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INFLUENCE OF pH ON ACUTE LETHALITY OF LONG CHAIN UNSATURATED FATTY ACIDS TO RAINBOW TROUT

by

© SIRI TOOKWINAS, B.Sc.(FISHERIES)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

ENVIRONMENTAL SCIENCE

CIVIL ENGINEERING

EDMONTON, ALBERTA

SPRING, 1980

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled INFLUENCE OF pH ON ACUTE LETHALITY OF LONG CHAIN UNSATURATED FATTY ACIDS TO RAINBOW TROUT submitted by SIRI TOOKWINAS, B.Sc.(FISHERIES) in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in ENVIRONMENTAL SCIENCE.

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Supervisor

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Date..... Dec. 14, 1979 .....

ABSTRACT

Long chain unsaturated fatty acids are ubiquitous compounds in municipal and industrial wastewaters due to their occurrence in lipids as components of triglycerides. In order to evaluate the acute toxicity of long chain unsaturated fatty acids, laboratory monitoring programs have to be developed. Two long chain unsaturated fatty acids, oleic and linoleic, were tested on rainbow trout (Salmo gairdneri Richardson) fingerlings to study the effects of pH on acute lethality.

pH affected the acute lethality of each toxicant but was more pronounced with oleic acid than with linoleic acid during a 96 hour test period. In terms of LC50 values the toxicity of linoleic acid was about 5 to 8 times greater than oleic acid depending upon the test pH.

This study indicates that the toxicity of long chain unsaturated fatty acids to fish depends on environmental factors, such as pH, as well as acid molecular structure (ie. number of double bonds), and physicochemical properties of the acid (ie. degree of ionization of the acid and the alkalinity of test water).

The median lethal concentrations of long chain unsaturated fatty acids to rainbow trout determined by this study are higher than the long chain fatty acid concentrations reported for treated effluents from some wastewater treatment plants in North America.

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GLOSSARY

Application factor = a decimal fraction used to multiply LC50's of organism species for different pollutants in order to estimate the concentration of these pollutants that would be harmless to these organism species in nature (Warren, 1971).

Esterification = The formation of an ester by the chemical reaction of an acid with an alcohol; e.g. the action of ethanol on acetic acid to form ethyl acetate and water (Uvarov and Chapman, 1976).

Incipient lethal level = the concentration of a toxicant at which 50 percent of the test organisms can live for an indefinite time (APHA, 1975).

Maximum allowable toxicant concentration (MATC) = the concentration of toxic waste that may be present in the receiving water without causing significant harm to its productivity and all its various uses (APHA, 1975).

Median lethal concentration (LC50) = the concentration of toxicant that produces 50 percent mortality at a definite time period (APHA, 1975 and Sprague, 1973).

Median survival time (MST) = the time required to produce 50 percent mortality of test organisms for each concentration of toxicant (Duangsawasdi, 1977).

Safe concentration (SC) = the maximum concentration of a toxicant that has no observable harmful effects after long-term exposure over one or more generations (APHA, 1975).

Saponification = the hydrolysis of an ester; the term is often confined to the hydrolysis of an ester using an alkali, thus forming a salt (soap in the case of some of the higher fatty acids) and the free alcohol (Uvarov and Chapman, 1976).

I. Introduction

Fatty acids are a part of cellular lipids which are commonly found in all types of cells. Lipids are present in every type of plant and animal cell, and often represent a large part of the total constituents of specialized organs of plants and animals. Therefore, several kinds of fatty acids and their derivatives (ie. phosphoglycerides and triglycerides) are used in a wide variety of industries.

Owing to the occurrence of fatty acids in every living cell, they form a major proportion of the waste material from living cells. For example, fatty acids are a major component of raw domestic sewage. When present in solution as anions, fatty acids act as surfactants (soaps). The specific physicochemical properties of fatty acids contribute to their influence on biological systems. Fatty acids and their derivatives are directly toxic to aquatic organisms through various mechanisms as discussed in the subsequent chapters.

The toxic effects of fatty acids on living organisms are extremely complicated and very little research work has been reported in this area. Consequently, the general aim of the present research was to experimentally study the toxic effects of these acids on rainbow trout and thereby derive some understanding of the environmental significance of these compounds as they occur in municipal and industrial effluents.

The specific objectives were:

1. To evaluate the acute lethality of long chain unsaturated fatty acids (oleic and linoleic) as a function of pH to rainbow trout fingerlings.
2. To compare the acute lethality of these acids to rainbow trout fingerlings with long chain fatty acid concentrations in treated effluents.
3. To evaluate, from the literature, the toxicity mechanism of oleic and linoleic acids to fish.
4. To study the sources of variability in laboratory procedures used to assess the acute toxicity of long chain unsaturated fatty acids.

A literature review, chaper 1, was first carried out on the characteristics and environmental significance of long chain fatty acids and the properties of fatty acids. The review also summarizes the properties and concentration of fatty acids found in typical sewage discharges (treated effluent) in North America. The toxic effects are also presented.

Chapter 2 deals with the experimental program and the methodology employed in separating the various effects of the fatty acids on rainbow trout fingerlings. The variables investigated were the pH levels and the stability of test concentration of fatty acids during static bioassay.

The discussion and evaluation of the test results are presented in Chapter 3. The possibility of toxicity of long

chain fatty acids due to concentration levels found in treated effluents are also discussed.

Finally, Chapters 5 and 6 deal with the conclusions and recommendations, respectively.

A. Characteristics and Environmental Significance of Long Chain Fatty Acids

Oxidation of Fatty Acids

Fatty acids vary in chain length from acetic acid (C_2) to lignoceric acid (C_{24}) and include various derivatives and isomers (Wakil, 1970 and White *et al.*, 1973), such as saturated, unsaturated, hydroxy and branched-chain acids. Although the most common fatty acids contain 16 or 18 carbon atoms (Sawyer and McCarty, 1978 and Gunstone, 1967), fatty acids of different chain lengths (both even and odd) also exist but are generally found at lower levels in cellular systems than the C_{16} and C_{18} fatty acids.

Long chain fatty acids, combined as triglycerides, provide the long term storage form of energy for higher animals. The most important mechanism by which these storage fatty acids are degraded is a process which occurs in a step-wise manner and is known as β-oxidation (Gurr and James, 1971).

Conn and Stumpf (1972) reported that in 1952, Green in Wisconsin and Lynen in Munich announced the separation, isolation, and purification of the five enzymes responsible

for the β-oxidation of fatty acids. Five reactions of these enzymes are integrated into a cyclical scheme. Each turn of the cycle removes a two carbon unit and the process can be repeated over and over again until the carbon chain has been completely oxidized to two carbon fragments (Gurr and James, 1971).

As mentioned previously, "energy - rich" molecules can be yielded by this oxidation. The energetics of β-oxidation was shown by Conn and Stumpf (1972) and Davies and Littlewood (1979). For example, in the total combustion of palmitic acid (equation 1), considerable energy is released (Figure 1.1).

$$C_{16}H_{32}O_2 + 23\ O_2 \longrightarrow 16CO_2 + 16H_2O + (-2{,}340\ \text{Kcal}) \quad \ldots(1)$$

palmitic acid

When palmitic acid is degraded enzymatically, one energy-rich bond of ATP is required for the primary activation. Four steps of the cycle are repeated seven times which yields energy plus acetyl-CoA. Acetyl-CoA can be subsequently oxidized to CO_2 and H_2O by means of the Krebs cycle (citric acid cycle). The net total energy from this route is -940 kcal/mole (129 ATP*-7.3 Kcal). Therefore, the efficiency of energy conversation in fatty acid oxidation is about 40 percent (Stryer, 1975). Hence, it becomes clear, why, fat is an effective source of available food energy.

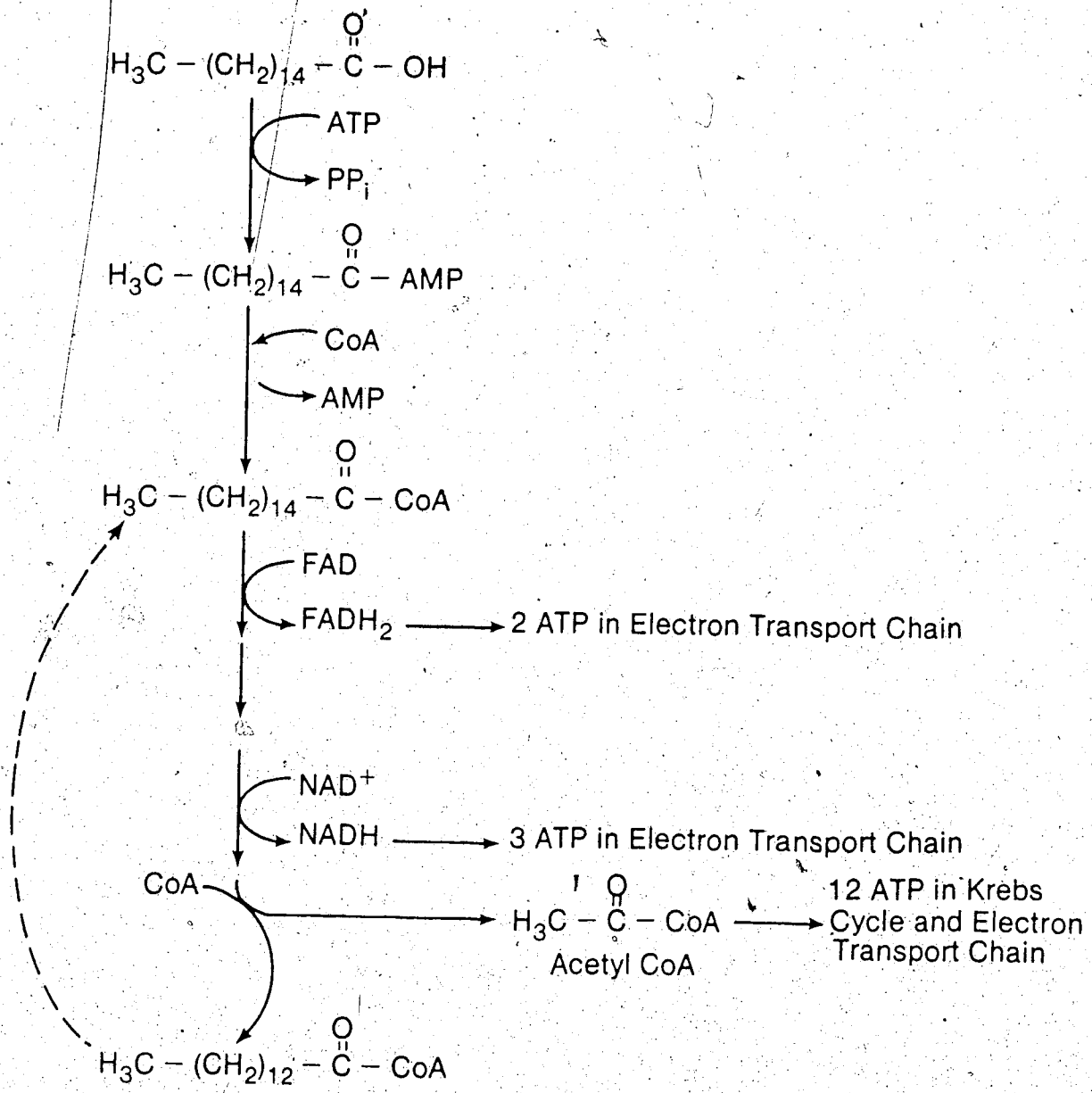


Figure 1.1 .Fatty Acid Oxidation (Davies and Littlewood, 1979)

Synthesis of Fatty Acids

Fatty acids are built up by successive condensations of 2-carbon units (acetyl-CoA). The degradation of fatty acids is in many respects a reversal of their synthesis, although different enzymes are used. The end product of fatty acid degradation (oxidation of fatty acid) is acetyl-CoA, the same molecule that serves as the starting material for their synthesis. However, the pathway is not an exact reversal, since malonyl CoA is the additive component used in synthesis (Gurr and James, 1971, Gunstone, 1967 and Davies and Littlewood, 1979). Also, it is important that synthesis and oxidation pathways of molecules such as fatty acids differ, so that the two pathways can be regulated independently.

Fatty acid synthesis begins when two 2-carbon units condense to form a 4-carbon fatty acid (Figure 1.2). The second turn of the cycle adds two more carbons, converting the 4-carbon fatty acid to a 6-carbon linear molecule. The 6-carbon chain grows to an 8-carbon chain, and this stepwise addition continues until the fatty acid typically contains 16 or 18 carbon atoms (Davies and Littlewood, 1979). Special reactions are needed to form the rare fatty acids that contain odd numbers of carbon atoms. If the original acid has an odd number of carbon atoms such as propionic, then the resulting long chain fatty acid is also an "odd-chain acid". If an original branched chain is used, then a branched chain fatty acid results. The overall chemistry of

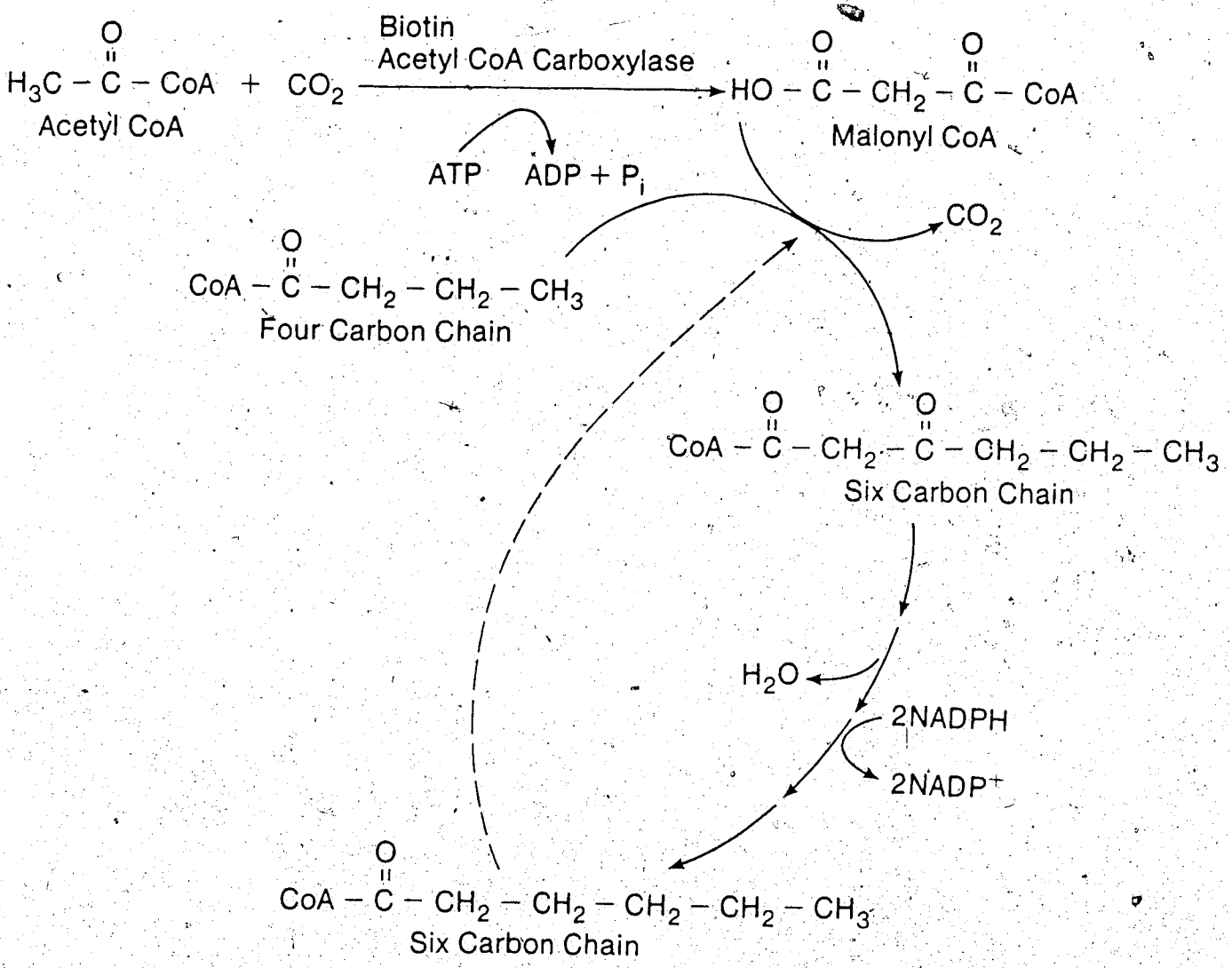


Figure 1.2 Fatty Acid Synthesis (Davies and Littlewood, 1979)

fatty acid synthesis in higher plants is the same as in animals, yeasts and bacteria (Gurr and James, 1971).

B. Chemistry of Fatty Acids

Fatty acids consist of carbon atoms arranged in a straight chain with an alkyl group, ($-CH_3$), at one end and a carboxyl group, (-COOH), at the opposite end. The naturally occurring fatty acids are divided into saturated acids and unsaturated acids on the basis of the presence or absence of double bonds in their hydrocarbon chains (Markley, 1967). All saturated fatty acids contain the maximum possible number of hydrogen atoms, while unsaturated compounds contain fewer hydrogen atoms and at least one unsaturated bond between carbons. Unsaturated fatty acids can contain one (mono-saturated) or more double bond (poly-unsaturated) (Davies and Littlewood, 1979). Naturally occurring saturated fatty acids that have from one to eight carbon atoms are liquid, whereas those with more than eight carbon atoms are solids. Stearic acid (18:0) has a melting point of 70°C but, with the introduction of one double bond, as in oleic acid (18:1), the melting point drops to 14°C. The addition of more double bonds further lowers the melting point, such as for linoleic acid (18:2) which has 2 double bonds and a melting point of -5°C (Conn and Stumpf, 1972 and Fasman, 1975).

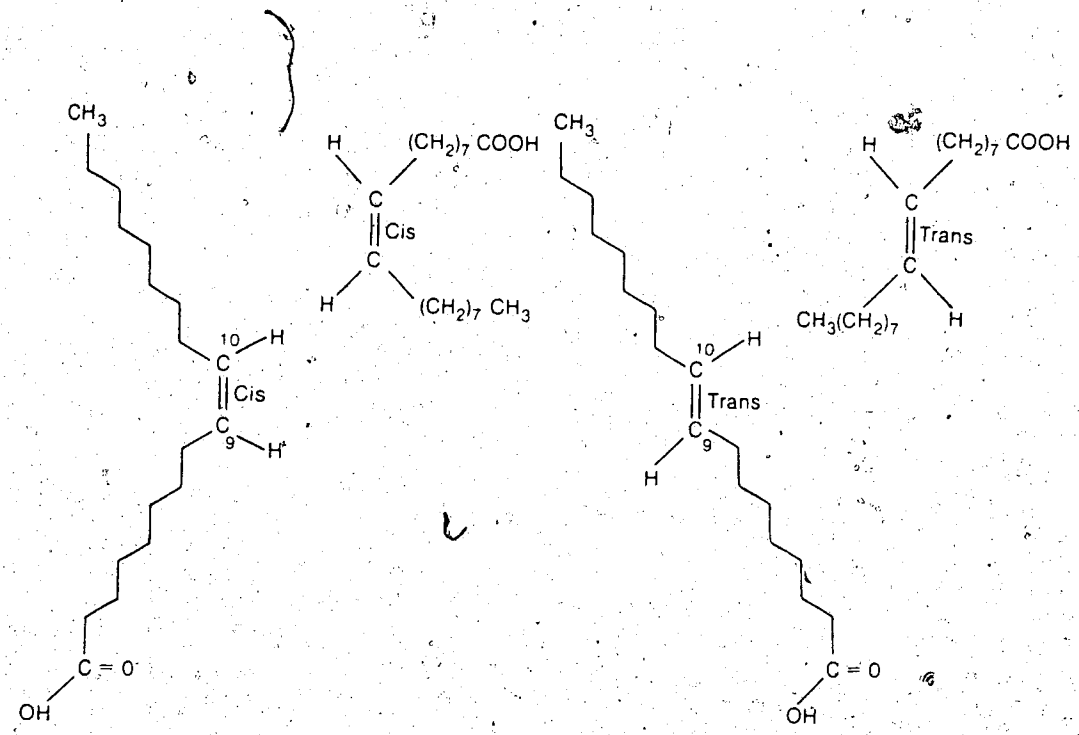
When a double bond is found in the hydrocarbon chain of a fatty acid, geometric isomerism occurs. If the radicals

which are being considered are on the same side of the bond, the compound is called "cis", if on opposite sides, "trans" (Harper et al., 1977 and Stenhagen, 1966). This can be illustrated with oleic, linoleic and elaidic acids (Figure 1.3).

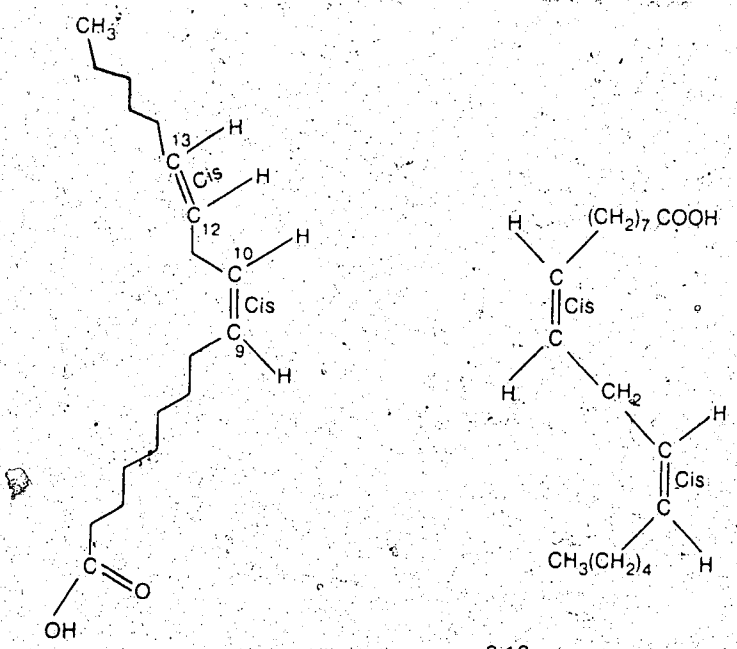
Physical Properties of Fatty Acids

The solubility of the fatty acids is greater than that of the corresponding glycerides. They are soluble in all the common polar and nonpolar organic solvents (Kirschenbauer, 1960 and Markley, 1967). The lower members are also soluble in water. With increasing chain length the acids become quite insoluble in water. Caprylic acid (8:0) at 30°C still has a solubility of 1.0 (g per 100 g of water), whereas stearic acid (18:0) has a solubility of 0.00034 at 30°C (Fasman, 1975) (Tables 1.1 and 1.2). The unsaturated acids are much more soluble in organic solvents than the corresponding saturated acids, and the solubility increases with increasing degrees of unsaturation (Kirschenbauer, 1960 and White et al., 1968).

Several factors limit fatty acid solubility in various solvents. First, the solubility will be increased with increasing temperature (Bailey, 1950, Fasman, 1975 and Markley, 1967). Second, the solubility will be increased when the pH of solution is increased. The most reliable and the most extensive solubility data on the saturated and unsaturated fatty acids have been reported by Bailey (1950)



Oleic Acid (Δ^9 C18:1) Elaidic Acid (Δ^9 C18:1)



Linoleic Acid ($\Delta^{9,12}$ C18:2)

Figure 1.3 Geometric Isomerism of Oleic, Elaidic and Linoleic Acids (Conn and Stumpf, 1971 and Harper et al, 1977)

Table 1.1 Fatty Acids: Physical and Chemical Characteristics (Fasman, 1975)

Common Name	Scientific Name	Molecular Formula	Molecular Weight	Melting Point °C
Unsaturated				
Oleic acid	9-Octadecenoic	$C_{17}H_{23}COOH$	282.5	13.4
Linoleic acid	Octadecadienoic	$C_{12}H_{31}COOH$	280.5	-5.2 to -5.0
Palmitoleic acid	9-Hexadecenoic	$C_{15}H_{29}COOH$	254.4	-0.5 to +0.5
Saturated				
Palmitic acid	Hexadecanoic	$C_{15}H_{31}COOH$	256.4	63.1
Stearic acid	Octadecanoic	$C_{17}H_{35}COOH$	284.5	69.6
Cyprylic acid	Octanoic	$C_7H_{15}COOH$	144.2	16.7
Propanoic acid	Propionic	C_2H_5COOH	74.1	-22.0
Acetic acid	Ethanoic	CH_3COOH	60.1	16.7
Formic acid	Methanoic	$HCOOH$	46.0	8.4

Table 1.2 Solubilities of Saturated Fatty Acids in Solvents at Various Temperature (Bailey, 1950)

g/acid/100 g water

Temp. °C	2-Butanone	Ethyl acetate	Butyl acetate	Chloro-form	Carbon tetra-chloride	1,2-Dichlo-roethane	Ethyl alchol 95%	Ethyl alcohol 99.4%	n-Butyl alcohol	Isopropyl alcohol	Glacial acetic acid
					PALMITIC ACID						
0	0.90	0.8	1.5	2.9	0.6	-	0.85	1.89	1.9	2.4	-
10	3.09	2.2	3.8	6.0	1.8	-	2.10	3.20	4.2	4.6	-
20	8.57	6.1	8.9	15.1	5.8	0.6	4.93	7.21	10.5	10.9	2.14
30	20.6	17.6	23.4	36.4	21.5	6.0	16.7	23.9	30.0	32.3	8.11
40	66.1	53	69	91	72	39.7	73.4	94.2	84	94	51.7
50	228	203	226	250	212	187	287	320	243	270	313
60	2390	2340	2330	1820	1590	1650	2280	2600	1960	2460	2280
					STEARIC ACID						
0	0.25	-	<0.1	0.4	-	-	0.24	0.42	-	0.1	-
10	1.01	-	0.2	2.0	0.2	-	0.65	1.09	0.2	0.4	-
20	2.99	0.5	1.6	6.0	2.4	-	1.13	2.25	1.6	2.0	0.12
30	8.34	5.2	8.1	17.5	10.7	1.0	3.42	5.42	9.0	10.0	1.68
40	24.8	21.6	28.7	48.7	36.4	10.0	17.1	22.7	36.2	38.1	7.58
50	84.7	78	97	124	108	70	83.9	105	111	118	74.8
60	344	348	350	365	325	280	365	400	370	422	485

and Fasman (1975) (Table 1.3). The relative solubilities of unsaturated fatty acids with different solvents are somewhat different from that for saturated fatty acids. The unsaturated fatty acids, especially oleic and linoleic acids differ from the saturated fatty acids. Oleic and linoleic acids are relatively insoluble in the polar solvents (ie. chloroform, CH_3Cl) at low tempertures and relatively soluble in weakly polar (ie. carbon disulfide) or non-polar solvents (ie. carbon tetrachloride, CCL_4) (Bailey, 1950). The solubility curves of oleic acid in various solvents are shown on Figure 1.4.

The fatty acids dissociate in aqueous solution like any other weak acid. The equilibrium expression for the dissociation in water may be written (Daniels and Alberty, 1966 and Conn and Stumpf, 1972).

$$RCOOH \longrightarrow RCOO^- + H^+$$

$$Ka = \frac{[H^+][RCOO^-](Y\pm)^2}{[RCOOH]Yha}$$

where:

Ka = The thermodynamic acid dissociation constant

Y± = ionic activity coefficient

Yha = activity coefficient of the undissociated weak acid

Table 1.3 Solubilities of Fatty Acids in Water (Fasman, 1975)

g/acid/100 g water

Acids	Temperature (°C)				
	0°C	20°C	30°C	45°C	60°C
Palmitic Acids	0.00046	0.00072	0.00083	0.0010	0.0012
Stearic Acids	0.00018	0.00029	0.00034	0.00042	0.0005

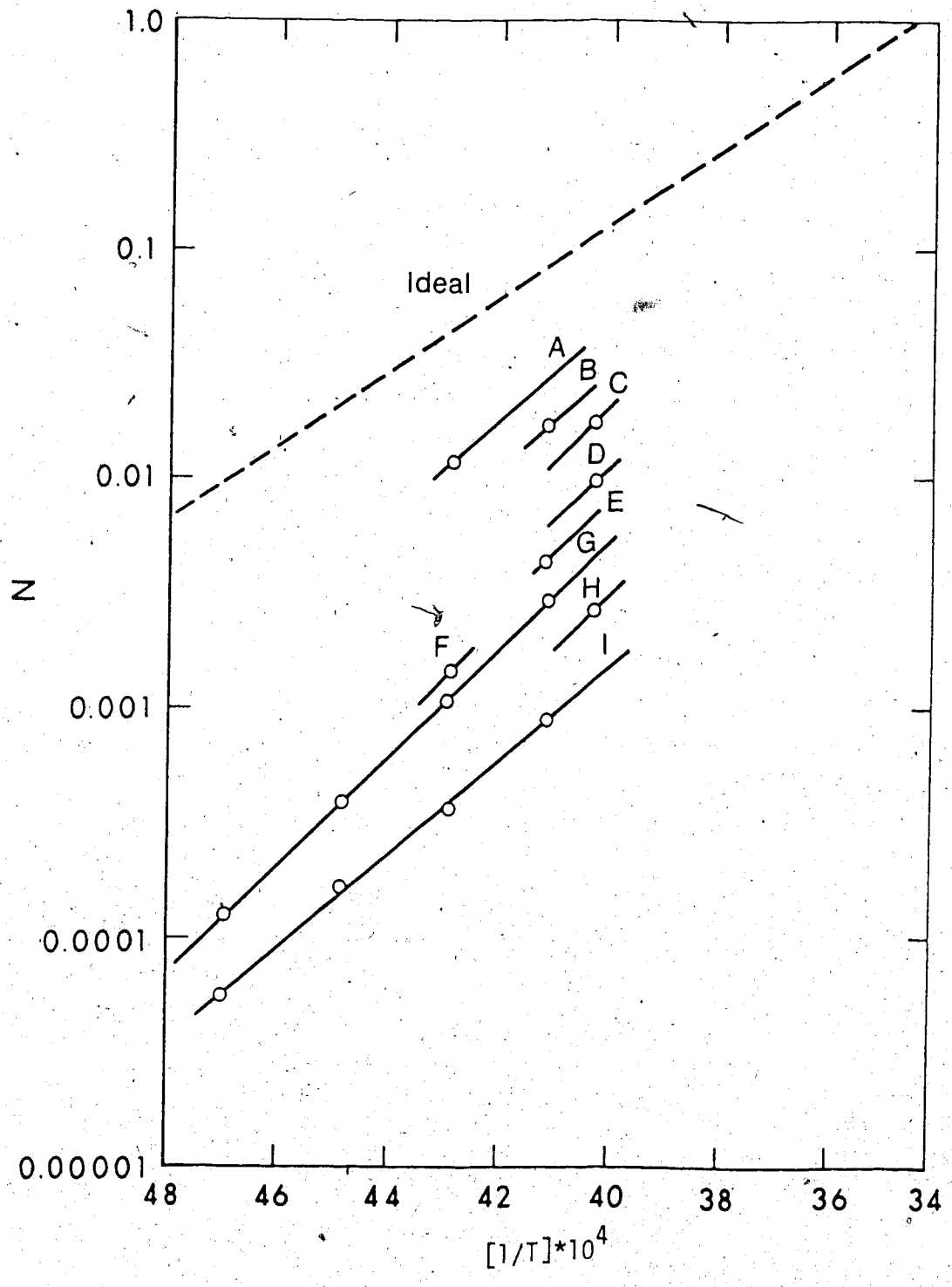


Figure 1.4 Solubility of Oleic Acid in Various Solvents (Bailey, 1950)

T = Temperature ^{o}K
N = Normality of Oleic Acid
(A) ether; (B) toluene; (C) butyl alcohol; (D) dichloroethane; (E) carbon disulfide; (F) commercial hexane; (G) acetone; (H) methyl acetate; and (I) methyl alcohol (Bailey, 1950)

For many practical purposes such as interpreting titration curves it is more convenient to use the apparent acid dissociation constant Ka', which is expressed in terms of concentrations.

