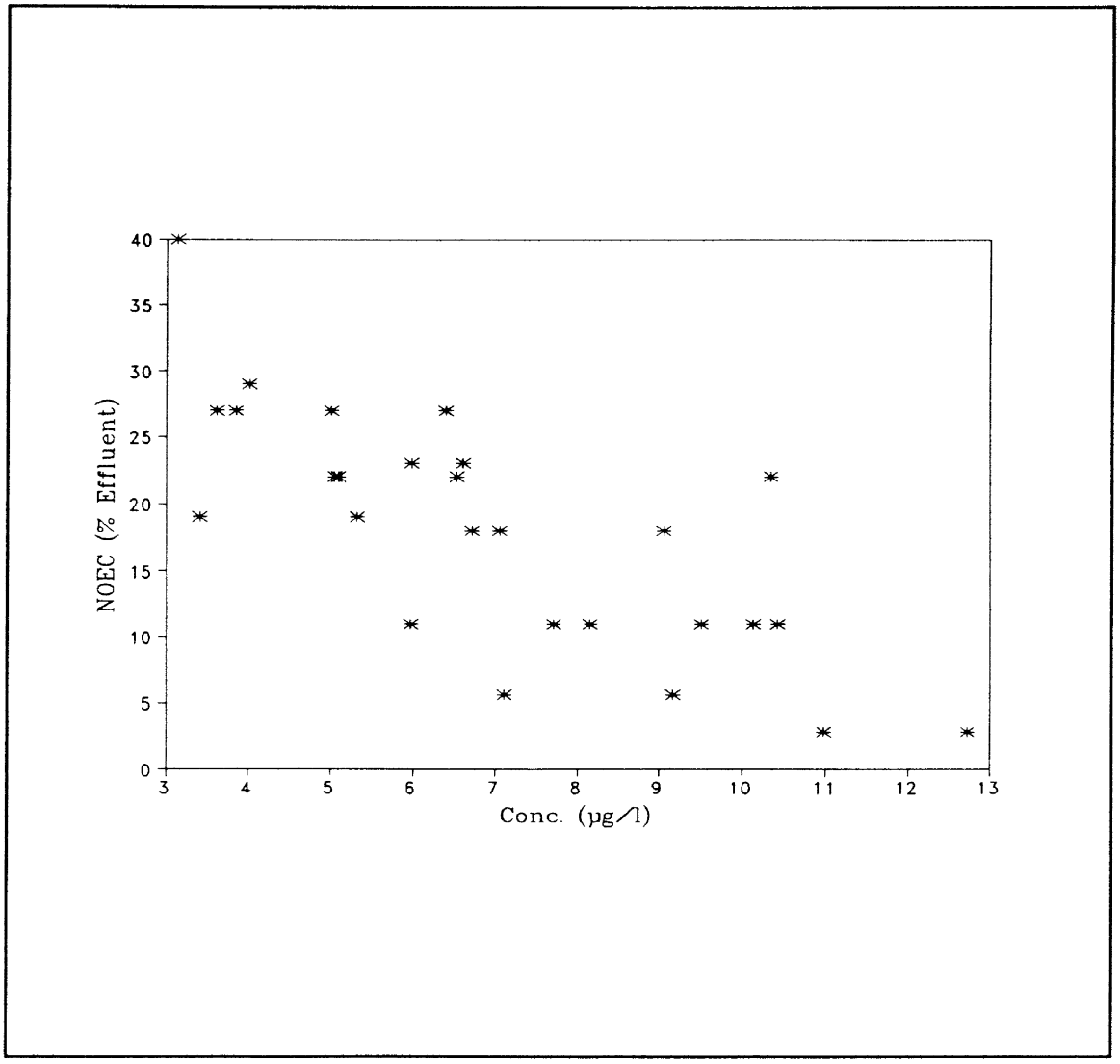

Figure 14. NOEC of *Photobacterium phosphoreum* for Refinery B as a function of amount of material bracketed by HPLC standards of 14 and 16 carbon atoms.

Ceriodaphnia dubia assays

There were slightly more significant correlations of *Ceriodaphnia dubia* survival and reproduction with amounts in chromatographic regions than were present in the fathead minnow results. The results of Pearson's correlations for *Ceriodaphnia dubia* are shown in Appendix D. The highest r value was 0.649 for survival NOEC against indices of C12-C14. Even though many correlations were significant, the sign of the correlation was positive in every case except one. In effect, amount of material bracketed by HPLC standards was inversely related to the toxicity response displayed by *Ceriodaphnia*. Therefore, greater quantitiies of substances bracketed by standards was associated with lower toxicity to *Ceriodaphnia dubia*. This trend is unusual because if a substance is causing toxicity by a generalized narcosis mechanism, more toxicant exposed to the organism should generally produce greater toxic effects. However, because of the great number of compounds present in a final effluent, the possibility exists that some type of interaction is taking place to cause this reverse trend from what would normally be expected. Veith, et al. (1982) showed that toxicity began to decrease for highly lipophillic chemicals with a log P starting near 5. The same phenomena may be occuring in *Ceriodaphnia* as seen with the positive correlations around standards C10, C12, and C14. The reversal of correlation coefficient signs appears

to indicate processes other than simple additive toxicity taking place in *Ceriodaphnia dubia* survival and reproduction.

Ceriodaphnia reproduction NOEC again showed some positive correlations (r=0.600) towards higher index values between 1400 and 1600 (Appendix D).

Figure 15 and 16 show survival and reproduction data for *Ceriodaphnia dubia* 7-day toxicity tests. Mean percent survival for Refinery A was much less than for Refinery B at all test concentrations except for the highest one. Reproduction was lower for Refinery A at low concentrations. This data indicates some differences in quality of the final effluent that may lead to lower correlations.

