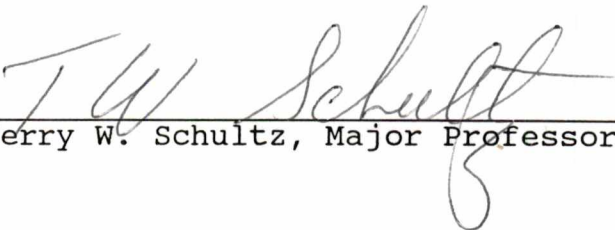
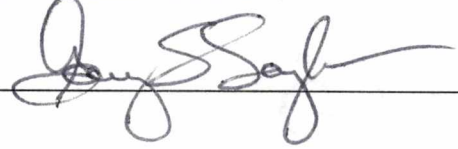


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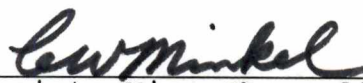
I am submitting herewith a thesis written by Anna P. Bearden entitled "The quantitative effects of nonpolar narcosis to Tetrahymena pyriformis and Escherichia coli." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.


Dr. Terry W. Schultz, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:


Associate Vice Chancellor
and Dean of the Graduate School

**THE QUANTITATIVE EFFECTS OF NONPOLAR NARCOTICS ON
TETRAHYMENA PYRIFORMIS AND *ESCHERICHIA COLI***

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Anna P. Bearden

August, 1996

DEDICATION

I would like to dedicate this thesis to my parents, Bill and Patti Bearden who taught me to work hard, to play hard, and to always be true to myself.

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ABSTRACT

Nonpolar narcosis, the most basic toxicity mechanism, is thought to be nonspecific interaction with a cellular membrane, due to the accumulation in the hydrophobic phases of the cell or organism. This mechanism is described by QSARs using hydrophobicity, measured by $\log K_{OW}$ (the 1-octanol/water partition coefficient). In this manuscript, QSARs for multiple endpoints from the no effect concentration to 100% lethality using hydrophobicity have been developed for a protozoan and bacteria population, *Tetrahymena pyriformis* and *Escherichia coli*, respectively. For *Tetrahymena*, both acute and chronic data were generated and compared using the acute to chronic ratio. A bacterial toxicity assay was developed using *Escherichia coli* that mimics the protozoan growth inhibition assay. These two species show good correlation in toxicity with hydrophobicity for the selected nonpolar narcotics. The potency to one species could be used to determine the potency of the other. In addition, it is possible to predict toxicity of endpoints based on other endpoints within a species. Using a population kinetics assay, the generation times of *Tetrahymena* affected by nonpolar narcotics with varying hydrophobicity and molecular structure were observed. Using chemicals with low, intermediate, and high $\log K_{OW}$, the response of *Tetrahymena* varied. Under the influence of the low $\log K_{OW}$ chemicals, there was a direct relationship between toxicant concentration and growth rate. For the high $\log K_{OW}$ chemicals, the lag phase in growth increased with toxicant concentration. Additionally, the generation times were identical to control growth with the high $\log K_{OW}$ chemicals. A median response was seen for the intermediate chemicals. It is hypothesized that the variation in population growth kinetics seen in this study may be due to different placement of the toxicant within the cellular membrane.

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LIST OF ABBREVIATIONS

ACR, Acute to Chronic Ratio

f, Fisher statistic

IGC_X, concentration which caused X% growth inhibition

K_{ow}, 1-octanol/water partition coefficient

LC_X, concentration which caused X% lethality

MATC, Maximum Acceptable Toxicant Concentration

mM, millimoles per liter

NOEC, No-Observable-Effects-Concentration

Pr > f, level of significance

QSAR, Quantitative Structure-Activity Relationship

r², coefficient of determination

s, square root of the mean square for error

SAS, Statistical Analysis System

CHAPTER 1

INTRODUCTION

In the modern industrial world, there are hundreds of thousands of synthetic organic chemicals. Hazards have been assessed for only a small percentage of these. Due to constraints in both time and money, realistically testing every single one is not feasible. Because of these constraints, structure-activity relationships have been used to predict the hazard of untested chemicals using the toxic potency of previously tested chemicals. Quantitative Structure-Activity Relationships (QSARs) have been shown to be reliable tools for use in hazard assessment of organic chemicals (Bradbury, 1994). Toxicology-based QSAR methodology is grounded on the premise that the toxic potency of a given group of chemicals is related to molecular descriptors. These molecular descriptors are quantitations of either molecular structure (e.g., molecular weight) or physiochemical properties (e.g., hydrophobicity). Moreover, descriptors may be derived experimentally or computer generated through tools such as molecular orbital quantum chemical software. Groups of toxicants used for QSAR analysis can be defined by the classical organic chemistry groupings (Clements and Nabholz, 1994; Clements *et al.*, 1994) or their mechanism of toxic action (Bradbury and Lipnick, 1990; Schultz *et al.*, 1990a).

Although many QSARs are developed based on classic organic chemistry groupings such as alcohols, ketones, or aldehydes, the most robust and meaningful QSARs are based on mechanism of toxic action (Cronin and Dearden, 1995). The problem arises when producing QSARs based on chemical class since chemicals that fall into the same "class" have completely different mechanisms of action and different potencies. One example are the alcohols. Many simple aliphatic alcohols such as ethanol and butanol are considered nonpolar narcotics, the most basic mechanism, a reversible process (Veith *et al.*, 1983; Schultz *et al.*, 1990b). On the other hand, 2-buten-1-ol exhibits

proelectrophilicity (Veith *et al.*, 1989; Schultz and Tichy, 1993) and is activated by alcohol dehydrogenase to become a soft electrophile (Klopman, 1974) nonreversibly binding to cellular thiols.

While acute toxicity has been classified in a number of ways (Schultz *et al.*, 1990a; Nendza and Russom, 1991; Verhaar *et al.*, 1992), it falls into one of two broad cause-and-effect categories: baseline or bioreactive. The interaction of toxicants with living systems depends upon a plethora of physical and/or chemical properties (Nendza and Russom, 1991) and often involves a poorly defined molecular site of action. Many different mechanisms of toxic action have been identified, including a wide range from reversible inhibition of cellular activity to chemical interactions which create or destroy chemical bonds inside cells, thereby, disrupting biochemical pathways or causing mutations in DNA (Bradbury, 1994). Each of these mechanisms, although identified, are not well defined nor understood. There are very few specific cellular or enzymatic pathways which have been identified that are affected by specific organic chemicals or specific mechanisms of action. These pathways are very complex and are not the subject of this thesis. Instead, the author of this thesis has chosen to identify chemicals from a single mechanism of toxic action, nonpolar narcosis, and study their effect on two different populations.

Narcosis is the most common mode of toxic action (Bradbury and Lipnick, 1990). Narcosis can be separated into several different types or mechanisms. Type I narcosis (Veith and Broderius, 1990) is nonpolar narcosis and presumably the most basic toxicity mechanism (Schultz, 1989). This mechanism is defined by nonspecific interactions with cellular membranes, due to the accumulation of chemical in the hydrophobic phases of the cell or organism (van Wezel and Opperhuizen, 1995). Because general narcosis represents reversible interactions between the chemical and membrane, the different types of narcotic toxicity can be described with QSARs using hydrophobicity, measured

by $\log K_{OW}$, the 1-octanol-water partition coefficient. Nonpolar narcotics have been shown over and over to be modeled by their hydrophobicity alone (Hansch *et al.*, 1989; Schultz, 1990b) with a theoretical correlation of one. Due to the relative simplicity in modeling nonpolar narcosis, the potency of chemicals eliciting their response by other toxic mechanisms is often compared to this baseline mechanism (Lipnick *et al.*, 1987).

Biologically, nonpolar narcosis is a reversible process. Organisms (e.g., mammals, fish, crustaceans, algae and protozoa) when placed in sublethal concentrations will recover when transferred to fresh media or the chemical is diluted (Schultz, 1989). Veith and Broderius (1990) have described nonpolar narcotics as the general slow down of cellular activity resulting in eventual death. Type I narcotics, when studying respiratory-cardiovascular inhibition in rainbow trout caused a decrease in metabolic rate, an approximate 50% decrease in heart rate, and a reduced respiratory function (McKim *et al.*, 1987).

Various studies have investigated many different possibilities for the exact site of action for narcosis, but interpretation has been difficult since almost all the endpoints studied have shown to be affected by nonpolar narcotic toxicity. For example, these chemicals seem to affect cellular pH, Na^+/K^+ gradients, protein function, increase in cell membrane volume, and possibly Ca^{2+} toxicity (Bangham *et al.*, 1965; Beavan *et al.*, 1982; Bolen and Sando, 1992; Leao and van Uden, 1984; Seeman and Roth, 1972; Nicotera *et al.*, 1992).

Franks and Lieb (1990) have proposed that narcosis disrupts protein-membrane interactions. Although they have produced multiple studies providing indirect evidence, no direct evidence has shown protein-membrane interactions to be disrupted. For example, the luciferase enzyme from *Photinus pyralis* emits light when the enzyme interacts with its substrate in the presence of cofactors. This highly specific enzyme has been shown to be competitively inhibited *in vitro* at the same 50% effect concentration

of the organism *in vivo*. A recent review by Sikkema *et al.* (1995) describes much of the evidence for the cellular membrane as the probable target for classic nonpolar narcotics, including monoaromatic hydrocarbons. In a separate paper, Sikkema *et al.* (1994) studied the effects of different chemicals on the membrane. These data begin to show that certain chemicals probably can be found in the center of the lipid bilayer.

This thesis focuses on assessing the effects of baseline, nonpolar narcotic toxicants on unicellular eukaryotes and gram negative prokaryotes. In Chapter 2 of this thesis, toxicities to *T. pyriformis* have been quantitated and described. Chapter 3 describes toxicities to *E. coli* which is followed by a comparison of the bacterial and protozoan sensitivities. Chapter 4 describes the effect of nonpolar narcotic stressors on protozoan generation time. The culmination of these three studies begins to refine the vague but widely accepted definition of nonpolar narcosis as it relates to aquatic toxicology.