The apparent acid dissociation constant Ka' depends on the ionic strength of solution. As the electrolyte and acid concentration approach zero, Ka approaches Ka' (Daniels and Alberty, 1966).

When the dissociation fraction equals unity, i.e. the amount of undissociated acid equals the amount of anion, then the H^+ concentration equals the dissociation constant of the acid, which is described by the Henderson-Hasselbalch equation (Karlson, 1965, and Stryer, 1975).

$$pH = \log\frac{[RCOO^-]}{[RCOOH]} - \log Ka$$

$$= \log\frac{[RCOO^-]}{[RCOOH]} + pKa$$

$$pH = pKa$$

The pKa of the first member of the series, formic acid (1:0) is 3.79. Acetic acid (2:0) has a pKa of 4.75 (White et al., 1959). The pKa of most of the remaining fatty acids are about 4.75 to 5.0 (Conn and Stumpf, 1972). Stronger acids have lower pKa values and weaker acids have higher pKa values. Since the pKa of most fatty acids is about 4.75, the titration curve and the percentage of un-ionized

(undissociated) acid versus pH can be plotted (Figures 1.5 and 1.6).

The Surfactant Properties of Fatty Acid Salts

The term surfactant as defined by Levitt (1967) is a contraction of "surface-active agent". The term embraces those organic chemicals that assist in penetrating, wetting, emulsifying, dispersing, solubilizing, foaming and frothing, and finally washing and scouring. Some surfactants do not have all these properties. In others, they are more or less pronounced, according to their chemical composition. Surfactants include soaps which are derived from fats and oils by saponification with sodium hydroxide (Sawyer and McCarty, 1978).

The chemical property which these materials have in common is their possession of both hydrophobic or lipophilic (oil-soluble) and hydrophilic (water-soluble) properties. The hydrophobic ends of a surfactant molecule will tend to dissolve in hydrophobic material globules with the hydrophilic ends projecting out into the water phase (Hrudey, 1978). This property allows surfactants to be effective as cleaning agents because of their effect of emulsifying grease and dirt thereby allowing them to be rinsed out. As a consequence of these properties, surfactants can be used as oil dispersants for emulsifying and dispersing oil spills.

All sodium and potassium soaps (sodium and potassium

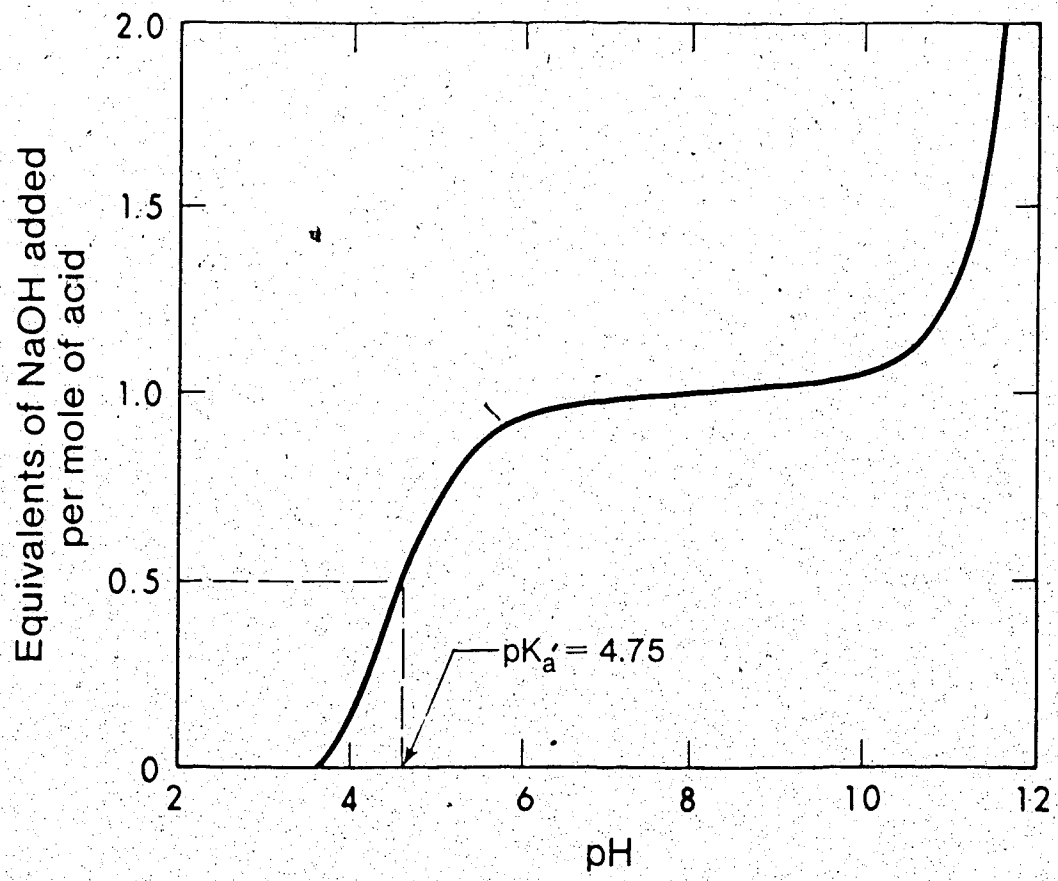


Figure 1.5 Titration of 0.004M Acetic Acid with Sodium Hydroxide at 25°C (Daniels and Alberty, 1966)

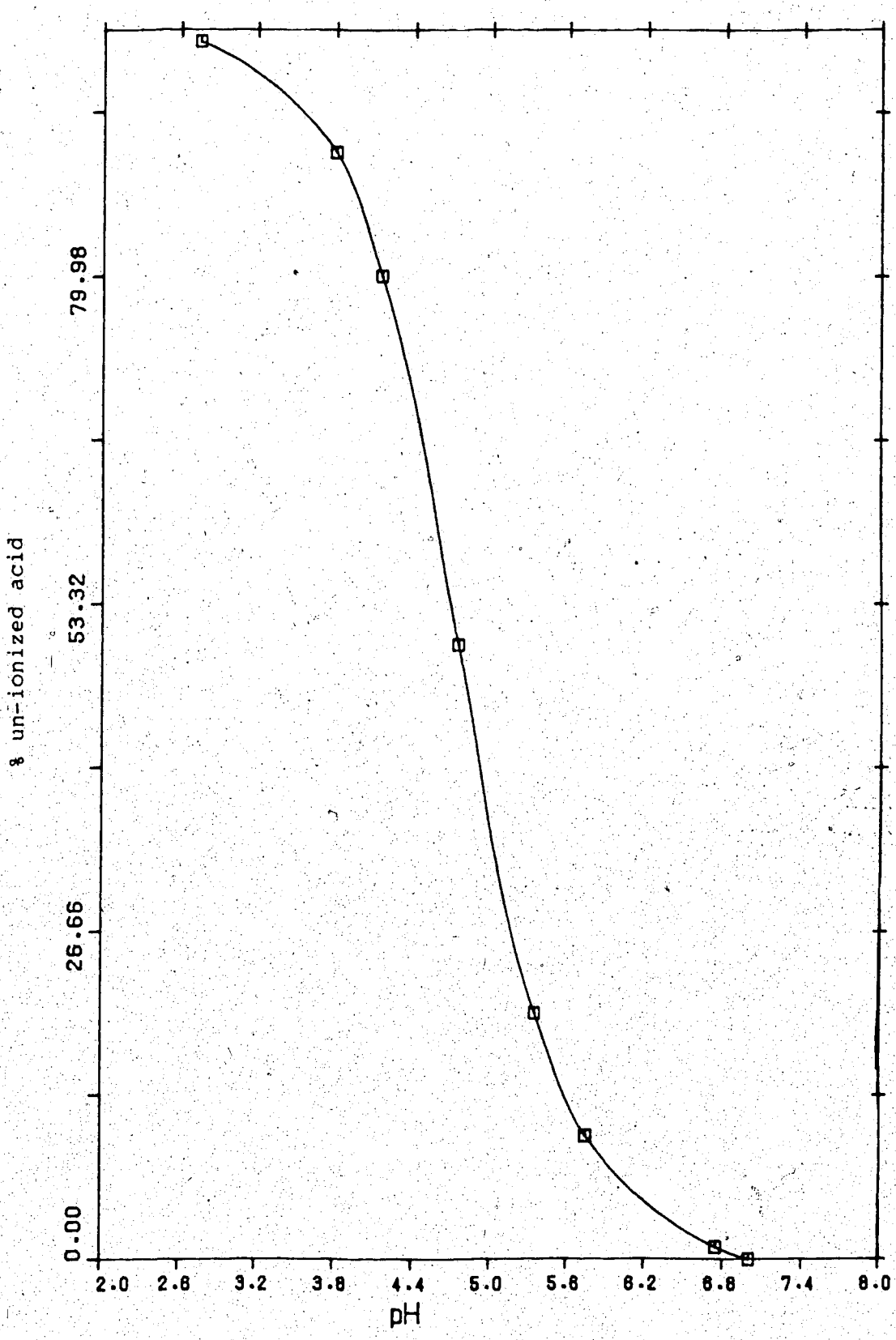


Figure 1.6 Percentage of Un-ionized Fatty Acids in Solution vs pH Calculated from pKa values of 4.75

salts of fatty acids) are soluble to some degree in water. However, if water is hard, the calcium, magnesium, and any other multivalent cations causing hardness will precipitate the soap in the form of metallic soap scum (Sawyer and McCarty, 1978).

$$2C_{17}H_{35}COONa + Ca^{2+} \longrightarrow (C_{17}H_{35}COO)_2Ca\downarrow + 2Na^{+} \quad \text{.....(2)}$$

(sodium stearate) (calcium stearate)

Therefore, soap must be added to precipitate all the ions causing hardness before it can act as a surfactant.

The physical properties of surfactants take place at the interface (Levitt, 1967): that is, at the surface or area of contact between two different phases or forms of matter. Of the five types of interface; solid/solid, solid/liquid, solid/gas, liquid/liquid, liquid/gas, only the solid/liquid and liquid/liquid are of concern in terms of soap surfactants.

Since 1945 a wide variety of synthetic detergents, commonly called syndets (Sawyer and McCarty, 1978) have been accepted as substitutes for soap. Their major advantage is that they do not form insoluble precipitates as readily with the ions causing hardness. The synthetic surfactants are of three major types anionic, nonionic and cationic (Levitt, 1967 and Kirschenbauer, 1960).

C. Industrial Uses and Sources of Fatty Acids

Sources

Various lipids, including the glyceride esters or fats, the glycerophosphoric acid esters or phosphatides, and related fatty compounds, occur in almost every type of plant and animal cell and often represent a large part of the total constituents of specialized parts such as seeds, nuts, tubers, animal livers, intermuscular connective tissue, the abdominal cavity, and subcutaneous connective tissue (Markley, 1960). We can extract these animal and vegetable fats and oils and obtain such substances as corn oil, coconut oil, cottonseed oil, palm oil, tallow, bacon grease, and butter (Table 1.4).

Industrial Uses

Several kinds of fatty acids and derivatives are used in a wide variety of industries, as follows:

1. Surface-Active Derivatives, Soaps, and Detergents.
2. Protective and Decorative Coatings: Fatty acids are used for alkyd resins, fatty acid-modified epoxy resins, and other primary coating products (Markley, 1967).
3. Petroleum Industry: The petroleum industry is a large consumer of chemicals derived from fats, principally surfactants which are used in such operations as drilling and primary recovery of oil, secondary recovery of oil, demulsification of crude petroleum, refining,

Table 1.4 Composition of Major Fats and Oils (Pattison, 1968)

Acid	Animal grease	Animal tallow	Castor oil	Coconut oil	Cottonseed oil	Linseed oil	Sardine oil	Soybean oil	Tall oil
Saturated under C_{12}				15					
Lauric				45					
Myristic	1	3		19	1		4		
Palmitic	28	28		10	22	6	11	10	7
Stearic	11	20	3	3	3	4	4	2	
Saturated above C_{18}								2	
Oleic	55	45	8	6	30	26	16	26	44
Linoleic	5	4	3	2	44	22	15	52	37
Linolenic						42		8	
Ricinoleic			86						
Unsaturated above C_{18}							38		11
Other unsaturated							12		1

*Approximate

storage, and transportation of oil (Markley, 1967).

4. Textile, Paper and Leather Industries: Fatty acids are used in various processes in these industries, such as for lubricants, softeners and finishing agents in the textile industry; in processing and coating papers of all types in the paper industries and in dyeing, fat-liquoring and finishing of leather products in the tanning industry (Markley, 1967 and Pattison, 1968).

5. Cosmetics and Pharmaceuticals: Fatty acids and their derivatives are widely used in cosmetic formulation (Pattison, 1968), such as, lotions, rouges, creams, ointments, shampoos, powders, mascaras, and hair [illegible]. These chemicals have appreciable versatility [illegible]edients of pharmaceutical products (Markley, 1[illegible]

6. Fo[illegible] Products: Fatty acids have found limited ap[illegible]cation in foods in comparison to their co[illegible]esponding derivatives (Pattison, 1968). Fatty chemicals, particularly certain fatty esters, are used as additives and ingredients in many foods (Markley, 1968).

7. Agriculture: Fatty acids and their derivatives are used as components of insecticides, herbicides, nematocides and rodenticides.

8. Rubber Industry: Fatty acids are used in the manufacturing processes for many synthetic elastomers, prima[illegible]ly as components of the emulsifiers used in

emulsion polymerization (Pattison, 1968).

9. Miscellaneous Uses: Fatty acids and their derivatives can be used as components of various products and industrial processes, such as, mineral flotation processes, candlemaking, plastic additives, plasticizers and in testing methods for physical and chemical properties (Markley, 1967 and Pattison, 1968).

D. Fatty Acids in Wastewater

Viswanathan et al. (1962) concluded that the principal fatty acids in domestic wastewater and sludge were the saturated myristic, palmitic, and stearic acids, and the unsaturated oleic and linoleic acids. These acids usually comprised over 90 percent of the total fatty acids in wastewater.

The total fatty acid concentrations were found to be from 1.01 to 1.40 mg/L (average = 1.28 mg/L) in paper mill effluent (Keith, 1976). A wide range of fatty acid concentrations were detected in kraft pulp and papermill effluent on the north shore of Lake Superior. Palmitic, stearic, arachidic, behenic, lignoceric, oleic and linoleic acid were found to be the major components (more than 0.1 mg/L). Myristic, pentadecanoic, heptadecanoic and palmitoleic acid were found to be the minor components (less than 0.1 mg/L) (Fox, 1977).

A wastewater survey of a modern catfish processing plant revealed total waste loads of 0.77 kg of grease/1,000

fish process (Markley, 1974). Grease concentrations were found to be from 35,070 mg/L to 35,900 mg/L and from 70,900 mg/L to 77,240 mg/L in waste loads of beef rendering waste and pork rendering waste, respectively (Dirasian, 1970).

Mineral oil concentrations were found to be from 0.8 to 9.6 mg/L (average 4.18 mg/L) and vegetable oil concentration was found to be 1.15 mg/L in the final effluent of the Upper Tame Basin. A sewage sludge analysis of the Upper Tame Basin revealed total waste loads of 56.25 g of mineral oil per kg of dry solids, 20.71 g of vegetable oil per kg of dry solids, 6.7 g of higher fatty acids per kg of dry solids and 35.5 g of lower fatty acids per kg of dry solids (Bennett et al., 1973). The studies of Farrington and Quinn (1973), Fedorak (1975) and Coleman (1975) have shown that long chain fatty acids (14:0 to 18:2) are the major fatty acids in raw wastewater and treated effluent. The distribution of these long chain fatty acids are shown in Table 1.5.

Hopwood and Rosen (1972) studied protein and fat recovery from effluents and revealed total wastewater concentrations of 8,390 mg/L and 33 mg/L of fat from soap cracking and barometric condenser water of slaughter house and poultry packing wastewater and plants respectively. The fatty acids are found to combine as triglyceride esters. The fatty acid esters are rapidly hydrolyzed to yield free fatty acids (Heukelekian and Mueller, 1958). Data obtained by Heukelekian and Mueller (1958) showed that oleic, palmitic and stearic acids were degraded at about the same rate.

Table 1.5 Fatty Acid Distribution in Raw Wastewater and Treated Effluents

Location (Sample Type)	Fatty Acid Concentration, mg/L (% of Total Fatty Acids) Myristic 14:0[a]	Palmitic 16:0	Palmitoleic 16:1	Stearic 18:0	Oleic 18:1	Linoleic 18:2	Total Fatty Acids	Total Lipid	References
Field's Point, Rhode Island (Secondary effluent)	0.32 (5.7%)	2.01 (36%)	0.58 (10.4%)	1.11 (19.9%)	1.33 (23.8%)	0.22 (3.9%)	5.58	-	Farrington and Quinn, 1975
West Warwick, Rhode Island (Secondary effluent)	0.06 (5.5%)	0.46 (42.2%)	Trace	0.31 (19.3%)	0.27 (24.8%)	0.06 (5.5%)	1.09	-	Farrington and Quinn, 1975
	0.37 (5.0%)	2.32 (31.2%)	Trace	0.93 (12.5%)	2.83 (38.1%)	0.86 (11.6%)	7.43	-	Farrington and Quinn, 1975
East Providence, Rhode Island (Secondary effluent)	Trace	2.38 (35.5%)	Trace	1.81 (27.0%)	2.14 (31.9%)	0.40 (6.0%)	6.71	-	Farrington and Quinn, 1975
Edmonton Gold Bar Plant (Secondary effluent)	0.104 (8.8%)	0.230 (19.5%)	0.123 (10.4%)	0.384 (32.5%)	0.199 (16.9%)	0.060 (5.1%)	1.19	7.7	Coleman, 1975
Edmonton Anaerobic Lagoons (Mixed Raw Sewage)	11 (2.5%)	114 (26%)	17.2 (3.9%)	74.8 (17.0%)	194 (44%)	24.2 (5.5%)	440	780	Fedorak, 1975

Viswanathan *et al.* (1962) observed, however, that oleic acid was degraded much more rapidly than any of the saturated fatty acids and that stearic acid was practically nondegradable.

Loehr and Roth (1968) found that the rate of degradation of long chain, saturated fatty acids decreased with increased chain length. They also observed that the unsaturated acids were metabolized more rapidly than their saturated counterparts. Data by Malaney and Gerhold (1969) indicated that the long chain saturated fatty acids were metabolized more slowly than either the shorter chain acids or the long chain unsaturates. Novak and Carlson (1969) found that the degradation of long chain fatty acids constituted the rate-limiting step in anaerobic digesters when COD removal or gas production was found to be maximized. The rate of fatty acid metabolism was limited by metabolic, not physical, considerations. They also found that the production of saturated fatty acid intermediates could occur during metabolism of soluble 18-carbon unsaturated fatty acids, but did not occur when saturated acids were metabolized.

Schaeffer *et al.* (1977) concluded that fats, oils and greases (FOG) were particularily resistant to anaerobic digestion, and when present in sludge they caused excessive accumulation in digesters and clogged the pores of filters. FOG could cause sheens, surface and sediment deposits, and exerted a long term BOD which could deplete the dissolved

oxygen in streams.

E. Toxicity of Fatty Acids

Leach and Thakore (1973) studied the identification of the constituents of kraft pulping effluent that were toxic to juvenile coho salmon (Oncorhynchus kisutch) and found that over 80 percent of the toxicity was caused by resin acid soaps and the remaining toxicity (18 percent) was contributed by sodium salts of the unsaturated fatty acids: palmitoleic, oleic, linoleic, and linolenic acid. Bioassay data showed that only sodium salts of the unsaturated fatty acids (linoleic = 3.57 mg/L, oleic = 1.53 mg/L, linolenic = 1.08 mg/L and palmitoleic = 1.02 mg/L) contributed measurably to unbleached whitewater (UWW) toxicity. Salts of the saturated fatty acids (lauric = trace, myristic = trace, palmitic = 1.89 mg/L, stearic = 1.02 mg/L, arachidic = 0.80 mg/L and behenic = trace) were not toxic to salmon at the concentrations found, nor when tested individually at 20 mg/L. A synthetic mixture of the sodium salts of toxic, unsaturated acids at the correct concentrations in water had toxicity-concentration characteristics identical to the fatty acid fraction isolated from UWW. There was a rapid decline in the toxicity of fatty acid soaps when diluted below their concentration in 100 percent UWW; at 90 percent concentration, fish were distressed and disoriented, but were not killed. The lethal thresholds for fatty acid soaps (except oleic acid) were found to be in the range of 8 and 9

mg/L. The toxicity of C_{18} fatty acid sodium salts decreased with diminishing degree of unsaturation in the order linolenate, linoleate, and oleate. Palmitoleate, a C_{16} mono-unsaturated acid, was the most toxic fatty acid soap tested. The median lethality (LC50) of these compounds to juvenile coho salmon are presented in Table 1.6.

Van Horn et al. (1950) cited that minimum lethal concentrations for minnows were found to be 5 mg/L fatty acid soaps. Leach and Thakore (1975) concluded that the concentrations of epoxy stearic acid encountered in the kraft pulpmill bleach plants effluent analyzed were very high relative to other fatty acids. It is possible that this compound might be formed by reactions of bleaching chemicals with an oleic acid derivative present in a process additive, such as a pitch dispersant or a defoamer. The 96-hour LC50 of epoxy-stearic to juvenile rainbow trout (Salmo gairdnerii) was found to be 1.5 mg/L.

Curtis et al. (1974) found that saturated fatty acids toxicity to brine shrimp larvae increased sharply from 6:0 (6 carbon saturated acid) to a maximum for 12:0 (12 carbon saturated acid) and then declined. Neither LC50 values nor other comparative toxicity figures could be determined for 14:0, 16:0 and 18:0 acids because of their increasing insolubility in the brine shrimp medium. However, all three of these acids were less toxic than the 12:0 acid. For unsaturated fatty acids, toxicity to the brine shrimp increased with the degree of unsaturation; 18:1 and 20:1

Table 1.6 Acute Lethality of Fatty Acids to Fish

Toxicants	LC50, mg/L (time period as indicated) against Juvenile Coho Salmon	Minnows	Juvenile Rainbow Trout	References
Palmitoleate 16:1	9.4*1	-	-	Leach & Thakore, 1973
Oleate 18:1	15.0*2	-	-	Leach & Thakore, 1973
Linoleate 18:2	11.0*1	-	-	Leach & Thakore, 1973
Linolenate 18:3	9.5*1	-	-	Leach & Thakore, 1973
Fatty Acids Soap		5*3	-	Van Horn et al, 1950
Expoxy-Stearic*4 18:0	-	-	1.5*3	Leach & Thakore, 1975

*1 = 4-Hour LC50
*2 = 24-Hour LC50
*3 = 96-Hour LC50
Expoxy Stearic*4 = They synthesized this fatty acid by oxidizing oleic in $CHCl_3$ with m-chloroperbenzoic acid at 20°C for 4 hours

unsaturated acids showed little toxicity but 18:2, 18:3 and 20:4 acids were highly toxic.

Galbraith et al. (1971) studied the toxicity of fatty acids (8:0 to 18:3) to microorganisms. The result indicated that linoleic (18:2) and linolenic (18:3) acids were the most toxic and caprylic (8:0) was the least toxic (Table 1.7). Sohode et al. (1974) indicated that triglyceride monohydroperoxide at 1 µmole/ml showed almost complete inhibitory effect on the growth of Escherichia coli.

Saxena and Thorsteinson (1971) reported that saturated fatty acids with a chain length of 10-13 carbon atoms showed the greatest toxicity, and oleic, linoleic and linolenic were the most toxic of the unsaturated acids on yellow fever mosquito larvae (Aedes aegypti). A similar relationship between the constituion of fatty acids and their acute toxicity to the aphid (Aphis rumicus) was reported by Tattersfield (1927).

The toxicity of fatty acids and lipids to higher animals were reported by Cunningham and Lawrence (1973). They indicated that the 96-hour LD50 (median effective dose, the least dosage that should be expected to kill 50 percent of animals that received it) of chlorinated wheat lipids in male, Wistar rats was found to be 28.2 g/kg compared to 20.2 g/kg for chlorinated oleic acid, 17.2 g/kg for chlorinated linoleic acid (Table 1.8). Chlorinated corn oil had a 96-hour LD50 of 5.7 g/kg in female rats compared to 11.3 g/kg in males. Mays (1972) also indicated that intravenous

Table 1.7 Acute Lethality of Fatty Acids to Brine Shrimp Larvae (Curtis et al., 1977) and Microorganisms (Galbraith et al., 1971)

Fatty Acids	96-hr LC50 (mg/L) against Brine Shrimp Larvae	MIC* (mm) against Bacillus megaterium	MIC* (mm) against Pseudomonas phaseolicola
Caproic (6:0)	>0.30	-	-
Caprylic (8:0)	0.24	2.00	> 3.00
Capric (10:0)	0.036	1.00	> 1.20
Lauric (12:0)	0.005	0.15	> 1.20
Myristic (14:0)	>0.027	0.15	> 1.20
Palmitic (16:0)	>0.014	0.30	> 1.20
Stearic (18:0)	>0.020	0.40	> 1.20
Oleic (18:1), cis	>0.087	0.05	> 1.20
Elaidic (18:1), tran	-	0.20	> 1.20
Linoleic (18:2)	0.033	0.02	> 1.20
Linolenic (18:3)	0.024	0.02	> 1.20
Eicosenic (20:1)	0.030	-	-
Arachidonic (20:4)	0.015 - 0.020	-	-

*MIC = minimum inhibitory concentration after 24hours in millimole of fatty acids per 9.0 ml of 0.5% nutrient broth in capped 150 x 15 mm test tubes and initial 10^6 microorganism cells per ml

Table 1.8 96-Hour LD50 of chlorinated Lipids and Unsaturated Fatty Acids to Rats (Cunningham and Lawrence, 1977)

Toxicants	96-hr LD50 g of toxicant per kg of rat
Chlorinated wheat flour lipid	28.2
Chlorinated oleic (18:1)	20.2
Chlorinated linoleic (18:2)	17.2
Chlorinated linolenic (18:3)	10.2

injection of free fatty acids (FFA) produced unconsciousness in experimental animals: I. FFA caused a gradual cessation of respiratory activity of brain cortex slices incubates with glucose and II. Serum albumin (SA) obviated the inhibition oxidation caused by FFA anions.

F. Toxicity of Other Surfactants

The information on the aquatic toxicity of long chain fatty acids is limited. Since these compounds can function as anionic surfactants, the toxicity of other common anionic surfactants, ABS (alkylbenzene sulfonates) and LAS (linear alkyl sulfonates) were reviewed. The synthetic anionic surfactants are generally sodium salts which ionize to yield Na^+ plus a negatively charged, surface-active ion (Sawyer and McCarty, 1978). The common ones are all sulfates and sulfonates.

In the past ABS was derived from propylene and was resistant to biological attack because of the branched-chain structure of the alkyl groups and because the benzene rings are attached principally to tertiary carbon atoms of the branched-chain groups (Sawyer and McCarty, 1978). This material is now made largely from normal (straight-chain) paraffins, and thus the alkyl chain is not branched and the benzene ring is attached primarily to secondary carbon atoms. This latter material has been labeled LAS which can be degraded under aerobic conditions.

The acute toxicity of LAS to aquatic organisms has been reviewed by Swisher *et al.* (1964), and Kimerle *et al.* (1977). Swisher *et al.* (1964) reported that LAS were relatively toxic to fish (bluegill fingerlings) when tested under static bioassay. The median tolerance limit as a 96-hour LC50 was found to be around 3.0 mg/L, 0.64 mg/L for the C_{12} and C_{14} homologs of LAS, respectively. They also reported that these materials were so readily degraded by bacterial attack that bluegill fingerlings lived with no trouble in effluents from laboratory continuous flow activated sludge units being fed 100 mg/L or more of either product. The longer homologs and more terminal isomers of these chemicals, which are the more toxic, are also more rapidly degraded under bacterial action (Kimerle *et al.*, 1977). Median lethal concentration (LC50) of LAS to water fleas (*Daphnia magna*) and fathead minnows (*Pimephales promelas*) may range from 0.5 to 50 mg/L depending mainly upon the chain length of the particular homolog (Kimerle *et al.*, 1977).