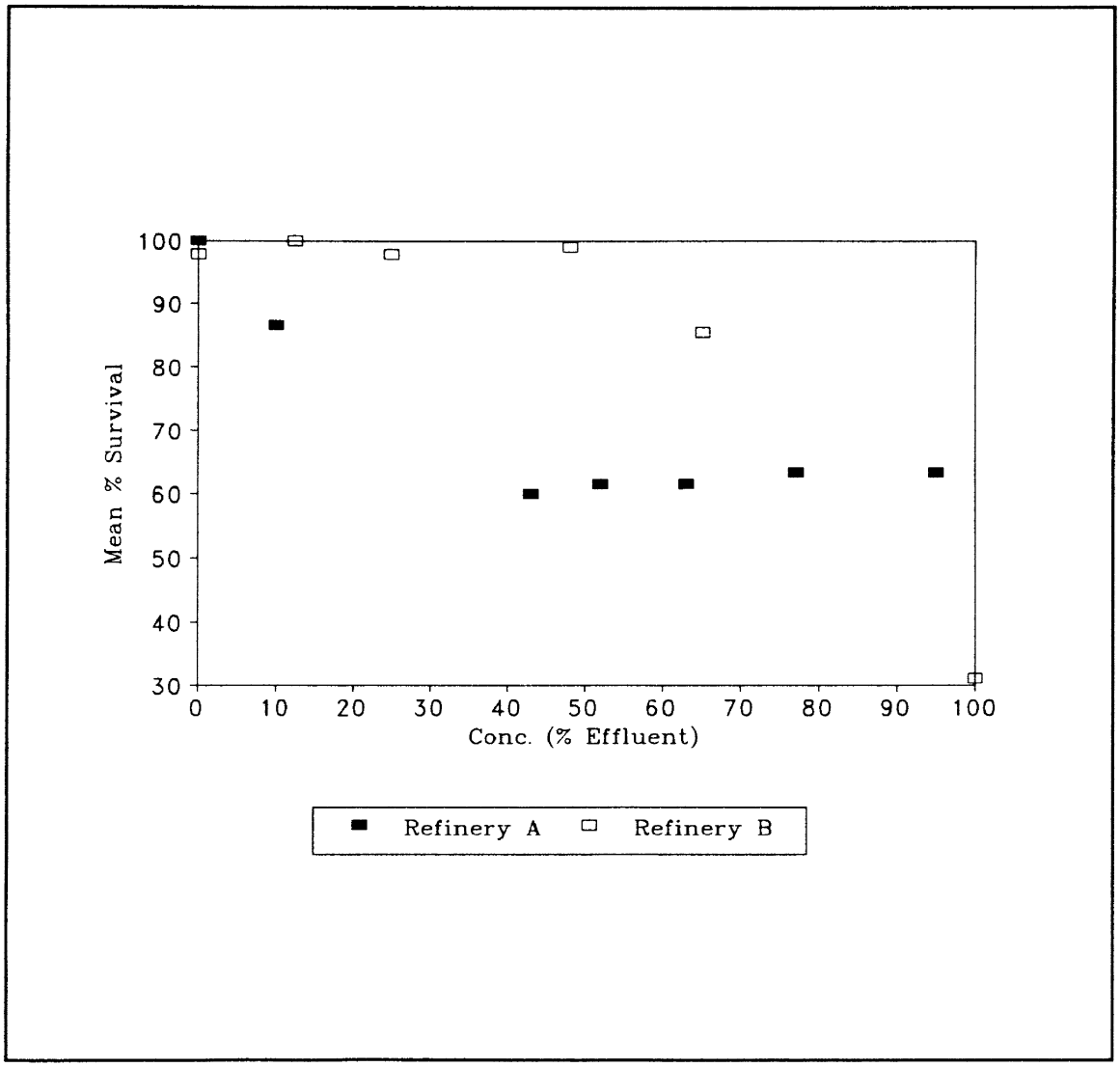

Figure 15. Mean survival of *Ceriodaphnia dubia* exposed to various concentrations of Refinery A and B final effluent.

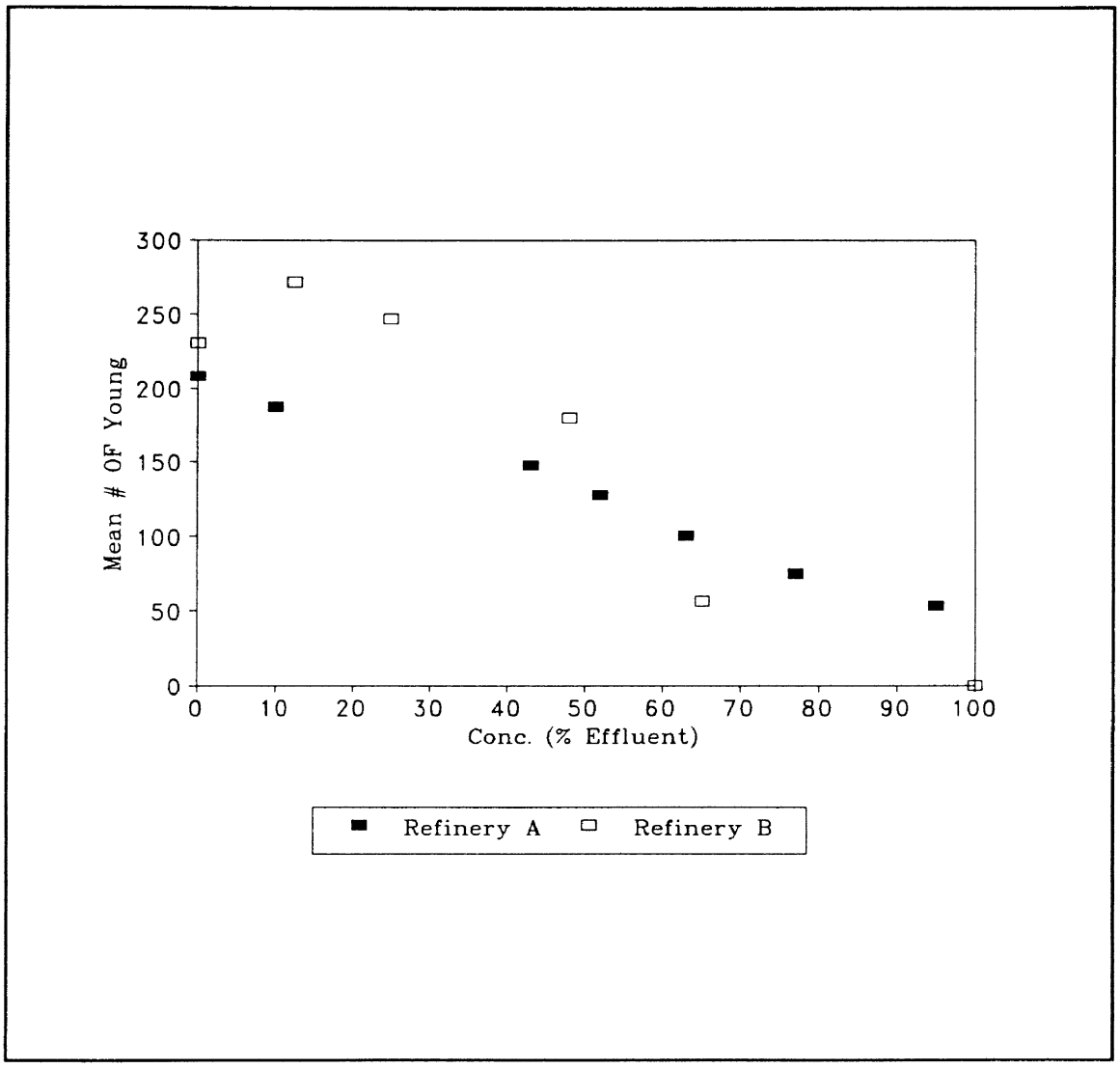

Figure 16. Mean number of *Ceriodaphnia dubia* produced as a function of concentration of Refinery A and B final effluent.