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CHAPTER 2

QUANTITATION OF *TETRAHYMENA PYRIFORMIS* TOXICITY WITH NONPOLAR NARCOTICS

INTRODUCTION

Tetrahymena is an ubiquitous freshwater Ciliophora. This pear-shaped organism typically measures 50 x 30 μm , has a generation time of 3 to 4 hr, and filter feeds. The biology of this organism is described in the *Biology of Tetrahymena* edited by Elliot (1973). At a recent conference (1996) in Berlin, Germany, "Workshop on a Protozoan Test Protocol with *Tetrahymena* in Aquatic Toxicity Testing", the great advantages of *Tetrahymena* as an assessment organism were discussed. It was pointed out that *Tetrahymena* has excellent advantages as a test organism due to its ease in culturing, short generation, and well studied biology. *Tetrahymena* is also uncommon in that it is a single cell; therefore, easy to manipulate experimentally, but also it is an independent, eukaryotic whole organism.

In the laboratory, *Tetrahymena* can be cultured axenically both easily and economically. While it is possible to monitor an abundance of toxicological end-points, the two most common are acute mortality (survivability) and population growth impairment. In acute survivability tests, populations of organisms are exposed to step concentrations of toxicant for a specified period of time. Because of the difficulty in discerning "live" from "dead" cells, survivability assays with *Tetrahymena* tend to lack the desired quantitative precision and can be labor intensive and awkward.

Population growth impairment with *Tetrahymena* can be measured directly or indirectly. Direct measurements include manual and electronic counting. Indirect

measurements, whether on organismal or biochemical criteria, are based on changes in optical density. While the direct methods, especially electronic counting, are more accurate, the indirect methods, especially organism-based optical density measurements, are less labor intense.

This study has employed both an acute mortality and a population inhibition toxicity assay using *Tetrahymena* to develop structure-activity relationships for a group of nonpolar narcotics. Quantitative Structure Activity Relationships (QSARs) are linear models that relate toxicity to molecular descriptor data for a representative set of chemicals in an effort to predict the toxicity of untested chemicals. Molecular descriptor data are often in the form of physiochemical or quantum chemical parameters. These models have been established as valid methods in hazard assessment (Bradbury, 1994).

According to McFarland (1970), chemical toxicity is a combination of penetration into the biophase and the interaction of the toxicant with the site of action. The importance of the penetration and/or interaction parameters depends on the bioreactivity of the toxicant. McFarland's principle is represented mathematically as the following generic QSAR equation:

$$\log (\text{toxicity}^{-1}) = A \log (\text{penetration}) + B \log (\text{interaction}) + C,$$

Penetration is the movement of the toxicant to the site of action and is quantified by the 1-octanol-water partition coefficient. The interaction term is the interaction of the chemical with the site of action. This parameter is quantified by stereoelectronic properties.

Nonpolar narcosis is believed to be the baseline mechanism of toxic action, such that the excess toxicity of other mechanisms is related to nonpolar narcosis. Because there is a large diversity in reactivity between mechanisms, the QSARs that describe their toxicity also vary. It is well established that nonpolar narcotics model by penetration alone (Hansch *et al.*, 1989). Therefore, the B term for this group is zero.

One purpose of this study was to compare QSARs based on endpoints that span the range from no effect to 100% effect. The most commonly reported endpoint is the acute 50% endpoint. Of interest for nonpolar narcotics is whether linear relationship exists between chemical concentration and the biological response. This relationship is in opposition to toxicants having a threshold-type response that requires a critical concentration, yielding an all or nothing response, or chemicals that cause exponential increase in biological response relative to a linear increase in concentration. In order to test this question, a series of QSARs was developed using both acute and chronic endpoints of varying sensitivities. Their slopes were compared to determine whether the biological response varies directly with concentration. The intercepts were observed in order to determine the relative sensitivity of endpoints.

Of special interest to regulatory agencies, such as the Environmental Protection Agency, are both acute and chronic toxicity data. For those environmental contaminants in which toxicity testing has been done, acute lethality data is predominant (Kenega, 1986). Many of the environmental hazards that exist are sublethal concentrations of different xenobiotics for which the dangers are not known. The major reason for the lack of knowledge about chronic effects is the inherent problem in experimental design and the difficulty in quantifying sublethal effects. As part of this investigation, data generated from the acute lethality assay and from the "chronic" population growth inhibition test are used to determine an acute-to-chronic ratio for *Tetrahymena* in an effort to determine whether chronic data could be estimated from acute lethality data.

The chemicals chosen for the toxicity testing were selected from the classical nonpolar narcotic groups: aliphatic alcohols, aromatic hydrocarbons, ketones, and ethers. This section reports *Tetrahymena* toxicity using both an acute and chronic test and has compared different endpoints, spanning a range from a NOEC (no observable effect concentration) to a 100% effect concentration. If the theoretical description of nonpolar

narcosis is accurate, then for purposes of regulation, toxicity endpoints should be interchangeable, and the choice of toxicity endpoint should not matter. In other words, based on data from one endpoint, toxicity for different endpoints should be predictable and calculable.

MATERIALS AND METHODS

Chemical Selection & Hydrophobicity:

When choosing chemicals for testing, multiple criteria were used for selection. First, the chemicals were members of one of the four major classical nonpolar narcotic groups: aromatic hydrocarbons, aliphatic alcohols, ketones, and ethers. Also, the chemicals spanned a hydrophobicity, [i.e., the 1-octanol/water partition coefficient, ($\log K_{OW}$)] range from -1 to 5.0 with approximate increments of 0.5 log units. Above 5.0, the time required for these chemicals to equilibrate is much longer than the duration of the toxicity tests used. Finally, the chemicals were chosen to have only measured $\log K_{OW}$ values as taken from Sangster (1993).

TETRATOX:

This 40-hr assay is described in Schultz *et al.* (1990). Briefly, this toxicity test uses the protozoan, *Tetrahymena pyriformis*, to study the population growth inhibition induced by chemical stress. 1.5×10^3 cells are inoculated at T_0 and allowed to incubate at $27 \pm 1^\circ\text{C}$. Spectrophotometric absorbance is measured at 540 nm after 40 hr. For each chemical, a range finding test is first done to determine the chemical concentration ranges such that zero and complete inhibition are seen using a minimum of 5 different step concentrations. Three separate definitive tests are then completed in duplicate with fresh stock solutions prepared in dimethyl sulfoxide (DMSO) between 10 thousand mg/L and straight chemical without dilution. Positive (i.e., *Tetrahymena* grown in media without chemical) and negative (i.e., flasks with no cells or chemical) controls are set up

with each experiment. All tests are performed in foam stoppered 250 mL Erlenmeyer flasks filled with 50 mL of proteose peptone amended media (Schultz *et al.*, 1990).

16-Hr Acute Test:

This water-based acute assay is a 16-hr test (Schultz *et al.*, 1994). This assay examines cessation of ciliary movement and cell lysis with the aid of a dissecting light microscope. Cells (1×10^3) are added to 16 x 125 mm sterile test tubes containing 10 mL of 0.1X nutrient-free Hank's isotonic salt solution. Stock solutions were made fresh in DMSO with each test in between 10 mg/L and straight chemical. The chemical is added in 10-step concentrations to begin initial range finding tests. Tests are repeated until similar results (i.e., percent mortality) are obtained, at least three times in a row. Due to the nature in evaluating the acute assay, only three endpoints (acute NOEC, LC₅₀, and LC₁₀₀) can be accurately determined.

Statistical Procedures:

The toxicity data were analyzed statistically using probit analysis of Statistical Analysis System (SAS) software (SAS Inc., 1989), where chemical concentration represents the dependent variable and population inhibition (i.e., absorbance normalized to control absorbance) or percent mortality represented the independent variable. The 5, 15, 50, 85, and 95% predicted growth inhibitions were extracted from the probit analysis in addition to the chronic estimated no effect and 100% effect. The IGC₅₀ was chosen since this value represents the predicted value with the smallest 95% confidence limit and, therefore, will have the least variability. The IGC₁₅ and IGC₈₅ were chosen to represent the endpoints between 14% and 86%, where dose-response relationships are theorized to be linear (Rand, *et al.*, 1995). The IGC₀₅ and IGC₉₅ were chosen to predict the chronic estimated no effect and 100% growth inhibition. In addition to those endpoints displayed in this analysis, the regression analysis using the 99% and 1% were

performed. These endpoints had large confidence limits that resulted in linear models with poor coefficients of determination.

These data were then mathematically transformed into the log of the inverse of the mM concentration. Any further reference to data compiled from this method is made as the IGC_X , representing the growth inhibition concentration, where X% of growth is inhibited or by the LC_X , representing the concentration which kills X% of the cells.

QSARs were developed using the least squares linear regression procedure of general linear model of SAS, where either the IGC_X or LC_X were regressed as $\log K_{ow}$ as the dependent variable. The resulting models were measured for quality by the coefficient of determination (r^2). In order to compare the slopes of QSARs to each other, an option under general linear model of SAS (SAS Inc., 1989) was used to perform a *t*-test. The resulting probability value indicated the probability that the difference between two values is zero. A *p* value < 0.05 indicated different slopes. In order to determine that the values of the intercepts represent the correct order corresponding to increasing IGC_X or LC_X , the Spearman Correlation test was performed under the correlation procedure of SAS (SAS Inc., 1989).

Acute to Chronic Ratios:

The acute to chronic ratio (ACR) for this manuscript has been defined by a linear regression (SAS Inc., 1989) which compares LC_{50} to the estimated chronic NOEC using data from Table 1.

RESULTS & DISCUSSION

Table 1 is a summary of the hydrophobicity and toxicity data for each of the 14 nonpolar narcotics examined. A cursory examination of these data reveals that potency increases as hydrophobicity increases. Due to a narrow toxicity range, complete chronic testing of 1-dodecanol was not done.

Table 2 presents the QSARs generated for each of the ten toxicity endpoints for *Tetrahymena*, along with the corresponding r^2 statistics. The slopes of these models were compared statistically (Table 3). Although there is a trend for the slopes to increase with sensitivity of the endpoint, the chronic endpoints are similar to each other, and the acute endpoints are similar to one another (Table 4).

All of the chronic endpoints have slopes statistically different from the acute endpoints. Although, the IGC₉₅ and IGC₀₅ are outside the 14-86% cut-off for dose-response linearity, their use gives an adequate prediction of the 100% effect and NOEC for the purposes of this analysis. This analysis implies for the reported endpoints, that either a single chronic or acute QSAR can be used to predict *Tetrahymena* toxicity.

The acute endpoints consistently have larger slopes than do the chronic endpoints. The different media utilized in the assays may explain the differences in slope between the acute and chronic endpoints. The 16-hr acute test uses a water-based media, whereas TETRATOX uses an organic-based media. Jaworska and Schultz (1994) showed that the theoretical slope for nonpolar narcotics is 1.00. The presence of organic components in the media causes the slope to be < 1.00 , due to interactions of the toxicant with the organic component of the media.