The acute toxicity of ABS to fish was reported by many researchers. First, Hendenson *et al.* (1959) reported the results of bioassays with ABS in both hard and soft dilution waters. 96-hour LC50 values for fathead minnows (*Pimephales promelas*) were on the average 6.6 mg/L (range 3.6 to 9.2 mg/L) in soft water and 4.3 mg/L (range 3.5 to 5.1 mg/L) in hard water for four ABS compounds frequently used in household syndets. Tests with 100 percent ABS in soft water

using bluegills as test organisms produced the following LC50's: 24 hr = 8.2 mg/L, 48 hr = 7.5 mg/L, 96 hr = 5.6 mg/L. The similar acute toxicity test results were reported by Cairns and Scheier (1962). The 96-hour LC50 result of the acute bioassay tests with Lepomis macrochirus Raf. and L. gibbosus (Linn.) were found on the average 17.3 ± 0.13 mg/L (range 17.15 to 17.44 mg/L) and 21.89 mg/L, respectively which were quite comparable to those of Hendenson et al. (1959) (Table 1.9). The toxicity of ABS was not greatly affected by the hardness of dilution water. The results also indicated that there was no significant difference between short-term batch tests and continuous flow.

Cairns and Scheier (1962) also reported the biological safe concentration (BSC) of ABS for both fish which were on the average 5.42 ± 0.5 mg/L (range 5.18 to 6.31 mg/L). Surber and Thatcher (1963) also found that concentrations below 8 mg/L ABS produced very little mortality on some aquatic invertebrate (Hydropsychidae). The 30 day bioassay toxicity of ABS to bluegill (Lepomis macrochirus) was conducted by Lemke and Mount (1963). The 30 day LC50 ranged between 15.5 and 18.3 mg/L of ABS. The difference between the 24 hours and 30 day LC50 ranged between 6.5 and 0.5 mg/L.

1.9 Acute Lethality of LAS and ABS to Fish and Water Fleas

Acute Lethality (96-hr LC50, mg/L)

Organism	LAS	ABS	References
Water Fleas (Daphnia magna)	0.5	-	Kimerle et al., 1977
Bluegill Fingerlings*	0.64 - 3.0	-	Swisher et al., 1964
Pimephales promelas Raf. (bluegill sunfish)	50	4.3 - 6.6	Kimerle et al. 1977 Hendenson et al., 1958
Lepomis macrochirus Raf. (bluegill sunfish)	-	17.3	Cairns and Scheier, 1962
Lepomis gibbosus (Linn.) (pumpkinseed sunfish)	-	21.89	Cairns and Scheier, 1962

* No scientific name reported.

II. Experimental Design

A. Statement of the Problem and Objectives of the Study

The long chain fatty acids are ubiquitous compounds in municipal and industrial wastewaters because of their occurrence in lipids as components of the triglycerides. These compounds are basically insoluble in water in the acid form, but sodium salts of the long chain fatty acids (soaps) can disperse in water. The fatty acid salts exhibit surfactant properties because of their combination of hydrophilic and hydrophobic properties into one molecule. The surfactant properties of fatty acids are very sensitive to pH because the undissociated fatty acids are virtually insoluble. However, it is the surfactant properties of fatty acid salts which would lead to an expectation of acute toxicity to fish, based on the known toxicity of synthetic surfactants.

A review of the literature for information on the toxicity of fatty acids or fatty acid salts was conducted. Only two references were located which specifically reported toxicity for individual fatty acids to fish (Leach and Thakore, 1973 and 1975).

Given a background of apparent lack of information on the toxicity of fatty acids and the major influence which pH exerts upon the physical behaviour of long chain fatty acids, there appeared to be a need to evaluate the acute

toxicity of long chain fatty acids as a function of pH. Specifications for the regulatory application of acute toxicity testing allow a relatively wide range of test pH values. Hence, a significant influence of pH on an observed toxicity would have a major bearing on the interpretation of test results.

Therefore, this project was proposed to conduct 96 hour static acute lethal bioassays using rainbow trout fingerling and the common long chain fatty acids (oleic and linoleic). It was hoped that the results would provide insight into the possible contribution which fatty acids would provide to acute lethal toxicity of municipal and industrial wastewaters. As well, information was sought on the sensitivity of acute lethality evaluation of the fatty acids to test pH conditions.

B. Methodology

Fish Holding and Feeding Conditions

Fish used in this study were rainbow trout (Salmo gairdneri Richardson) fingerlings of either sex with weights and lengths as indicated in Table 2.1. The longest fish in each test were not more than 1.5 times the length of the shortest, as required by Standard Methods (APHA, 1975).

The fish were transferred from the Toxicology Laboratory, Environmental Protection Service, Edmonton, Alberta to an acclimation room and were held at 9°C in a

Table 2.1 Weight and Length of Test Fish

Toxicants	Test No.	Parameters	Means	Ranges	Standard Deviations
Oleic Acid	1	Weight*	0.80	0.29-2.10	0.37
		Length*	4.28	3.30-6.00	0.54
	2	Weight	0.97	0.30-3.19	0.50
		Length	4.57	3.50-7.00	0.67
	3	Weight	0.85	0.30-1.82	0.33
		Length	4.51	3.50-5.60	0.50
	4	Weight	0.52	0.18-1.25	0.23
		Length	3.83	2.90-5.30	0.52
	5	Weight	0.55	0.29-0.90	0.18
		Length	4.09	3.60-4.70	0.34
Average		Weight	1.22	0.18-3.19	0.56
		Length	4.81	2.90-6.00	0.76
		Length	4.81	2.90-6.00	0.75
Linoleic Acid	1	Weight	0.92	0.25-2.30	0.49
		Length	4.30	3.10-5.80	0.73
	2	Weight	0.84	0.70-1.10	0.34
		Length	4.35	4.10-4.80	0.58
	3	Weight	1.10	0.50-2.19	0.41
		Length	4.71	3.50-6.00	0.56
	4	Weight	1.63	0.52-2.70	0.96
		Length	5.08	3.60-6.20	0.52
Average		Weight	1.12	0.25-2.70	0.36
		Length	4.61	3.10-6.20	0.36

*unit, Weight = gram
Length = centimeter

1,000 litre fiberglass holding tank at the Zoology Department, University of Alberta. They received a continuous flow of dechlorinated tap water (EDTA hardness as $CaCO_3$ = 116 mg/L, conductivity = 225 µmho/cm, pH = 6.70, total alkalinity as $CaCO_3$ = 36 mg/L, total residual = 180.8 mg/L and turbidity = 0.5 J.T.U.). Holding tanks were illuminated with fluorescent lights which were electrically switched on and off with a timer to keep a photoperiod cycle with appropiate twilight periods similar to the local conditions.

The fish were fed once daily at the rate of about 3 percent of body weight. Fish were maintained in very good health and no significant mortality occurred. The fish were allowed to acclimate to these holding conditions for at least 2 weeks prior to experimentation. Feeding was stopped 48 hours before starting the experiment and the fish were transferred to the experimental rooms. Water temperature in the tanks was maintained constant at 12±1°C by a room temperature control.

Experimental Conditions

Test Tanks

The test tanks were circular polyethylene tanks, 49 cm. in diameter and 100 litres in volume. Nineteen test tanks were placed in a temperature controlled room. Clean plastic bags containing 40 litres of water sample were placed in the test tanks at the start of each experiment. The bags acted

as lining to prevent residual contamination of tanks and were discarded after each experiment.

Stock Solutions Preparation

Fatty acids themselves are not soluble in water. Therefore, a fresh stock solution of fatty acids (oleic and linoleic acids) were directly prepared by reacting the fatty acids with sodium hydroxide. The amount of fatty acids in solution was carefully controlled to provide an accurate concentration of toxicity required in each of the experiments.

Dilution Water Preparation

Normally, EDTA hardness of dechlorinated tap water would precipitate some of fatty acids, resulting in a decrease in concentration and toxicity of the original fatty acids. Therefore, the dechlorinated tap water was diluted with an equal volume of distilled water to obtain the required dilution water for this study. The EDTA hardness as $CaCO_3$ was reduced from 116 mg/L to 60 mg/L.

Experimental Procedures

To determine the effects of pH on the acute lethality of oleic and linoleic acids to rainbow trout fingerlings, a series of experiments was designed using the following procedures.

Acute Lethality Studies

Rainbow trout fingerlings which were acclimated for 2 weeks in the holding tank to 9°C and were tested with each fatty acid at 3 levels of pH (6.5, 7.5 and 9.0). Five acid concentrations were tested at each pH. Three control pH tanks and one blank tank were set at the same time. Totally, 19 tanks were tested in each experiment.

In each experiment, 190 rainbow trout fingerlings of either sex were equally distributed in a random manner into 19 test tanks (10 fish per tank). Experiments with each fatty acid at each pH level were replicated (five replicated tests for oleic acid and four replicated tests for linoleic acid). All data from each fatty acid at each pH were pooled for statistical analysis. All tests were run for a 96 hour period by starting the test on Monday morning and running static bioassay until Friday morning.

Treated fish in each test tank were checked for mortality at 0.5, 1, 2, 4, 8, 24, 48, 72 and 96 hours. Dead fish were removed from the test tanks and time of death, weight and length of each fish were recorded. At the end of the 96 hour period, all fish that survived in each test tank were sacrificed and weight and length were recorded.

Test Water Chemical Characteristics

Water chemistry characteristics of test water in each test tank were checked for dissolved oxygen, pH and temperature at 12 hour intervals and EDTA hardness,

alkalinity, ammonia-nitrogen and ether extractable matter (EEM) were checked at 24 hour intervals.

The procedures for water chemistry analysis were as follows:

1. Dissolved oxygen and temperature were measured with a Dissovled Oxygen Meter (YSI Model 54A oxygen meter).
2. pH was measured with a pH meter (Hach portable pH meter model 164000).
3. EDTA hardness was measured with Standard Method 309B, EDTA titrimetric method (APHA, 1975), using EDTA analytical reagent grade and eriochrome black T commercial indicator (Univer, Hach Chemical Co.).
4. Alkalinity was measured with Standard Method 403 (APHA, 1975), using phenolphthalalein and mixed bromocresol green-methyl red indicators and 0.02 N H_2SO_4 titrant.
5. Ammonia-nitrogen was measured with a Spectronic 20 (Bausch and Lomb), according to the nesslerization method 418B (APHA, 1975) using zinc sulfate solution, stabilizer reagent (rochelle salt solution), nessler reagent (HgI_2 + KI in KOH) and standard ammonia solution.
6. Fatty acids were extracted by intimate contact with petroleum ether in a continuous liquid-liquid extraction unit (Figure 2.1 and Plate 2.1). The procedure was as follows:

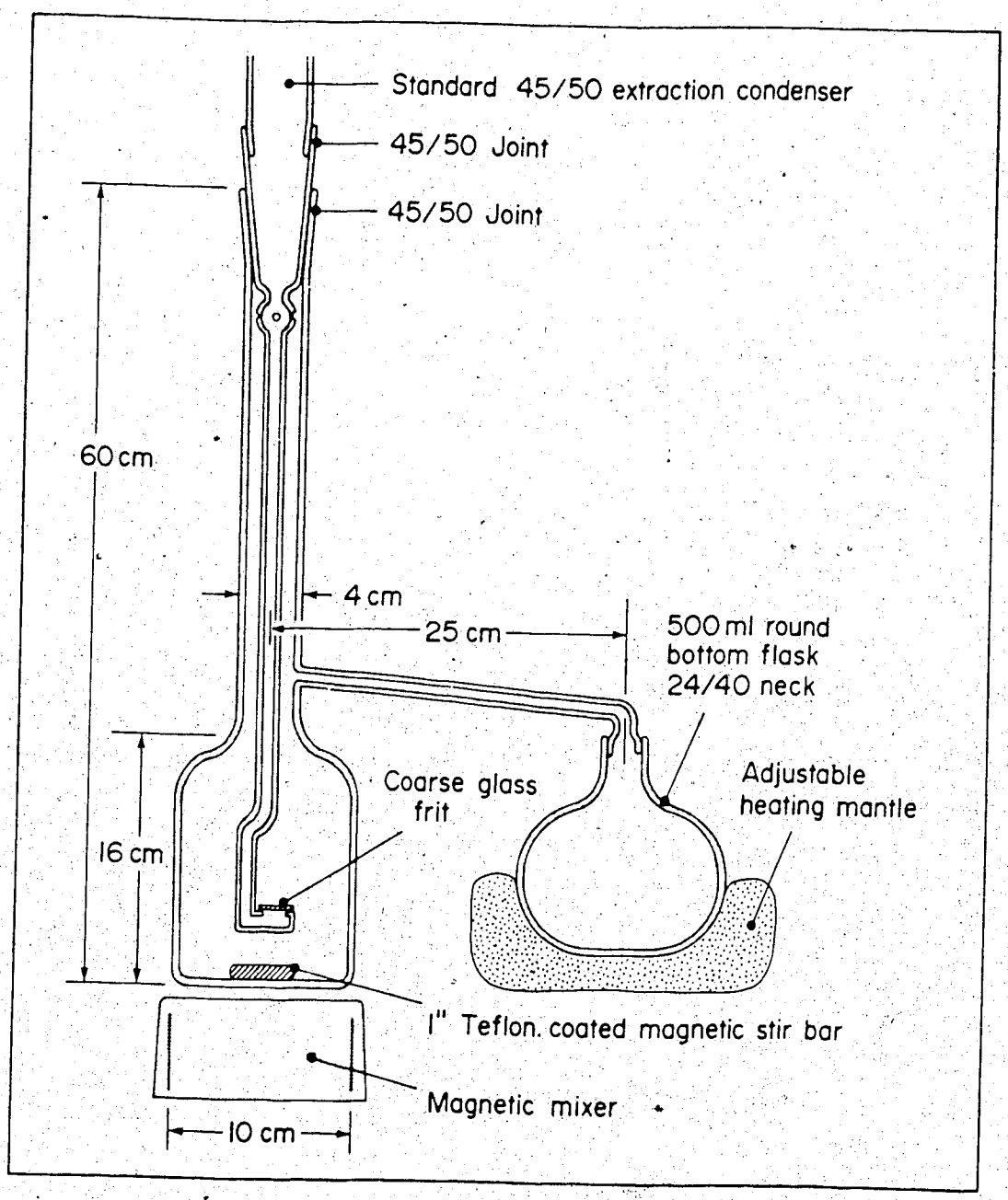


Figure 2.1 Continuous Liquid-Liquid Extraction Apparatus

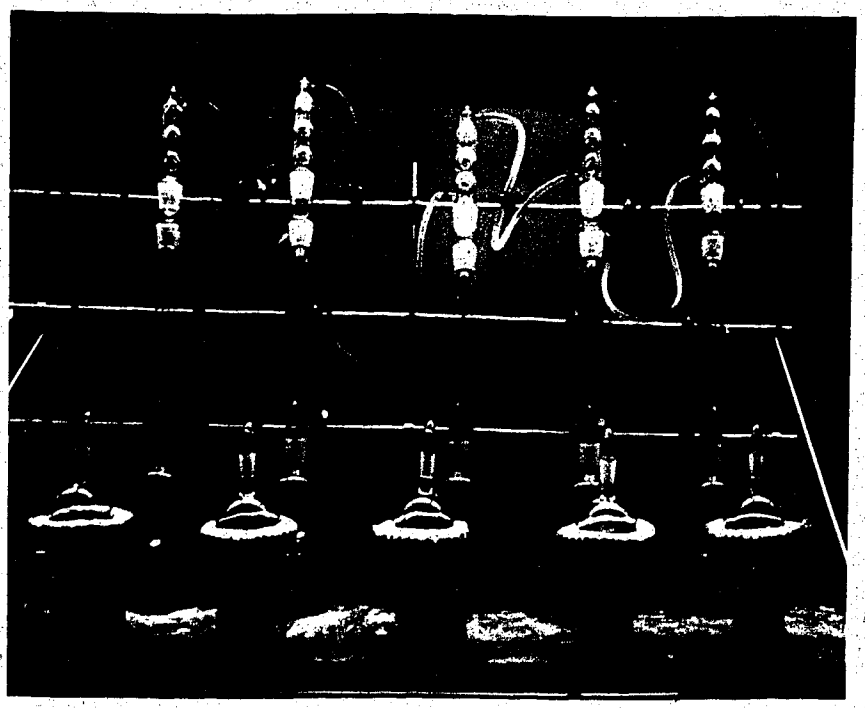


Plate 2.1 Continuous Liquid-Liquid Extraction Unit

a. One liter of water sample was collected in the extraction bottle. The sample was acidified with 20 mL of 1:1 HCl in order to preserve the samples. They were then stored in a cooler at $5^{0}C$.

b. Approximately 200 mL of petroleum ether was added into a 500 mL round bottom flask and the extraction unit was set up as shown in Figure 2.1 and Plate 2.1.

c. Petroleum ether was added to the extraction unit until the solvent (petroleum ether) just began to overflow into the 500 mL flask via the side arm of the extractor.

d. The magnetic stirrer and heater were turned on to a suitable rate until the solvent began to boil gently. The solvent vapour travelled up to the condensors and the condensed solvent dropped down the center tube to the bottom of the extractor. The extraction unit was then left running for about 16 hours.

e. The ether extractable matter (EEM) in the samples was extracted by the solvent over the 16 hour period and overflowed into the flask via the side arm of the extractor.

f. The flask was disconnected and the extractor was carefully tipped forward to drain about 2/3 of the petroleum ether layer on the top of the water sample through the side arm into the 500 mL flask.

g. The ether solution was filtered through #40 filter paper into a clean 500 mL flat bottom flask. The second flask was placed on a rotary evaporator (Buchi Rotovapor-R) connected to a nitrogen gas cylinder at a temperature not exceeding 60°C in order to evaporate the solvent (about 5 min.).

h. 3 successive 10 mL portions of petroleum ether were added to the 500 mL flask. The solvent was swirled and transferred to a preweighed 50 mL flask (accurate to 0.1 mg) using a disposable pipet. The 50 mL flasks were placed back on the rotary evaporator and evaporated to dryness (about 3 min.).

i. The flasks were placed into an oven at 105°C in order to remove any water (less than 5 min.) and then were placed into a dessicator for one hour.

j. The dried flasks were weighed and the weight difference calculated in milligrams in order to get the original ether extractable matter (EEM) concentration as mg/L of sample.

Due to the capacity limited availability of extraction units, only four water samples and a blank sample were collected for EEM analysis each day. Of the four water samples, three were of the middle toxicity concentrations in each pH level and were collected each day. The fourth sample was collected from alternate concentrations as indicated by the schedule in Figure 2.2. This schedule was continued for

Initial (0 hr.)

C.L.	pH levels		
	6.5	7.5	9.0
1	x		
2			
3	x	x	x
4			
5			
pH Control Blank		x	

24 hours

C.L.	pH levels		
	6.5	7.5	9.0
1		x	
2			
3	x	x	x
4			
5			
pH Control Blank		x	

48 hours

C.L.	pH levels		
	6.5	7.5	9.0
1			x
2			
3	x	x	x
4			
5			
pH Control Blank		x	

24 hours

C.L.	pH levels		
	6.5	7.5	9.0
1			
2	x		
3	x	x	x
4			
5			
pH Control Blank		x	

96 hours

C.L.	pH levels		
	6.5	7.5	9.0
1*			
2		x	
3	x	x	x
4			
5*			
pH Control Blank		x	

C.L. = Concentration Levels (mg/L)
x = Values Measured (mg/L)
1* = The Lowest Concentration at Each pH Test Condition
5* = The Highest Concentration at Each pH Test Condition

Figure 2.2 Ether extractable Matter (EEM) Analysis Schedule for First Test in Series

subsequent tests in the series by starting at the next sample location in the figure.

The three samples of middle concentration of each pH level on the first and last day were saved for fatty acids identification. The fatty acids were identified by gas chromatography (Hewlett - Packard, 5736A), using the external standard technique with a flame ionization detector (FID) and esterification as per the method of Metcalfe et al. (1968). The gas chormatography conditions were as follows:

Column : Glass, 1/4" ID., 6 ft. Long

Column Packing : 5% SP 2330, 100/120 Chromosorb W AW

Temperature : Oven 185°C

Injection Inlet 250°C

Detector 300°C

Gas Flow Rate : Carrier (N_2) 30 mL/min.

H_2 30 psig.

Air 27 psig.

Sample Size : 2 µL

Attenuation : 2^4 = 16

Chart Speed : 0.5 cm/min.

Data representing the chemical, physical and biological parameters were collected for each bioassay test. A test was considered complete either when all the fish had died or when a period of 96 hours was passed regardless of whether all the fish had died. Means and standard deviations were

subsequently calculated for each set of data. However, pH was averaged from H^+ concentration (activity) and then converted back to pH values. The ranges of the pH values are also presented.

For any experiment, the missing values of EEM at each pH level were calculated as indicated in the following example for the first test in series. The EEM which were analyzed for each experiment are presented in Table 2.2. The missing values of EEM at each pH level were calculated by applying the percentage EEM decrease, indicated by the measured values for the middle concentration or the single extra concentration, to the known initial concentration (Table 2.3).

Calculation of Median Lethal Concentration

The median lethal concentration LC50 (the concentration of fatty acids that produced 50 percent mortality at a specific time period) and its 95 percent confidence interval were calculated at 96 hour period for each experiment by using the trimmed Spearman-Karber method (Hamilton et al., 1977). Comparison of the LC50 value for each pH level was done by two-way analysis of variance ($p \geq 0.05$) (Bennett and Franklin, 1954).

LC[illegible]s obtained for individual tests on each acid we[illegible] an unpaired student t-test ($p \geq 0.05$) for e[illegible]H series.

[illegible]om every replicated test of each acid at

Table 2.2 Ether Extractable Matter (EEM) Analysis for First test in Series (Concentration mg/L)

pH 8.78 Test Condition

F.A.C.A.*1	Time Period (hrs.)					EEM*2
	0	24	48	72	96	
42.0	-	-	36.5	-	-	-
56.0	-	-	-	-	-	
75.0	75.3	70.2	65.2	58.2	44.0	63.19
100.0	-	-	-	-	-	-
135.0	-	-	-	-	-	-
Blank	2.1	2.0	1.8	2.0	2.2	2.28 (.66)*3

pH 7.10 Test Condition

F.A.C.A.	Time Period (hrs.)					EEM
	0	24	48	72	96	
24.0	-	23.0	-	-	-	-
32.0	-	-	-	-	23.9	-
42.0	43.5	40.3	36.4	30.2	25.6	35.51
56.0	-	-	-	-	-	-
75.0	-	-	-	-	-	-

pH 6.47 Test Condition

F.A.C.A.	Time Period (hrs.)					EEM
	0	24	48	72	96	
18.0	18.5	-	-	-	-	-
24.0	-	-	-	19.8	-	-
32.0	32.2	24.8	18.2	14.8	9.60	19.80
42.0	-	-	-	-	-	-
56.0	-	-	-	-	-	-

F.A.C.A.*1 = Fatty Acids Concentration Added
EMM*2 = Ether Extractable Matter by Interpolation from Zero Hour to Ninety-six Hour Period
3* = From Averaged Mean Value (S.D.)

Table 2.3 EEM Calculation Procedure (Example for First Test in Series)

pH 8.78 Test Condition

F.A.C.A.	Time Period (hrs.) 0	% *1	24	%	48	%	72	%	96	%	EEM *3
42.0	42.2	100.4	39.3	93.6	(36.5)	(86.93)	32.6	77.6	24.6	58.4	35.45
56.0	56.2	100.4	52.4	93.6	48.7	86.93	43.5	77.6	32.8	58.64	47.28
75.0	(75.3)	(100.4)	(70.2)	(93.6)	(65.2)	(86.93)	(58.2)	(77.6)	(44.0)	(58.64)	(63.31)
100.0	100.4	100.4	93.6	93.6	86.9	86.93	77.6	77.6	58.6	58.64	84.4
135.0	135.5	100.4	126.4	93.6	117.4	86.93	104.8	77.6	-*2	-	121.32

pH 7.10 Test Condition

F.A.C.A.	Time Period (hrs.) 0	% *1	24	%	48	%	72	%	96	%	EEM *3
24.0	24.9	103.57	(23.0)	(95.83)	20.8	86.67	17.3	71.9	17.9	74.69	20.63
32.0	33.1	103.97	30.7	95.83	27.7	86.67	23.0	71.9	(23.9)	(74.69)	27.69
42.0	(43.5)	(103.57)	(40.3)	(95.95)	(36.4)	(86.67)	(30.2)	(71.9)	(25.6)	(60.95)	(35.36)
56.0	57.9	103.57	53.7	95.95	48.5	86.67	40.3	71.9	34.1	60.95	47.13
75.0	77.7	103.57	71.9	95.95	65.0	86.67	53.9	71.9	-*2	-	67.57

pH 6.47 Test Condition

F.A.C.A.	Time Period (hrs.) 0	% *1	24	%	48	%	72	%	96	%	EEM *3
18.0	(18.5)	(102.78)	14.0	77.5	10.2	56.88	14.6	82.5	5.4	30	12.69
24.0	24.7	102.78	18.6	77.5	13.7	56.88	(19.8)	(82.5)	7.2	30	17.01
32.0	(32.2)	(100.63)	(24.8)	(77.50)	(18.2)	(56.88)	(14.2)	(46.25)	(9.6)	(30)	(19.53)
42.0	42.3	100.63	32.6	75.5	23.9	56.88	19.4	46.25	12.6	30	25.84
56.0	56.4	100.63	43.4	75.5	31.6	56.88	*2	-	-	-	43.70

Number in brackets from EEM Analysis (Table 2.2)

*1 = The percents vary from F.A.C.A.

*2 = Complete mortality 24 hours before that time period

*3 = EEM Interpolation Values

each of the test pH values were pooled and then the geometric mean was determined.

Median survival time (MST, time required to produce 50 percent mortality for each concentration of fatty acids) and its 95 percent confidence interval were calculated according to the method of Litchfield (1949) and subsequent mathematical calculation of a linear regression analysis. A mortality curve for both acids as constructed between log median survival time (MST) and log concentration of acid.

The rate of mortality (the reciprocal of the median survival time, 1/MST) for oleic and linoleic acids in each concentration were calculated at each pH level.

pH Control Method for Dilution Water

At pH 6.5; 50 mg/L of $NaHCO_3$ was added as a buffer and the pH was adjusted with 1N HCl at 12 hour intervals.

At pH 7.5; 100 mg/L of $NaHCO_3$ was added and the pH was adjusted with 1N NaOH at 12 hour intervals.

At pH 9.0; 100 mg/L of Na_2CO_3 was added and the pH was adjusted with 1N HCl and NaOH at 12 hour intervals.

Every test tank was aerated using an air compressor with an airstone in the center of the tank. Dissolved oxygen was checked every 12 hours and the air flow was adjusted for maintaining the dissolved oxygen in the tank above 9 mg/L.

III. Results and Discussion

A. Results

Test Fatty Acids Composition

The chemical compositions of oleic acid and lower grade linoleic acid are presented in Tables 3.1 and 3.2 and with the corresponding gas chromatograms (using internal standard technique) in Figures 3.1 and 3.2. From these results, it can be concluded that the purity of oleic acid was 87 percent. The major remaining components were linoleic acid (18:2 = 8 percent), palmitic acid (16:0 = trace). The purity of linoleic acid was 70 percent. The major remaining components were oleic acid (18:1 = 29 percent) and palmitic acid (16:0 = trace).

The results from gas chromatograpic identification (using external standard technique) of the test compounds at the beginning and at 96 hours of experiments are given in Tables 3.3, 3.4 and 3.5. The test oleic acid exibited oleic acid (18:1), linoleic acid (18:2) palmitic acid (16:0) and myristic acid (14:0). The 70 percent test linoleic acid exibited linoleic acid (18:2), oleic acid (18:1) and palmitic acid (16:0) and a trace of stearic acid (18:0). The 95 percent test linoleic acid contained linoleic acid (18:2) and oleic acid (18:1) (Figures 3.3, 3.4 and 3.5).