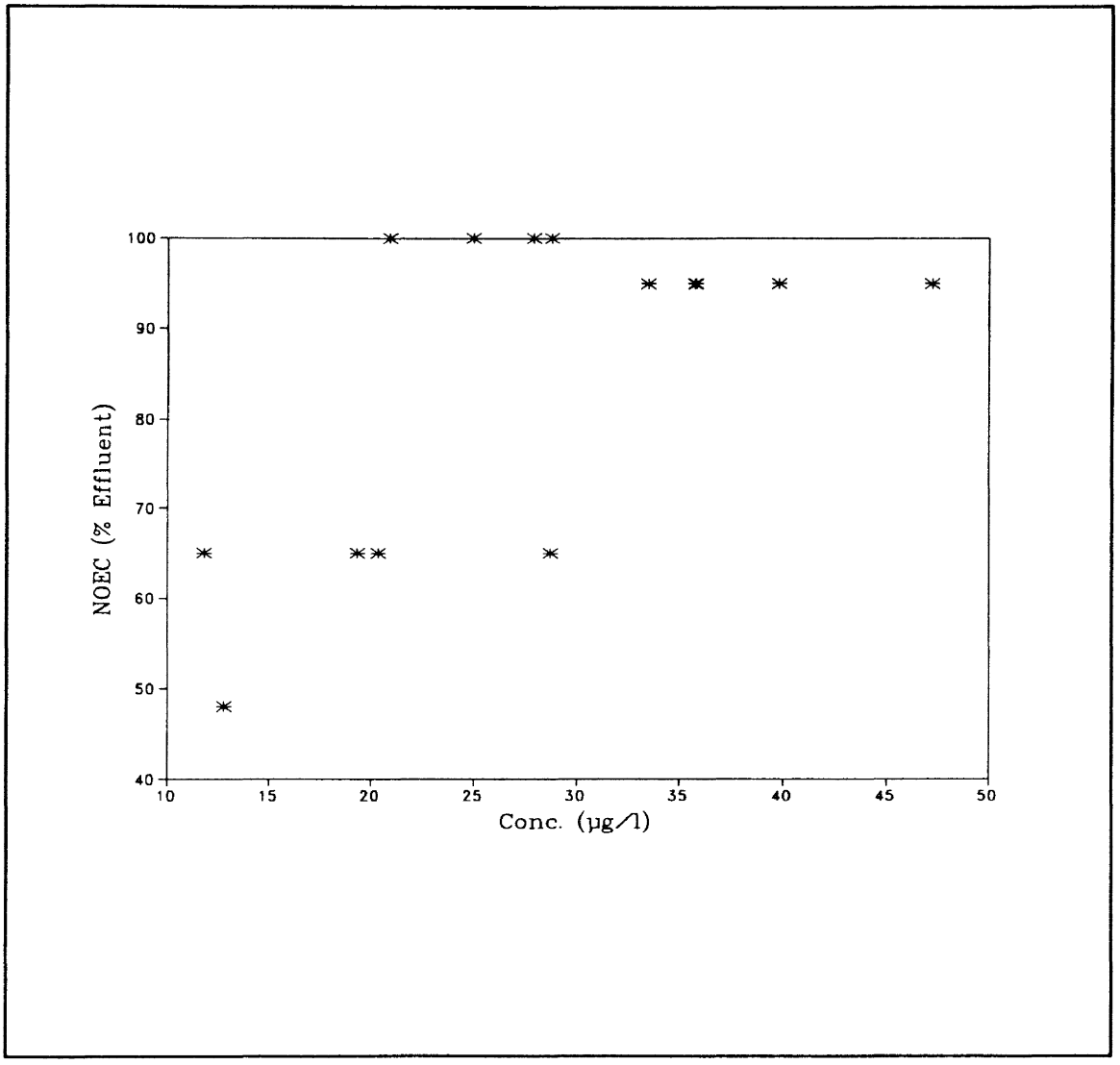

Figure 17. *Ceriodaphnia dubia* survival NOEC as a function of amount of material bracketed by HPLC standards of 12 and 14 carbon atoms.

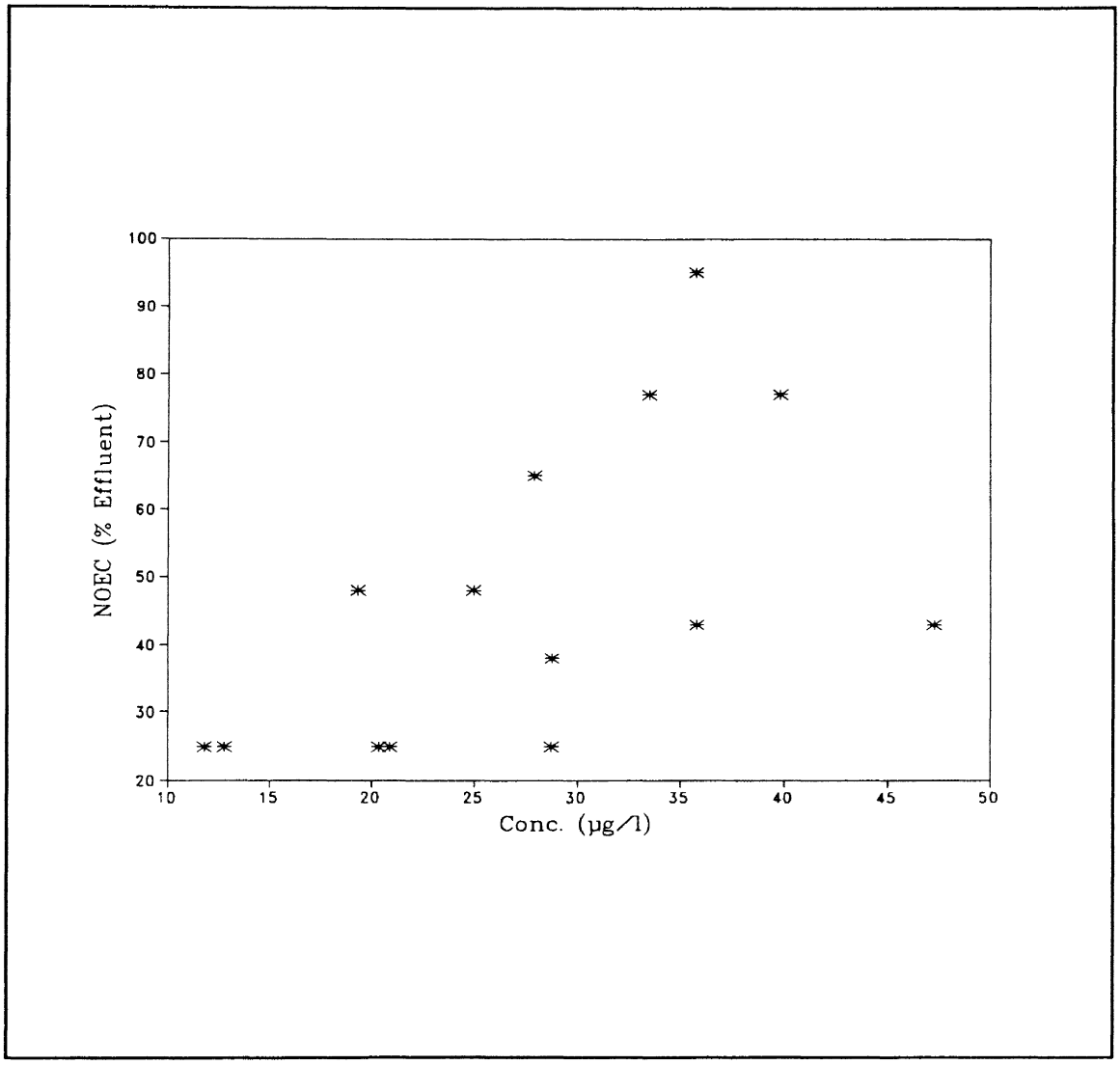

Figure 18. *Ceriodaphnia dubia* reproduction NOEC as a function of amount of material bracketed by HPLC standards of 12 and 14 carbon atoms.