The relative sensitivity of an endpoint can be assessed by observation of the intercept of the QSAR. The intercepts of the acute endpoints are consistently more negative than the intercepts of the chronic endpoints, indicating the acute endpoints are more sensitive than the chronic ones. The Spearman correlation determines whether a list of numbers rank in the correct order. The chronic and acute data were analyzed separately with the Spearman correlation. For both groups, the correlation coefficient was 1.00 indicating a perfect rank order. In other words, sensitivity, as measured by the absolute value of the intercept, is in the correct order relative to the endpoint (i.e., the IGC₉₅ > IGC₈₅ > IGC₅₀ > IGC₁₅ > IGC₀₅, etc.).

The results of the ACR analysis are shown in Eq. 2.9. By exponentiating the intercept and dividing by the slope, the ACR for this data is approximately 3.40.

$$\begin{aligned} \text{population growth NOEC} &= (0.83) \text{LC}_{50} + 0.45; & \text{Eq. [2.11]} \\ r^2 &= 0.97, s = 0.26, f = 413.54, \text{Pr} > f = 0.0001 \end{aligned}$$

The ACR model shown in Eq. 2.11 was generated based on Kenega (1982b) who defined the ACR as the acute lethality concentration (LC_{50}) divided by the chronic no observable effects concentration (NOEC) or maximum acceptable toxicant concentration (MATC). The MATC was determined as the mean of the highest concentration at which none of the measured endpoints are affected and the lowest concentration causing significant effects on at least one of the endpoints for fish (Kenega, 1982b). By taking the antilog of the intercept and divided by the slope (Eq. 2.11), the ACR is approximately 3.40. The slope of this equation is expected to be less than one based on the above analysis comparing the slopes for acute and chronic endpoints since as shown in Table 3, the acute endpoints had larger slopes than did the chronic endpoints. Equation 2.11 is consistent with the ACR for nonpolar narcotics found by Jones and Schultz (1995) for fathead minnow.

The merit of the ACR lies in using known ACR values for a particular species-toxicant pair for predicting probable chronic responses of similar species or toxicants (Giesy and Graney, 1989). For almost 90% of the chemicals assessed, the difference between the acute LC_{50} and the chronic NOEC is less than two orders of magnitude (Kenega, 1982a). Therefore, the use of the ACR approach has become a useful predictive tool.

The QSARs developed herein relate toxicity to hydrophobicity. The QSARs presented in Table 3, along with the slope comparisons in Table 4, and ACR ratio analysis indicate for *Tetrahymena* that the toxicity of any endpoint can be predicted based on a single endpoint. If narcotics affect the functioning of membranes as endorsed

by Franks and Lieb (1984), then hydrophobicity describes the ability of toxicants to enter biological membranes. If narcotics enter cells through passive diffusion, then as the chemical concentration increases, there should be a linear relationship between toxicant concentration and biological response. Because any endpoint can be predicted based on another endpoint, there is a direct relationship between the biological response and log K_{OW} . This relationship is in opposition to toxicants that have threshold-type response that require a critical concentration yielding an all-or-nothing response or chemicals which cause exponential increase in biological response relative to a linear increase in concentration.

This observation gives merit to the idea that nonpolar narcosis is the baseline mechanism and that toxicity is based on passive diffusion through the cellular membrane.

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APPENDIX

TABLE 1 Toxicities, CAS Numbers, and Hydrophobicities for the Selected Endpoints

Name	CAS Number ^a	Log K _{ow}	LC _{NO}	LC ₅₀	LC ₁₀₀	IGC ₉₅	IGC ₈₅	IGC ₅₀	IGC ₁₅	IGC ₀₅	Estimated NOEC	Estimated IGC ₁₀₀
1. methanol	67-56-1	-0.77	-2.97	-3.04	-3.19	-2.62	-2.75	-2.52	-2.38	-2.44	-2.29	-2.77
2. ethanol	64-17-5	-0.31	-2.75	-2.78	-2.94	-2.44	-2.37	-2.20	-2.06	-1.88	-1.99	-2.35
3. acetone	67-64-1	-0.24	-2.65	-2.68	-2.71	-2.06	-2.12	-2.00	-1.96	-1.80	-1.71	-2.22
4. 2-butanone	78-93-3	0.29	-1.84	-1.92	-2.14	-1.96	-1.99	-1.75	-1.61	-1.30	-1.05	-1.92
5. 2-pentanone	107-87-9	0.91	-1.54	-1.72	-1.76	-1.59	-1.54	-1.44	-1.30	-1.12	-0.84	-1.61
6. 1-pentanol	71-41-0	1.56	-1.15	-1.23	-1.45	-1.26	-1.20	-1.07	-0.89	-0.73	-0.66	-1.27
7. anisole	100-66-3	2.11	-0.57	-0.62	-0.74	-0.79	-0.72	-0.57	-0.34	-0.10	0.04	-0.81
8. 1-heptanol	111-70-6	2.57	-0.11	-0.18	-0.41	-0.02	0.02	0.11	0.22	0.30	0.37	-0.08
9. 1-octanol	111-87-5	3.15	-0.64	0.51	0.16	0.73	0.78	0.88	0.57	1.09	1.20	0.60
10. 1,2-dichlorobenzene	95-50-1	3.38	1.17	0.87	0.62	0.47	0.49	0.53	0.83	0.60	0.61	0.47
11. 2-decanone	693-54-9	3.60	0.72	0.59	0.49	0.41	0.61	0.58	0.90	0.89	1.10	0.42
12. n-butylbenzene	104-51-8	4.26	1.31	1.30	1.22	1.17	1.20	1.25	1.31	1.35	1.35	1.17
13. n-amyIbenzene	538-68-1	4.90	1.57	1.56	1.55	1.63	1.68	1.79	1.95	2.07	2.17	1.57
14. 1-dodecanol ^b	112-53-8	5.13	2.53	2.49	2.46	--	--	2.16	--	--	2.30	2.06

^aChemical Abstract Registry number^bDue to a narrow toxicity range, complete testing was not possible

TABLE 2 QSARs for *Tetrahymena* Endpoints

Endpoint	Slope	Intercept	r ²	Equation #
1. estimated NOEC	0.75	-1.59	0.98	1-1
2. IGC ₀₅	0.75	-1.70	0.98	1-2
3. IGC ₁₅	0.76	-1.86	0.99	1-3
4. IGC ₅₀	0.77	-2.00	0.99	1-4
5. IGC ₈₅	0.79	-2.14	0.98	1-5
6. IGC ₉₅	0.77	-2.15	0.98	1-6
7. estimated IGC ₁₀₀	0.77	-2.16	0.99	1-7
8. LC _{NO}	0.89	-2.34	0.98	1-8
9. LC ₅₀	0.89	-2.43	0.99	1-9
10. LC ₁₀₀	0.90	-2.59	0.99	1-10

TABLE 3 Results of *t*-tests for Slope Comparisons. A p-value < 0.05 Indicated "Different"

	Estimate NOEC	Estimate IGC ₁₀₀	IGC ₀₅	IGC ₁₅	IGC ₅₀	IGC ₈₅	IGC ₉₅	LC _{NO}	LC ₅₀	LC ₁₀₀
estimate NOEC	---	same	same	same	same	same	same	different	different	different
estimate IGC ₁₀₀		---	same	same	same	same	same	different	different	different
IGC ₀₅			---	same	same	same	same	different	different	different
IGC ₁₅				--	same	same	same	different	different	different
IGC ₅₀					---	same	same	different	different	different
IGC ₈₅						--	same	different	different	different
IGC ₉₅							---	different	different	different
LC _{NO}								---	same	same
LC ₅₀									---	same
LC ₁₀₀										---

TABLE 4 Results of *t*-tests for Slope Comparisons

	Estimate NOEC	Estimate IGC ₁₀₀	IGC ₀₅	IGC ₁₅	IGC ₅₀	IGC ₈₅	IGC ₉₅	LC _{NO}	LC ₅₀	LC ₁₀₀
estimate NOEC	---	0.41	0.97	0.75	0.52	0.41	0.56	0.0007	0.0008	0.0004
estimate IGC ₁₀₀		---	0.46	0.64	0.86	0.97	0.84	0.009	0.010	0.006
IGC ₀₅			---	0.79	0.56	0.45	0.60	0.0014	0.0016	0.0009
IGC ₁₅				---	0.77	0.63	0.80	0.003	0.003	0.002
IGC ₅₀					---	0.83	0.98	0.005	0.006	0.004
IGC ₈₅						---	0.82	0.01	0.02	0.01
IGC ₉₅							---	0.008	0.008	0.005
LC _{NO}								---	0.97	0.89
LC ₅₀									---	0.86
LC ₁₀₀										---

CHAPTER 3

QUANTIFICATION OF *ESCHERICHIA COLI* TOXICITY TO NONPOLAR NARCOTICS

INTRODUCTION

While acute aquatic toxicity has been classified in a number of ways (Schultz *et al.*, 1990a; Nendza and Russom, 1991; Verhaar *et al.*, 1992), it occurs in one of two broad cause-and-effect classifications: baseline or bioreactive. Nonpolar narcosis is considered baseline mechanism (Schultz, 1989) and has been recently reviewed by van Wezel and Opperhuizen (1995). Because nonpolar narcosis toxicity depends only on toxicant uptake, the modeling of toxic potency of nonpolar narcotics yields simple 1-octanol/water partition ($\log K_{OW}$) dependent quantitative structure-activity relationships (QSARs). These QSARs are consistent regardless of test system or endpoint (Hansch *et al.*, 1989).

Unfortunately for nonpolar narcosis similar to many toxicity mechanisms, a specific molecular pathway or site of action has not been identified. In the past, nonpolar narcosis has been considered a general decrease in cytoplasmic activity (Veith and Broderius, 1990). Different studies have investigated diverse possibilities for the site but interpretation has been difficult since almost all the responses studied have been affected by nonpolar narcotic toxicity (Franks and Leib, 1990). Specifically, neutral organic chemicals seem to affect cellular pH, Na^+/K^+ gradients, protein function, increase in cell membrane volume, and possibly Ca^{2+} toxicity (Bangham *et al.*, 1965; Beavan *et al.*, 1982; Bolen and Sando, 1992; Leao and van Uden, 1984; Seeman and Roth, 1972; Nicotera *et al.*, 1992).

The use of prokaryotes as model systems in scientific experiments has long been an accepted method for investigation of almost all aspects of biological research.