Table 3.1 Test Oleic Acid Composition

Approximate Percentage Composition		
Linoleic 18:2	Oleic 18:1	Palmitic 16:0
8.0	87.0	Trace

Table 3.2 70 Percent Test Linoleic Acid Composition

Approximate Percentage Composition		
Linoleic 18:2	Oleic 18:1	Palmitic 16:0
70.0	29.0	Trace

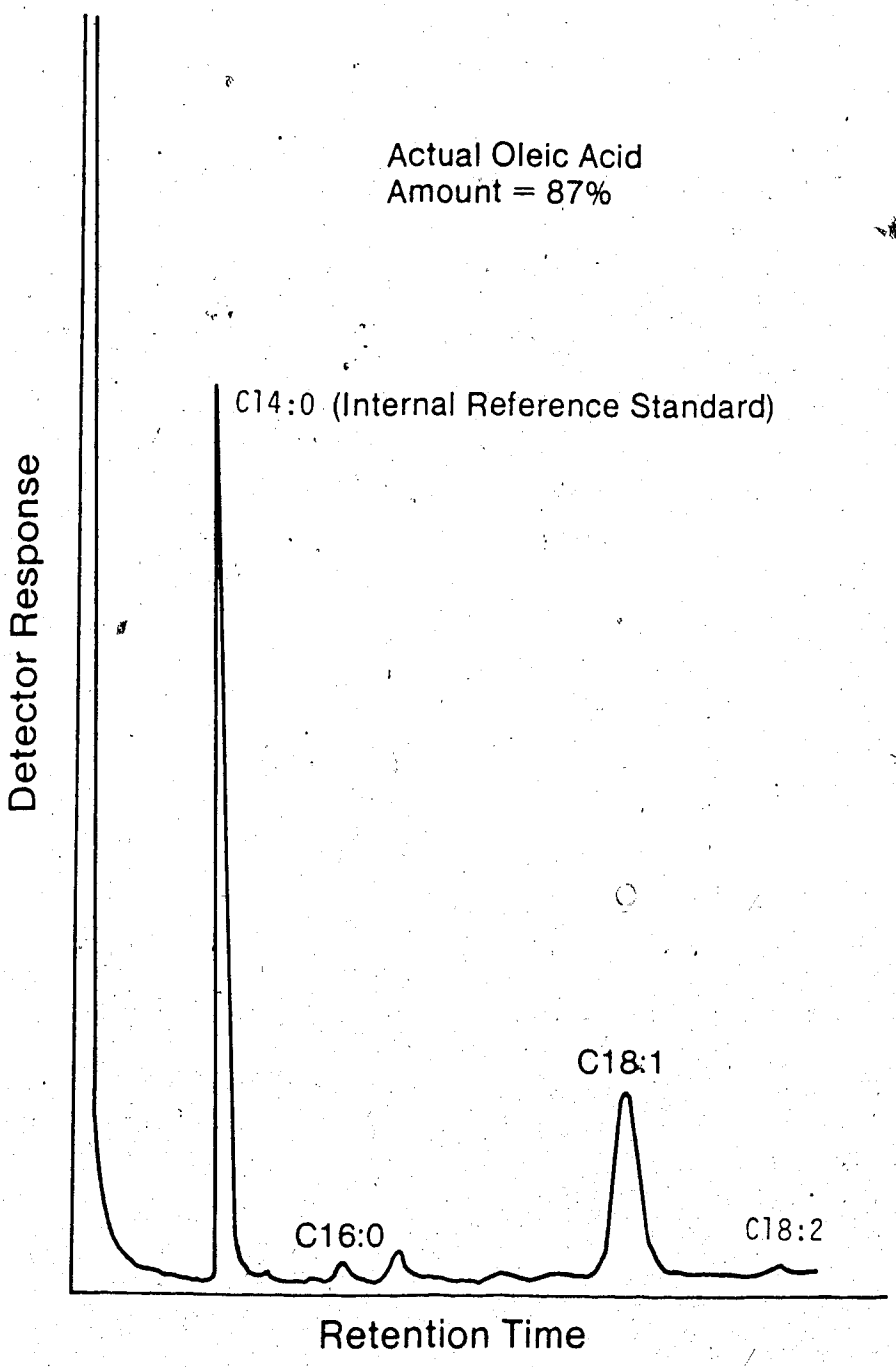


Figure 3.1 Chromatograms of Test Oleic Acid Composition

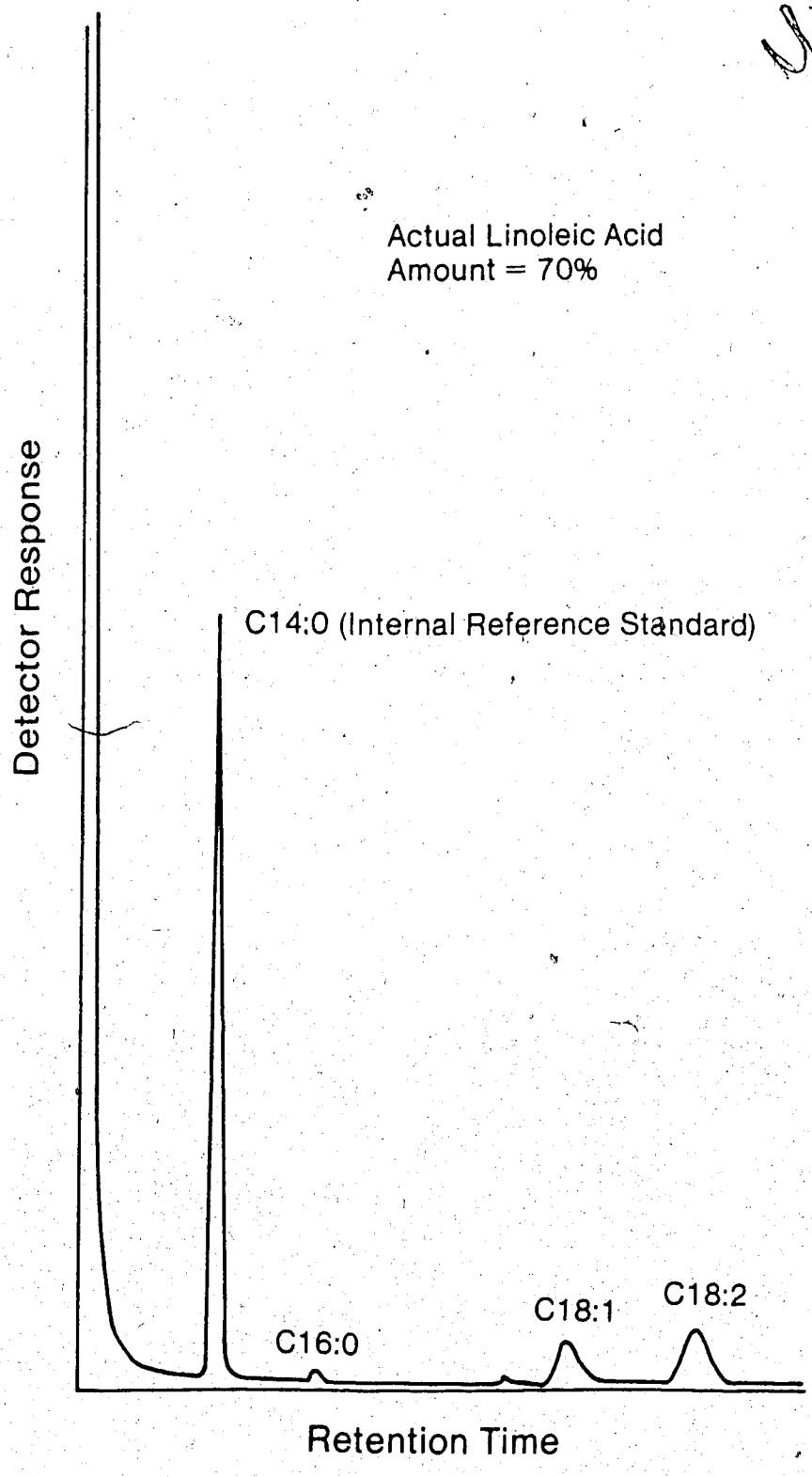


Figure 3.2 Chromatograms of 70 Percent Test Linoleic Acid Composition

Table 3.3 Average Values of Test Oleic Acid Composition at Zero Hour and Ninety-six Hour Period; Test IV and V

Total Fatty Acid Concentration, mg/L as EEM (% of Total Fatty Acids)

pH of Water (Ranges)	Time												
	Zero Hours							Ninety-Six Hours					
	EEM	Myristic 14:0	Palmitic 16:0	Oleic 18:1	Linoleic 18:2	Total Acid	Percentage Recovery	EEM (S.D.)	Palmitic 16:0	Oleic 18:1	Linoleic 18:2	Total Acid	Percentage Recovery
8.69 (8.4-9.0)	73.8	0.79±.07 (2.6%)	1.33±.14 (4.6%)	25.23±2.53 (87.8%)	1.43±.32 (5%)	28.78	48.75	37.7	0.46±.01 (5%)	8.75±.31 (95%)	-	9.21	30.54
7.09 (6.8-7.5)	50.9	-	0.83±.05 (4.7%)	16.27±.3 (91.3%)	0.73±.09 (4.1%)	17.83	43.79	11.4 (3.32)	-	1.79±.63 (100%)	-	1.79	19.71
6.51 (6.45-6.65)	23.3	-	-	5.49±.46 (100%)		5.49	29.45	-	-	4.54±.77 (100%)	-	4.54	23.16

Table 3.4 Average Values of 70 percent Test Linoleic Acid Composition at Zero Hour and Ninety-six Hour Period

Total Fatty Acid Concentration, mg/L as EEM
(% of Total Fatty Acids)

	Time												
	Zero Hour							Ninety-six Hours					
pH of Water (Ranges)	EEM	Palmitic 16:0	Stearic 18:0	Oleic 18:1	Linoleic 18:2	Total Acid	Percentage Recovery	EEM	Palmitic 16:0	Oleic 18:1	Linoleic 18:2	Total Acid	Percentage Recovery
8.74 (8.4-9.0)	45.8	0.97±.087 (3.6%)	-	7.35±.58 (27.4%)	18.49±1.72 (69%)	26.81	73.17	34.2	0.61±.1 (3.6%)	5.49+.02 (32.1%)	11.02±.24 (64.4%)	17.12	62.57
8.64 (8.32-9.0)	34.8	0.58±.05 (3.5%)	0.18±.02 (1.1%)	4.75±.48 (28.5%)	11.15±1.1 (66.9%)	16.66	59.84	14.7	0.27+.01 (10.3%)	0.89±.05 (33.8%)	1.47±.27 (55.9%)	2.63	22.36
7.0 (6.6-7.5)	29.9	0.5±.02 (3.5%)	-	3.92±.18 (27.4%)	9.91±.54 (69.2%)	14.33	59.91	11.1	-	1.55±.06 (37.6%)	2.57±.03 (62.4%)	4.12	46.40
6.99 (6.6-7.5)	18.7	0.27±.02 (3.7%)	-	2.16±.17 (29.3%)	4.95±.34 (67.1%)	7.38	49.33	5.6	-	-	0.07±.001 (100%)	0.07	1.56
6.49 (6.45-6.5)	17.7	-	-	-	-	-	-	16.1	-	0.73±.11 (26.1%)	2.07±.46 (73.9%)	2.80	21.74
6.49 (6.45-6.5)	10.8	-	-	-	-	-	-	4.2	-	-	0.056 (100%)	0.056	1.67

Table 3.5 Average Values of 95 Percent Test Linoleic Acid Composition at Zero Hour and Ninety-six Hour Period

Total Fatty Acid Concentration, mg/L as EEM (% of Total Fatty Acid)

	Time									
	Zero Hour					Ninety-Six Hours				
pH of Water (Ranges)	EEM (S.D.)	Oleic 18:1	Linoleic 18:2	Total Acid	Percentage Recovery	EEM (S.D.)	Oleic 18:1	Linoleic 18:2	Total Acid	Percentage Recovery
8.79 (8.6-9.0)	20.9 (3.18)	0.36±.08 (7.9%)	4.19±.87 (92.1%)	4.55	27.28	6.5 (0.71)	-	0.99±.18 (100%)	0.99	19.04
7.0 (6.7-7.6)	12.6 (1.27)	0.23±.16 (7.7%)	2.77±1.43 (92.3%)	3.00	29.76	4.5	*-	-	-	-
6.5	10.2	-	1.1±1.8 (100%)	1.10	13.35	1.4	-	-	-	-
6.5	5.2	-	0.87±.09 (100%)	0.87	20.91	3.6	-	-	-	-

* Fatty acids esters were not detected.

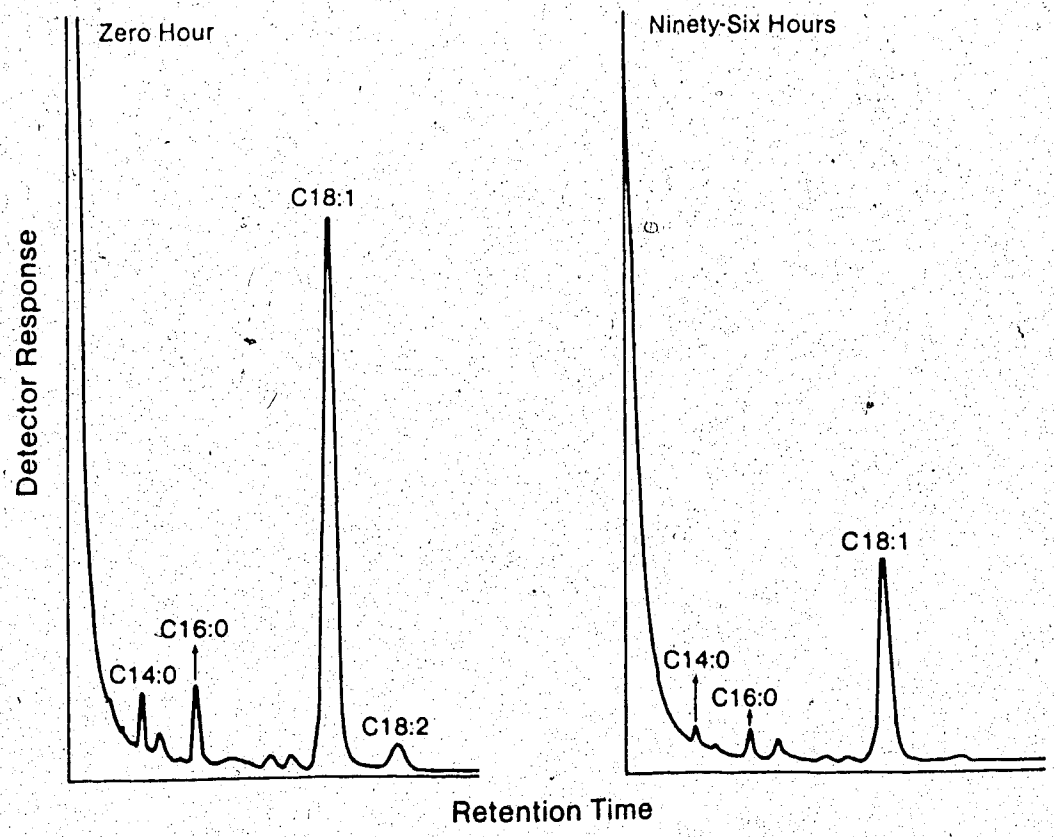


Figure 3.3 Chromatograms of Oleic Acid Toxicity Test at the Zero Hour and Ninety-Six Hour Period at a pH of 8.69 (Sample diluted to 10 ml in Isooctane)

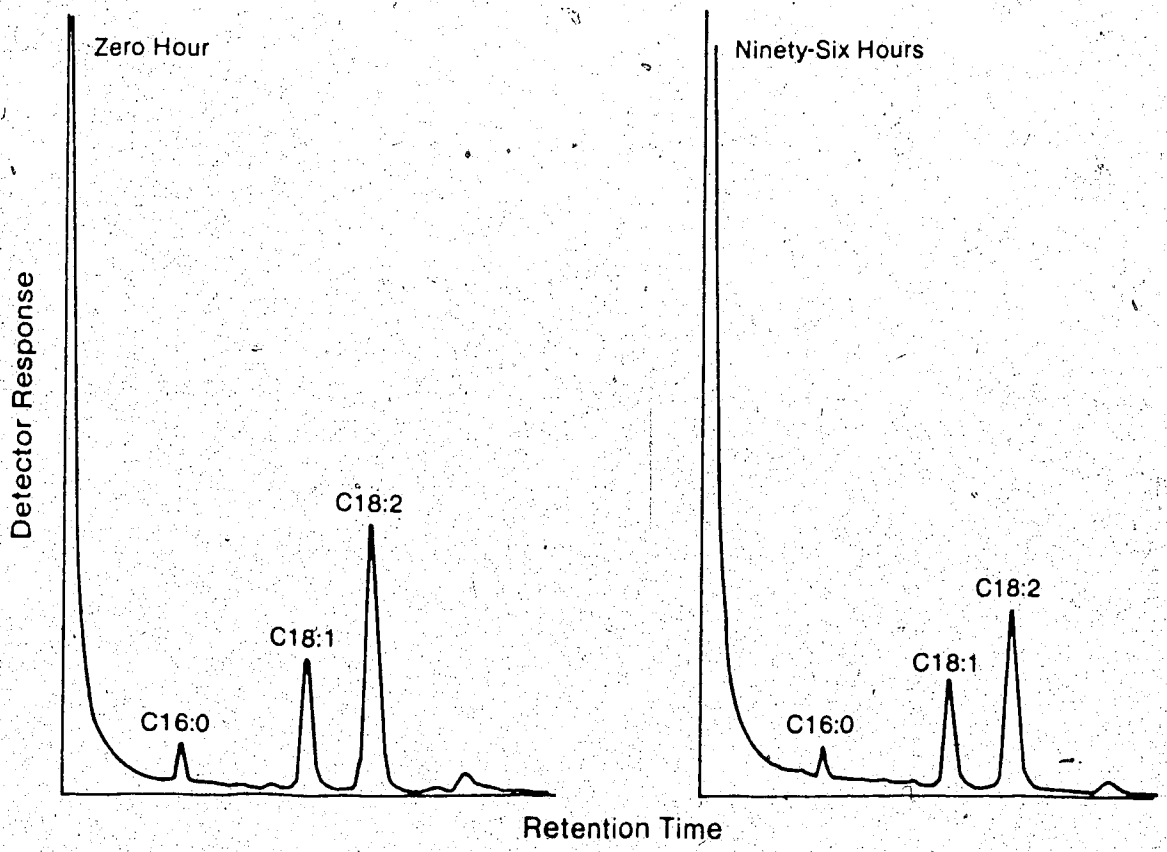


Figure 3.4 Chromatograms of 70 Percent Linoleic Acid Toxicity Test at the Zero Hour and Ninety-six Hour Period at a pH of 8.74 (Sample diluted to 10 ml in Isooctane)

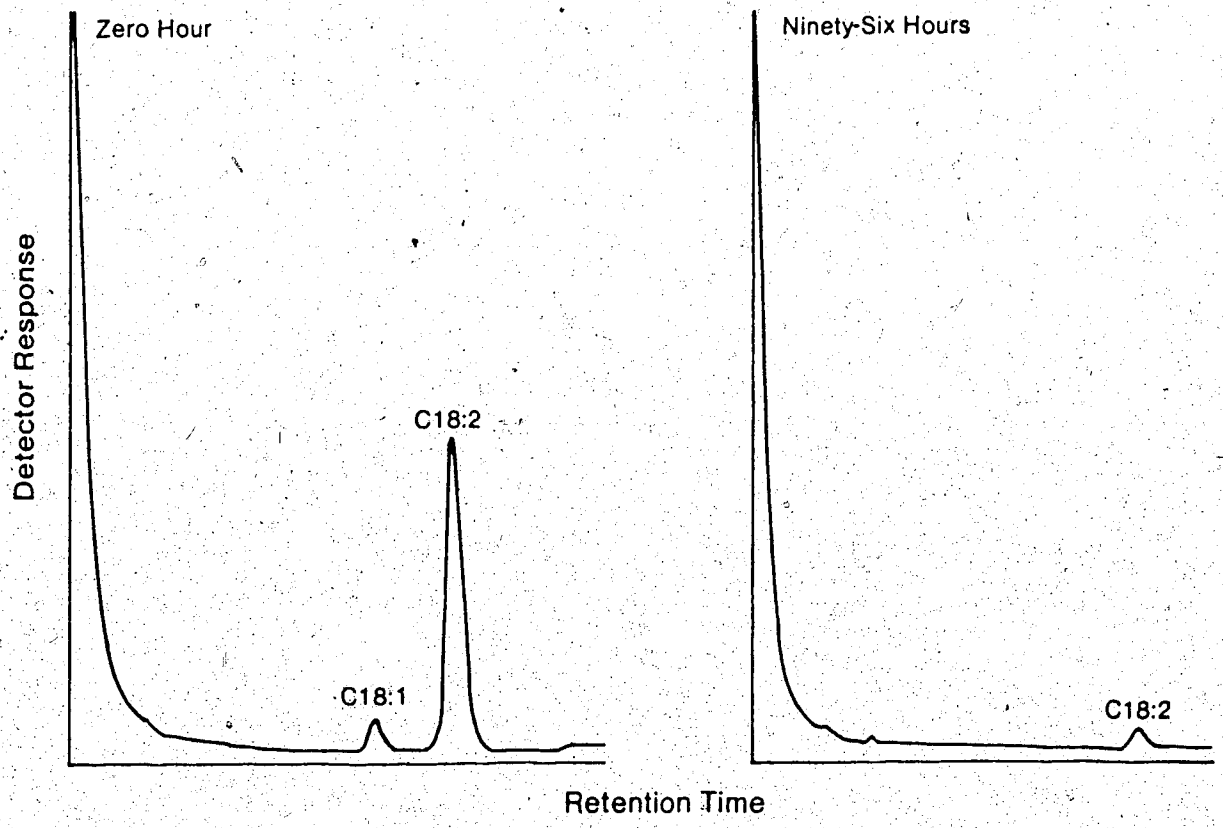


Figure 3.5 Chromatograms of 95 Percent Linoleic Acid Toxicity Test at the Zero Hour and Ninety-six Hour Period at a pH of 8.79 (Sample diluted to 5 ml in Isooctane)

Acute Lethality Studies

The median lethal concentration values (LC50) and 95 percent confidence intervals for oleic and linoleic acids at three pH levels are presented in Tables 3.6 and 3.7, respectively. The 96-hour LC50 values are expressed as milligrams of ether extractable matter (EEM) (mg/L). The 96-hour LC50 of five replicated tests of oleic acid is presented as the geometric mean in Table 3.8, whereas the 96-hour LC50 geometric mean values of two replicated tests of 70 percent acid purity and two duplicated tests of 95 percent acid purity are given in Table 3.9.

It should be noted that the mortality of fish in 70 percent linoleic acid test I was more than 50 percent at the lowest test concentration (Table A6 in appendix). Therefore, this mortality data has to be taken out, as required by Standard Methods (APHA, 1975).

From these results, it can be seen that the LC50 values of oleic acid are approximately 5 to 8 times greater than the LC50 values of linoleic acid at the same pH level. The LC50 values of 70 percent linoleic acid are approximately 1.5 to 2.5 times greater than the LC50 values of 95 percent linoleic acid at the same pH level, indicating that linoleic acid is the predominant active agent in the linoleic acid test mixtures.

The 95 percent confidence intervals of LC50 geometric mean values for oleic acid to rainbow trout are presented in Table 3.10. These confidence interval values represent the

Table 3.6 96-Hour LC50 Values and 95 Percent Confidence Intervals at 10% Trim for Oleic Acid to Rainbow Trout Fingerlings

LC50 Concentration, mg/L as EEM

	Test I			Test II			Test III		
	pH			pH			pH		
	8.78 (8.5-9.0)	7.10 (6.6-7.5)	6.47 (6.4-6.6)	8.74 (8.40-9.0)	7.13 (6.75-7.50)	6.45 (6.4-6.5)	8.63 (8.3-9.0)	7.12 (6.8-7.6)	6.47 (6.45-6.50)
96-Hour LC50	60.4 (50.7-71.9)	48.6 (43.6-54.2)	22.7 (18.4-27.9)	75.8 (61.5-92.9)	44.7 (35.5-56.6)	23.4 (18.4-29.7)	74.6 (60.9-91.4)	48.6 (42.1-56.1)	26.9 (22.9-31.8)
Fish Length (cm)	4.25	4.33	4.30	4.52	4.80	4.50	4.64	4.38	4.38
Standard Deviation	0.41	0.62	0.61	0.55	0.79	0.62	0.52	0.54	0.44
Fish Weight (gm)	0.74	0.78	0.89	0.89	1.17	0.90	0.91	0.85	0.80
Standard Deviation	0.27	0.41	0.43	0.35	0.69	0.44	0.36	0.42	0.28
EDTA Hardness (mg/L as $CaCO_3$)	61.24	60.08	60.45	62.53	61.97	61.97	60.97	60.97	62.43
Standard Error of Mean	1.53	0.82	0.61	1.24	0.55	0.55	1.56	0.02	1.68
Dissolved Oxygen (mg/L)	9.21	9.25	9.32	9.52	9.53	9.64	9.49	9.51	9.53
Standard Error of Mean	0.06	0.08	0.03	0.11	0.13	0.22	0.22	0.29	0.32
Total Alkalinity (mg/L as $CaCO_3$)	125.26	85.33	61.06	111.42	90.58	57.23	117.24	99.19	60.03
Standard Error of Mean	1.32	0.66	0.72	5.73	6.39	1.82	4.08	3.88	1.14

Table 3.6 (continued)

	Test IV			Test V		
	pH			pH		
	8.46 (8.1-9.0)	7.09 (6.8-7.6)	6.52 (6.5-6.6)	8.76 (8.4-9.0)	7.11 (6.8-7.5)	6.51 (6.45-6.55)
96-Hour LC50	86.0 (73.1-101.0)	49.1*1 (48.0-51.9)	35.3 (30.7-40.7)	93.1 (81.3-106.6)	42.6 (34.4-52.8)	29.8 (25.3-35.2)
Fish Length (cm)	3.75	3.95	3.75	4.00	4.32	3.90
Standard Deviation	0.43	0.57	0.52	0.35	0.29	0.26
Fish Weight (gm)	0.49	0.56	0.46	0.45	0.69	0.42
Standard Deviation	0.18	0.22	0.22	0.13	0.17	0.18
EDTA Hardness (mg/L as $CaCO_3$)	60.13	60.49	62.25	60.43	61.08	61.03
Standard Error of Mean	0.68	0.99	0.99	1.05	0.32	0.78
Dissolved Oxygen (mg/L)	9.37	9.41	9.42	9.51	9.56	9.53
Standard Error of Mean	0.11	0.09	0.11	0.06	0.04	0.05
Total Alkalinity (mg/L as $CaCO_3$)	121.74	99.72	59.23	114.06	97.48	58.95
Standard Error of Mean	0.90	1.16	0.59	0.56	1.04	0.32

*1 = at untrimmed calculable

Table 3.7 96-Hour LC50 Values and 95 Percent Confidence Intervals at 10% Trim for Linoleic Acid to Rainbow Trout Fingerlings

LC50 Concentration, mg/L as EEM

	Test I*1			Test II		
	pH			pH		
	8.74 (8.3-9.0)	7.06 (6.6-7.5)	6.49 (6.45-6.5)	8.70 (8.3-9.0)	7.04 (6.6-7.5)	6.49 (6.45-6.55)
96-Hour LC50	24.9 (19.6-31.2)	10.3 (8.4-13.7)	-	26.7 (21.3-33.4)	9.2 (7.4-11.5)	6.4 (5.8-7.1)
Fish Length (cm)	4.08	4.26	4.62	4.18	4.49	4.55
Standard Deviation	0.71	0.75	0.64	0.67	0.48	0.42
Fish Weight (gm)	0.76	0.91	1.11	0.75	0.92	0.89
Standard Deviation	0.44	0.50	0.49	0.39	0.29	0.21
EDTA Hardness (mg/L as $CaCO_3$)	59.63	59.73	60.17	59.75	59.61	60.04
Standard Error of Mean	0.66	0.50	1.70	1.19	1.13	1.28
Dissolved Oxygen (mg/L)	9.62	9.60	9.66	9.66	9.65	9.73
Standard Error of Mean	0.23	0.40	0.26	0.16	0.17	0.34
Total Alkalinity (mg/L as $CaCO_3$)	117.45	94.78	50.68	116.29	92.15	50.31
Standard Error of Mean	2.14	3.29	0.64	4.35	5.23	0.83

*1 Test I and II = 70% Acid; Test III and Test IV = 95% Acid

Table 3.7 (continued)

	Test III			Test IV		
	pH			pH		
	8.76 (8.4-9.0)	6.99 (6.7-7.5)	6.51 (6.5-6.6)	8.82 (8.4-9.0)	7.05 (6.75-7.60)	6.51 (6.5-6.6)
96-Hour LC50	10.6 (8.2-13.6)	8.2 (6.6-10.2)	5.7*2	8.6 (6.8-10.9)	6.1 (4.9-5.6)	4.8 (4.0-5.7)
Fish Length (cm)	4.57	4.81	4.76	5.08	5.02	5.13
Standard Deviation	0.54	0.58	0.49	0.57	0.45	0.53
Fish Weight (gm)	1.00	1.15	1.14	1.42	1.39	1.52
Standard Deviation	0.40	0.42	0.37	0.47	0.38	0.51
EDTA Hardness (mg/L as $CaCO_3$)	61.82	60.47	60.61	59.40	59.90	59.71
Standard Error of Mean	1.74	1.33	0.42	0.86	1.17	0.76
Dissolved Oxygen (mg/L)	9.53	9.49	9.61	9.67	9.54	9.48
Standard Error of Mean	0.11	0.06	0.13	0.22	0.12	0.07
Total Alkalinity (mg/L as $CaCO_3$)	114.34	96.03	50.56	113.08	95.54	57.72
Standard Error of Mean	2.94	3.25	0.63	1.32	4.37	0.90

*2 = 95% Confidence Interval Not Calculable.

Table 3.8 LC50 Geometric Mean Values and Standard Deviations for Oleic Acid to Rainbow Trout Fingerlings

LC50 concentration, mg/L as EEM

	pH		
	8.65 (8.1-9.0)	7.12 (6.6-7.6)	6.46 (6.4-6.6)
96-Hour LC50	77.1 (65.4-90.9)	46.8 (43.8-50.0)	27.2 (22.7-32.7)
Fish Length (cm)	4.23	4.36	4.17
Standard Error of Mean	0.37	0.30	0.32
Fish Weight (gm)	0.70	0.74	0.69
Standard Error of Mean	0.22	0.29	0.24
EDTA Hardness (mg/L as $CaCO_3$)	60.24	61.12	61.41
Standard Error of Mean	0.64	1.12	0.29
Dissolved Oxygen (mg/L)	9.39	9.51	9.43
Standard Error of Mean	0.09	0.15	0.07
Total Alkalinity (mg/L as $CaCO_3$)	118.64	94.26	59.05
Standard Error of Mean	0.94	0.01	1.05

Table 3.9 LC50 Geometric Mean Values and Ranges for Linoleic Acid to Rainbow Trout Fingerlings

LC50 Concentration, mg/L as EEM

	70% Acid Purity pH			95% Acid Purity pH		
	8.73 (8.3-9.0)	7.06 (6.6-7.5)	6.49 (6.45-6.55)	8.79 (8.4-9.0)	7.10 (6.7-7.6)	6.51 (6.5-6.6)
96-Hours LC50 (Ranges)	25.8 (24.9-26.7)	9.8 (9.2-10.3)	6.4* (5.8-7.1)	9.5 (8.6-10.6)	7.1 (6.1-8.2)	5.2 (4.8-5.6)
Fish Length (cm)	4.13	4.38	4.59	4.83	4.92	4.95
Standard Error of Mean	0.07	0.16	0.05	0.36	0.15	0.26
Fish Weight (gm)	0.76	0.92	1.00	1.21	1.27	1.33
Standard Error of Mean	0.01	0.01	0.16	0.30	0.17	0.27
EDTA Hardness (mg/L as $CaCO_3$)	59.94	59.71	60.42	60.82	60.19	60.22
Standard Error of Mean	0.84	0.32	1.54	1.20	1.07	0.53
Total Alkalinity (mg/L $CaCO_3$)	117.07	93.97	50.58	113.73	95.79	53.40
Standard Error of Mean	2.29	3.05	0.70	2.11	3.70	1.50
Dissolved Oxygen (mg/L)	9.68	9.53	9.63	9.62	9.48	9.55
Standard Error of Mean	0.20	0.08	0.22	0.11	0.05	0.08

*Only 1 test and LC50 Value from Trimmed Spearman-Karber Method

Table 3.10 96-Hour LC50 Geometric Mean Values and 95 Percent Confidence Intervals for Oleic Acid to Rainbow Trout Fingerlings

pH of Test Water (Ranges)	LC50 Concentration, mg/L as EEM LC50 Geometric Mean	Confidence Interval
8.65 (8.1-9.0)	77.1	73.9 - 80.4
7.12 (6.6-7.6)	46.8	43.8 - 49.8
6.46 (6.4-6.6)	27.2	24.5 - 30.6

LC50 range within which 19 out of 20 test results should fall. These values represent a reasonably narrow range about the LC50 geometric mean values. Normally, the LC50 values of any bioassay tests would vary from test to test depending upon many variable factors. First, the difference in susceptibility of individual organisms is the main limiting factor (Warren, 1971). Second, the laboratory conditions will vary from time to time. When considreing LC50 values for the natural aquatic environment, some more variable factors would occur resulting in a wider confidence interval around the true LC50 value. For example, the natural aquatic environment will differ from the laboratory conditions. Therefore, knowledge of the confidence interval values as an indication of the variability of LC50 determination is important to enable wise usage to be made of LC50 values for regulatory purposes. Thus the confidence intervals as well as the geometric mean values should be considered for regulatory purposes.