Pimephales promelas assays

Overall fathead minnow toxicity in the final effluent was not correlated with the overall HPLC Index of the effluent. The correlation as shown by Pearson's r was a quite low in most cases. However, the correlation with the amounts in the chromatographic regions bracketed by ketoalkane standards was more substantial in the case of Fathead Minnow growth. Correlations with chromatographic standards were inconclusive (Appendix E). Correlations of *Pimephales promelas* growth NOEC and IC50 with the amount in chromatographic region bracketed by C10-C12 standards were statistically significant ($p \leq 0.05$) However, there was no significant linear correlation of fathead minnow survival with amounts of any of the chromatographic standards. Compounds in region 4 are bracketed by standards having 10 to 12 carbon atoms. The relationship betweeen *Pimephales promelas* growth IC50 as a function of concentration of C10-C12 material (Figure 19) reveals higher concentration associated with higher toxicity. Compounds of this size may encompass substituted aromatics such as alkylated benzenes and naphthalenes. These results agree with the results obtained by Johnson (1990) in which it was concluded that the toxic portion of refinery effluents are bracketed by molecules ranging from toluene to acenaphthene, although that research looked specifically at 96 hour acute toxicity and this study deals with 7 day chronic toxicity to

Pimephales promelas.

Previous research has provived evidence that growth is the most sensitive parameter in *Pimephales promelas* early life stage toxicity tests (Call et al., 1985). *Pimephales promelas* growth provided the only significant correlations between amounts bracketed by HPLC standards and toxicity as seen in (Appendix E). There was an the absence of any significant correlations between HPLC indices and toxicity to *Pimephales promelas* (Appendix E). Figures 20 and 21 show the efect of Refinery final effluent on growth and survival of fathead minnows. Both refineries produced similar effects upon this organism.

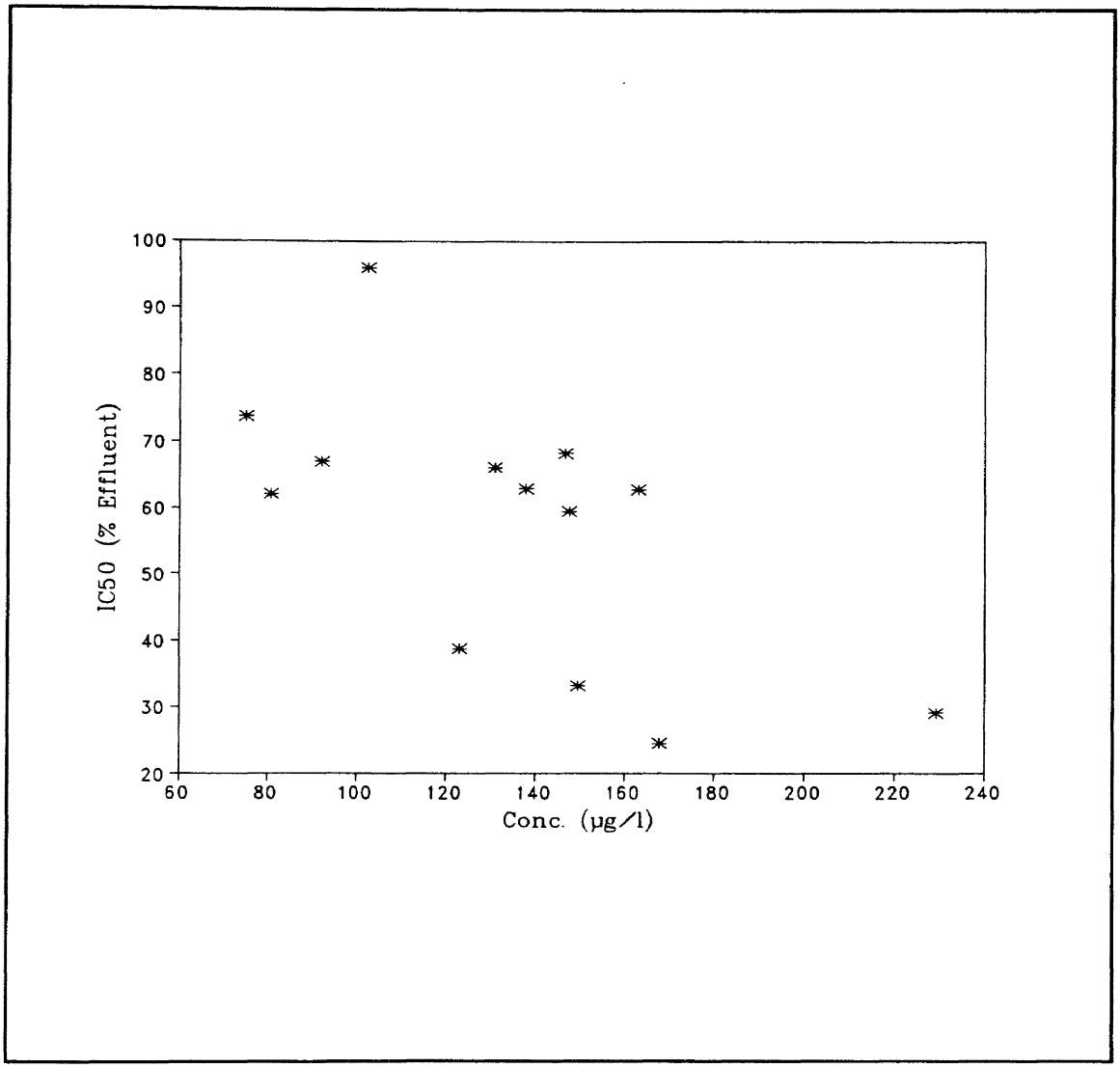

Figure 19. *Pimephales promelas* growth IC50 as a function of amount of material bracketed by HPLC standards of 10 and 12 carbon atoms.