Escherichia coli is one species of bacteria which has been extensively studied and is well characterized (Nanninga, 1985). The use of bacteria in toxicity testing, both axenic culture and mixed naturally occurring cultures (Martinez *et al.*, 1989) has been well-established. The 5- to 30-min *Vibrio fischeri* (formally *Photobacterium phosphoreum*) luminescence inhibition acute assay, better known as the acute Microtox assay has been widely used (Bulich, 1979). Other bacterial assays measure a wide variety of endpoints, some of which include the monitoring of population growth (Nenzda and Seydel, 1988), CO₂ production (Jardin *et al.*, 1990), oxygen consumption, enzyme biosynthesis (Dutton *et al.*, 1990), and glucose mineralization (Retuna *et al.*, 1989).

Although each of these studies provide useful information on the relative toxicity towards a particular species of bacteria, when comparing this data to other organisms tested in different test systems or to the same organism with different endpoints, there are often discrepancies that can be attributed to differences in protocols, toxicokinetics, or toxicodynamics. Even something as basic as the organic component of the media can result in discrepancies. Jaworska and Schultz (1994) have shown for nonpolar narcotics that the slope of a QSAR developed with log K_{OW} as the independent variable will vary with the organic content of the medium. For water-based assays, the theoretical slope is 1.00. For organic-based assays, the theoretical slope will be less than one, since the organic component of the medium will bind some of the test chemicals, thereby, removing it from access to the organism.

The combination of all of these questions was the catalysis for the development of a bacterial toxicity assay that mimics the well-established TETRATOX assay (Schultz *et al.*, 1990b) for comparative purposes. The resulting data, therefore, allows direct

comparison since both assays use similar endpoints and growth medium, two of the biggest differences between different toxicity assays.

The purposes of this research were 1) to develop an *E. coli* toxicity assay; 2) to use the assay to assess the toxicity of ten selected nonpolar narcotics; 3) to develop QSARs with different toxicity endpoints and compare these different endpoints, and 4) to compare the bacterial toxicity with *Tetrahymena* toxicity.

METHODS AND MATERIALS

I. Development of *E. coli* Toxicity Assay.

A. Assay Parameters

TETRATOX (Schultz *et al.*, 1990b) was the basis for this assay. Certain alterations which reflect differences between bacteria and protozoa were necessary. Under the growth conditions described below, the optimal temperature for growth is $26 \pm 1^\circ\text{C}$. Seventy-five mM Tris Base/HCl buffer was used with an initial pH of 7.5. All tests were performed in foam-stoppered 250 mL Erlenmeyer flasks occupied with 50 mL of media.

With an inoculum of 4.5×10^3 cells/mL, the bacteria grow in log growth for 16-17 hr after a lag phase of approximately 30 min. With a generation time of 45-60 min, a minimum of 16 generations span.

Because of the short generation time of the bacteria and actual time required to read each individual assay, an additional step was included in the bacterial test procedure. At T₁₅, 250 μL of 8 N HCl was added to every flask. This addition caused the pH of the media to quickly drop below a pH of 4.00, increasing the generation time to many hours.

Population inhibition was measured spectrophotometrically at 540 nm. A positive control flask containing no chemical was used with every assay in addition to a negative control that contained neither bacteria nor chemical. When control flask grew above 0.85, experiments were discarded.

Linear regression analysis with cells/mL as the dependent variable and absorbance as the independent variable was performed by the general linear model procedure of Statistical Analysis Software, SAS, (SAS Inc., 1989). The following model was generated:

$$\begin{aligned} \text{Cell Number} &= (\text{Absorbance}) 2.89 \times 10^6 - 6.33 \times 10^5; \\ r^2 &= 0.98, s = 9.5 \times 10^5, f = 1.5 \times 10^3, \text{Pr}>f = 0.0001 \end{aligned} \quad \text{Eq. [3.1].}$$

B. Validation of E. coli Toxicity Test

In order to show that population growth inhibition can be achieved under the above described toxicity assay, the following experiment was performed using bacteria grown under the presence of butylbenzene to measure the growth of bacteria with time.

At time zero, flasks of media were inoculated in triplicate with 4.5×10^3 cells/mL and either 0.11, 0.14, and 0.17 mM of butylbenzene. The time points chosen *a priori* were hours 10, 11, 12, 13, 14, 14.75, 15.25, and 16. These times reflect the time period where spectrophotometric absorbance can be measured and cells grow linearly.

During the theoretical conditions of the toxicity test, flasks are neither aerated nor agitated. Both aeration and agitation increase bacterial growth rates. In order to read a flask on the spectrophotometer, it is necessary to both remove the foam stopper, allowing oxygen to enter the flask and to gently swirl the flask to homogenate the culture, thereby agitating it. If flasks are read hourly, this degree of both aeration and agitation allow increased growth rates of the cultures. To mimic the conditions of the toxicity test, each flask was only read one time and discarded.

Table 1 shows the population growth models and the corresponding generation times for *E. coli* grown in the presence of butylbenzene. By dividing $\ln 2$ by the slope, the generation time was achieved. QSAR development and *t*-test comparisons were performed in the general linear model procedure of SAS (SAS Inc., 1989). Between the four conditions, there was a mean generation time of 52 min.

A dose-response relationship was seen for bacterial growth in butylbenzene. These results also indicate, that for a given chemical concentration, differential response is not due to a decreased growth rate of the bacteria, but instead is the result of a lag in growth. The only difference among the models in Table 1 are intercepts. The absolute value of these intercepts increases with chemical concentration and also coincides with increasing lag times.

II. *E. coli* Toxicity Testing.

Ten toxicants were evaluated using the *E. coli* test. These ten were selected as the best representative group covering the log K_{OW} range of -1 to 5.0 and representing the classic nonpolar groups.

First, a range-finding test of at least six step concentrations of test chemicals, including duplicate control flasks was done to determine the appropriate chemical concentrations. A minimum of three definitive tests performed in duplicate were then completed. Positive and negative controls were set up with each experiment. A fresh stock solution of between 5000 mg/L and straight chemical were made for each test. At T_0 , flasks were inoculated with chemical and 4.5×10^3 bacteria/mL, and then allowed to incubate at $26 \pm 1^\circ \text{C}$ for 15 hours. After this time, the flasks were acid stopped with 250 μL of 8N HCl and read on the spectrophotometer at 540 nm.

Data were recorded as mg/L and analyzed statistically using probit analysis of SAS (SAS Inc., 1989). Chemical concentration represented the dependent variable and population inhibition (i.e., absorbance normalized to control values) represented the independent variable. The growth inhibition concentrations for the endpoints described below were taken from the probit analysis. These data were converted to mM and mathematically transformed into the log of the inverse of mM resulting in IGC_X or $\log (\text{mM})^{-1}$.

From the probit analysis, endpoints of interest can be chosen from the 1% to 99% predicted growth inhibition concentration. The 5, 15, 50, 85, and 95% predicted growth inhibitions were chosen. The IGC_{50} was chosen since this value represents the predicted value with the smallest 95% confidence limit, and, therefore, will have the least variability. The IGC_{15} and IGC_{85} were chosen to represent the endpoints between 14% and 86% where dose-response relationships are theorized to be linear (Rand *et al.*, 1995). The IGC_{05} and IGC_{95} were chosen to predict the no effect and 100% growth inhibition. In addition to those endpoints shown in this analysis, the regression analysis using the 99% and 1% were performed. These endpoints were deemed unreliable, since they had exceedingly large confidence limits and poor coefficients of determination.

The estimated no effect and 100% population inhibition were observed directly from the raw data. The initial cell inoculum used in this assay does not register an absorbance value on the spectrophotometer at 540 nm. The bacteria must divide approximately eight times before the cells give an accurate reading. Because the cells must go through these divisions, the estimated "100% effect" may not exactly be the amount of chemical that causes complete growth inhibition. The defined "no effect" was the estimated value where growth equaled the control growth.

QSARs were developed for each endpoint. Using the general linear model procedure of SAS least squares regression, the toxic potency of each endpoint was regressed against $\log K_{OW}$ to develop the relationships. The slopes of these models were compared to each other using a *t*-test that compares slope values. A *p* value < 0.05 indicated slopes of differing values. Moreover, the Spearman Correlation test was performed in order to determine that the values of the intercepts represent the correct order corresponding to increasing IGC_X .

RESULTS

QSARs for E. coli Toxicity:

Table 2 shows the toxicity data for *E. coli* population inhibition. QSARs representing each of the endpoints of interest are shown in Table 3, along with their respective r^2 statistic. N-amylbenzene was consistently less toxic than predicted and showed the largest residual for each of the models. This chemical has a log K_{OW} of 4.90 that approaches the aquatic solubility limit for acute toxicity, a value we estimate by a log K_{OW} of 5.00, and has not come to equilibrium in the aqueous solution in the short time of the assay. Because of this, n-amylbenzene was removed from further analysis.

Tables 4 and 5 show the results of slope comparisons for the *E. coli* QSARs. There is a direct relationship between the slope value of the QSAR and the endpoint. The NOEC and IGC₀₅, which have identical slopes, exhibited statistical differences from the estimated IGC₁₀₀ and approached statistical significance from the IGC₉₅ (p-value). As noted previously, dose-response relationships are theorized as being linear between 86% and 14% (85% and 15% reported in this study) (Rand *et al.*, 1995). The estimated no effect and 100% effect, in addition to the predicted 5% and 95%, are all outside of this range. Each of the QSARs within the linear range has slope values that are statistically similar.

Spearman correlation determines whether a list of numbers rank in the correct order. For these data, the correlation coefficient was 1.00 indicating a perfect rank order. In other words, sensitivity as measured by the absolute value of the intercept is in the correct order relative to the endpoint (i.e., the IGC₉₅ > IGC₈₅ > IGC₅₀ > IGC₁₅ > IGC₀₅, etc.).

Comparative Toxicity:

The TETRATOX assay was described in Chapter 2. Briefly, this toxicity test uses the protozoan, *Tetrahymena pyriformis*, to study the population growth inhibition

induced by chemical stress. Tests were performed in foam-stoppered 250 mL Erlenmeyer flasks filled with 50 mL of proteose-peptone-based medium. Population density was estimated spectrophotometrically as absorbance at 540 nm after 40 hr of incubation at $27 \pm 1^\circ\text{C}$. Table 6 summarizes the *T. pyriformis* population growth impairment data previously reported in Chapter 2.