Median survival time (MST), 95 percent confidence intervals and rate of mortality values for fish exposed to oleic and linoleic acid at three test pH are presented in Tables 3.11, 3.12 and 3.13. MST values at the adjusted pH 9.0 test condition are little higher than that for the adjusted pH 7.5 and 6.5 test condition. However, MST values at the adjusted test pH 7.5 and 6.5 test condition are approximately the same. The MST values increase with decreasing fatty acid concentration and range from 2.30 to

Table 3.11 Median Survival Time (MST), 95 Percent Confidence Intervals and Rate of Mortality (1/MST) of Oleic Acid with Rainbow Trout Fingerlings

pH of Test Water (Ranges)	F.A.C.A.*1 mg/L	EEM mg/L (S.D.)	MST hrs.	Rate of Mortality 1/hrs.
8.58 (8.1-9.0)	100.0	83.16 (4.89)	71.5*2	0.014
8.78 (8.4-9.0)	135.0	127.35 (8.64)	6.98 (5.72-8.52)	0.143
7.09 (6.6-7.6)	75.0	51.20 (7.61)	90.0*2	0.011
7.08 (6.8-7.6)	100.0	75.25 (20.65)	14.0 (11.74-16.98)	0.071
7.32 (7.0-7.6)	135.0	133.0 (1.41)	2.15 (1.8-2.68)	0.465
6.5 (6.4-6.6)	42.0	31.52 (3.31)	93.0*2	0.011
6.48 (6.4-6.6)	56.0	48.02 (4.24)	17.1 (14.98-20.47)	0.058
6.49 (6.45-6.6)	75.0	74.0	4.15 (3.89-5.36)	0.241

F.A.C.A.*1 = Fatty Acids Concentration Added
*2 = 95% Confidence Not Calculable

Table 3.12 Median Survival Time (MST), 95 Percent Confidence Intervals and Rate of Mortality (1/MST) of 70 Percent Linoleic Acid with Rainbow Trout Fingerlings

pH of Test Water (Ranges)	F.A.C.A.* mg/L	EEM mg/L (S.D.)	MST hrs.	Rate of Mortality 1/hrs.
8.74 (8.4-9.0)	56.0	46.07 (4.36)	23.1 (21.0-25.41)	0.043
8.75 (8.45-9.0)	100.0	97.80 (2.05)	12.4 (10.09-14.31)	0.081
7.0 (6.6-7.5)	32.0	27.58 (3.29)	7.25 (5.66-9.38)	0.138
7.12 (6.8-7.5)	56.0	56.0 (2.12)	3.75 (3.38-4.27)	0.267
7.5	100.0	96.50	2.30 (2.05-2.58)	0.435
6.49 (6.45-6.5)	5.6	8.59 (0.48)	36.1 (28.74-46.74)	0.028
6.49 (6.45-6.5)	10.0	10.14 (0.33)	10.9 (8.25-14.39)	0.092
6.48 (6.4-6.5)	18.0	17.62 (0.16)	5.2 (4.22-6.62)	0.192
6.5	32.0	32.0	4.2 (3.76-4.95)	0.238
6.5	56.0	51.42	3.70 (3.45-4.11)	0.270

F.A.C.A.* = Fatty Acids Concentration Added

Table 3.13 Median Survival Time (MST), 95 Percent Confidence Intervals and Rate of Mortality (1/MST) of 95 Percent Linoleic Acid with Rainbow Trout Fingerlings

pH of Test Water (Ranges)	F.A.C.A.*1 mg/L	EEM mg/L (S.D.)	MST hrs.	Rate of Mortality 1/hrs.
8.79 (8.6-9.0)	18.0	14.90 (7.10)	17.0 (14.1-20.2)	0.059
8.84 (8.6-9.0)	56.0	52.30 (4.53)	4.4 (3.42-5.99)	0.227
7.0 (6.7-7.6)	10.0	9.67 (4.21)	22.1*2	0.045
6.94	18.0	19.20 (3.82)	4.8 (3.82-5.67)	0.208
7.14 (6.7-7.6)	32.0	31.70 (3.11)	3.2 (2.34-4.38)	0.313
6.5	5.6	8.52 (1.53)	12.0 (8.89-16.20)	0.083
6.5	10.0	12.50 (1.77)	5.0 (4.10-6.10)	0.200
6.5	18.0	20.70	2.9 (2.54-3.42)	0.339

F.A.C.A.*1 = Fatty Acids Concentration Added
*2 = 95% Confidence Not Calculable

93.0 hours. In this study, the different acid concentrations were used at the three pH levels. Therefore, the MST values could not validly be compared to indicate the effect of test pH on acute lethality of both acids to rainbow trout.

Rate of mortality values (1/hrs) increase with increasing acid concentration. The rate of mortality's trends are approximately the same in the three pH test condition.

Mortality curves for oleic and linoleic acid to rainbow trout fingerling at three test pH are constructed using log median survival times and log concentrations (Figures 3.6, 3.7 and 3.8). Each point represents the median survival time (in hours) of fish at each concentration and vertical bars represent 95 percent confidence intervals.

pH affected the acute lethality of oleic and linoleic acid. For both fatty acids, the 96-hour LC50 values in all three test pH levels are found significantly different ($P \geq 0.05$, two-way analysis of variance). However, the difference at each replicated test of each acid is not significant at the 5 percent level by two-way analysis of variance.

The 96-hour LC50 values obtained at each of the test pH values were found to be significantly different ($P \geq 0.05$, unpaired student t-test) between the two acids.

As well, the 70 and 95 percent linoleic acid, 96-hour LC50 values were found to be different for the adjusted pH 9.0 test condition, at the 5 percent significance level by unpaired student t-test. However, at the adjusted pH 7.5

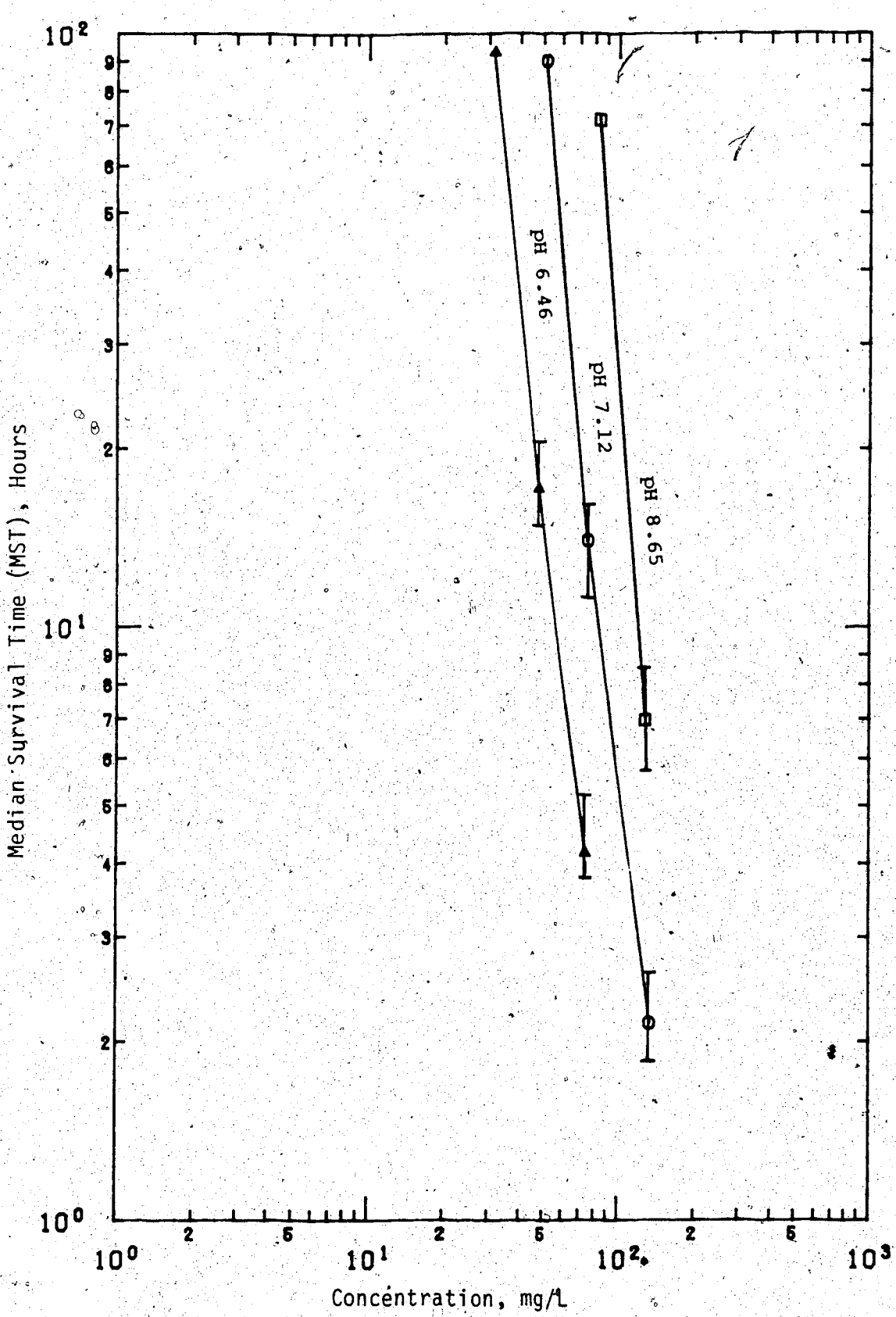


Figure 3.6 Mortality Curves of Oleic Acid to Rainbow Trout Fingerlings (Vertical Bars represent 95 percent confidence intervals)

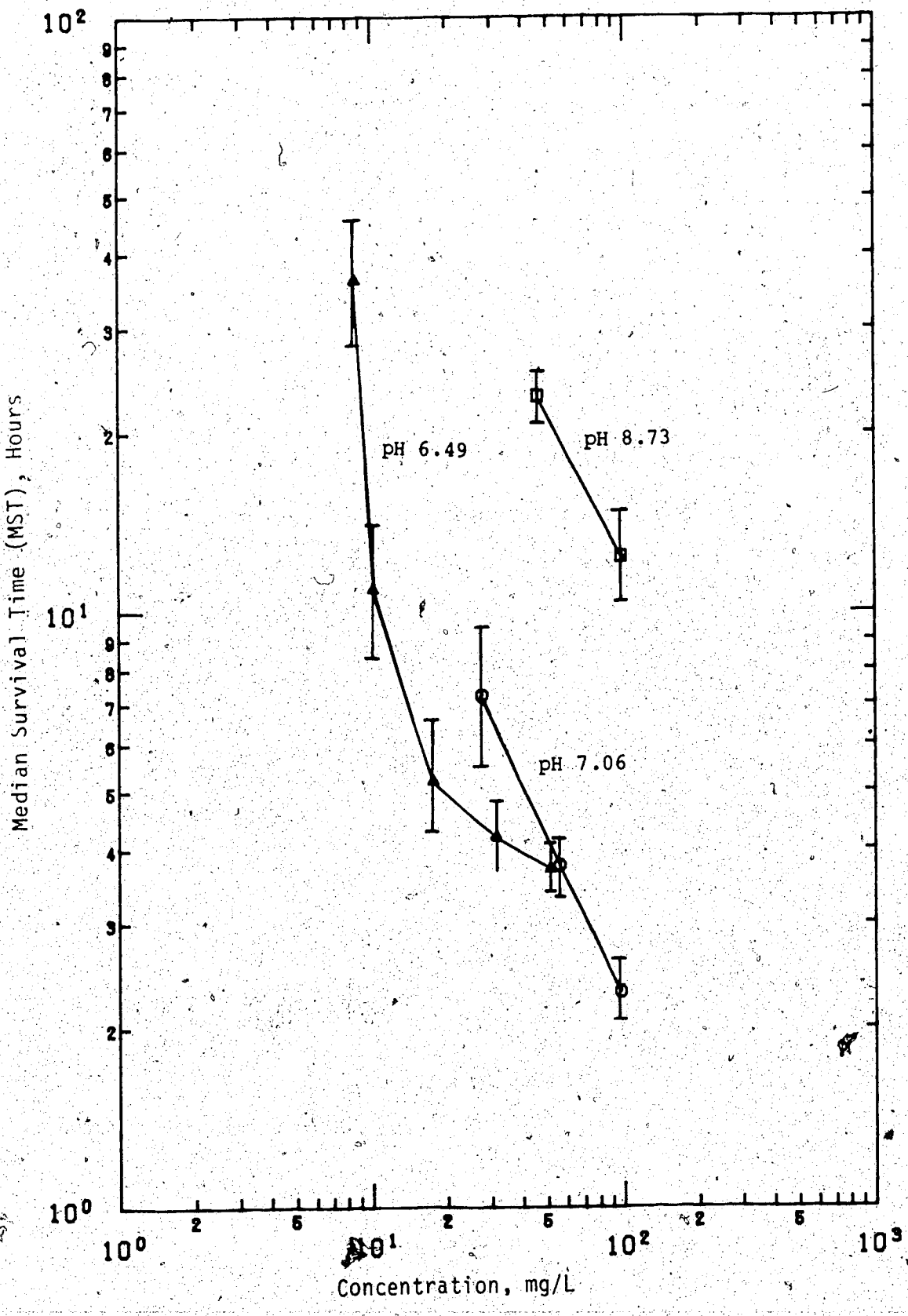


Figure 3.7 Mortality Curves of 70 Percent Linoleic Acid to Rainbow Trout Fingerlings (Vertical Bars represent 95 percent confidence in intervals)

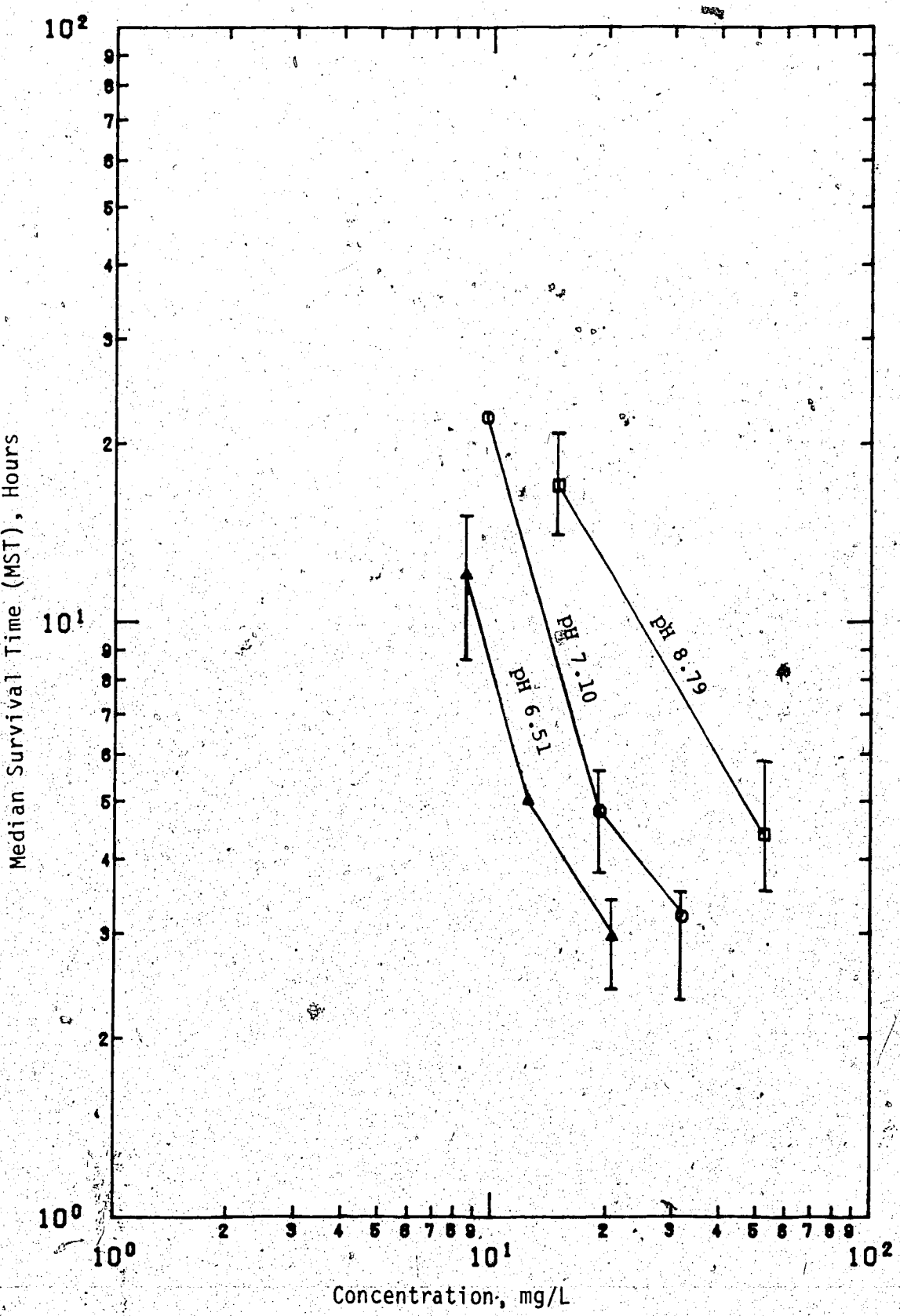


Figure 3.8 Mortality Curves of 95 Percent Linoleic Acid to Rainbow Trout Fingerlings (Vertical bars represent 95 percent confidence intervals)

test condition, the LC50 values were found significantly different at the 1 percent significance level. The difference at the adjusted pH 6.5 test condition were not significant at the 5 percent level by unpaired student t-test. Figure 3.9 summarizes the difference in 96-hour LC50 geometric mean values of oleic and linoleic acids at each pH level.

Characteristics of Test Water.

The characteristics of test water for both experiments are presented in Table A10 to A21 (in appendix). The list of approximate values are: dissolved oxygen greater than 9.0 mg/L, EDTA hardness approximately 60.0 mg/L as $CaCO_3$, water temperature 12±1°C, ammonia-nitrogen less than 0.25 mg/L and total alkalinity approximately 120 mg/L, 90 mg/L, and 59 mg/L as $CaCO_3$ at the adjusted pH 9.0, 7.5 and 6.5 test conditions, respectively. Only alkalinity at the adjusted pH 9.0 and 7.5 test conditions were increased every day by the effect of pH readjustment. Tables 3.14 to 3.16 summarize the phenolphthalein and total alkalinity of both experiments from the first day to the last day of the experiment (96 hours) (Figure 3.10).

B. Discussion

Acute Lethality Studies

The results from this study indicates that pH alters

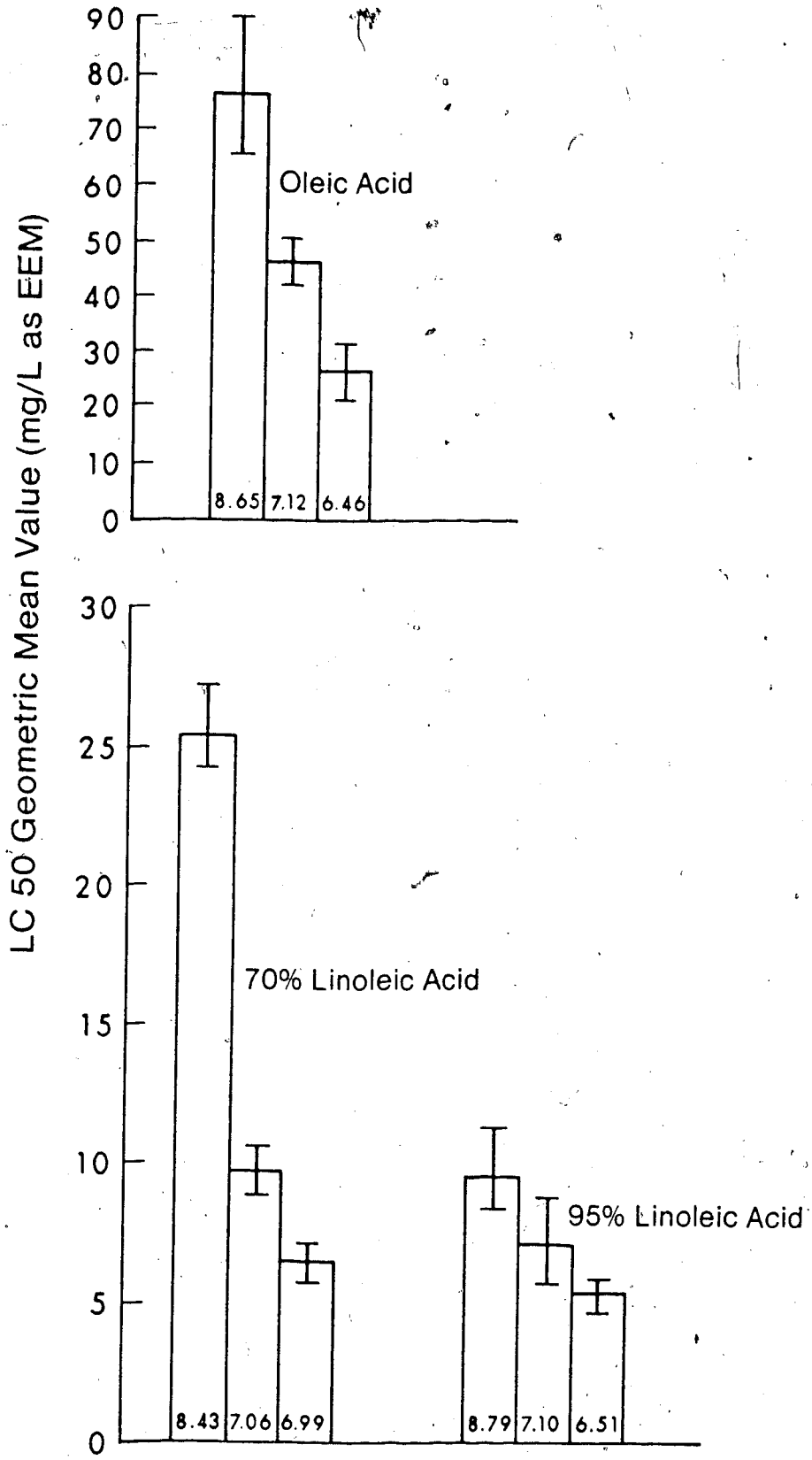


Figure 3.9 LC50 Geometric Mean Values of Oleic and Linoleic Acid with Rainbow Trout Fingerlings (Number in histograms indicate pH test condition)

Table 3.14 Phenolphthalein and Total Alkalinity (mg/L as $CaCO_3$) of Oleic Acid Toxicity Test from Zero Hour to Ninety-six Hour Period

	Time of Experiment									
	Zero Hour		24 Hours		48 Hours		72 Hours		96 Hours	
	Alkalinity				Alkalinity		Alkalinity		Alkalinity	
pH (Ranges)	P	Total	P	Total	P	Total	P	Total	P	Total
8.65 (8.1-9.0)										
Mean	24.32	105.70	27.10	118.28	26.20	122.50	26.30	125.70	12.00	127.41
Standard Error of Mean	2.13	2.36	1.98	2.95	2.03	2.10	1.98	2.35	1.03	3.24
7.12 (6.6-7.6)										
Mean	-	82.35	-	86.33	-	91.20	-	99.48	-	102.30
Standard Error of Mean	-	2.17	-	1.98	-	2.20	-	3.10	-	3.52
6.46 (6.4-6.6)										
Mean	-	50.20	-	51.31	-	50.98	-	51.48	-	52.08
Standard Error of Mean	-	2.12	-	1.98	-	2.32	-	2.56	-	1.87

Table 3.15 Phenolphthalein and Total Alkalinity (mg/L as $CaCO_3$) of 70 Percent Linoleic Acid Toxicity Test from Zero Hour to Ninety-six Hour Period

	Time of Experiment									
	Zero Hour		24 Hours		48 Hours		72 Hours		96 Hours	
	Alkalinity				Alkalinity		Alkalinity		Alkalinity	
pH (Ranges)	P	Total	P	Total	P	Total	P	Total	P	Total
8.73 (8.3-9.0)										
Mean	27.84	111.60	30.30	115.66	28.87	117.74	28.56	121.56	17.0	127.38
Standard Error of Mean	2.26	2.06	1.88	2.53	2.14	1.73	0.54	1.78	2.58	3.99
7.06 (6.6-7.5)										
Mean	-	87.42	-	90.35	-	93.51	-	102.51	-	106.80
Standard Error of Mean	-	1.68	-	1.31	-	2.87	-	4.81	-	5.09
6.49 (6.45-6.55)										
Mean	-	49.52	-	52.23	-	50.06	-	50.83	-	50.10
Standard Error of Mean	-	0.94	-	0.67	-	0.67	-	1.73	-	1.26

Table 3.16 Phenolphthalein and Total Alkalinity (mg/L as $CaCO_3$) of 95 Percent Linoleic Acid Toxicity Test from Zero Hour to Ninety-six Houf Period

	Time of Experiment									
	Zero Hour		24 Hours		48 Hours		72 Hours		96 Hours	
	Alkalinity				Alkalinity		Alkalinity		Alkalinity	
pH (Ranges)	P	Total	P	Total	P	Total	P	Total	P	Total
8.79 (8.4-9.0)										
Mean	30.11	106.29	29.80	113.72	28.55	116.04	27.43	119.15	11.07	121.40
Standard Error of Mean	1.11	2.25	1.00	1.35	0.50	0.43	0.48	0.82	0.93	2.50
7.10 (6.7-7.6)										
Mean	-	87.92	-	94.25	-	98.93	-	106.80	-	103.18
Standard Error of Mean	-	2.22	-	0.94	-	1.14	-	3.58	-	2.90
6.51 (6.5-6.6)										
Mean	-	53.67	-	53.09	-	53.39	-	53.74	-	54.20
Standard Error of Mean	-	2.27	-	2.56	-	0.64	-	2.71	-	0.77

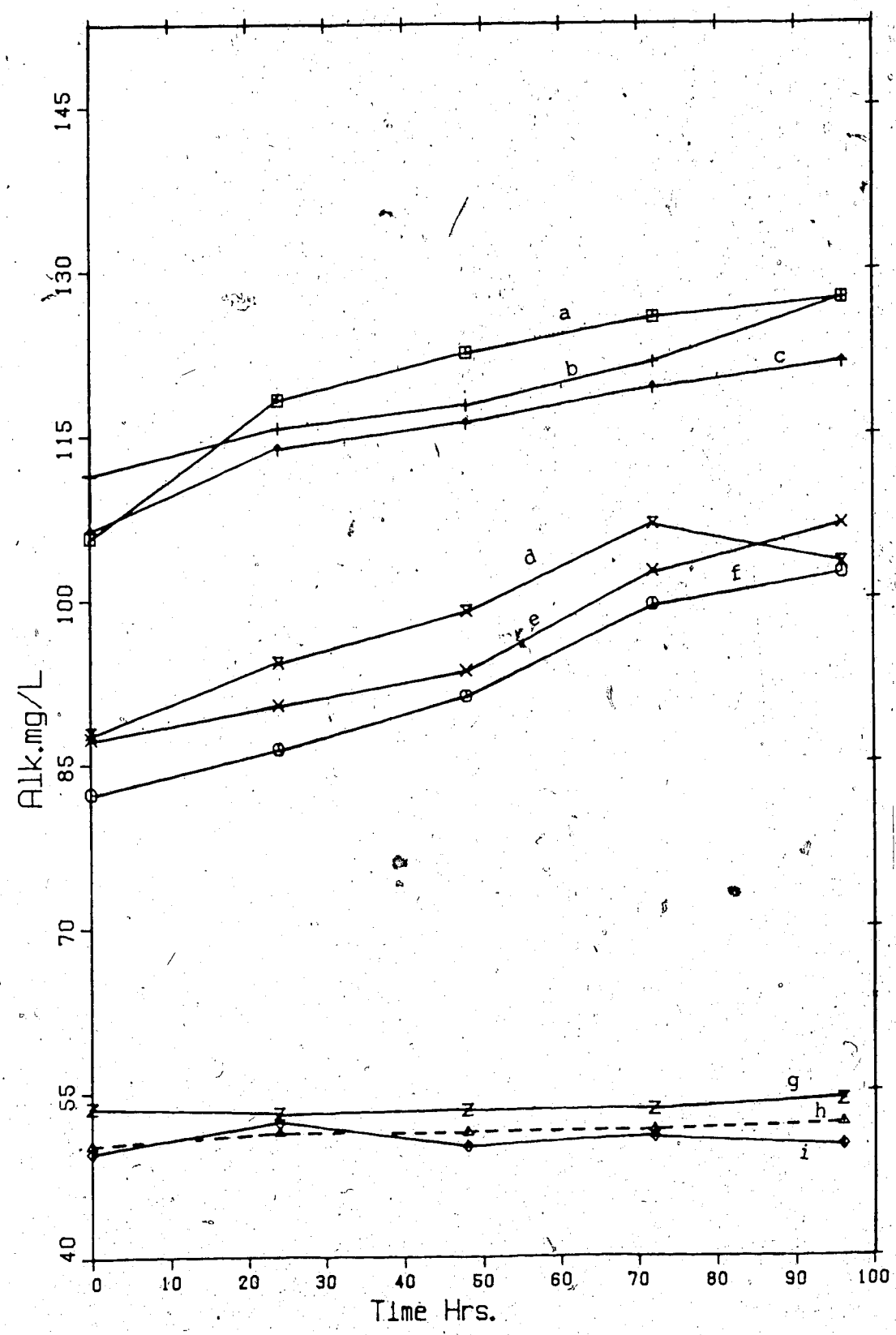


Figure 3.10 Relationship of Alkalinity Values of Test Water to the Time Period of Experiment

a = Oleic Acid Toxicity Test at a pH of 8.65
b = 70% Linoleic Acid Toxicity Test at a pH of 8.73
c = 95% Linoleic Acid Toxicity Test at a pH of 8.79
d = 95% Linoleic Acid Toxicity Test at a pH of 7.10
e = 75% Linoleic Acid Toxicity Test at a pH of 7.06
f = Oleic Acid Toxicity at a pH of 7.12
g = 95% Linoleic Acid Toxicity Test at a pH of 6.51
h = Oleic Acid Toxicity Test at a pH of 6.46
i = 75% Linoleic Acid Toxicity Test at a pH of 6.49

the toxicity of oleic and linoleic acids to rainbow trout fingerlings. The effect of pH on the susceptibility of fish, as indicated by LC50 (Tables 3.6 to 3.9) were observed in the first 96 hour period.