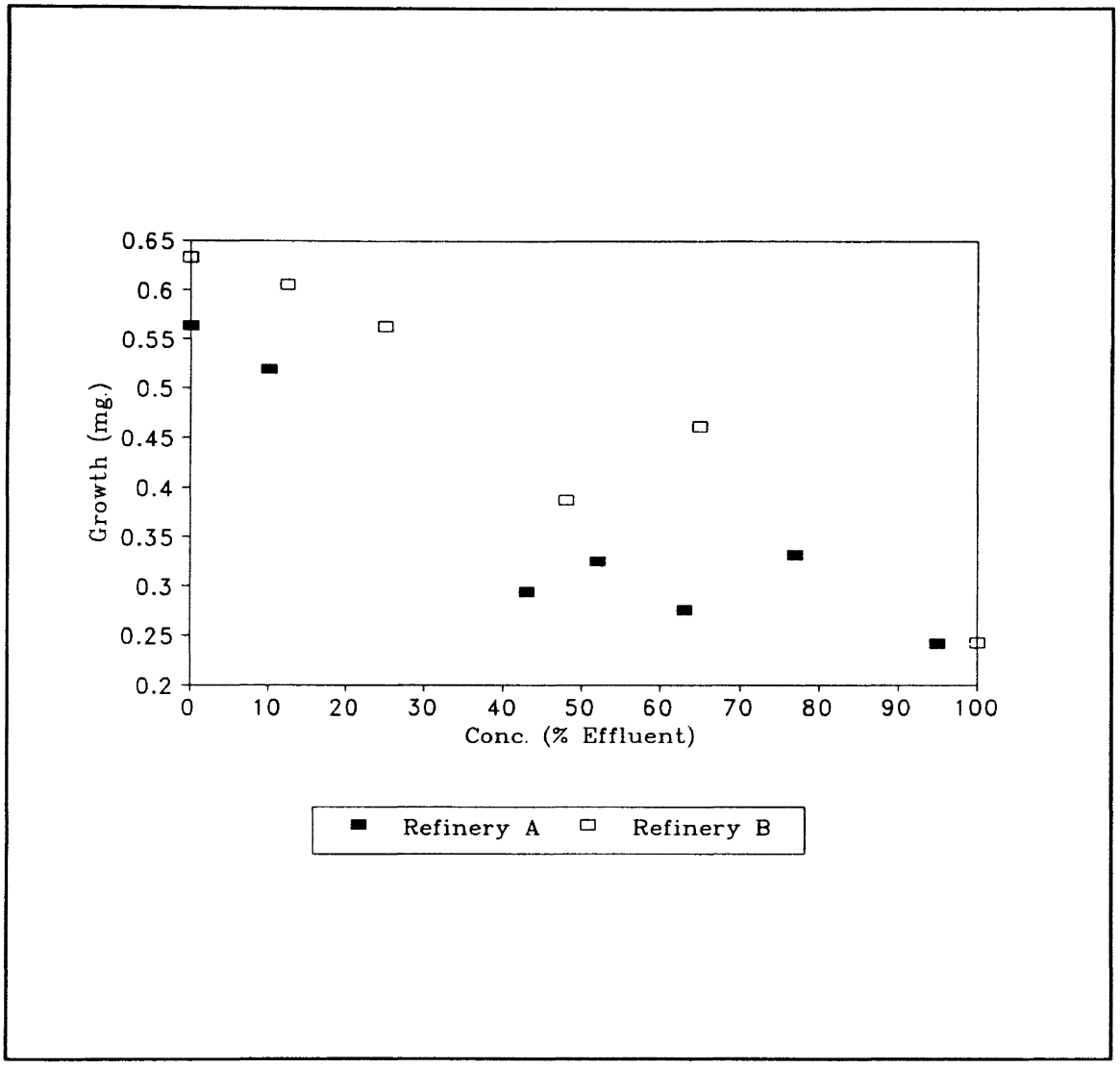

Figure 20. Mean *Pimephales promelas* growth for Refinery A and B final effluent.

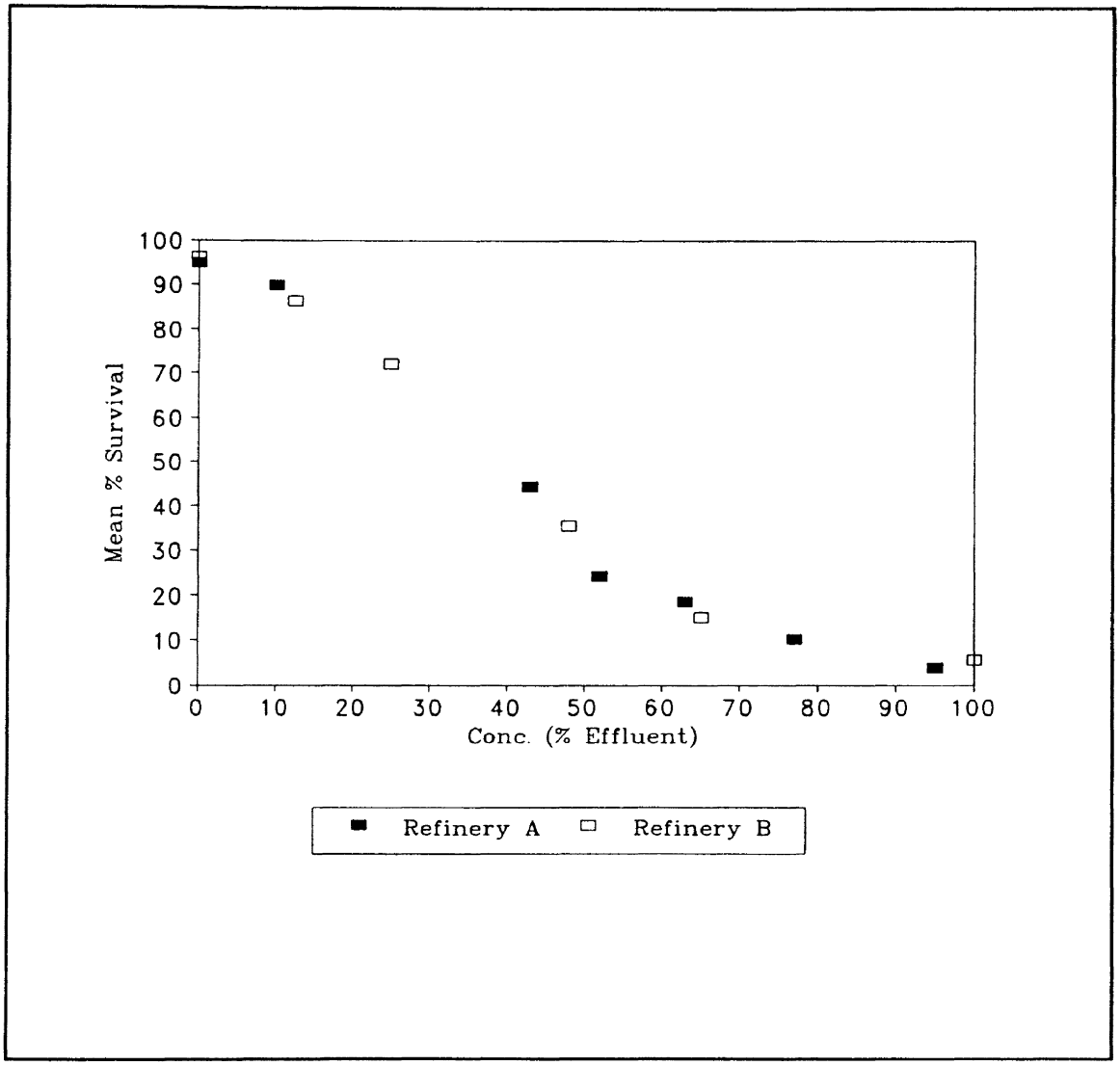

Figure 21. Mean *Pimephales promelas* survival for Refinery A and B final effluent.

CHAPTER V

SUMMARY

The primary objective of the experiment was based on the hypothesis that the concentration of chromatographically separated fractions would be negatively correlated with toxic effects upon *Photobacterium phosphoreum*, *Ceriodaphnia dubia*, and *Pimephales promelas*. This hypothesis was based on the fundamental toxicological tenet that quantity and duration of exposure to a toxicant was directly related to adverse toxic response.

The results of this study detected some fairly significant correlations between quantity of specific fractions of the organic compounds in the refinery final effluents and adverse response measured. However, there was no consistent pattern of correlation between the variables. Neither the HPLC nor the Kovats indices exhibited a high correlation in all samples.

Based upon the results of the study, it would appear that the mode of action of the organic contaminants in the refinery wastewater was more complicated than originally assumed. If the toxic effect was due to narcosis alone then one would expect to see each chromatographic fraction strongly

negatively correlated with toxic response measured. Since the data did not support this concept, it must be concluded that the effect of refinery wastewater upon aquatic organisms is not due to one simple mode of action but is due to several mechanisms in combination. The combined effects of the organic contaminants within the refinery wastewater are apparently too complicated to be consistently predicted with a single physical-chemical chromatographic retention index.