From observation of the slope values (Tables 3 and 6) for the two species, the protozoan values are slightly greater than the bacterial slopes, although not statistically different. On the other hand, the intercept values are very similar and are essentially equal for the IGC₀₅, IGC₅₀, IGC₈₅, IGC₉₅, and estimated IGC₁₀₀. These comparisons indicate that the species have equal sensitivity, as indicated by the intercepts.

The 50% effects concentration is the most commonly reported end-point. Toxic potency between the two test systems was further examined by the linear regression analysis, comparing the IGC₅₀ of bacteria with that of the protozoan; this analysis yielded the model:

$$E. coli \text{ IGC}_{50} = 0.97 (T. pyriformis \text{ IGC}_{50}) - 0.04;$$

$$n = 10, r^2 = 0.99, s = 0.14, F = 588.07, Pr > F = 0.0001 \quad \text{Eq. [3.20]}$$

The r^2 values for Eq. [3.20] reveals excellent agreement between observed and modeled IGC₅₀ values. A slope of near unity and an intercept of near zero is evidence that toxic potency for nonpolar narcotics measured in the *E. coli* and *T. pyriformis* assays differ little, if any.

DISCUSSION

Although each of QSARs shown in Table 3 generated with *E. coli*, toxicity and hydrophobicity for nonpolar narcotics yielded quality models, not all the endpoints yielded slope values similar to each of the others. The endpoints IGC₁₅, IGC₅₀, and

IGC₈₅ (Table 3) could be used to estimate the potency in another end-point. The estimated NOEC and IGC₁₀₀ and the predicted IGC₀₅ and IGC₉₅ did not yield slope values that were statistically similar to all the endpoints, and, therefore, should not be used to estimate the toxicity of other endpoints. The predicted 5% and 95% inhibition in this analysis were adequate predictions of the NOEC and estimated 100%.

Results noted in Chapter 2 for regressions of toxic potencies measured for the *T. pyriformis* and hydrophobicity (see Table 6) indicated that for *T. pyriformis*, any endpoint could be used interchangeably. This observation was seen for those outside of the range where the dose-response is linear (Rand *et al.*, 1995). The relative sensitivity of an endpoint can be assessed by observation of the intercept of the QSAR. As shown in Chapter 2 for *T. pyriformis*, the sensitivity of endpoints increased with the per cent inhibition caused by a particular endpoint.

The results reported in Eq. [3.20] suggest that potency measured for either *E. coli* and *T. pyriformis* end-points can be used to predict potency for the other assay for nonpolar narcotics. Schultz *et al.* (1990a) observed this for a limited number of alcohols and ketones. As anticipated from the results of Hansch *et al.* (1989), good correlation should be observed between species for the baseline narcosis mechanism of action.

McFarland's probabilistic model of toxicity (McFarland, 1970) notes that the expression of chemical toxicity is a multistep process. The first occurrence is the movement of the chemical to the molecular site of action. The second is interaction of the chemical with the site. Movement of the chemical to the site of action typically requires the crossing of biological membranes. This process, one of passive diffusion, is modeled by hydrophobicity. In contrast, interaction of the chemical with the site of action is more complicated and considered to be a nonhydrophobic-dependent process.

The majority of neutral organic chemicals exhibit the nonpolar narcosis mechanism of toxic action. Such chemicals cause only reversible physiological alterations and are

considered narcotics. They exhibit an absence of irreversible binding to either macromolecules or membranes, and, regardless of molecular structure, exhibit acute toxicities directly related to $\log K_{OW}$. This mechanism of toxic action has been the subject of several reviews, the most recent from van Wezel and Opperhuizen (1995).

In the past, nonpolar narcosis has been considered a general decrease in cytoplasmic activity (Veith and Broderius, 1990). This vague definition arose from the fact that for many years biochemical toxicologists have searched for and been unsuccessful in finding a specific enzymatic pathway or molecular site of action for these chemicals (Franks and Lieb, 1990). Franks and Lieb (1990) have endorsed the hypothesis that narcotics affect the functioning of membranes by affecting protein-lipid interactions. They have studied the effects of firefly luciferase competitive inhibition by narcotics (Franks and Lieb, 1984) and the effects of anesthetics on activation of a potassium current and on inhibition of an acetylcholine-receptor-mediated current (Franks and Lieb, 1994). In a recent review by Sikkema *et al.* (1995), the membrane as the probable target for many hydrocarbons has been discussed in detail.

The outer covering of *E. coli* consists of two distinctly different layers, including the outer membrane and inner membrane, both of which are lipid bilayers containing embedded proteins. The two membrane layers are separated by a periplasmic space (Neidhardt *et al.*, 1990). The outer membrane is covered with lipopolysaccharide (LPS), a compound containing a hydrophobic fatty acid region and a hydrophilic polysaccharide region. The polysaccharide region extends into the environment (Mizushima, 1985) and acts as a sort of boundary layer against some toxicants (Britton *et al.*, 1988). Due to LPS layer in addition to the phospholipids heads and the porins, the outer surface is hydrophilic in nature (Neidhardt *et al.*, 1990).

Tetrahymena are covered with a multi-layer membrane structure called a pellicle (Elliott and Kennedy, 1973) and are covered with proteins and glycoproteins, typical of

many eukaryotic cells (Alberts *et al.*, 1994). Much of the cell surface is covered with the immundominant membrane glycoprotein (i-antigen) anchored to the membrane via phosphatidylinositol linkages (Smith and Doerder, 1992; Ron *et al.*, 1992). In addition to glycoprotein, the cell surface of this ciliate is coated by a cytoskeleton lattice made up of basal bodies associated with the ciliary rows (Jerka-Dziadosz *et al.*, 1995).

Even with the distinct differences between the outer surface of the two species, they exhibited similar toxicity to nonpolar narcotics. These results give merit to the hypothesis that nonpolar narcosis occurs via passive diffusion through the cellular membrane (Franks and Lieb, 1990).

The QSARs developed here relate toxicity to hydrophobicity. If narcotics affect the functioning of membranes as endorsed by Franks and Lieb (1984), then hydrophobicity describes the ability of toxicants to enter biological membranes. If narcotics enter cells through passive diffusion, then as the chemical concentration increases, there should be a linear relationship between toxicant concentration and biological response. Because endpoints can be predicted based on other endpoints between 86 and 14%, there is a direct relationship between the biological response and $\log K_{OW}$. This relationship is in opposition to toxicants that have threshold-type response that require a critical concentration yielding an all or nothing response, or chemicals which cause exponential increase in biological response relative to a linear increase in concentration.

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APPENDIX

TABLE 1 Population Growth Models and the Corresponding Generation Times for
E. coli Grown with Different Concentrations of BUTYLBENZENE

Estimated IGC _X	mM	Slope	Intercept	r ²	Generation time (minutes)	Lag phase relative to control (hours)	Equation #
Control	0	0.74	-6.42	0.98	56.19	N/A	2-2
50	0.11	0.85	-8.72	0.98	48.92	2.71	2-3
75	0.14	0.77	-8.74	0.94	54.00	3.01	2-4
95	0.17	0.86	-11.11	0.99	48.35	5.45	2-5

TABLE 2 *E. coli* Data from Toxicity Testing

Name	CAS Number	Log K _{ow}	IGC ₉₅	IGC ₈₅	IGC ₅₀	IGC ₁₅	IGC ₀₅	Estimated IGC ₁₀₀	Estimated NOEC
1. ethanol	64-17-5	-0.31	-2.67	-2.60	-2.450	-2.22	-2.00	-2.90	-1.64
2. 2-butanone	78-93-3	0.29	-2.08	-2.01	-1.86	-1.63	-1.41	-2.26	-1.18
3. 2-pentanone	107-87-9	0.91	-1.48	-1.41	-1.26	-1.03	-0.79	-1.50	-0.54
4. 1-pentanol	71-41-0	1.56	-1.20	-1.13	-0.98	-0.75	-0.52	-1.40	-0.36
5. anisole	100-66-3	2.11	-0.71	-0.64	-0.51	-0.32	-0.16	-0.92	0.03
6. 1-heptanol	111-70-6	2.57	-0.12	-0.06	0.08	0.28	0.47	-0.33	0.67
7. 1,2-dichlorobenzene	95-50-1	3.38	0.33	0.38	0.48	0.61	0.71	0.26	1.17
8. 2-decanone	693-54-9	3.60	0.40	0.46	0.59	0.78	0.94	0.35	0.94
9. n-butylbenzene	104-51-8	4.26	0.88	0.91	0.98	1.07	1.13	0.79	1.53
10. n-amy/benzene	538-68-1	4.90	0.66	0.72	0.87	1.10	1.33	0.53	1.57

TABLE 3 QSARs for *E. coli* Population Growth Endpoints

Endpoint	Slope	Intercept	r ²	Equation #
estimated NOEC	0.69	-1.34	0.98	2-6
IGC ₀₅	0.69	-1.59	0.98	2-7
IGC ₁₅	0.72	-1.83	0.99	2-8
IGC ₅₀	0.75	-2.08	0.99	2-9
IGC ₈₅	0.77	-2.24	0.99	2-10
IGC ₉₅	0.77	-2.31	0.99	2-11
estimated IGC ₁₀₀	0.79	-2.50	0.99	2-12

TABLE 4 p-values for the QSAR Slope Comparisons. Results of *t*-tests for Slope Comparisons

	Estimate NOEC	Estimate IGC ₁₀₀	IGC ₀₅	IGC ₁₅	IGC ₅₀	IGC ₈₅	IGC ₉₅
estimate NOEC	---	0.03	0.19	0.47	0.19	0.11	0.08
estimate IGC ₁₀₀		---	0.02	0.12	0.34	0.52	0.60
IGC ₀₅			---	0.45	0.18	0.10	0.08
IGC ₁₅				--	0.55	0.36	0.30
IGC ₅₀					---	0.76	0.67
IGC ₈₅							0.90
IGC ₉₅							---

TABLE 5 Slope Comparisons for the *E. coli* QSARs. A p-value > 0.05

Indicated "Same"

	Estimate NOEC	Estimate IGC ₁₀₀	IGC ₀₅	IGC ₁₅	IGC ₅₀	IGC ₈₅	IGC ₉₅
estimate NOEC	---	different	same	same	same	same	same
estimate IGC ₁₀₀		---	different	same	same	same	same
IGC ₀₅			---	same	same	same	same
IGC ₁₅				--	same	same	same
IGC ₅₀					---	same	same
IGC ₈₅						--	same
IGC ₉₅							---

TABLE 6 QSARs for *T. pyriformis* Population Growth Endpoints

Endpoint	Slope	Intercept	r ²	Equation #
1. estimated NOEC	0.75	-1.59	0.98	3-13
2. IGC ₀₅	0.75	-1.70	0.98	3-14
3. IGC ₁₅	0.76	-1.86	0.99	3-15
4. IGC ₅₀	0.77	-2.00	0.99	3-16
5. IGC ₈₅	0.79	-2.15	0.98	3-17
6. IGG ₉₅	0.77	-2.14	0.98	3-18
7. estimated IGC ₁₀₀	0.77	-2.16	0.99	3-19

CHAPTER 4

POPULATION GROWTH KINETICS OF *TETRAHYMENA PYRIFORMIS* EXPOSED TO SELECTED NONPOLAR NARCOTICS

INTRODUCTION

The "classic" aquatic toxicity assay entails populations of organisms exposed to varying concentrations of a toxicant for a specified period of time. Causality is the primary assumption associated with this type of assay. The expression of the biological response as a function of the exposure concentration is another assumption. Ideally, the measured response is associated with molecular events that result from toxicant exposure. However, in aquatic toxicology these molecular events are for the most part unknown. Typical aquatic toxicity data reports only the concentration of a test chemical required for a percentage of organism to exhibit a defined response. Such potency data, although informative, do not provide any information describing the kinetics of either the organismal growth or the toxicant fate.