A similar pattern of pH effects to that observed in this study has been reported by other investigators using different toxicants on various fish species. Kraft pulp and paper mill effluent (Ladd, 1969, and Mcleay et al., 1979), and antimycin (Marking, 1975) are more toxic in acid solutions, whereas zinc (Mount, 1966) is more toxic at alkaline pH values. However, kraft pulp and paper mill effluents are also more toxic at the extreme alkaline pH values (pH > 10) (Mcleay et al., 1979). The rapid mortalities at the extreme alkaline pH values may be due to the inability of rainbow trout to tolerate such pH extremes (Jordan and Lloyd, 1964 and Eicher, 1946).

According to Lloyd and Jordan (1964), who had been studying factors affecting the resistance of rainbow trout to acid waters, pH values below 5.0 might be harmful to rainbow trout since death could be brought about by acidaemia. The rate of mortality was raised by the presence of free carbon dioxide in the acid water. Several investigators have stressed that two possible toxic factors may arise from acid waste discharge. First, if the water has a high bicarbonate content, sufficient free carbon dioxide may be liberated by the acid to kill fish even though the pH value does not fall to a level which would be dangerous in

the absence of carbon dioxide (Doudoroff and Katz, 1950 and Lloyd and Jordan, 1964). The amount of carbon dioxide required to kill rainbow trout is about 100 mg/L if the dissolved oxygen content of the water is at the air-saturation value (Alabaster et al., 1957). Second, if the water has a low bicarbonate content, the pH value of the water may be reduced to a lethal level without liberating a harmful concentration of carbon dioxide (Lloyd and Jordan, 1964). The pH values below 4.0 are likely to be toxic to fish (Doudoroff and Katz, 1950). Lloyd and Jordan (1964) also suggested that the resistance of fish to acids might also be influenced by the calcium content of the water.

Hardness and alkalinity of water appear to be involved in toxicity of weak acids and heavy metals. Copper and zinc (Clarke, 1974 and Mount, 1974) and ground-wood mill effluent (Middelraad and Wilson, 1975) decreased in toxicity markedly with increasing hardness.

Resin acids and long chain fatty acids are the major and minor contributors to the toxicity of unbleached kraft mill effluents, respectively (Leach and Thakore, 1973, Rogers, 1973 and Mueller and Walden, 1974). Their toxicity are also decreased markedly with increasing hardness and alkalinity (Mcleay et al., 1979). A direct dependence is only observed when the hardness or alkalinity of test water exceeds 100 mg/L as $CaCO_3$.

For this study, the test water was soft (hardness = 60 mg/L as $CaCO_3$). Therefore, the calcium, magnesium and any

other ions causing hardness would not influence the precipitation of the sodium salt of fatty acid. The total alkalinity was less than 100 mg/L as $CaCO_3$ in the adjusted pH 6.5 and pH 7.5 test conditions and more than 100 mg/L as $CaCO_3$ (= 115 mg/L) in the adjusted pH 9.0 test condition. It can be noted that the alkalinity might appear to be responsible for the decrease in the toxicity of fatty acids to rainbow trout fingerlings at the adjusted pH 9.0 test condition (Figure 3.11). However, the high LC50 values (less toxicity) at the adjusted pH 9.0 test condition might be mainly caused by another mechanism which will be discussed later.

The dissolved oxygen was kept above 9.0 mg/L by aeration. This aeration contributed to the precipitation of toxicant on the surface of test water. Consequently, the concentration of oleic and linoleic acid decreased gradually from the first day to the last day of the experiment. The higher initial concentrations decreased more rapidly than the lower initial concentrations. Therefore, the toxicity concentration data were interpolated directly from the first day to the last day data, as mentioned in the experimental design.

Other water characteristics are not clearly implicated in the toxicity results. Dissolved ammonia, or ammonium hydroxide at concentrations of 2 to 7 mg/L as NH_3 is toxic to fish. The toxicity of ammonia salts is much less harmful than the base in the same water, and its toxicity can be

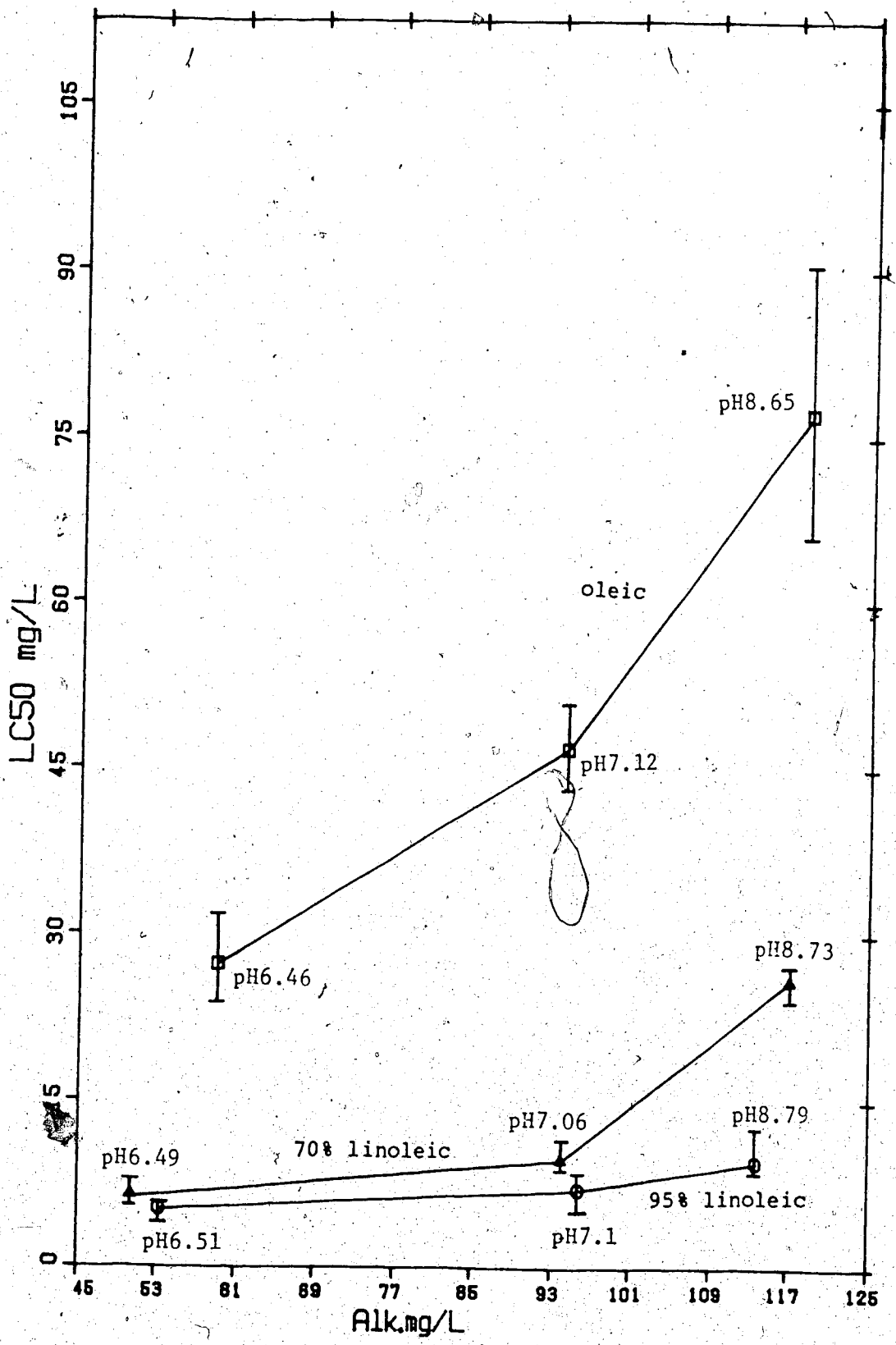


Figure 3.11 Relationship of Alkalinity Values of Test Water to LC50 Geometric Mean Values of Oleic and Linoleic Acids with Rainbow Trout Fingerlings (Vertical bars represent standard deviation for oleic acid and ranges for linoleic acid)

reduced or eliminated by the addition of acids (Doudoroff, 1950). Hrudey (1978) indicated that ammonia in water would begin an equilibrium between NH_3 and NH_4^+ with dependence upon pH of water. The higher pH will favour NH_3 and lower pH will favour NH_4^+. Undissociated ammonia will exert the toxic effect upon aquatic organisms. The recommended safe concentration of undissociated ammonia to aquatic organism is 0.02 mg/L. A plot of the total ammonia concentrations which will produce an undissociated ammonia concentration of 0.02 mg/L with different pH is presented in Figure 3.12. The ammonia level of test water in this study was less than 0.25 mg/L as NH_3. Therefore, this ammonia levels observed would not be toxic to rainbow trout fingerlings.

Physiological Effect of pH on Toxicity of Long Chain Fatty Acids

The passage of toxicants across gill membranes in fish was reported by Hunn and Allen (1974). They found that this passage was governed by physical processes and was predictable from the dissociation constant of toxicants (pKa) and lipid solubility of the chemical. For example, a weak acid was least toxic to fish at pH 9.5. Similar results were reported by Marking (1975), as previously mentioned. The ionization (pH-partition) theory which support the Hunn and Allen's Theory, was reported by Jollow and Brodie (1972) and Loomis (1970). Fatty acids which are weak acids, ionize in alkaline solutions and the ionized form is not expected

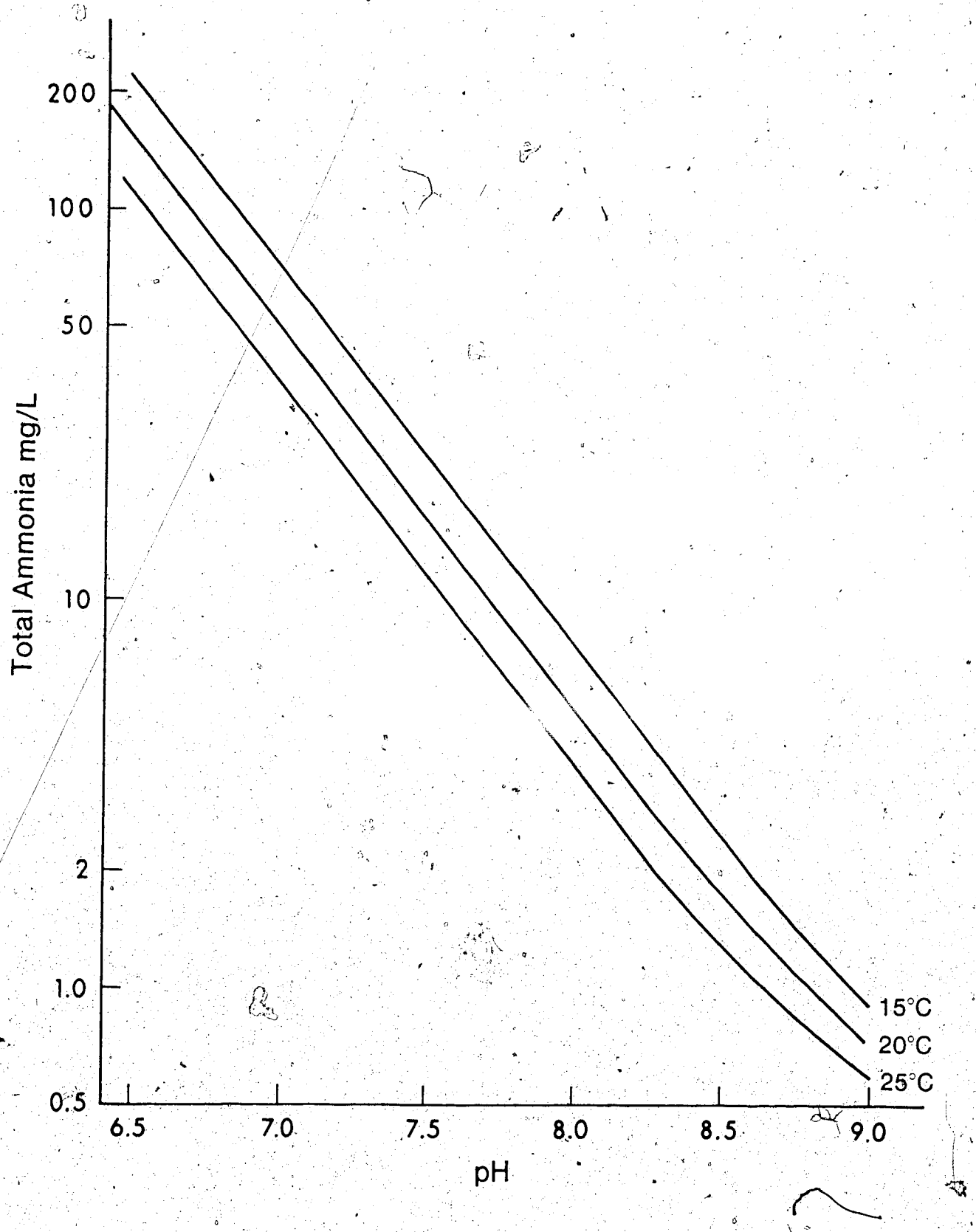


Figure 3.12 Total Ammonia Required to Produce an Un-ionized Ammonia Concentration of 0.02 mg/L (Hrudey, 1978)

to pass through cell membranes as readily as the more lipid-soluble un-ionized form that exists at the lower pH. According to this theory and the ionization curve of fatty acids (Figure 1.6), oleic and linoleic acid would ionize completely at the adjusted pH 7.5 and 9.0 test conditions. The ionized form would not be expected to pass through a rainbow trout's cell membrane. Therefore, the toxicity of these acids would decrease gradually with increasing pH of the test water (Figure 3.13) if the un-ionized acid was the predominant form. The LC50 value and the percentage of un-ionized fatty acids were inversely proportional and nearly log linear throughout the pH range from 6.4 to 6.8 (Figure 3.14).

From the Figure 1.6, oleic and linoleic acids would ionize about 98 percent at adjusted pH 6.5 test condition. The 2 percent un-ionized form of these acids would exist in the water and might translocate readily across the gill tissue and epithelial cells. The higher toxicity at the adjusted pH 6.5 test condition might be due to a physical accumulation of un-ionized form between the gill filaments, followed by transfer to the epithelium and mucous to inhibit oxygen transfer by some mechanism. Marking (1975) discussed the significance of the translocation of the un-ionized form of antimycin, a weak acid, across the gill tissue of fish. He found that the toxicity of antimycin was related to the concentration of un-ionized molecules and toxicity of antimycin decreased gradually from pH 6.5 to 8.5 with carp,

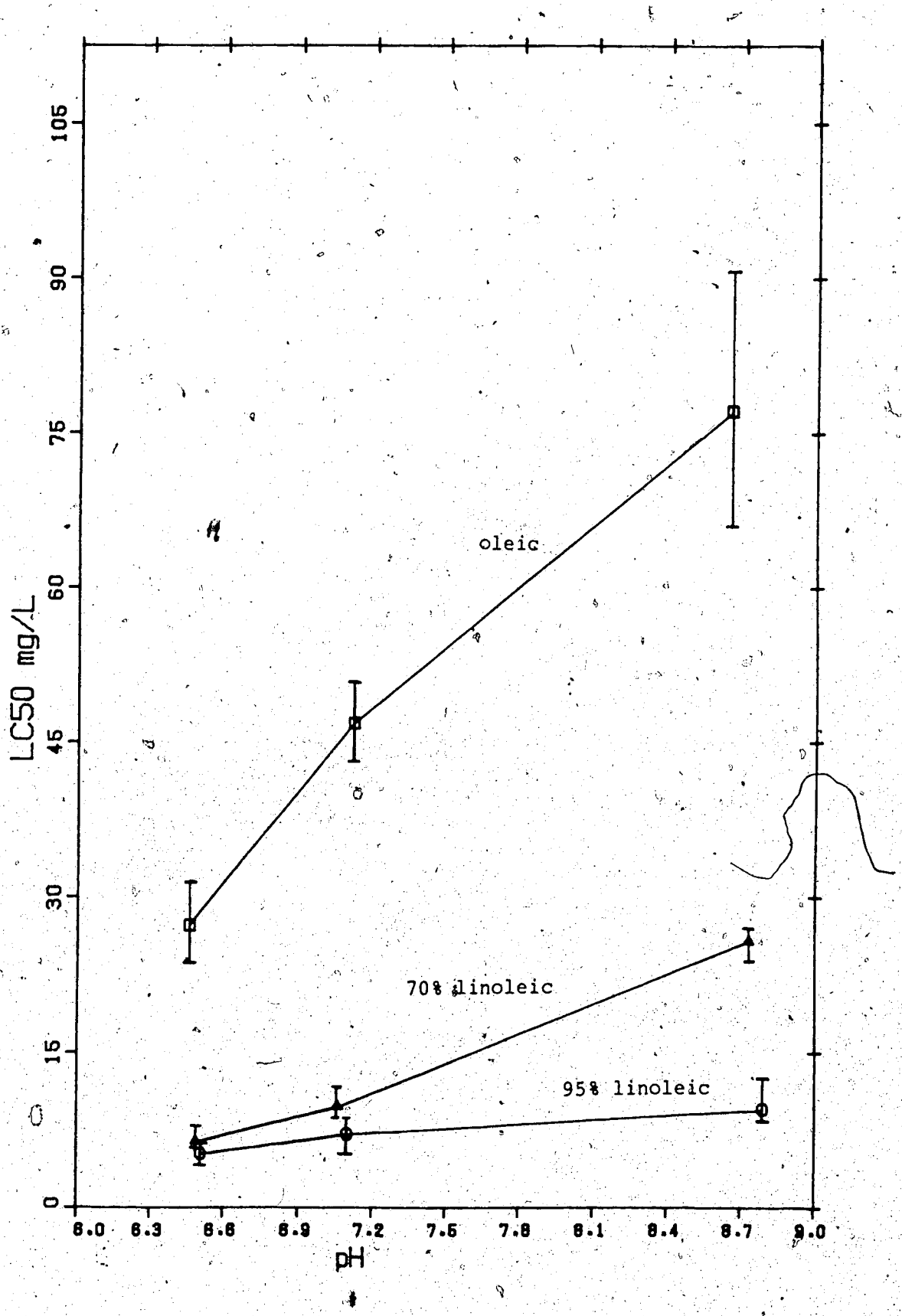


Figure 3.13 Relationship of pH Values to LC50 Geometric Mean Values of Oleic and Linoleic Acids with Rainbow Trout Fingerlings (Vertical bars represent standard deviations for oleic acid and ranges for linoleic acid)

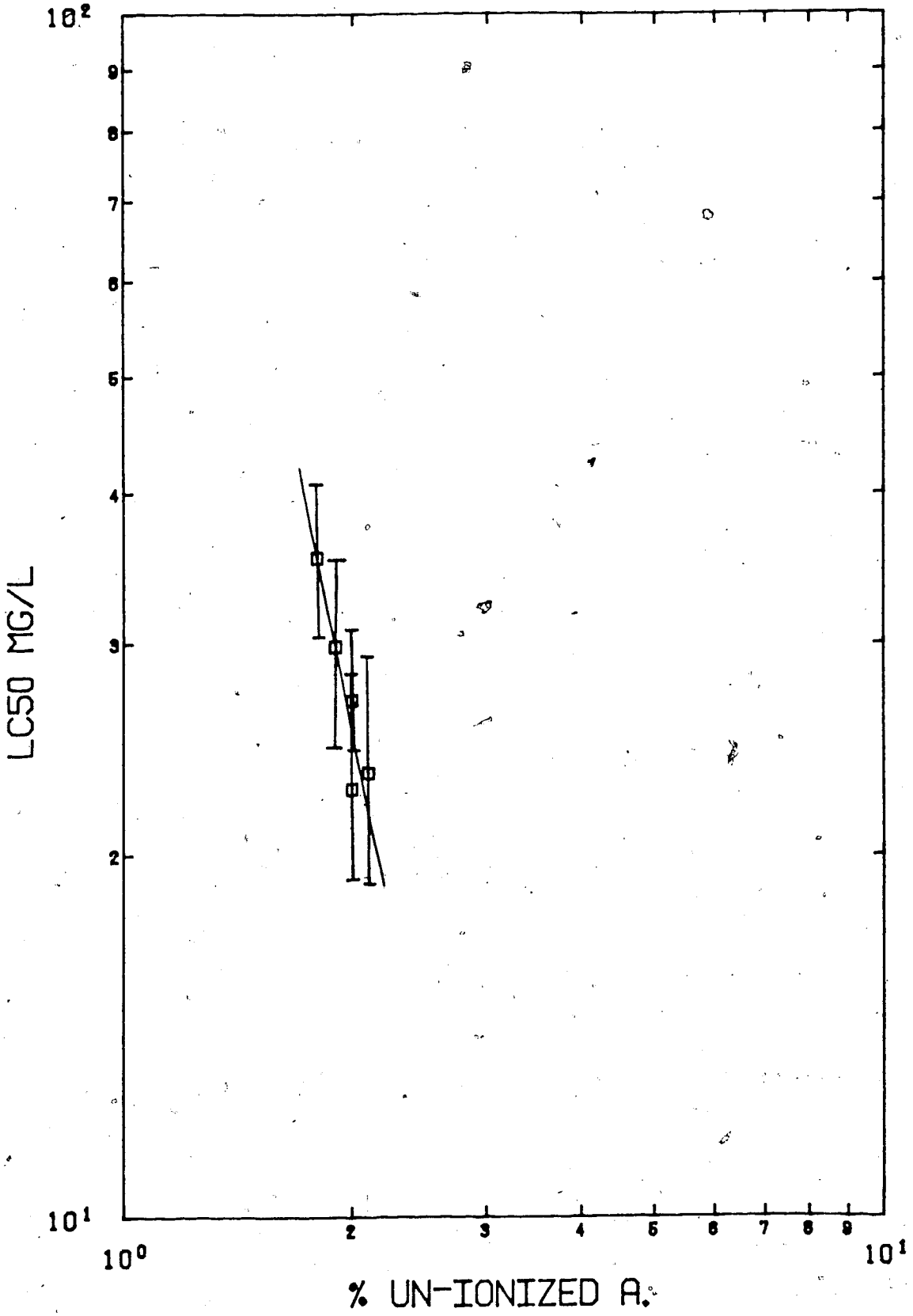


Figure 3.14 Relationship of Percentage of Un-ionized Fatty Acid at the Adjusted pH 6.5 Test Condition to LC50 Value of Oleic Acid with Rainbow Trout Fingerlings (Vertical bars represent 95% confidence interval)

green sunfish and bluegill. However, the un-ionized fatty acids at the adjusted pH 6.5 test condition is only 2 percent. Such a low percentage of un-ionized fatty acids might not concentrate sufficiently to cause an inhibition of oxygen transfer in the gill chamber.

Another physiological action of acids was reported by Lloyd and Jordan (1964). They noticed that rainbow trout developed a slightly opaque film over their bodies, but not on the gills in a solution of low pH (3.0 to 3.3). No visible damage either to the epithelial cells, or to any other gill tissue occurred. Also, in one experiment, eight rainbow trouts were overturned in a solution of pH value 3.8 at the end of a 24-hour experiment, but these fish recovered after being transferred to clean water (pH of approximately 8.2), showing that no permanent damage had been done. Lloyd and Jordan (1964) also indicated that those rainbow trout which had overturned in acid solutions had venous blood pH values lower than those of the controls (median values of 7.00 and 6.65 compared with 7.20 and 7.21, respectively). It was more likely, therefore, that acidaemia would be the cause of death in acidic test water. A similar pattern of results was reported by Jonas et al. (1962). They found that when hydrochloric acid was injected into the venous system of rainbow trout, fish died if the venous blood pH value was less than 6.85 one minute after injection.

The lowest pH test condition of this study was about 6.5 which is much higher than Lloyd and Jordan's test pH

(3.0 to 3.3). The difference of H^+ concentration (activity) is about 10^3. Therefore, the effects should be dramatically different in severity. However, the trend of physiological action of acids might be the same.

The Toxicity of Oleic versus Linoleic Acids

The results from this study indicated that linoleic acid (18:2) was more toxic to rainbow trout fingerlings than oleic acid (18:1) at the same pH level. The recent studies of Leach and Thakore (1973) indicated that the toxicity of C_{18} fatty acid sodium salts to fish decreased with diminishing degree of unsaturation in the order linolenate,, linoleate, and oleate.

A similar pattern of toxicity observed in this study was also reported by other investigators in long chain fatty acids to aquatic organisms. Curtis et al. (1974) indicated that the toxicity of unsaturated fatty acids to brine shrimp larvae increased with the degree of unsaturated ; 18:1 and 20:1 unsaturated acids showed little toxicity, but 18:2, 18:3 and 20:4 acids were highly toxic.

Galbraith et al. (1971) and Galbraith and Miller (1973a, 1973b and 1973c) studied the physicochemical effects of long chain fatty acids on bacterial cells and bacterial respiration. They found that the unsaturated fatty acids were more active than the saturated fatty acids and the activity was dependent on; (a) isomerism, where elaidic acid (trans isomer, 18:1) required higher concentrations than

oleic acid (cis isomer, 18:1) and (b) double bonding, where linoleic acid was more effective than oleic acid.

Comparison of the Measured LC50 Value to the LC50 Value from Other Investigations

The median lethal concentration (LC50) from this study are not inconsistent with those of Leach and Thakore (1973). Long chain fatty acids of the study by Leach and Thakore were prepared in form of sodium salt of acid as same as the toxicant solution preparation of this study. The pH of test water was not reported. The LC50 value of long chain fatty acids to juvenile coho salmon which were calculated from the data of Leach and Thakore (1973), produced the following: 24-hour LC50 of oleate = 15.0 mg/L, 4-hour LC50 of linoleate = 11.0 mg/L (Table 1.6).

From this study, 96-hour LC50 value of oleic acid to rainbow trout fingerlings is 27.2 mg/L as EEM at the pH of 6.46 which is higher than that value of Leach and Thakore (1973). However, the 96-hour LC50 value of linoleic acid to rainbow trout fingerlings is 5.2 mg/L as EEM at the pH of 6.51 which may be similar to that value of Leach and Thakore (1973).

Test Oleic and Linoleic Acids Composition

The results from this study indicated that oleic and linoleic acids initially contained some shorter chain fatty acids as impurities.

The gas chromatographic analysis confirm the loss of fatty acids from solution during the 96 hours test duration. However, the results from this study indicated that the composition of fatty acids at zero hour and ninety-six hour of each acid at three pH levels were similar. Therefore, pH did not apparently influence the loss of individual long chain unsaturated fatty acids (oleic and linoleic). Also, the lack of an observable difference in fatty acid compositions from start to finish indicated that the loss was due to precipitation (physical loss) rather than microbial degradation.

Esterification Procedure Evaluation

The percentage recovery of fatty acids in the esterification procedure ranges from 19.71 to 48.75 percent for oleic acid, from 1.96 to 73.17 percent for 70 percent linoleic acid and from 13.35 to 27.28 percent for 95 percent linoleic acid. The percentage recovery is very low in the 70 percent linoleic acid, because the sample size is only 4.4 mg/L as EEM (Table 3.4 and Figure 3.4). The sample size influence on boron trifluoride-methanol procedure for preparing fatty acid methyl esters was reported by Solomon *et al.* (1974). They indicated that the overall percentage recovery of triglycerides methyl esters of the sample sizes of 50, 100, 200, 350 mg were found to be 60.7, 92.1, 95.8 and 98.4 percent, respectively. Therefore, it is readily apparent that sample size is a dominant factor in the

recovery of the methyl esters from lipid. The percentage recovery decreases markedly with decreasing sample size and becomes less acceptable as the sample size becomes smaller.

In this study, most of sample size concentrations are lower than 50 mg/L as EEM. For example, the percentage recovery of fatty acid methyl esters at the sample size of 45.18 mg/L as EEM is 73.17 percent while for a sample size of 34.8 mg/L as EEM, methyl ester recovery is 59.84 percent. It is apparent that the esterification recovery on this study is comparable to trend reported by Solomon et al. (1974).