This study utilized 100 percent methanol extracts for HPLC and methylene chloride for GC analysis. The possibility exists that this experiment may not have extracted the chronically toxic materials in the case of HPLC analysis or the acutely toxic compounds used for GC analysis. Other factors such as ammonia may be contributing some of the unexplained toxicity. Further studies could alter the extraction and elution procedures to ensure that all of the toxic materials are available for chromatographic separation.

The original objective of the study was to evaluate the possibility of predicting the combined toxic effects of a complex unknown mixture. However, the lack of consistent correlations provides support for the notion that the original objective was too complicated for the experimental design employed.

One modification that could make the QSAR relationship more reliable involves using a simpler mixture composed of a limited number of chemicals. A fundamental requirement for a

successful QSAR is the toxic response must be caused by a single mechanism. Predicting the toxicity of such a mixture would possibly be more successful because of the low likelihood that more than one mechanism is responsible for the observed toxic response.

Another possible revision consists of focusing on only the organic compounds in the mixture because they are the only class of chemicals responding to the chromatographic index. The presence of inorganic compounds complicates the QSAR because toxicity caused by inorganic substances is not accounted for when using a chromatographic index. Therefore, removing the inorganic substituents, through use of ion-exchange methods, prior to measuring the index would eliminate the inorganics as a reason for obtaining a low quality QSAR.

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APPENDIXES

APPENDIX A

CHROMATOGRAPHIC DATA

AMOUNTS OF MATERIAL BRACKETED BY 2-KETOALKANE STANDARDS ADSORBED ONTO C18 FOR REFINERY A AND B[a]

Sample	1	2	3	4	5	6
A8591t	73.07	285.69	126.67	34.53	7.72	1.48
A8791t	91.60	328.44	146.88	40.77	13.13	2.23
A8791	103.04	346.85	167.48	53.86	12.98	1.84
A8991t	170.99	453.89	215.04	72.28	18.99	3.61
A8991	110.93	307.55	143.73	45.87	10.49	1.94
A91691	126.71	339.03	162.28	64.47	13.89	1.04
A91891	135.60	395.46	209.54	79.21	14.56	2.00
A92091	79.20	352.86	210.17	85.66	18.81	2.14
A101491	104.23	296.04	151.86	61.60	13.90	1.55
A101691	87.35	296.60	161.52	60.12	13.25	1.77
A101891	107.48	256.95	123.93	41.40	8.60	2.84
A12092	88.52	288.76	162.27	46.44	13.21	2.08
A12292	102.05	293.90	148.23	42.54	12.25	1.62
A12492	114.18	311.94	157.48	41.95	10.32	1.37
A21092	82.65	201.85	91.55	24.56	8.45	2.02
A21292	69.20	231.00	120.62	33.61	9.08	1.24
A21492	99.41	302.23	156.75	44.29	11.22	1.19
B82091	150.47	356.30	151.33	29.41	3.62	0.76
B82292	155.73	318.45	150.97	25.92	3.14	0.39
B82492	156.12	324.00	155.40	36.72	5.01	0.86
B92692	145.80	306.79	139.47	26.86	3.41	0.50
B92892	126.93	249.99	130.97	27.12	5.33	2.13
B102291	258.83	432.31	215.83	58.22	9.06	1.83
B102491	306.42	501.74	225.23	45.17	6.40	2.56
B102691	279.32	247.63	192.87	36.48	3.85	2.07
B111391	195.65	412.79	197.36	42.89	6.54	2.86
B111891	96.49	299.53	167.96	39.59	5.05	1.27
B111991	150.71	406.60	249.71	58.20	5.10	1.46
B112191	124.48	384.21	250.60	60.65	10.34	1.07
B112391	141.35	387.97	212.93	49.13	9.51	1.77
B121091	155.02	372.87	176.10	53.41	10.98	2.97

APPENDIX A (Continued)

Sample	1	2	3	4	5	6
B121291	174.80	398.53	207.69	37.95	8.16	3.78
B121491	141.98	358.22	208.45	41.69	10.14	3.43
B121791	141.18	369.15	208.36	43.36	10.42	3.43
B12892	223.30	501.51	317.88	52.12	7.06	1.58
B13092	164.01	480.38	247.87	46.17	6.73	0.91
B2192	191.23	497.85	262.65	48.38	7.11	0.99
B22791	88.91	442.66	211.12	54.53	12.73	0.92
B22992	127.05	469.81	192.79	43.35	9.17	0.48
B3392	122.59	433.36	172.04	37.10	5.97	1.50
B32492	237.96	460.38	178.86	27.77	7.73	1.82
B32692	260.62	484.99	173.51	20.75	5.98	1.00
B32892	250.67	489.19	171.53	26.52	6.61	0.64
B430	70.48	284.82	425.45	263.70	92.08	26.66
B52	103.64	390.93	566.52	336.89	117.15	29.78
B55	32.99	253.19	368.24	304.21	61.00	18.41
B324	51.69	164.57	156.44	85.68	31.08	20.40
B326	63.98	258.64	292.20	183.33	90.77	53.85
B328	69.67	342.98	396.84	227.52	90.46	22.27
A48	37.84	107.94	188.12	213.88	114.83	45.40
A410	33.10	84.76	149.51	176.59	119.53	49.23
B526	355.60	757.50	354.60	140.70	93.20	44.90
B528	413.70	938.10	445.00	161.00	100.10	31.43
B61	358.80	833.60	405.03	137.29	76.58	26.42

[a] Values given in μg/l.

APPENDIX A (Continued)

HPLC INDICES FOR REFINERIES A AND B FINAL EFFLUENT ADSORBED ONTO C18

Sample	1	2	3	4	5	6	Total
A8591t	540.7	709.6	884.1	1083.5	1252.9	1543.9	734.1
A8791t	543.6	709.2	883.6	1071.3	1255.9	1545.5	733.9
A8791	545.8	705.9	878.3	1081.2	1260.2	1547.6	738.4
A8991t	554.7	706.7	876.7	1078.4	1253.0	1544.0	730.6
A8991	553.0	707.3	878.6	1082.8	1253.8	1544.4	729.6
A91691	554.4	706.3	870.1	1084.5	1271.0	1553.2	733.9
A91891	551.1	701.7	872.1	1088.9	1260.8	1547.9	742.9
A92091	536.7	696.3	869.1	1084.0	1264.5	1549.8	767.3
A101491	552.1	702.1	869.3	1083.2	1264.9	1550.0	742.1
A101691	545.5	699.5	872.8	1083.9	1261.4	1548.2	750.3
A101891	559.0	705.9	876.9	1085.6	1235.3	1535.2	727.0
A12092	546.9	698.4	882.5	1075.7	1257.8	1546.4	746.9
A12292	551.5	704.0	882.4	1075.3	1261.6	1548.3	734.9
A12492	553.6	703.9	884.9	1080.5	1261.5	1548.3	731.1
A21092	558.1	708.6	884.7	1068.8	1246.5	1540.7	721.8
A21292	546.1	701.4	883.4	1077.4	1261.0	1548.0	741.3
A21492	549.5	701.7	882.9	1079.6	1265.8	1550.4	737.7
B82091	494.4	700.4	887.5	1068.1	1255.4	1492.7	709.7
B82291	510.7	695.7	890.7	1068.4	1267.8	1498.9	707.8
B82491	510.0	695.2	881.8	1066.0	1260.6	1495.3	712.9
B92491	509.4	697.5	887.7	1067.5	1264.5	1497.3	708.0
B92691	522.1	695.5	880.7	1063.7	1253.2	1491.6	698.6
B92891	512.4	691.2	885.7	1057.2	1232.9	1481.5	715.4
B102291	519.9	693.4	877.5	1063.1	1256.5	1493.2	706.0
B102491	520.8	698.0	886.6	1065.2	1232.8	1581.4	694.6
B102691	506.0	672.4	888.2	1070.9	1220.1	1475.1	682.2
B111391	519.3	695.3	884.3	1063.6	1229.1	1479.6	713.1
B111891	493.7	688.1	881.9	1067.4	1249.8	1489.9	739.2
B111991	499.1	683.9	882.2	1073.9	1245.6	1487.8	740.3
B112191	493.9	681.0	881.0	1060.9	1271.2	1500.6	751.6
B112391	498.4	689.1	882.5	1057.6	1258.7	1494.3	734.0
B121091	503.7	695.8	873.5	1055.9	1247.4	1488.7	723.7
B121291	506.0	691.5	889.1	1054.6	1226.7	1478.4	720.8
B121491	501.8	686.4	886.7	1050.9	1239.5	1484.7	734.0
B121791	500.3	687.8	885.5	1051.2	1240.5	1485.2	733.7
B12892	516.6	682.4	891.8	1066.1	1253.4	1491.7	731.0
B13092	495.9	691.9	888.6	1064.6	1266.1	1498.0	728.7
B2192	500.5	690.9	888.9	1064.4	1265.6	1497.8	725.7
B22792	478.5	695.4	878.9	1052.1	1276.5	1503.2	725.3
B22992	487.6	701.8	883.3	1055.1	1280.0	1505.0	725.3