Neutral industrial organic chemicals exhibit the narcosis mode of acute toxic action (Bradbury and Lipnick, 1990). Such chemicals cause only reversible physiological alterations that are directly related to the 1-octanol/water partition coefficient (K_{OW}). Nonpolar narcosis is the mechanism of baseline toxicity (Schultz, 1989) and is deemed the arresting of general cytoplasmic activity (Veith and Broderius, 1990). Because nonpolar narcosis depends only on the passive uptake of the chemical, the modeling of toxic potency of nonpolar narcotics is consistent regardless of the test system (Hansch *et al.*, 1989). This mechanism of toxic action has been the subject of several reviews, the most recent from van Wezel and Opperhuizen (1995).

Sprague (1971) stated that reproduction impairment should be regarded as among the most important parameters in determining relative hazard. He further noted, even with invertebrates and algae, tests of sublethal effects such as growth and development are more meaningful than mortality endpoints. Reproductive ability appears to be one of the most sensitive sublethal responses (Sprague, 1970; 1971). Population growth impairment is the reduction in population density under specified conditions (e.g., time, temperature, etc.). With microorganisms such as bacteria, protozoa, and algae, this reduction may be the result of mortality of a percentage of the initial inoculum, a reduction in the rate of cell division, or an extension in the lag phase of the growth curve.

Other investigators have studied the kinetics of microorganism growth under the influence of nonpolar narcotics but most have used low log K_{OW} (i.e., < 3.0) aliphatic alcohols, especially ethanol, as the model chemicals for such analysis (Sauvant *et al.*, 1995; Sawada and Nakamura, 1986; Nendza and Syedel, 1988a). These alcohols represent one of the major classes of nonpolar narcotics. However, alcohols differ in physicochemical properties (e.g., more hydrophilic) from other neutral organic chemicals such as aromatic hydrocarbons. The results have shown that with increasing chemical concentration, microorganisms increase their generation time. If as stated previously, nonpolar narcosis is thought to be the general arrest of cytoplasmic activity (Veith and Broderius, 1990), then this result is the expected one.

Studies of fish acute toxic syndromes (McKim *et al.*, 1987) have characterized nonpolar narcosis syndrome of spinally transected trout. One-octanol and MS-222 were used as models. The overall response of fish to these two nonspecific narcotics was a remarkable slowing of all respiratory-cardiovascular functions (McKim *et al.*, 1987).

Surak *et al.* (1976) and Sauvant *et al.* (1995) have reported that *Tetrahymena pyriformis*, when inhibited by chemical stress, exhibited a lag phase in growth followed

by generation times equivalent to control. Neither study used nonpolar narcotics, and again only used either a single model chemical or only a few toxicants to describe this phenomenon. Sauviant *et al.* (1995) actually observed, for some selected metals and an assortment of acids and alcohols, both this lag phase response and the outcome described previously, where there is a differential growth rate with increasing toxicant concentration. Neither study gave an explanation for such a phenomenon and did not relate this lag phase to any molecular property, such as hydrophobicity or molecular structure.

If the cellular membrane is the site of action for narcotics, as hypothesized by Franks and Lieb (1990), then only using one molecular structural type or a narrow range of hydrophobicities may not accurately describe the response of organisms to nonpolar narcotics. As predicted by the Fluid Mosaic Model of Singer and Nicholson (1972), cellular membranes are made up of a bilayer of amphipathic phospholipids with proteins embedded in this bilayer. The structure and function of all cells are dependent on the maintenance of this bilayer.

Just as membranes have hydrophilic, hydrophobic, and amphipathic components, environmental toxicants can have similar properties. To assume that hydrophilic xenobiotics will interact in the same way as hydrophobic ones may not be an accurate assumption. The following experiments described herein have attempted to investigate this problem by exploring the effects of nonpolar narcotics with varying hydrophobicity and molecular structure on the population growth kinetics of *T. pyriformis*.

The specific aims of this project were to develop an assay to study the generation times of log phase *T. pyriformis* and to use this assay to describe its growth kinetics under the stress of a group of nonpolar narcotics with varying log K_{OW} and molecular structure. The results of these experiments suggest possibly that *T. pyriformis* acclimate to some nonpolar narcotics.

MATERIALS AND METHODS

Growth Medium:

The growth medium used is the same as used for the TETRATOX 40-hr population growth inhibition assay (Schultz *et al.*, 1990). All cell growth and experiments are performed in 250 mL foam-stoppered Erlenmeyer flasks. All incubations described herein were performed at $27^{\circ} \pm 1^{\circ}\text{C}$. All growth media used was equilibrated to $27^{\circ} \pm 1^{\circ}\text{C}$ prior to use.

Inoculation Flask Preparation:

In order to achieve an inoculation flask in log growth, the following procedure was followed. 1.5×10^3 cell/mL stationary phase *T. pyriformis* were incubated for 24 hr. Following incubation, varying amounts from 5.0×10^2 to 4.0×10^3 of cells were transferred to secondary inoculation flasks and incubated for 24 hr.

The volume required to result in 1.2×10^3 cell/mL in the experimental flasks was determined by electronically counting the secondary inoculation flasks, using a Coulter Counter Zm. Maximum volume of *T. pyriformis* used was 1.0 mL.

Test Chemical Preparation:

Six different nonpolar narcotics were chosen to be evaluated in the growth rate assay. The chemicals selected were ethanol, acetone, 1-pentanol, anisole, 2-decanone, and butylbenzene. These represent at least one of each of the four major groups of nonpolar narcotics and represent the range of $\log K_{OW}$ from -0.31 to 4.26. Stock solutions of chemicals were made in dimethylsulfoxide in 10, 25, or 100 thousand mg/L or dispensed directly without dilution.

Growth rate assay procedure:

Five to seven-step chemical concentrations and a control containing no chemical were tested in duplicate. At T_0 , samples were taken immediately after chemical and *T.*

pyriformis were dispensed. After an incubation time of 13 hr, samples were taken hourly for 5 hr. The experimental flasks were returned to the environmental chamber between samplings. Each sample consisted of diluting 2 mL of *T. pyriformis* culture into 50 mL of 10% Isoton. Six electronic counts using the Coulter Counter Model Zm were taken of each sample. Mean counts were used to determine the generation times.

Linear regressions were performed using the $\ln$ (cell count) as the dependent variable and time in hours as the independent variable. The doubling time in hours was determined by dividing $\ln 2$ by the slope of this regression model. In order to compare the slope (i.e., generation times) of the linear models, *t*-tests were also performed. All statistical procedures were performed with the general linear model procedure of Statistical Analysis Software, SAS (SAS Inc., 1989). A *p*-value of < 0.05 was considered the cut-off for statistical significance.

If the *t*-tests comparing slopes (i.e., generation time) resulted in statistical similarity, the approximate lag phase was calculated. These times were calculated by subtracting the intercept of the growth model from the control intercept and then dividing by the slope of the given model.

RESULTS

Among the six experiments, the mean doubling time for the controls was 3.26 hr with a range from 3.01 to 3.45 hr.

Ethanol and acetone represent the "low" $\log K_{OW}$ (i.e., < 0.00) chemicals in this set of toxicants. The growth models and statistical comparisons for these chemicals are shown in Tables 1-3 for ethanol and Tables 4-6 for acetone. The growth kinetics of *T. pyriformis* resulting from growth in ethanol and acetone (Tables 1 and 4) are very similar. For both of these chemicals, there is a direct relationship between the concentration of the chemical and the generation time of *T. pyriformis*. Observation of

the intercepts shows no observable relationship between the endpoint and the intercept value. Because there is no relationship between intercept and concentration, it is assumed that no lag phase in growth occurred.

One-pentanol and anisole represent the "intermediate" log K_{OW} chemicals (i.e., > 0.00 and < 3.00). The doubling times and statistical analyses for these chemicals are shown in Tables 7-9 and 10-12 for 1-pentanol and anisole, respectively. Cells inhibited by approximately $< 35\%$ for 1-pentanol and $< 50\%$ for anisole grew with either visually identical generation times or with statistically similar generation times to control flasks containing no chemical. For cells inhibited $> 35\%$ or $> 50\%$ for 1-pentanol and anisole, the doubling times were longer than control growth. Close examination of the data for 1-pentanol (Table 7) demonstrates for the 25% and 35% inhibition the generation times are similar to control, and the intercepts decrease relative to the control intercept. Because of these two observations, it is possible to calculate the approximate lag phase (Table 7) for the 25% and 35% inhibition endpoints. For anisole, there is an indirect relationship between the intercepts of the growth models and the chemical concentration (Table 10). Lag times were calculated for the three endpoints that had cell doubling time comparable to control.

Two-decanone and butylbenzene represent the "high" log K_{OW} chemicals (i.e., log $K_{OW} > 3.00$) and are considered hydrophobic chemicals. The growth models and statistical comparisons for these two chemicals are shown in Tables 13-15 for 2-decanone and Tables 16-19 for butylbenzene. The growth kinetics for *T. pyriformis* inhibited by both of these chemicals indicate that, for cells inhibited up to approximately 85%, are growing at either visually identical or statistically similar rates to the control growth. By observing Tables 13 and 16, the y-intercepts of the growth models are the principal differences among endpoints. This variance implies that, instead of differential growth rates, the *T. pyriformis* exhibited a lag phase in growth followed by doubling

time equal to control growth. This lag phase increased with larger concentrations of either 2-decanone or butyl benzene.