Comparison of Measured LC50 Value to Fatty Acid Concentration in Treated Effluents

The result from this study indicated that LC50 values of oleic and linoleic acid to rainbow trout fingerlings are higher than long chain fatty acid concentrations in treated effluent (secondary effluent) from some waste treatment plants in North America. The results by Coleman (1975) indicated that total long chain fatty acid concentration in the Edmonton Gold Bar Treatment Plant's effluent was found to be 1.19 mg/L. The oleic and linoleic acids concentrations were found to be 0.199 mg/L (16.9 %) and 0.06 mg/L (5.1 %), respectively (Table 1.5). The pH of treated effluent was found to be 7.52 (range 7.1 to 7.9). These concentations are lower than LC50 values for oleic and linoleic acids to

rainbow trout at the adjusted pH 7.5 test condition (Tables 3.6 and 3.7).

Farrington and Quinn (1973) also analyzed the fatty acid distribution in treated effluent at Rhode Island waste treatment plants by using gas-liquid chromatography and an esterification as per the method of Metcalfe *et al.* (1968). These results indicated that the total fatty acid concentrations in treated effluent were found to be 5.58 mg/L, 6.71 mg/L and 4.26 mg/L (average from 1.09 and 7.43 mg/L) at Field's Point, East Providence and West Warwick waste treatment plant, respectively. The oleic and linoleic acids concentrations in these treated effluents were found to be lower than 2.83 mg/L and 0.86 mg/L, respectively (Table 1.5). The pH of treated effluents was not reported. These treated effluent concentrations are lower than LC50 values for oleic and linoleic acids to rainbow trout at the adjusted pH 7.5 test condition (Tables 1.5, 3.6 and 3.7).

However, the long chain fatty acid concentration in treated effluent might be higher than the safe concentration (SC), and maximum allowable toxicant concentration (MATC) for oleic and linoleic acids to rainbow trout. The safe concentration (SC) and maximum allowable toxicant concentrations (MATC) for aquatic organism as an application of toxicity bioassay results have been proposed. Warren (1971) discussed the various variable limiting factors in the development of application factors for safe concentration from laboratory bioassay results. First, the

difference in nature of aquatic organism is the main limiting factor. Second, the condition in the laboratory must be different from natural aquatic environment. Many investigators have suggested the use of an arbitrary application factor of 0.1 to 0.3 to multiply the 48-hour LC50 in estimating a safe concentration (Warren, 1971).

For this study, if an arbitrary application factor of 0.1 was selected, the safe concentration for oleic acid and linoleic acid to rainbow trout fingerlings would be 4.07 and 0.67 mg/L as EEM at a pH of 7.06 and 7.10, respectively. The safe concentration for linoleic is lower than the linoleic acid concentration in the treated effluent from West Warwick, Rhode Island waste treatment plant. Therefore, it can be concluded that the linoleic acid concentration in that treated effluent may be toxic to rainbow trout provided exposure is for an indefinite time period. This, of course, provides no consideration for dilution of the effluent in receiving waters. On the other hand, it should be noted that effluent from food processing industries may exibit much higher concentrations of linoleic and oleic acids.

As well, the percentage recovery of long chain fatty acid esters in the esterification method for gas chromatography analysis were not reported by Coleman (1975) or Farrington and Quinn (1973). The sample sizes in both reports by these workers were less than 10 mg/L (Table 1.5). From the reports by Solomon *et al.* (1974) and the esterification procedure evaluation by this study, it can be

estimated that the percentage recovery of long chain fatty acid esters from the reports by Coleman (1975) and Farrington and Quinn (1973) might be less than 50 percent. Therefore, the actual amount of oleic and linoleic acids in these treated effluents might be actually higher than the safe concentration for oleic and linoleic acids to rainbow trout. Further evaluation of treated effluent data would be needed to address this matter.

The effect of the treated effluent on the fatty acid content of river water was reported by Coleman (1975). Coleman (1975) analysed the long chain fatty acids in the upstream, sewage effluent site and 62.8 km downstream from the sewage effluent site of North Saskatchewan River. The results indicated that treated effluent had little effect on the fatty acid content of the river water. The total long chain fatty acid concentrations were found to be 0.773 mg/L, 1.19 mg/L and 0.319 mg/L upstream of the sewage effluent discharge, at the discharge site and downstream of the discharge on the North Saskatchewan River, respectively. The pH of water were found to be 8.07 (range 7.8 to 8.3), 7.52 (range 7.1 to 8.4) and 7.91 (range 7.7 to 8.4) at upstream, discharge site and downstream, respectively. The long chain fatty acid distribution in the North Saskatchewan River from three sample sites are presented in Table 3.17. The total fatty acid concentration in the river water upstream of the sewage effluent discharge was higher than that at downstream of the discharge. The long chain fatty acid concentration

Table 3.17 Fatty Acid Distribution in North Saskatchewan River Water (Coleman, 1975)

Fatty Acid Concentration, mg/L
(% of Total Fatty Acids)

Sample Site	Lauric 12:0	Myristic 14:0	Palmitic 16:0	Palmitoleic 16:1	Stearic 18:0	Oleic 18:1	Linoleic 18:2	Linolenic 18:3	Total Fatty Acids	Total Lipid
Upstream	0.003 (0.4%)	0.023 (2.9%)	0.569 (73.6%)	0.029 (3.8%)	0.10 (12.9%)	0.033 (4.3%)	0.016 (2.1%)	-	0.773	2.2
Edmonton Gold Bar Plant	-	0.104 (8.8%)	0.230 (19.5%)	0.123 (10.4%)	0.384 (32.5%)	0.199 (16.9%)	0.060 (5.1%)	-	1.19	7.7
Downstream 68.2 km from Gold Bar Plant	0.014 (4.4%)	0.022 (6.9%)	0.045 (14.1%)	0.038 (11.9%)	0.116 (36.4%)	0.039 (12.2%)	0.025 (7.8%)	0.02 (6.3%)	0.319	1.7

was found to be high (1.77 mg/L) in the bottom sediment at the downstream site (Coleman, 1975). However, as previously mentioned in the esterification evaluation, it can be concluded that the actual amount of fatty acids for such low concentrations might not be difference in the upstream and downstream river sites.

No investigation has been reported on the toxicity of long chain fatty acids to benthic organism. However, the toxicity of long chain fatty acids to brine shrimp larvae (swimming invertebrate) was reported by Curtis et al. (1974). They found that 96-hour LC50 of long chain fatty acids (6:0 to 20:1) to brine shrimp larvae were found to be on the range from 0.014 to 0.30 mg/L (Table 1.7) which are less than the total long chain fatty acid concentration in the bottom sediment downstream of the effluent discharge in the North Saskatchewan River. The pH of test water was not reported by Curtis et al. (1974).

IV. Summary and Conclusions

This study can be summarized as follows:

1. Two long chain unsaturated fatty acids; oleic acid (cis-9- Octadecenoic with a basic structure of $C_{18}H_{34}O_2$, 18:1) and linoleic acid (cis, cis-9, 12- Octadecatrienoic with a basic structure $C_{18}H_{30}O_2$, 18:2) which are common long chain unsaturated fatty acids in the natural environment, were tested on rainbow trout (Salmo gairdneri Richardson) fingerlings to study the effects of pH on acute lethality.
2. pH affected the acute lethality of each long chain unsaturated fatty acid (as indicated by LC50 values), but was more pronounced with oleic acid than with linoleic acid during a 96 hour period. In general, fish died faster as pH decreased and slower as pH increased between the pH range of 6.47 to 8.84.
3. pH would influence the degree of acid ionization. Fatty acids which are weak acids, ionize in alkaline solutions. The ionized fatty acid is not expected to pass through cell membranes as readily as the more lipid-soluble un-ionized fatty acid which exists in an acid solution. An observed decrease in toxicity at high pH levels might be attributed to a high alkalinity. The higher observed toxicity at pH 6.5 may be caused by enhanced passage by the un-ionized acids through the cell membrane of the fish. The mode of toxic action for

such absorbed fatty acids requires further elaboration.

4. In terms of the LC50 values, the toxicity of linoleic acid was found to be about 5 to 8 times greater than oleic acid depending upon the test pH. The difference in toxicity between linoleic and oleic acids may be attributed to an increase in the number of double bonds. An increase in the number of double bonds may affect the compound's ability to penetrate cell membranes and reach the biochemical target receptor in the body of fish.
5. The results from this study indicates that if the pH of treated effluents is about 6.5, the long chain unsaturated fatty acid concentration in treated effluents from some waste treatment plants may be toxic to rainbow trout provided exposure is for an indefinite time period.

From the results observed in this study, it is concluded that the 96-hour LC50 for oleic acid to rainbow trout are 77.1 (range 65.4 to 90.9), 46.8 (range 43.8 to 50.0) and 27.2 (range 22.7 to 32.7) mg/L as EEM at a pH of 8.65, 7.12 and 6.46, respectively and the 96-hour LC50 values for linoleic acid to rainbow trout are 9.5 (range 8.6 to 10.6), 7.1 (range 6.1 to 8.2) and 5.2 (range 4.8 to 5.6) mg/L as EEM at a pH of 8.79, 7.10 and 6.51, respectively.

V. Recommendations

1. Further studies on the effect of pH on toxicity of fatty acids to fish and other aquatic organisms is strongly recommended. The bioassay should be conducted as follows:
   a. A 24-hour period bioassay should be conducted and the pH of the water should be readjusted every hour or every other hour.
   b. The sites of action and biochemical target receptor in fish or other aquatic organisms should be investigated in order to determine the cause of fish kill.
2. The toxicity of long chain fatty acids in treated effluent to aquatic organism should be evaluated in order to derive the median lethality (LC50) value, safe concentration and maximum allowable toxicant concentration of these compounds in treated effluents.

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APPENDIX

Table A 1 Bioassay Mortality Data of Oleic Acid Toxicity Test I

pH of Test Water											
8.78 (8.5 - 9.0)				7.10 (6.6 - 7.5)				6.47 (6.4 - 6.6)			
F.A.C.A.* mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality
42.0	35.45	10	0	24.0	20.63	10	0	18.0	12.69	10	0
56.0	47.28	10	40	32.0	27.48	10	0	24.0	17.01	10	0
75.0	63.31	10	40	42.0	35.36	10	0	32.0	19.53	10	50
100.0	84.4	10	90	56.0	47.13	10	30	42.0	25.84	10	70
135.0	121.32	10	100	75.0	67.57	10	100	56.0	43.70	10	100
pH control tank		10	0	pH control tank		10	0	pH control tank		10	0
Blank tank		10	0								

F.A.C.A.* = Fatty Acids Concentration Added

Table A 2 Bioassay Mortality Data of Oleic Acid Toxicity Test II

pH of Test Water											
8.74 (8.40 - 9.0)				7.13 (6.75 - 7.5)				6.45 (6.4 - 6.5)			
F.A.C.A.* mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality
42.0	32.80	10	0	42.0	25.10	10	0	18.0	9.00	10	0
56.0	42.54	10	10	56.0	33.78	10	20	24.0	13.12	10	0
75.0	51.82	10	20	75.0	44.40	10	60	32.0	16.73	10	40
100.0	79.02	10	50	100.0	103.10	10	100	42.0	26.86	10	50
135.0	130.00	10	100	135.0	134.00	10	100	56.0	44.93	10	100
pH control tank		10	0	pH control tank		10	0	pH control tank		10	0
Blank tank		10	0								

F.A.C.A.* = Fatty Acids Concentration Added

Table A 3 Bioassay Mortality Data of Oleic Acid Toxicity Test III

pH of Test Water											
8.63 (8.3 - 9.0)				7.12 (6.8 - 7.6)				6.47 (6.45 - 6.50)			
F.A.C.A.* mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality
42.0	34.70	10	0	42.0	23.3	10	0	24.0	18.52	10	0
56.0	47.00	10	20	56.0	33.98	10	10	32.0	25.24	10	50
75.0	60.20	10	30	75.0	47.39	10	40	42.0	31.65	10	70
100.0	78.60	10	50	100.0	66.18	10	100	56.0	52.80	10	100
135.0	132.00	10	100	135.0	132.0	10	100	75.0	74.0	10	100
pH control tank		10	0	pH control tank		10	0	pH control tank		10	0
Blank tank		10	0								

F.A.C.A.* = Fatty Acids Concentration Added

Table A 4 Bioassay Mortality Data of Oleic Acid Toxicity Test IV

pH of Test Water											
8.46 (8.1 - 9.0)				7.09 (6.8 - 7.6)				6.52 (6.5 - 6.6)			
F.A.C.A.* mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality
42.0	35.50	10	0	32.0	21.04	10	0	18.0	17.36	10	0
56.0	47.04	10	10	42.0	27.67	10	0	24.0	22.14	10	0
75.0	60.12	10	10	56.0	36.39	10	0	32.0	28.61	10	20
100.0	87.60	10	50	75.0	47.40	10	10	42.0	35.16	10	50
135.0	127.57	10	100	100.0	54.78	10	100	56.0	51.60	10	100
pH control tank		10	0	pH control tank		10	0	pH control tank		10	0
Blank tank		10	0								

F.A.C.A.* = Fatty Acids Concentration Added

Table A 5 Bioassay Mortality Data of Oleic Acid Toxicity Test V

pH of Test Water											
8.76 (8.4 - 9.0)				7.11 (6.8 - 7.5)				6.51 (6.45 - 6.50)			
F.A.C.A.* mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality
42.0	35.02	10	0	32.0	9.47	10	0	18.0	16.76	10	0
56.0	43.14	10	0	42.0	26.28	10	0	24.0	19.92	10	20
75.0	65.21	10	10	56.0	32.71	10	40	32.0	22.98	10	30
100.0	81.46	10	30	75.0	53.28	10	60	42.0	34.06	10	50
135.0	134.60	10	100	100.0	76.93	10	100	56.0	42.84	10	100
pH control tank		10	0	pH control tank		10	0	pH control tank		10	0
Blank tank		10	0								

F.A.C.A.* = Fatty Acids Concentration Added

Table A6 Bioassay Mortality Data of 70 Percent linoleic Acid Toxicity Test I

pH of Test Water											
8.74 (8.3 - 9.0)				7.06 (6.6 - 7.5)				6.49 (6.45 - 6.5)			
F.A.C.A.* mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality
18.0	13.50	10	0	10	6.90	10	0	5.6	8.25	10	60
32.0	22.78	10	40	18	10.64	10	60	10.0	10.37	10	100
56.0	42.00	10	100	32	25.25	10	100	18.0	17.73	10	100
100.0	99.25	10	100	56	54.50	10	100	32.0	32.00	10	100
180.0	175.0	10	100	100	96.50	10	100	56.0	51.47	10	100
pH control tank		10	0	pH control tank		10	0	pH control tank		10	0
Blank tank		10	0								

F.A.C.A.* = Fatty Acids Concentration Added

Table A 7 Bioassay Mortality Data of 70 Percent linoleic Acid Toxicity Test II

pH of Test Water											
8.70 (8.30 - 9.0)				7.04 (6.6 - 7.5)				6.49 (6.45 - 6.5)			
F.A.C.A.* mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality
10.0	9.96	10	0	5.6	5.04	10	0	1.8	4.86	10	0
18.0	17.08	10	10	10.0	7.06	10	10	3.2	5.24	10	20
32.0	26.16	10	50	18.0	10.69	10	80	5.6	8.93	10	100
56.0	49.15	10	100	32.0	29.90	10	100	10.0	9.90	10	100
100.0	96.35	10	100	56.0	57.50	10	100	18.0	17.50	10	100
pH control tank		10	0	pH control tank		10	0	pH control tank		10	0
Blank tank		10	0								

F.A.C.A.* = Fatty Acids Concentration Added

Table A 8 Bioassay Mortality Data of 95 Percent linoleic Acid Toxicity Test III

pH of Test Water											
8.76 (8.4 - 9.0)				6.99 (6.7 - 7.5)				6.51 (6.5 - 6.6)			
F.A.C.A.* mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality
5.6	5.20	10	0	3.2	4.12	10	0	1.0	2.32	10	0
10.0	7.32	10	0	5.6	5.34	10	0	1.8	3.81	10	0
18.0	9.85	10	60	10.0	6.69	10	40	3.2	4.28	10	0
32.0	28.50	10	100	18.0	16.50	10	100	5.6	7.43	10	100
56.0	49.10	10	100	32.0	29.50	10	100	10.0	11.25	10	100
pH control tank		10	0	pH control tank		10	0	pH control tank		10	0
Blank tank		10	0								

F.A.C.A.* = Fatty Acids Concentration Added

Table A 9 Bioassay Mortality Data of 95 Percent linoleic Acid Toxicity Test IV

pH of Test Water											
8.82 (8.40 - 9.0)				7.05 (6.75 - 7.6)				6.5			
F.A.C.A.* mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality	F.A.C.A. mg/L	EEM mg/L	Number of Fish	96-hr Percentage Mortality
5.6	5.84	10	0	3.2	4.04	10	0	1.8	3.86	10	0
10.0	7.38	10	50	5.6	5.44	10	50	3.2	4.10	10	50
18.0	19.95	10	100	10.0	12.65	10	100	5.6	9.60	10	100
32.0	32.50	10	100	18.0	21.90	10	100	10.0	13.75	10	100
56.0	55.50	10	100	32.0	33.90	10	100	18.0	20.70	10	100
pH control tank		10	0	pH control tank		10	0	pH control tank		10	0
Blank tank		10	0								

F.A.C.A.* = Fatty Acids Concentration Added

Table A10 The Average Values of Water Characteristics of Oleic Acid Toxicity Test from Test I to Test V

Adjusted pH	F.A.C.A.* mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$		NH_3-N $\bar{x} \pm$ S.D. mg/L as N
						P	Total	
9.0	42.0	34.65 ± 1.11	9.32 ± 0.39	8.68 (8.4 – 9.0)	60.66 ± 2.18	26.62 ± 8.48	118.61 ± 7.36	0.13 ± 0.04
	56.0	45.4 ± 2.35	9.32 ± 0.38	8.66 (8.4 – 9.0)	60.57 ± 2.62	26.42 ± 8.38	118.58 ± 9.46	0.13 ± 0.05
	75.0	60.13 ± 5.12	9.36 ± 0.38	8.58 (8.1 – 9.0)	60.60 ± 2.39	27.17 ± 8.49	119.35 ± 8.78	0.13 ± 0.06
	100.0	76.22 ± 15.77	9.53 ± 0.32	8.58 (8.1 – 9.0)	60.22 ± 2.49	26.11 ± 8.12	119.52 ± 8.48	0.15 ± 0.05
	135.0	129.1 ± 5.06	9.41 ± 0.35	8.78 (8.4 – 9.0)	59.14 ± 2.33	31.34 ± 2.82	117.15 ± 9.60	0.17 ± 0.05
	Control	–	9.56 ± 0.25	8.70 (8.4 – 9.0)	58.05 ± 3.01	25.5 ±8.42	120.47 ± 9.16	0.15 ± 0.06
7.55	24.0	20.63	9.36 ± 0.45	7.11 (6.6 – 7.6)	60.50 ± 3.33	–	85.52 ± 3.79	0.19 ± 0.11
	32.0	19.29 ± 9.07	9.51 ± 0.56	7.11 (6.6 – 7.6)	60.43 ± 2.48	–	94.54 ± 9.34	0.17 ± 0.05
	42.0	26.61 ± 5.99	9.56 ± 0.58	7.11 (6.6 – 7.6)	60.68 ± 1.87	–	95.89 ± 8.45	0.16 ± 0.03
	56.0	35.44 ± 7.35	9.64 ± 0.38	7.09 (6.6 – 7.6)	60.74 ± 1.61	–	95.50 ± 8.20	0.17 ± 0.04
	75.0	50.11 ± 11.22	9.38 ± 0.43	7.09 (6.6 – 7.6)	60.42 ± 2.02	–	96.17 ± 8.0	0.16 ± 0.07
	100.0	71.94 ± 23.46	9.76 ± 0.43	7.08 (6.8 – 7.6)	61.55 ± 2.19	–	95.98 ± 6.69	0.16 ± 0.03
	135.0	119.8 ± 20.08	9.38 ± 0.81	7.32 (7.0 – 7.6)	63.50 ± 0.14	–	86.25 ± 8.84	0.07 ± 0.05
	Control	–	9.48 ± 0.39	7.07 (6.6 – 7.6)	60.59 ± 1.52	–	95.64 ± 9.0	0.16 ± 0.05
6.5	18.0	13.96 ± 3.90	9.35 ± 0.81	6.49 (6.4 – 6.6)	61.46 ± 2.01	–	59.20 ± 2.27	0.15 ± 0.03
	24.0	17.40 ± 3.67	9.49 ± 0.51	6.48 (6.4 – 6.6)	61.18 ± 1.65	–	57.38 ± 2.16	0.14 ± 0.04
	32.0	21.61 ± 4.50	9.51 ± 0.68	6.48 (6.4 – 6.6)	61.35 ± 1.99	–	59.76 ± 2.69	0.13 ± 0.03
	42.0	29.45 ± 4.76	9.38 ± 0.48	6.50 (6.4 – 6.6)	61.18 ± 1.89	–	59.12 ± 2.79	0.14 ± 0.03
	56.0	45.06 ± 3.79	9.41 ± 0.35	6.48 (6.4 – 6.6)	61.89 ± 1.68	–	58.41 ± 2.75	0.17 ± 0.04
	75.0	59.24	10.0	6.49	65.0	–	60.40	0.21
	Control	–	9.71 ± 0.36	6.49 (6.4 – 6.6)	61.25 ± 2.10	–	59.81 ± 2.34	0.16 ± 0.03
	Blank	2.28 ± 0.66	9.45 ± 0.42	6.52 (6.3 – 7.4)	60.44 ± 1.64	–	20.66 ± 1.06	0.22 ± 0.02

*F.A.C.A. = Fatty Acid Concentration Added

Table All Water Characteristics of Oleic Acid Toxicity Test I

Adjusted pH	F.A.C.A. mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ P	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ Total	NH_3-N $\bar{x} \pm$ S.D. mg/L as N
9.0	42.0	35.45	9.16 ± 0.26	$8.5^{8.77}-9.0$	62.18 ± 2.37	28.44 ± 7.96	124.76 ± 8.36	0.14 ± 0.1
	56.0	47.28	9.18 ± 0.24	$8.5^{8.77}-9.0$	61.74 ± 1.99	27.72 ± 8.25	125.64 ± 11.08	0.14 ± 0.1
	75.0	63.31	9.18 ± 0.25	$8.6^{8.79}-9.0$	62.98 ± 1.46	28.04 ± 10.4	126.66 ± 9.72	0.14 ± 0.07
	100.0	84.4	9.29 ± 0.30	$8.55^{8.77}-9.0$	59.96 ± 2.97	26.96 ± 11.31	126.0 ± 11.31	0.16 ± 0.08
	135.0	121.32	9.26 ± 0.24	$8.6^{8.79}-9.0$	59.34 ± 3.68	32.03 ± 3.27	123.25 ± 10.77	0.20 ± 0.06
	control	-	9.20 ± 0.39	$8.5^{8.77}-9.0$	61.50 ± 2.53	23.08 ± 8.40	128.20 ± 11.98	0.16 ± 0.09
7.5	24.0	20.63	9.18 ± 0.25	$6.6^{7.11}-7.5$	60.50 ± 3.33	-	85.52 ± 3.79	0.19 ± 0.11
	32.0	27.48	9.18 ± 0.30	$6.6^{7.11}-7.5$	59.46 ± 2.67	-	85.24 ± 4.86	0.16 ± 0.02
	42.0	35.36	9.30 ± 0.30	$6.6^{7.11}-7.5$	59.16 ± 1.26	-	86.04 ± 5.73	0.14 ± 0.07
	56.0	47.13	9.36 ± 0.35	$6.6^{7.11}-7.5$	61.22 ± 2.01	-	84.26 ± 3.31	0.22 ± 0.08
	75.0	67.57	9.25 ± 0.32	$6.6^{7.05}-7.5$	60.05 ± 2.78	-	85.58 ± 4.24	0.23 ± 0.07
	control	-	9.23 ± 0.36	$6.6^{7.11}-7.5$	60.54 ± 1.10	-	84.04 ± 7.08	0.16 ± 0.08
6.5	18	12.69	9.31 ± 0.20	$6.4^{6.47}-6.5$	60.32 ± 1.94	-	60.40 ± 0.89	0.15 ± 0.07
	24	17.01	9.35 ± 0.17	$6.4^{6.46}-6.6$	60.52 ± 1.68	-	60.44 ± 0.61	0.16 ± 0.02
	32	19.53	9.30 ± 0.22	$6.4^{6.46}-6.6$	60.30 ± 1.70	-	62.14 ± 1.66	0.14 ± 0.07
	42	25.84	9.36 ± 0.45	$6.4^{6.47}-6.5$	59.70 ± 1.58	-	61.22 ± 1.61	0.16 ± 0.04
	56	43.70	9.30 ± 0.54	$6.4^{6.49}-6.6$	61.39 ± 1.79	-	61.10 ± 2.37	0.15 ± 0.08
	control	-	9.28 ± 0.44	$6.4^{6.46}-6.6$	60.94 ± 1.86	-	61.20 ± 1.53	0.14 ± 0.09
	blank	2.28 ± 0.66	9.50 ± 0.42	$6.45^{6.64}-6.8$	60.12 ± 1.31	-	21.40 ± 1.49	0.21 ± 0.07

Table A12 Water Characteristics of Oleic Acid Toxicity Test II.

Adjusted pH	F.A.C.A. mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ P	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ Total	NH_3-N $\bar{x} \pm$ S.D. mg/L as N
9.0	42.0	32.80	9.44 ± 0.38	$8.4^{8.72}-9.0$	59.42 ± 1.47	23.64 ± 8.75	113.66 ± 4.81	0.14 ± 0.04
	56.0	42.54	9.46 ± 0.36	$8.45^{8.72}-9.0$	59.78 ± 2.97	24.98 ± 8.55	113.30 ± 8.10	0.14 ± 0.04
	75.0	51.82	9.42 ± 0.40	$8.45^{8.72}-9.0$	59.40 ± 0.31	24.16 ± 8.92	114.52 ± 8.02	0.15 ± 0.06
	100.0	79.02	9.68 ± 0.34	$8.45^{8.72}-9.0$	59.30 ± 0.16	24.80 ± 2.51	114.40 ± 8.44	0.15 ± 0.08
	135.0	130.0	9.60	55.89	55.80	28.80	101.20	0.15
	control	-	9.48 ± 0.33	$8.4^{8.72}-9.0$	61.24 ± 1.98	24.74 ± 7.88	115.24 ± 7.82	0.20 ± 0.02
7.5	42.0	25.10	9.46 ± 0.38	$6.75^{7.07}-7.5$	61.98 ± 2.28	-	95.52 ± 9.30	0.19 ± 0.03
	56.0	33.78	9.42 ± 0.35	$6.75^{7.06}-7.5$	62.40 ± 1.40	-	94.0 ± 8.29	0.20 ± 0.02
	75.0	44.40	9.46 ± 0.46	$6.80^{7.10}-7.5$	61.14 ± 3.09	-	94.22 ± 8.93	0.21 ± 0.04
	100.0	103.10	9.73 ± 0.37	$6.8^{7.06}-7.5$	62.63 ± 2.15	-	89.17 ± 9.30	0.17 ± 0.04
	135.0	134.0	9.60	7.5	64.50	-	80.0	0.03
	control	-	9.56 ± 0.36	$6.75^{7.06}-7.5$	60.76 ± 2.14	-	93.68 ± 8.87	0.17 ± 0.04
6.5	18.0	9.0	9.74 ± 0.43	$6.40^{6.45}-6.5$	62.66 ± 2.42	-	58. 6 ± 3.49	0.16 ± 0.09
	24.0	13.12	9.46 ± 0.36	$6.4^{6.45}-6.5$	61.92 ± 1.77	-	58.48 ± 3.10	0.17 ± 0.08
	32.0	16.73	9.52 ± 0.42	$6.4^{6.45}-6.5$	61.22 ± 0.75	-	57.46 ± 3.34	0.15 ± 0.07
	42.0	26.86	9.50 ± 0.41	$6.4^{6.45}-6.5$	61.74 ± 1.84	-	58.94 ± 3.16	0.16 ± 0.07
	56.0	44.93	9.97 ± 0.47	$6.4^{6.45}-6.5$	62.33 ± 1.1	-	54.43 ± 0.91	0.20 ± 0.04
	control	-	9.58 ± 0.41	$6.4^{6.45}-6.5$	61.34 ± 0.89	-	57.58 ± 3.45	0.20 ± 0.03
	blank	2.34 ± 0.35	9.60 ± 0.53	$6.4^{6.56}-6.8$	58.92 ± 2.21	-	20.32 ± 0.99	0.21 ± 0.03

Table A13 Water Characteristics of Oleic Acid Toxicity Test III

Adjusted pH	F.A.C.A. mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x}$ + S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ P	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ Total	NH_3-N $\bar{x} \pm$ S.D. mg/L as N
9.0	42.0	34.70	9.22 ± 0.39	8.55 8.3 - 9.0	59.10 ± 0.02	26.0 ± 10.3	118.44 ± 4.12	0.12 ± 0.09
	56.0	47.00	9.36 ± 0.38	8.62 8.4 - 9.0	60.84 ± 2.68	27.22± 10.11	118.76 ± 4.81	0.07 ± 0.05
	75.0	60.20	9.48 ± 0.33	8.61 8.3 - 9.0	60.88 ± 1.87	26.48± 10.38	119.28 ± 4.59	0.07 ± 0.36
	100.0	48.60	9.60 ± 0.34	8.58 8.3 - 9.0	60.48 ± 2.68	26.90± 10.24	119.72 ± 5.16	0.09 ± 0.06
	135.0	132.00	9.80	8.82	57.60	56.10	110.0	0.17
	control	-	9.38 ± 0.40	8.67 8.3 - 9.0	61.32 ± 1.89	28.10 ± 10.22	101.14 ± 3.83	0.05 ± 0.05
7.5	42.0	23.3	9.32 ± 0.33	7.10 6.8 - 7.6	59.52 ± 0.86	-	101.18 ± 3.84	0.13 ± 0.10
	56.0	33.98	9.30 ± 0.32	7.11 6.85 - 7.6	60.16 ± 0.98	-	100.74 ± 4.65	0.13 ± 0.10
	75.0	47.39	9.44 ± 0.32	7.11 6.85 - 7.6	60.80 ± 1.50	-	102.18 ± 4.37	0.08 ± 0.08
	100.0	66.18	9.50 ± 0.26	7.08 6.8 - 7.6	60.75 ± 0.64	-	99.37 ± 4.80	0.12 ± 0.08
	135.0	132.0	10.0	7.20	63.60	-	92.50	0.10
	control	-	9.46 ± 0.26	7.00 6.8 - 7.6	60.36 ± 1.61	-	60.74 ± 0.89	0.08 ± 0.07
6.5	24.0	18.32	9.28 ± 0.47	6.48 6.45 - 6.5	60.90 ± 2.62	-	60.00 ± 1.06	0.08 ± 0.06
	32.0	25.24	9.24 ± 0.48	6.48 6.45 - 6.5	61.32 ± 2.49	-	59.42 ± 1.12	0.09 ± 0.09
	42.0	31.65	9.44 ± 0.33	6.48 6.45-6.5	61.72 ± 1.13	-	60.60 ± 1.06	0.10 ± 0.08
	56.0	52.80	9.70 ± 0.42	6.45	63.20 ± 1.27	-	58.35 ± 0.07	0.21 ± 0.07
	75.0	74.0	10.0	6.45	65.0	-	61.20	0.21
	Control	-	9.48 ± 0.30	6.48 6.45-6.5	61.12 ± 1.55	-	60.4 ± 0.82	0.13 ± 0.08
	Blank	2.34 ± 0.52	9.78 ± 0.40	6.43 6.3 - 6.6	59.92 ± 0.47	-	22.42 ± 4.99	0.21 ± 0.09