APPENDIX A (Continued)

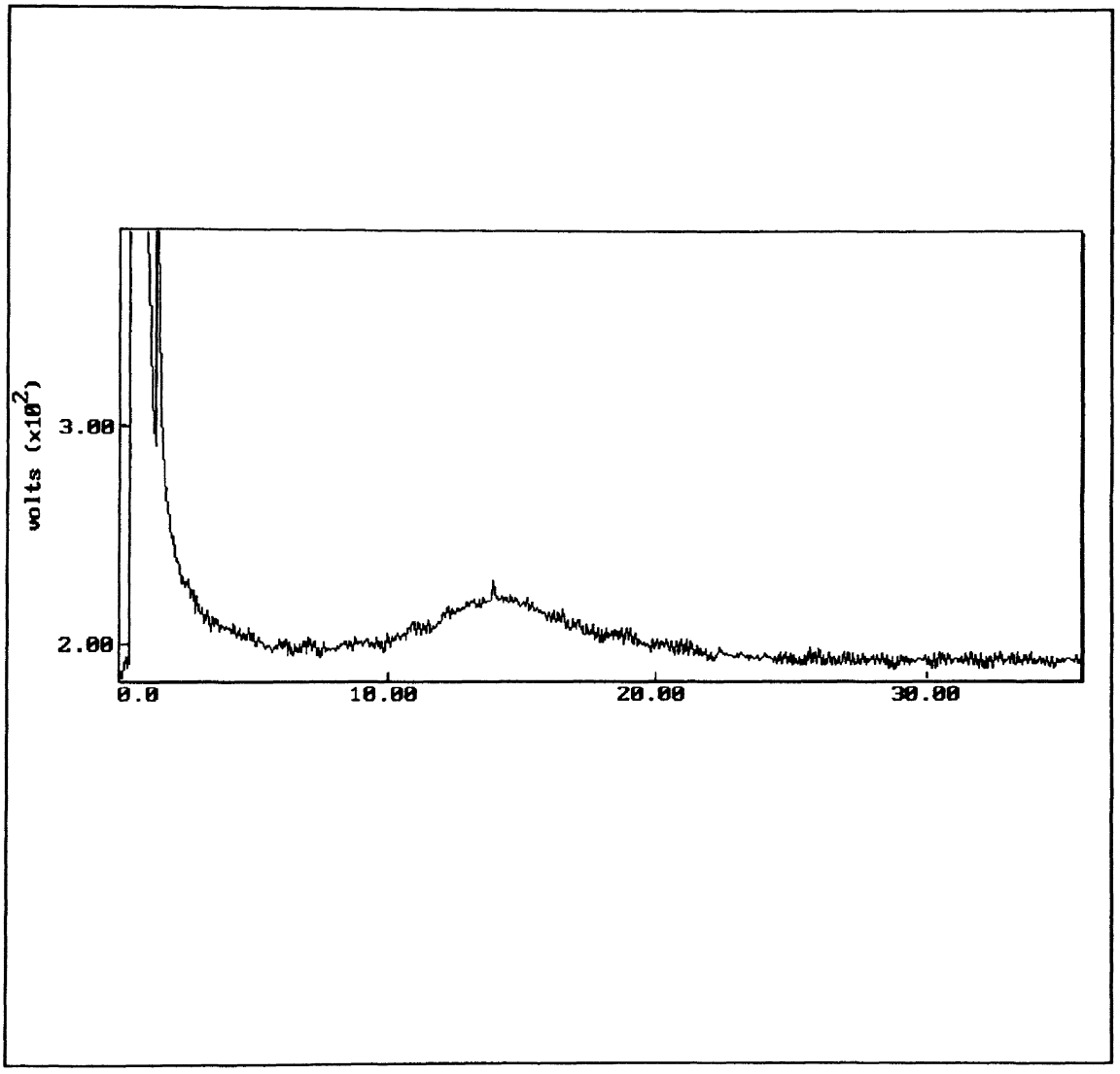
Sample	1	2	3	4	5	6	Total
B3392	489.1	703.2	884.5	1062.3	1249.9	1490.0	695.3
B32492	513.2	704.0	893.1	1046.5	1252.0	1491.0	695.3
B32692	514.9	707.3	898.6	1045.2	1261.2	1495.6	687.7
B32892	512.8	708.1	893.2	1050.1	1272.4	1501.2	690.7

GC INDICES FOR REFINERIES A AND B ADSORBED ONTO C18.

Sample	1	2	3	4	5	6	Total
B43	1346.4	1528.8	1695.6	1874.7	2085.7	2238.2	1699.1
B52	1309.8	1506.8	1709.6	1889.6	2092.9	2264.3	1616.7
B55	1322.2	1511.6	1705.5	1881.6	2090.6	2245.7	1326.8
B32	1354.4	1518.1	1678.1	1882.6	2105.1	2316.9	1642.1
B34	1363.4	1521.7	1686.8	1881.1	2104.1	2271.9	1683.3
B38	1367.4	1521.7	1691.2	1877.4	2088.8	2243.8	1676.3
A48	1292.7	1512.5	1709.6	1890.3	2084.7	2267.4	1819.4
A41	1340.4	1502.4	1708.8	1900.5	2100.0	2274.2	1887.6
B56	1311.2	1555.9	1710.5	1886.5	2074.3	2301.6	1709.1
B58	1325.4	1526.4	1695.6	1881.2	2091.3	2257.7	1693.9
B61	1306.8	1509.5	1683.8	1872.4	2088.6	2250.0	1687.9

APPENDIX A (Continued)

CAPILLARY GAS CHROMATOGRAM OF A REPRESENTATIVE REFINERY FINAL EFFLUENT ADSORBED ONTO C18



APPENDIX B

CHEMICAL ANALYSIS

SUMMARY OF WATER QUALITY ANALYSIS PERFORMED ON REFINERY A AND B FINAL EFFLUENTS[a]

	Refinery A	Refinery B
Alkalinity	88.3(66)	71.0(40)
Hardness	432.1(246)	400.5(190)
pH	7.8(0.9)	7.8(0.8)
Cond. (μmho/cm)	4238.8(4050)	6956.4(2060)
Total NH3	7.8(2.5)	25.4(4.9)
COD	77.6(89)	273.1(104)

[a] All values in mg/l unless otherwise noted. Values in parenthesis represent the magnitude of the range.