Table 19 culminates the data from Tables 1-18. There is a direct relationship between $\log K_{OW}$ and the degree of similarity of generation times to control growth. With increasing $\log K_{OW}$, the growth rate of *T. pyriformis* approaches the generation time of the control. As stated previously, for ethanol and acetone (i.e., low $\log K_{OW}$ chemicals), the generation times of the cells increased with chemical concentration. For 2-decanone and butylbenzene (i.e., high $\log K_{OW}$) the generation times for *T. pyriformis* grown in toxicant up to 85% of inhibitory concentration were the same as for control. For the intermediate $\log K_{OW}$ chemicals, an intermediate response was seen.

DISCUSSION

McFarland's (1970) basic QSAR model indicates that toxicity is a combination of the penetration of the toxicant to the site of action along with the interaction of the chemical with that site. Penetration is considered a passive diffusion process and is quantitated in QSARs by $\log K_{OW}$. It is well established for multiple species that nonpolar narcosis can be well predicted by this physiochemical property (Hansch *et al.*, 1989; Schultz *et al.*, 1990). The use of $\log K_{OW}$ suggests that the site of toxic action for narcosis is the cellular membrane, since this parameter is a ratio of the partitioning between 1-octanol (i.e., a model for membrane structure) and water.

Veith and Broderius (1990) define nonpolar narcosis as a general decrease in cytoplasmic activity. If this definition is accurate, then it would be expected that generation times of cellular systems would increase with increasing chemical concentration. Both Nendza and Seydel (1988b) and Sawada and Nakamura (1986) showed this type growth inhibition by ethanol in *E. coli*.

Other studies have demonstrated this type of relationship using *T. pyriformis* as the test organism with chemicals other than nonpolar narcotics. Included in these are Perkins and Ciereszko (1973), who studied the effects of crassin acetate, a toxin produced by coral; Rankin *et al.* (1977), who investigated the effects of the fungicide benomyl; Surak and Schifanella (1979), who studied the effects of α -tomatine, a tomato alkanoid; and Wiger (1985), who observed growth in the presence of lindane and Tween 80. Other investigators observed the variable grow rates of *T. pyriformis* caused by metals (Thrasher and Adams, 1972; Larsen and Nilsson, 1983; Larsen, 1989), amino-carcinogens (Roth, 1954), and acridine (Schultz *et al.*, 1981). Although these studies demonstrate protozoan growth rates that increase with toxicant stress, the majority of these assays were conducted over many hours to weeks (Roth, 1954; Perkins and Ciereszko, 1973; Rankin *et al.*, 1977; Surak and Schifanella, 1979; Schultz *et al.*, 1981; Wiger, 1985). *T. pyriformis* leave log growth and enter stationary phase after 15-24 hr (Cameron, 1973). Biotic factors such as the life stage of the organism, the size and age are important determinants in toxicity testing and should be kept constant (Cooney, 1995).

Of the studies that described growth kinetics resulting from nonpolar narcotics, both Nendza and Seydel (1988b) and Sawada and Nakamura (1986) studied the effect of alcohols, especially ethanol, on *E. coli* growth. They reported that for increasing chemical concentration, the generation times of the bacteria increased. The results reported in this manuscript indicate that the use of ethanol as the single toxicant or a narrow hydrophobicity range of toxicants to model nonpolar narcosis may not be entirely appropriate. Supporting these prior studies, the low log K_{OW} chemicals tested in this study exhibited the theoretical response, where the generation times increased with chemical dosage. For both ethanol and acetone, the variation in *T. pyriformis* growth was visible in the change in the slope terms representing doubling time.

For other chemicals, the generation time was not directly related to the chemical dosage. Instead (see Table 19), as the log K_{OW} increased, the generation times of *T. pyriformis* approached the identical doubling time of control flasks, where the high log K_{OW} chemicals actually had visibly analogous generation times as control. The differences between endpoints from the high log K_{OW} are seen in the decreasing intercepts representing increasing lag phases.

Determining the site of action for polar narcosis has been hampered by the abundance of studies demonstrating that nonpolar narcotics effect multiple cellular processes. For example, these chemicals seem to effect cellular pH, Na^+/K^+ gradients, protein function, increase in cell membrane volume, and possibly Ca^{2+} toxicity (Bangham *et al.*, 1965; Beavan *et al.*, 1982; Bolen and Sando, 1992; Leao and van Uden, 1984; Seeman and Roth, 1972; Nicotera *et al.*, 1992).

Franks and Lieb (1984) have endorsed the hypothesis that narcotics affect the interactions of membranes with proteins. They have studied the effects of firefly luciferase competitive inhibition by narcotics (Franks and Lieb, 1984), and the effects of anesthetics on activation of a potassium current, and on inhibition of an acetylcholine-receptor-mediated current (Franks and Lieb, 1994). In a recent review by Sikkema *et al.* (1995), the membrane as the probable target for many hydrocarbons was discussed in detail.

Recently, Sikkema *et al.* (1994) showed that with a group of monoaromatic hydrocarbons, the amount of membrane expansion of artificial liposomes increased with log K_{OW} . Seeman (1972) also showed, for some anesthetics, the increase in cell volume of erythrocytes was related to hydrophobicity. This evidence from Seeman (1972) and Sikkema *et al.* (1994) support McFarland's theoretical QSAR (1970) which uses log K_{OW} to describe the penetration of the chemical through the membrane as the proposed site of action.

To parallel the membrane expansion data, Sikkema *et al.* (1994) demonstrated that a group of aromatic hydrocarbons are thought to reside within the center of the membrane but not the outer portion of the membrane. Although the data reported by Sikkema *et al.* (1994) begin to show the membrane as the site of toxic action for nonpolar narcotics, the study ignores aliphatics and chemicals with $\log K_{ow} < 2.13$.

Other studies investigating alcohols suggest that more hydrophilic chemicals interact with the hydrophilic heads of the phospholipids, as compared to the hydrophobic aromatics studied by Sikkema *et al.* (1994). Rottenberg (1992) showed that some alcohols bind to the hydrophilic surface of the membrane. Paralleling this data is the study by Chiou *et al.* (1992), that showed some short-chained alcohols affected the hydration of the membrane.

Cells have multiple processes by which they adapt to chemical stress. Among these are changing the *trans/cis* ratio of fatty acids, alteration of the lipopolysaccharide region in gram-negative bacteria (Nikaido and Vaara, 1985), cross-linking of components of the cell wall of bacteria (Park *et al.*, 1988), and active secretion, especially important for drug resistance (Sikkema *et al.*, 1995).

A change in the degree of saturation or a transition from *cis* to *trans* fatty acids in membranes directly affects the membrane fluidity, which in turn affects membrane characteristics. Fluidity is greatest when highly unsaturated lipids are present and less when more saturated lipids are present. The degree of saturatedness will affect the membrane elasticity, as well as the aggregation and diffusion of protein and lipid components. A change in fluidity can also affect the permeability to water and ions (Chapman, 1993). *Tetrahymena* has been shown to adjust the lipid composition of its membrane in response to changing environmental conditions, such as temperature and nutrients (Umeki and Nozawa, 1993). Decreasing the growth temperature (Fukushima *et al.*, 1976) or starving cells (Umeki and Nozawa, 1993) results in an increase in

saturated fatty acids, such as cilienic, linoleic, and γ -linolenic acids and a decrease in unsaturated fatty acids, such as palmitic acid.

Ingram (1977) reported that *E. coli* grown in the presence of ethanol and acetone showed an increase in unsaturated fatty acids but more apolar and aromatic solvents, such as benzene and aniline resulted in an increase in saturated fatty acids. Studies with yeast also showed that, under the presence of ethanol, there was a decrease in saturated fatty acids (i.e., more fluidity) (Beavan *et al.*, 1992). On the other hand, Heipeper and deBont (1994) and Weber *et al.* (1994) showed that there was transition from *cis* to *trans* fatty acids (i.e., less fluidity) when cells were grown in phenol and toluene, respectively. It is interesting to note that the studies which used ethanol, an aliphatic, hydrophilic compound, and phenol which is aromatic and intermediate log K_{OW} , (log K_{OW} of 1.50; [Sangster, 1993]), showed opposite results. These discrepancies may be due to differences in the hydrophobicities or molecular structure of the test chemicals.

In addition to hydrophobicity, molecular structure could play an important role in determining the biological response to a given chemical. Although hydrophobicity is the important parameter in passive diffusion of the toxicant into the membrane, the physical shape of the molecule could also effect the response. Aliphatic chemicals with a more linear shape could feasibly fit between hydrophobic tails. Studies have shown that approximately one hexane molecule per phospholipid did not increase the membrane volume (White *et al.*, 1981; King *et al.*, 1985). On the other hand, cyclic compounds may further disrupt the functioning of the membrane, since their physical shape would interfere with the aliphatic nature of the fatty acid tails. Maher and Singer (1984) showed some drugs induced a redistribution of protein and lipid components within the membrane. Sheetz and Singer (1974) indicate that different amphipathic drugs collect in different layers of the membrane based on their charge and subsequently change

phospholipid interactions. As already noted, Sikkema *et al.* (1994) showed for a group of aromatics, the membrane volume increased.

The data presented in this study does not suggest that molecular structure is an important determinant in *T. pyriformis* growth rates. Butylbenzene and 2-decanone were the two high log K_{OW} chemicals studied herein and resulted in identical responses in *T. pyriformis*. Structurally, they are aromatic and aliphatic, respectively. Anisole, an aromatic ether, and 1-pentanol, an aliphatic alcohol, were the intermediate log K_{OW} toxicants tested. Log K_{OW} seems to be a better predictor of the growth kinetics, resulting from these two intermediate chemicals than molecular structure. These data suggest that the molecular structure was not as important as hydrophobicity in determining the growth kinetics of the protozoan.

Finally, the data reported herein indicate that when measuring the generation times of *T. pyriformis* exposed to different nonpolar narcotics of varying hydrophobicities, the response varies from a change in generation times to a change in lag phase with identical generation time. These differences may be due to differences in the placement of the toxicant within the cellular membrane. It is feasible to hypothesize a connection between the biochemical evidence discussed previously and the results of this study. If, for example, butylbenzene, a high log K_{OW} chemical which exhibited the lag phase response resides in the center of the membrane, then during the lag time, it is possible to envision that *T. pyriformis* exhibit a possible change from *cis* to *trans*, or more generally, a transition from unsaturated to more saturated fatty acids, resulting in decreased membrane fluidity and an adaptation to the chemical stress. Adjusting membrane fluidity could possibly restore the interactions between the phospholipids and the transmembrane proteins necessary for proper cell functioning that were disrupted by the presence of the hydrocarbon.