APPENDIX C

MICROTOX CORRELATIONS

PEARSON'S R VALUES FOR CORRELATION OF AMOUNT BRACKETED BY HPLC STANDARDS AGAINST TOXICITY VALUES FOR REFINERY A FINAL EFFLUENT

Standards	EC25	IC25	NOEC
C4-C6	-0.211	-0.353	-0.386
C6-C8	-0.444	-0.350	-0.660*
C8-C10	-0.336	-0.106	-0.562*
C10-C12	-0.197	0.122	-0.351
C12-C14	-0.474	-0.151	-0.329
C14-C16	-0.786*	-0.399	-0.156
C4-C16	-0.359	-0.250	-0.586*

* - $p \leq 0.05$

PEARSON'S R VALUES FOR CORRELATION OF AMOUNT BRACKETED BY HPLC STANDARDS AGAINST TOXICITY VALUES FOR REFINERY B FINAL EFFLUENT

Standards	EC25	IC25	NOEC
C4-C6	0.253	0.251	0.281
C6-C8	-0.551*	-0.451*	-0.382*
C8-C10	-0.521*	-0.515*	-0.311
C10-C12	-0.491*	-0.497*	-0.424*
C12-C14	-0.651*	-0.647*	-0.762*
C14-C16	-0.191	-0.264	-0.325
C4-C16	-0.400	-0.352	-0.239

* - $p \leq 0.05$

APPENDIX C (Continued)

PEARSON'S R VALUES FOR CORRELATION OF TOXICITY TO *PHOTOBACTERIUM PHOSPHOREUM* AGAINST HPLC INDICES FOR REFINERY A FINAL EFFLUENT

HPLC Index Region	EC25	IC25	NOEC
C4-C6	0.198	-0.095	0.300
C6-C8	-0.185	-0.486*	0.003
C8-C10	-0.008	-0.357	0.086
C10-C12	0.331	0.475	0.395
C12-C14	0.650*	0.173	0.192
C14-C16	0.652*	0.173	0.188
C4-C16	-0.044	0.395	0.160

* - $p \leq 0.05$

PEARSON'S R VALUES FOR CORRELATION OF TOXICITY TO *PHOTOBACTERIUM PHOSPHOREUM* AGAINST HPLC INDICES FOR REFINERY B FINAL EFFLUENT

HPLC Index Region	EC25	IC25	NOEC
C4-C6	0.568*	0.532*	0.494*
C6-C8	0.030	0.121	-0.054
C8-C10	0.020	0.159	0.273
C10-C12	0.460*	0.317	0.515*
C12-C14	-0.189	-0.044	-0.110
C14-C16	0.108	0.118	0.158
C4-C16	-0.568*	-0.537*	-0.460*

* - $p \leq 0.05$

APPENDIX C (Continued)

PEARSON'S R VALUES FOR CORRELATION OF TOXICITY TO *PHOTOBACTERIUM PHOSPHOREUM* AGAINST AMOUNT BRACKETED BY N-ALKANE GC STANDARDS

Standards	EC25	IC25	NOEC
C12-C14	-0.100	-0.008	-0.415
C14-C16	-0.101	0.049	-0.351
C16-C18	0.018	0.344	0.270
C18-C20	0.827*	0.572	0.787*
C20-C22	0.750	0.297	0.437
C22-C24	0.500	-0.404	-0.030
C12-C24	0.014	0.199	0.117
Indices			
C12-C14	0.425	-0.631	-0.384
C14-C16	0.350	-0.408	-0.333
C16-C18	0.455	0.788*	0.778*
C18-C20	0.505	0.325	0.345
C20-C22	0.475	-0.771*	-0.427
C22-C24	0.464	-0.496	-0.611
C12-C24	0.412	0.288	0.478

* - $p \leq 0.05$

APPENDIX D

CERIODAPHNIA CORRELATIONS

PEARSON'S R VALUES FOR CORRELATION OF TOXICITY TO *CERIODAPHNIA DUBIA* AGAINST AMOUNTS BRACKETED BY HPLC STANDARDS

Standards	Survival NOEC	Reproduction NOEC	Reproduction IC50
C4-C6	-0.547*	-0.430	0.301
C6-C8	0.060	-0.266	0.061
C8-C10	0.215	-0.225	0.039
C10-C12	0.617*	0.469	0.001
C12-C14	0.649*	0.575*	-0.041
C14-C16	-0.051	0.230	-0.355
C4-C16	-0.051	0.283	-0.074

* - $p \leq 0.05$

PEARSON'S R VALUES FOR CORRELATION OF TOXICITY TO *CERIODAPHNIA DUBIA* AGAINST HPLC INDICES FOR REFINERY FINAL EFFLUENT

HPLC Index Regions	Survival NOEC	Reproduction NOEC	Reproduction IC50
C4-C6	0.338	0.443	-0.251
C6-C8	0.254	0.458	-0.047
C8-C10	-0.415	-0.639*	-0.032
C10-C12	0.519	0.582*	-0.186
C12-C14	0.480	0.052	0.469
C14-C16	0.539*	0.600*	-0.228
C4-C16	0.792*	0.099	0.331

* - $p \leq 0.05$

APPENDIX E

FATHEAD MINNOW CORRELATIONS

PEARSON'S R VALUES FOR CORRELATION OF *PIMEPHALES PROMELAS* SURVIVAL AND GROWTH WITH QUANTITY OF MATERIAL BRACKETED BY HPLC STANDARDS

Standards	Growth		Survival	
	NOEC	IC50	NOEC	LC50
C4-C6	0.111	0.087	-0.059	-0.001
C6-C8	-0.065	-0.139	-0.172	-0.294
C8-C10	-0.155	-0.403	-0.136	-0.265
C10-C12	-0.547*	-0.642*	-0.404	-0.457
C12-C14	-0.411	-0.343	-0.079	-0.210
C14-C16	-0.341	-0.366	-0.021	-0.146
C4-C16	-0.089	-0.230	-0.179	-0.262

* - $p \leq 0.05$

PEARSON'S R VALUES FOR CORRELATION OF TOXICITY TO *PIMEPHALES PROMELAS* AGAINST HPLC INDICES FOR REFINERY FINAL EFFLUENT

HPLC Index Region	Growth		Survival	
	NOEC	IC50	NOEC	LC50
C4-C6	-0.029	0.123	0.160	0.281
C6-C8	-0.020	0.287	-0.003	0.043
C8-C10	0.517	0.485	0.401	0.343
C10-C12	-0.295	-0.151	-0.161	0.012
C12-C14	0.107	0.285	0.055	0.014
C14-C16	-0.172	0.064	0.023	0.172
C4-C16	0.323	-0.386	-0.022	-0.134

VITA

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Master of Science

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