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APPENDIX

TABLE 1 Linear Models for *T. pyriformis* Grown in ETHANOL and the Resulting Doubling Time

Chemical Concentration (mM)	IG%	Slope	Intercept	r ²	Doubling time (hr) ln2/slope	Lag phase relative to control (hr)
Control	0	0.23	0.09	0.86	3.01	N/A
108.53	25.7	0.16	0.94	0.99	4.33	N/A
217.06	39.6	0.15	0.46	0.94	4.62	N/A
254.99	60.3	0.12	0.76	0.99	5.78	N/A
318.74	70.3	0.11	0.50	0.93	6.30	N/A
424.99	81.6	0.04	0.64	0.93	17.33	N/A

TABLE 2 Comparisons of Slope Values for *T. pyriformis* Grown in the Presence of ETHANOL

Chemical concentration (mM)	Control	108.53	217.06	254.99	318.74	424.99
Control	--	0.05	0.05	0.007	0.002	0.0001
108.53		--	0.80	0.38	0.27	0.009
217.06			--	0.21	0.11	0.002
254.99				--	0.238	0.0008
318.74					--	0.04
424.99						--

TABLE 3 Comparisons and p-values of Slope Values for *T. pyriformis* Grown in the Presence of ETHANOL

Chemical concentration (mM)	Control	108.53	217.06	254.99	318.74	424.99
Control	--	different	different	different	different	different
108.53		--	same	same	same	different
217.06			--	same	same	different
254.99				--	same	different
318.74					--	different
424.99						--

TABLE 4 Linear models for *T. pyriformis* grown in ACETONE and the Resulting Doubling Time

Chemical Concentration (mM)	IG%	Slope	Intercept	r ²	Doubling time(hr) ln2/slope	Lag phase relative to control (hr)
Control	0	0.20	0.21	0.98	3.45	N/A
84.37	21.4	0.18	0.29	0.99	3.94	N/A
100.72	38.5	0.18	0.03	0.99	3.96	N/A
117.08	54.1	0.17	-0.21	0.99	4.05	N/A
149.79	64.6	0.14	-0.00	0.80	4.99	N/A
181.65	85.8	0.12	0.11	0.76	5.82	N/A

TABLE 5 Comparisons and p-values of Slope Values for *T. pyriformis* Grown in the Presence of ACETONE

Chemical concentration (mM)	Control	84.37	100.72	117.08	149.79	181.65
Control	--	0.26	0.24	0.16	0.01	0.0003
84.37		--	0.99	0.84	0.12	0.02
100.72			--	0.84	0.10	0.01
117.08				--	0.14	0.02
149.79					--	0.35
181.65						--

TABLE 6 Comparisons of Slope Values for *T. pyriformis* Grown in the Presence of ACETONE

Chemical concentration (mM)	Control	84.37	100.72	117.08	149.79	181.65
Control	--	same	same	same	different	different
84.37		--	same	same	same	different
100.72			--	same	same	different
117.08				--	same	different
149.79					--	same
181.65						--

TABLE 7 Linear Models for *T. pyriformis* Grown in the Presence of 1-PENTANOL
and the Resulting Doubling Times

Chemical concentration (mM)	IG%	Slope	Intercept	r ²	Doubling time (hr) ln2/slope	Lag time compared to control (hr)
Control	N/A	0.20	0.60	0.99	3.41	N/A
3.40	24.9	0.19	0.54	0.99	3.77	0.36
5.67	35	0.20	0.25	0.99	3.55	1.80
7.94	50	0.17	0.35	0.98	4.18	N/A
11.34	66.1	0.12	0.59	0.98	5.82	N/A
14.18	90.2	0.07	0.21	0.92	10.66	N/A

TABLE 8 Comparisons and p-values of Slope Values for *T. pyriformis* Grown in the
Presence of 1-PENTANOL

Chemical concentration (mM)	Control	3.40	5.67	7.94	11.34	14.18
Control	--	0.22	0.60	0.02	0.0001	0.0001
3.40		--	0.39	0.15	0.0001	0.0001
5.67			--	0.03	0.0001	0.0001
7.94				--	0.0004	0.0001
11.34						0.0009
14.18						--

TABLE 9 Comparisons of Slope Values for *T. pyriformis* Grown in the Presence of
1-PENTANOL

Chemical concentration (mM)	Control	3.40	5.67	7.94	11.34	14.18
Control	--	same	same	different	different	different
3.40		--	same	same	different	different
5.67			--	different	different	different
7.94				--	different	different
11.34					--	different
14.18						--

TABLE 10 Linear Models for *T. pyriformis* Grown in ANISOLE and the Resulting
Doubling Times

Chemical concentration (mM)	IG%	Slope	Intercept	r ²	Doubling time (hr) ln2/slope	Lag phase relative to control (hr)
Control	0%	0.20	0.98	0.99	3.43	N/A
0.46	26%	0.21	0.61	0.99	3.35	1.79
0.92	41%	0.20	0.48	0.97	3.47	2.53
1.39	53%	0.20	0.29	0.99	3.52	3.50
2.31	78%	0.18	-0.25	0.99	3.83	6.80
2.77	88%	0.15	-0.49	0.97	4.59	N/A

TABLE 11 Comparisons and p-values of Slope Values for *T. pyriformis* Grown in the Presence of ANISOLE

Chemical concentration (mM)	Control	0.46	0.92	1.39	2.31	2.77
Control	--	0.70	0.87	0.68	0.18	0.0001
0.46		--	0.57	0.41	0.10	0.0006
0.92			--	0.80	0.23	0.002
1.39				--	0.32	0.004
2.31					--	0.09
2.77						--

TABLE 12 Comparisons of Slope Values for *T. pyriformis* Grown in the Presence of ANISOLE

Chemical concentration (mM)	Control	0.46	0.92	1.39	2.31	2.77
Control	--	same	same	same	same	different
0.46		--	same	same	same	different
0.92			--	same	same	different
1.39				--	same	different
2.31					--	same
2.77						--

TABLE 13 Linear Models for *T. pyriformis* Grown in 2-DECANONE and the Resulting Doubling Times

Chemical concentration (mM)	IG%	Slope	Intercept	r ²	Doubling time (hr) ln2/slope	Lag phase relative to control (hr)
Control	0%	0.22	0.62	0.99	3.22	N/A
0.01	11.5	0.21	0.52	0.89	3.24	0.46
0.03	25.4	0.21	0.35	0.99	3.25	1.27
0.10	50.7	0.19	0.30	0.98	3.67	1.69
0.12	61.4	0.19	0.08	0.97	3.71	2.89
0.15	80.6	0.19	-0.53	0.81	3.55	5.91

TABLE 14 Comparisons and p-values of Slope Values for *T. pyriformis* Grown in the Presence of 2-DECANONE

Chemical concentration (mM)	Control	0.01	0.03	0.10	0.12	0.15
Control	--	0.95	0.92	0.47	0.33	0.48
0.01		--	0.97	0.50	0.37	0.53
0.03			--	0.52	0.39	0.55
0.10				--	0.95	0.89
0.12					--	0.80
0.15						--

TABLE 15 Comparisons of Slope Values for *T. pyriformis* Grown in the Presence of 2-
DECANONE

Chemical concentration (mM)	Control	0.01	0.03	0.10	0.12	0.15
Control	--	same	same	same	same	same
0.01		--	same	same	same	same
0.03			--	same	same	same
0.10				--	same	same
0.12					--	same
0.15						--

TABLE 16 Linear Models for *T. pyriformis* Grown in BUTYLBENZENE and the
Resulting Doubling Times

Chemical concentration (mM)	IG%	Slope	Intercept	r^2	Doubling time (hr) $\ln 2/\text{slope}$	Lag phase relative to control (hr)
Control	0%	0.23	0.56	0.98	3.04	N/A
0.034	15%	0.23	0.34	0.99	2.96	1.06
0.039	29%	0.24	0.12	0.99	2.92	1.86
0.042	41%	0.24	-0.14	0.99	2.86	2.91
0.045	88%	0.22	-1.49	0.99	3.12	9.26

TABLE 17 Comparisons and p-values of Slope Values for *T. pyriformis* Grown in the Presence of BUTYLBENZENE

Chemical concentration (mM)	Control	0.034	0.039	0.042	0.045
Control	--	0.686	0.455	0.237	0.689
0.034		--	0.812	0.536	0.467
0.039			--	0.646	0.288
0.042				--	0.151
0.045					--

TABLE 18 Comparisons of Slope Values for *T. pyriformis* Grown in the Presence of BUTYLBENZENE

Chemical concentration (mM)	Control	0.034	0.039	0.042	0.045
Control	--	same	same	same	same
0.034		--	same	same	same
0.039			--	same	same
0.042				--	same
0.045					--

TABLE 19 Between Chemical Comparisons for Slope Values Among 0, 25, and 85%
Inhibition

Chemical (log K _{ow})	Ethanol (-0.31)	Acetone (-0.24)	2-Pentanol (1.56)	Anisole (2.11)	2-Decanone (3.60)	Butylbenzene (4.26)
IGC _X 0% (control)	0.23	0.20	0.20	0.20	0.22	0.23
25% (+/- 5%)	0.16 different	0.18 different	0.19 same	0.21 same	0.21 same	0.23 same
55% (+/- 5%)	0.12 different	0.17 different	0.17 different	0.20 same	0.19 same	0.24 (40%) same
85% (+/- 5%)	0.04 different	0.12 different	0.07 different	0.15 different	0.19 same	0.22 same

VITA

Anna Pate Bearden was born in LaGrange, Georgia on April 24, 1972. At the age of six, she moved with her family to Columbia, S.C. In Irmo, S.C., she attended elementary, middle, and high school where graduated in the top 2% of her class. Her father is a marketing professor at the University of South Carolina and her mother manages a tennis club. She has one sister four years her younger. Anna graduated magna cum laude with a Bachelors of Science degree in Biological Sciences from Clemson University in May, 1994. Beginning in the fall of 1994 with a Graduate Research Assistantship, she entered the Life Sciences Graduate Program for Environmental Toxicology at The University of Tennessee. Upon finishing a Masters of Science degree, she will begin work for her Ph.D. also at The University of Tennessee in the Department of Ecology and Evolutionary Biology.