Table A14 Water Characteristics of Oleic Toxicity Test IV

Adjusted pH	F.A.C.A. mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ P	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ Total	NH_3-N $\bar{x} \pm$ S.D. mg/L as N
9.0	42.0	35.50	9.32 ± 0.44	8.4 $^{8.66}$ – 9.0	61.20 ± 1.57	28.0 ± 8.12	121.74 ± 6.24	0.18 ± 0.05
	56.0	47.04	9.34 ± 0.26	8.35 $^{8.51}$ – 9.0	60.0 ± 3.75	26.0 ± 8.17	122.24 ± 7.86	0.19 ± 0.04
	75.0	60.12	9.42 ± 0.46	8.1 $^{8.30}$ – 9.0	59.36 ± 3.52	25.56 ± 8.32	122.26 ± 8.64	0.22 ± 0.03
	100.0	87.60	9.54 ± 0.22	8.1 $^{8.30}$ – 9.0	60.24 ± 3.69	27.02 ± 8.32	122.28 ± 6.20	0.22 ± 0.03
	135.0	127.57	9.25 ± 0.26	8.45 $^{8.67}$ – 9.0	59.83 ± 0.42	30.48 ± 2.32	120.18 ± 7.65	0.23 ± 0.02
	control	–	9.48 ± 0.51	8.4 $^{8.66}$ – 9.0	62.0 ± 2.25	26.48 ± 9.30	122.54 ± 6.99	0.20 ± 0.04
7.5	32.0	21.04	9.34 ± 0.13	6.8 $^{7.10}$ – 7.6	60.70 ± 3.01	–	100.90 ± 7.59	0.22 ± 0.10
	42.0	27.67	9.32 ± 0.28	6.8 $^{7.10}$ – 7.6	61.92 ± 1.31	–	100.86 ± 7.04	0.20 ± 0.08
	56.0	36.39	9.36 ± 0.34	6.8 $^{7.09}$ – 7.6	60.28 ± 1.35	–	99.58 ± 6.21	0.17 ± 0.10
	75.0	47.40	9.52 ± 0.33	6.8 $^{7.09}$ – 7.6	59.16 ± 1.67	–	99.02 ± 6.19	0.20 ± 0.09
	100.0	54.78	9.4 ± 0.45	6.8 $^{7.07}$ – 7.6	60.40 ± 2.62	–	98.23 ± 5.83	0.19 ± 0.05
	control	–	9.56 ± 0.53	6.8 $^{7.09}$ – 7.6	61.48 ± 1.37	–	100.44 ± 6.38	0.21 ± 0.04
6.5	18.0	17.36	9.38 ± 0.49	6.5 $^{6.52}$ – 6.6	60.86 ± 0.50	–	59.04 ± 1.80	0.18 ± 0.05
	24.0	22.14	9.62 ± 0.41	6.5 $^{6.52}$ – 6.6	61.54 ± 1.38	–	59.82 ± 1.66	0.17 ± 0.02
	32.0	28.61	9.44 ± 0.54	6.5 $^{6.52}$ – 6.6	63.08 ± 1.35	–	59.88 ± 1.52	0.16 ± 0.02
	42.0	35.16	9.46 ± 0.48	6.5 $^{6.52}$ – 6.6	62.86 ± 1.74	–	58.90 ± 1.88	0.17 ± 0.03
	56.0	51.60	9.55 ± 0.50	6.5 $^{6.52}$ – 6.6	62.9 ± 1.49	–	58.53 ± 1.45	0.17 ± 0.05
	control	–	9.44 ± 0.56	6.4 $^{6.5}$ – 6.6	62.34 ± 1.98	–	60.50 ± 1.52	0.17 ± 0.08
	blank	2.34 ± 0.52	9.64 ± 0.49	6.4 $^{6.53}$ – 7.4	61.72 ± 1.29	–	20.76 ± 0.93	0.24 ± 0.02

Table A15 Water Characteristics of Oleic Acid Toxicity Test V

Adjusted pH	F.A.C.A. mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ P	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ Total	NH_3-N $\bar{x} \pm$ S.D. mg/L as N
9.0	42.0	35.02	9.44 ± 0.54	8.4 $^{8.74}$ 9.0	60.98 ± 2.15	27.0 ± 10.05	114.46 ± 8.02	0.09 ± 0.04
	56.0	43.14	9.46 ± 0.66	8.45 $^{8.74}$ 9.0	60.48 ± 2.03	26.14 ± 10.28	113.24 ± 9.61	0.09 ± 0.05
	75.0	65.21	9.58 ± 0.46	8.4 $^{8.69}$ 9.0	61.14 ± 2.65	31.6 ± 4.48	114.66 ± 8.86	0.09 ± 0.05
	100.0	81.46	9.56 ± 0.37	8.45 $^{8.71}$ 9.0	60.94 ± 1.84	24.8 ± 9.37	114.16 ± 6.52	0.12 ± 0.05
	135.0	134.60	9.53	9.0	58.60	30.98	113.80	0.09
	control	-	9.42 ± 0.55	8.45 $^{8.71}$ 9.0	61.8 ± 1.25	25.12 ± 9.43	115.82 ± 9.06	0.16 ± 0.06
7.5	32.0	9.47	9.58 ± 0.25	6.8 $^{7.12}$ 7.5	61.2 ± 1.90	-	97.48 ± 7.40	0.13 ± 0.05
	42.0	26.28	9.50 ± 0.51	6.8 $^{7.12}$ 7.5	60.8 ± 1.75	-	96.88 ± 7.35	0.13 ± 0.07
	56.0	32.71	9.58 ± 0.49	6.8 $^{7.09}$ 7.5	60.82 ± 1.54	-	98.90 ± 5.79	0.14 ± 0.0
	75.0	53.28	9.58 ± 0.48	6.8 $^{7.11}$ 7.5	61.06 ± 1.74	-	97.96 ± 6.01	0.1 ± 0.06
	100.0	76.93	9.58 ± 0.57	6.8 $^{7.11}$ 7.5	61.60 ± 2.18	-	96.18 ± 5.25	0.15 ± 0.04
	control	-	9.52 ± 0.61	6.8 $^{7.12}$ 7.5	61.50 ± 0.81	-	98.10 ± 6.91	0.17 ± 0.07
6.5	18.0	16.76	9.55 ± 0.59	6.45 $^{6.51}$ 6.6	61.98 ± 2.27	-	59.0 ± 2.34	0.1 ± 0.06
	24.0	19.92	9.50 ± 0.39	6.45 $^{6.5}$ 6.65	61.34 ± 2.24	-	59.36 ± 3.55	0.11 ± 0.08
	32.0	22.98	9.48 ± 0.36	6.45 $^{6.51}$ 6.65	60.82 ± 2.61	-	58.72 ± 3.18	0.11 ± 0.05
	42.0	34.06	9.52 ± 0.38	6.50	59.86 ± 1.42	-	59.10 ± 1.99	0.13 ± 0.05
	56.0	42.84	9.60 ± 0.54	6.45 $^{6.50}$ 6.54	61.13 ± 1.42	-	58.55 ± 2.58	0.11 ± 0.06
	control	-	9.58 ± 0.47	6.45 $^{6.51}$ 6.50	61.54 ± 1.62	-	59.36 ± 2.37	0.17 ± 0.10
	Blank	2.48 ± 0.89	9.60 ± 0.47	6.4 $^{6.47}$ 6.6	61.54 ± 0.82	-	20.42 ± 0.82	0.16 ± 0.06

Table A16 The Average Values of Water Characteristics of 70 Percent Linoleic Acid Toxicity Test from Test I and II

Adjusted pH	F.A.C.A. mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/l	pH and its ranges	Hard. $\bar{x} \pm$ S. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$		NH_3-N $\bar{x} \pm$ S.D. mg/L as N
						P	Total	
9.0	10.0	9.96	9.76 ± 0.36	8.69 / 8.3 – 9.0	60.76 ± 2.10	26.88 ± 7.5	117.80 ± 6.72	0.17 ± 0.08
	18.0	15.29 ± 2.53	9.67 ± 0.47	8.65 / 8.3 – 9.0	60.26 ± 2.04	27.49 ± 6.94	119.16 ± 5.99	0.15 ± 0.05
	32.0	24.43 ± 2.45	9.54 ± 0.46	8.64 / 8.32 – 9.0	58.78 ± 1.34	27.37 ± 4.49	119.93 ± 9.30	0.16 ± 0.06
	56.0	46.07 ± 4.36	9.43 ± 0.28	8.74 / 8.4 – 9.0	59.35 ± 1.30	26.08 ± 3.59	116.20 ± 4.43	0.15 ± 0.07
	100.0	97.80 ± 2.05	9.66 ± 0.43	8.75 / 8.45 – 9.0	60.55 ± 1.27	27.43 ± 1.24	115.35 ± 4.50	0.15 ± 0.03
	180.0	175.0	10.0	9.0	59.0	26.0	114.0	0.14
	control	–	9.40 ± 0.52	8.64 / 8.3 – 9.0	60.18 ± 1.22	25.11 ± 6.50	117.72 ± 4.46	0.22 ± 0.07
7.5	5.6	5.04	9.48 ± 0.51	6.97 / 6.6 – 7.5	59.44 ± 2.30	–	97.20 ± 13.15	0.20 ± 0.05
	10.0	6.98 ± 0.11	9.46 ± 0.37	6.99 / 6.6 – 7.5	59.51 ± 1.50	–	96.12 ± 9.22	0.17 ± 0.05
	18.0	10.67 ± 0.04	9.49 ± 0.35	6.99 / 6.6 – 7.5	59.49 ± 1.33	–	96.52 ± 8.76	0.19 ± 0.06
	32.0	27.58 ± 3.29	9.58 ± 0.44	7.0 / 6.6 – 7.5	60.11 ± 2.07	–	92.80 ± 6.43	0.20 ± 0.05
	56.0	56.0 ± 2.12	9.64 ± 0.54	7.12 / 6.8 – 7.5	60.0 ± 1.31	–	91.15 ± 4.69	0.19 ± 0.05
	100.0	96.50	10.30	7.5	59.40	–	90.0	0.10
	control	–	9.46 ± 0.48	6.99 / 6.6 – 7.5	59.70 ± 1.26	–	96.04 ± 7.83	0.22 ± 0.06
6.5	1.8	4.86	9.56 ± 0.51	6.47 / 6.45 – 6.5	61.62 ± 2.48	–	51.76 ± 1.73	0.22 ± 0.01
	32.	5.24	9.44 ± 0.44	6.48 / 6.45 – 6.55	60.80 ± 1.62	–	49.98 ± 0.18	0.23 ± 0.01
	5.6	8.59 ± 0.48	9.57 ± 0.54	6.49 / 6.45 – 6.5	58.7 ± 1.79	–	50.27 ± 2.38	0.20 ± 0.07
	10.0	10.14 ± 0.33	9.58 ± 0.43	6.49 / 6.45 – 6.5	59.58 ± 0.87	–	50.0 ± 1.22	0.19 ± 0.08
	18.0	17.62 ± 0.16	9.71 ± 0.60	6.48 / 6.4 – 6.5	58.9 ± 1.95	–	50.02 ± 1.67	0.15 ± 0.09
	32.0	32.0	9.47 ± 0.64	6.5	60.37 ± 2.37	–	50.83 ± 1.91	0.14 ± 0.08
	56.0	51.42	10.1	6.5	63.0	–	51.2	0.10
	control	–	9.45 ± 0.52	6.48 / 6.45 – 6.5	59.81 ± 0.75	–	50.28 ± 1.42	0.21 ± 0.08
	Blank	2.9 ± 0.37	9.39 ± 0.48	6.48 / 6.45 – 6.6	59.19 ± 1.08	–	20.07 ± 0.69	0.25 ± 0.06

Table A17 Water Characteristics of 70 percent Linoleic Acid Toxicity Test I

Adjusted pH	F.A.C.A. mg/L	EDM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$		NH_3-N $\bar{x} \pm$ S.D. mg/L as N
						P	Total	
9.0	18.0	13.5	9.62 ± 0.52	$8.3^{8.62}$ – 9.0	59.68 ± 2.25	27.72 ± 3.72	119.32 ± 1.93	0.14 ± 0.04
	32.0	22.7	9.46 ± 0.40	$8.32^{8.61}$ – 9.0	59.12 ± 1,78	25.80 ± 4.15	119.08 ± 4.32	0.15 ± 0.05
	56.0	42.99	9.44 ± 0.11	$8.55^{8.78}$ – 9.0	59.68 ± 1.42	27.10 ± 1.27	117.80 ± 5.02	0.15 ± 0.05
	100.0	99.25	0.57 ± 0.49	$8.6^{8.78}$ – 9.0	60.67 ± 1.53	27.0 ± 1.22	117.07 ± 3.56	0.15 ± 0.05
	180.0	175.0	10.0	9.0	59.0	26.0	114.0	0.14
	control	–	9.44 ± 0.61	$8.3^{8.61}$ – 9.0	60.0 ± 1.59	25.16 ± 7.88	118.36 ± 3.07	0.16 ± 0.04
7.5	10.0	6.90	9.40 ± 0,38	$6.6^{7.02}$ – 7.5	59.52 ± 2.07	–	96.64 ± 5.02	0.14 ± 0.06
	18.0	10.64	9.36 ± 0.27	$6.6^{7.01}$ – 7.5	59.88 ± 1.25	–	98.0 ± 6.23	0.16 ± 0.06
	32.0	25.25	9.38 ± 0.43	$6.6^{6.93}$ – 7.5	59.32 ± 2.25	–	96.28 ± 5.49	0.18 ± 0.04
	56.0	54.50	9.57 ± 0.57	$6.8^{7.02}$ – 7.5	60.53 ± 0.92	–	92.87 ± 3.92	0.18 ± 0.04
	100.0	96.50	10.3	7.5	59.4	–	90.0	0.10
	control	–	9.54 ± 0.53	$6.6^{7.01}$ – 7.5	59.84 ± 0.91	–	96.32 ± 4.40	0.19 ± 0.06
6.5	5.6	8.25	9.54 ± 0.62	$6.45^{6.48}$ – 6.5	59.16 ± 2.06	–	50.84 ± 3.25	0.15 ± 0.07
	10.0	10.37	9.64 ± 0.60	$6.45^{6.48}$ – 6.5	59.62 ± 1.15	–	50.0 ± 1.22	0.13 ± 0.06
	18.0	17.37	9.48 ± 0.53	$6.4^{6.47}$ – 6.5	58.68 ± 2.09	–	50.02 ± 1.87	0.12 ± 0.06
	32.0	32.0	9.47 ± 0.64	6.5	60.37 ± 2.37	–	51.33 ± 2.52	0.14 ± 0.08
	56.0	51.47	10.1	6.5	63.0	–	51.20	0.10
	control	–	9.44 ± 0.61	$6.45^{6.49}$ – 6.5	59.64 ± 0.86	–	50.84 ± 1.93	0.15 ± 0.05
	blank	3.06 ± 0.15	9.42 ± 0.45	$6.45^{6.50}$ – 6.6	59.54 ± 1.15	–	19.82 ± 0.56	0.21 ± 0.06

Table A18 Water Characteristics of 70 Percent Linoleic Acid Test II

Adjusted pH	F.A.C.A. mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ P	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$ Total	NH_3-N $\bar{x} \pm$ S.D. mg/L as N
9.0	10.0	9.96	9.76 ± 0.36	8.3 – 8.69 – 9.0	60.76 ± 2.10	26.88 ± 7.50	117.80 ± 6.72	0.17 ± 0.08
	18.0	17.08	9.72 ± 0.44	8.35 – 8.69 – 9.0	60.84 ± 1.86	27.26 ± 9.69	119.0 ± 8.77	0.15 ± 0.07
	32.0	26.16	9.62 ± 0.54	8.35 – 8.68 – 9.0	58.44 ± 0.77	28.94 ± 4.68	120.90 ± 13.16	0.17 ± 0.07
	56.0	49.15	9.40 ± 0.35	8.4 – 8.71 – 9.0	58.52 ± 0.87	27.40 ± 2.31	113.53 ± 1.10	0.16 ± 0.10
	100.0	96.35	9.80 ± 0.42	8.45 – 8.71 – 9.0	60.2	26.3	110.2	0.15
	control	–	9.36 ± 0.50	8.4 – 8.68 – 9.0	59.74 ± 5.76	25.06 ± 5.76	117.04 ± 5.84	0.28 ± 0.02
7.5	5.6	5.04	9.48 ± 0.51	6.6 – 6.97 – 7.5	59.44 ± 2.30	–	97.20 ± 13.15	0.20 ± 0.05
	10.0	7.06	0.52 ± 0.38	6.6 – 6.97 – 7.5	59.70 ± 2.72	–	95.60 ± 12.86	0.19 ± 0.02
	18.0	10.69	9.62 ± 0.40	6.6 – 6.97 – 7.5	59.10 ± 1.43	–	94.94 ± 11.30	0.22 ± 0.04
	32.0	29.90	9.90 ± 0.26	6.6 – 6.97 – 7.5	61.43 ± 0.85	–	87.0 ± 1.90	0.24 ± 0.04
	56.0	57.50	9.75 ± 0.64	6.8 – 7.09 – 7.5	58.4	–	86.0	0.23
	control	–	9.36 ± 0.50	6.65 – 6.97 – 7.5	60.36 ± 0.85	–	95.76 ± 10.88	0.25 ± 0.04
6.5	1.8	4.86	9.56 ± 0.51	6.45 – 6.47 – 6.5	61.62 ± 2.48	–	51.76 ± 1.73	0.22 ± 0.01
	3.2	5.24	9.44 ± 0.44	6.45 – 6.48 – 6.55	60.80 ± 1.62	–	49.98 ± 0.18	0.23 ± 0.01
	5.6	8.93	9.60 ± 0.52	6.5	58.24 ± 1.57	–	49.70 ± 1.16	0.25 ± 0.02
	10.0	9.90	9.75 ± 0.51	6.5	59.53 ± 0.51	–	50.0 ± 1.65	0.26 ± 0.02
	18.0	17.50	10.30 ± 0.28	6.5	60.0	–	50.0	0.29
	control	–	9.40 ± 0.45	6.45 – 6.48 – 6.55	59.98 ± 0.67	–	49.72 ± 0.57	0.27 ± 0.02
	Blank	2.74 ± 0.47	9.36 ± 0.55	6.45 – 6.47 – 6.50	58.84 ± 0.98	–	20.32 ± 0.77	0.29 ± 0.04

Table A19 The Average Values of Water Characteristics of 95 Percent Linoleic Acid from Test III and IV

Adjusted pH	F.A.C.A. mg/L	EDM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$		NH_3-N $\bar{x} \pm$ S.D. mg/L as N
						P	Total	
9.0	5.6	5.52 ± 0.45	9.56 ± 0.45	$8.4^{8.75}_{-}9.0$	59.84 ± 2.64	24.86 ± 8.43	115.38 ± 5.49	0.13 ± 0.05
	10.0	7.35 ± 0.04	9.59 ± 0.49	$8.4^{8.74}_{-}9.0$	61.72 ± 2.63	25.71 ± 8.17	114.91 ± 7.46	0.15 ± 0.07
	18.0	14.90 ± 7.14	9.53 ± 0.51	$8.6^{8.79}_{-}9.0$	60.49 ± 3.06	25.27 ± 7.36	115.14 ± 5.25	0.16 ± 0.08
	32.0	30.5 ± 2.83	9.63 ± 0.60	$8.5^{8.84}_{-}9.0$	59.65 ± 2.0	29.95 ± 1.86	112.77 ± 4.71	0.15 ± 0.06
	56.0	52.30 ± 4.53	9.80 ± 0.56	$8.6^{8.84}_{-}9.0$	62.40 ± 3.77	29.88 ± 1.83	110.45 ± 5.90	0.17 ± 0.04
	control	-	9.48 ± 0.57	$8.4^{8.75}_{-}9.0$	60.54 ± 2.32	24.78 ± 8.30	115.89 ± 6.56	0.21 ± 0.04
7.5	3.2	4.08 ± 0.06	9.48 ± 0.46	$6.7^{7.02}_{-}7.6$	61.68 ± 2.10	-	98.53 ± 7.09	0.20 ± 0.04
	5.6	5.39 ± 0.07	9.50 ± 0.55	$6.7^{7.02}_{-}7.6$	60.57 ± 1.72	-	98.07 ± 6.13	0.18 ± 0.07
	10.0	9.67 ± 4.21	9.42 ± 0.45	$6.7^{7.0}_{-}7.6$	59.44 ± 0.98	-	98.8 ± 7.80	0.19 ± 0.08
	18.0	19.20 ± 3.82	9.45 ± 0.48	6.94	58.92 ± 1.07	-	92.42 ± 5.63	0.15 ± 0.08
	32.0	31.70 ± 3.11	9.56 ± 0.59	$6.7^{7.14}_{-}7.6$	60.35 ± 1.43	-	91.13 ± 5.01	0.15 ± 0.07
	control	-	9.44 ± 0.50	$6.7^{7.02}_{-}7.6$	59.98 ± 1.53	-	98.25 ± 6.45	0.20 ± 0.03
6.5	1.0	2.32	9.64 ± 0.71	$6.5^{6.53}_{-}6.6$	61.06 ± 1.37	-	50.52 ± 1.44	0.23 ± 0.05
	1.8	3.84 ± 0.04	9.52 ± 0.57	$6.5^{6.51}_{-}6.6$	59.74 ± 2.08	-	54.44 ± 4.41	0.22 ± 0.06
	3.2	4.19 ± 0.13	9.52 ± 0.46	6.5	60.17 ± 1.45	-	54.66 ± 4.08	0.21 ± 0.05
	5.6	8.52 ± 1.53	9.51 ± 0.49	6.5	60.14 ± 1.46	-	53.78 ± 3.94	0.20 ± 0.07
	10.0	12.50 ± 1.77	9.47 ± 0.41	6.5	60.56 ± 1.95	-	53.27 ± 4.65	0.16 ± 0.06
	18.0	20.70	9.66 ± 0.38	6.5	59.65 ± 2.19	-	53.75 ± 3.75	0.18 ± 0.02
	control	-	9.36 ± 0.42	6.5	60.66 ± 2.40	-	53.7 ± 3.44	0.21 ± 0.04
	blank	2.56 ± 0.66	9.48 ± 0.48	$6.4^{6.44}_{-}6.45$	59.72 ± 1.67	-	19.82 ± 1.02	0.21 ± 0.03

Table A20 Water Characteristics of 95 Percent Linoleic Acid Toxicity Test III

Adjusted pH	F.A.C.A. mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$		NH_3-N $\bar{x} \pm$ S.D. mg/L as N
						P	Total	
9.0	5.6	5.2	9.60 ± 0.41	$\overset{8.79}{8.4 - 9.0}$	60.80 ± 3.26	22.90 ± 7.79	116.64 ± 6.39	0.15 ± 0.04
	10.0	7.32	9.62 ± 0.48	$\overset{8.79}{8.4 - 9.0}$	63.92 ± 1.15	24.94 ± 8.99	115.98 ± 7.88	0.15 ± 0.07
	18.0	9.85	9.43 ± 0.60	$\overset{8.79}{8.4 - 9.0}$	61.86 ± 2.90	25.20 ± 8.97	116.56 ± 6.28	0.16 ± 0.07
	32.0	28.50	9.40 ± 0.69	$\overset{8.72}{8.5 - 9.0}$	59.52 ± 0.77	30.73 ± 2.39	112.43 ± 6.07	0.15 ± 0.09
	56.0	49.40	9.60 ± 0.71	$\overset{8.72}{8.6 - 9.0}$	63.0 ± 4.24	30.60 ± 0.57	110.10 ± 7.07	0.18 ± 0.05
	control	-	9.48 ± 0.54	$\overset{8.79}{8.5 - 9.0}$	61.04 ± 3.13	24.34 ± 9.55	116.06 ± 6.48	0.23 ± 0.04
7.5	3.2	4.12	9.46 ± 0.48	$\overset{7.04}{6.7 - 7.5}$	62.20 ± 1.04	-	98.68 ± 7.21	0.20 ± 0.03
	5.6	5.36	9.48 ± 0.55	$\overset{7.03}{6.7 - 7.5}$	60.0 ± 2.09	-	99.06 ± 6.98	0.18 ± 0.07
	10.0	6.69	9.44 ± 0.48	$\overset{7.03}{6.7 - 7.5}$	59.30 ± 0.85	-	97.32 ± 5.94	0.20 ± 0.07
	18.0	16.50	9.48 ± 0.45	$\overset{6.94}{6.7 - 7.5}$	59.30 ± 1.47	-	92.73 ± 4.20	0.18 ± 0.07
	32.0	29.50	9.60 ± 0.85	$\overset{6.94}{6.7 - 7.5}$	61.55 ± 0.64	-	92.35 ± 3.89	0.20 ± 0.06
	control	-	9.40 ± 0.45	$\overset{7.03}{6.7 - 7.5}$	60.28 ± 1.64	-	98.72 ± 5.40	-
6.5	1.0	2.32	9.64 ± 1.37	$\overset{6.53}{6.5 - 6.6}$	61.06 ± 1.37	-	50.52 ± 1.44	0.23 ± 0.05
	1.8	3.81	9.58 ± 0.51	$\overset{6.51}{6.5 - 6.55}$	60.16 ± 0.43	-	51.18 ± 1.83	0.23 ± 0.05
	3.2	4.28	9.58 ± 0.45	6.5	60.16 ± 1.49	-	50.26 ± 1.15	0.23 ± 0.05
	5.6	7.43	9.43 ± 0.40	6.5	60.85 ± 2.57	-	49.68 ± 0.55	0.17 ± 0.05
	10.0	11.25	9.80 ± 0.28	6.5	60.80 ± 0.85	-	51.15 ± 0.07	0.18 ± 0.05
	Control	-	9.50 ± 0.49	6.5	61.42 ± 3.01	-	50.30 ± 1.35	0.23 ± 0.05
	Blank	2.58 ± 0.87	9.48 ± 0.57	$\overset{6.44}{6.40 - 6.45}$	60.50 ± 2.04	-	20.20 ± 0.96	0.21 ± 0.04

Table A21 Water Characteristics of 95 Percent Linoleic Acid Toxicity Test IV

Adjusted pH	F.A.C.A. mg/L	EEM $\bar{x} \pm$ S.D. mg/L	Dissolved Oxygen $\bar{x} \pm$ S.D. mg/L	pH and its ranges	EDTA Hardness $\bar{x} \pm$ S.D. mg/L as $CaCO_3$	Alkalinity $\bar{x} \pm$ S.D. mg/L as $CaCO_3$		NH_3-N $\bar{x} \pm$ S.D. mg/L as N
						P	Total	
9.0	5.6	5.84	9.52 ± 0.54	8.4 $^{8.71}$ – 9.0	58.88 ± 1.66	26.82 ± 9.51	114.12 ± 4.80	0.11 ± 0.06
	10.0	7.38	9.56 ± 0.55	8.4 $^{8.70}$ – 9.0	59.52 ± 1.47	26.48 ± 8.23	113.64 ± 7.80	0.14 ± 0.08
	18.0	19.93	9.48 ± 0.44	8.6 $^{8.79}$ – 9.0	58.22 ± 2.58	25.34 ± 6.44	113.72 ± 4.17	0.15 ± 0.10
	32.0	32.5	9.80 ± 0.40	9.0	60.13 ± 2.95	29.17 ± 1.05	113.10 ± 4.28	0.15 ± 0.04
	56.0	55.5	10.0 ± 0.57	9.0	60.25 ± 4.45	29.15 ± 2.76	110.80 ± 7.35	0.16 ± 0.05
	control	–	9.48 ± 0.66	8.4 $^{8.71}$ – 9.0	60.04 ± 1.29	25.22 ± 7.96	115.72 ± 7.40	0.20 ± 0.04
7.5	3.2	4.04	9.50 ± 0.50	6.75 – $^{7.01}$ 7.6	61.16 ± 2.86	–	98.34 ± 7.83	0.20 ± 0.04
	5.6	5.44	9.52 ± 0.61	6.75 – $^{7.01}$ 7.6	61.06 ± 1.30	–	97.08 ± 5.77	0.18 ± 0.08
	10.0	12.65	9.44 ± 0.50	6.73 – $^{6.98}$ 7.6	59.58 ± 1.18	–	100.28 ± 9.81	0.18 ± 0.1
	18.0	21.90	9.50 ± 0.56	6.75 – $^{6.94}$ 7.6	58.52 ± 0.49	–	92.10 ± 7.84	0.12 ± 0.08
	32.0	33,90	9.75 ± 0.64	7.6	59.15 ± 0.07	–	89.90 ± 7.35	0.10
	control	–	9.48 ± 0.59	6.75 – $^{7.01}$ 7.6	59.68 ± 1.54	–	97.78 ± 7.99	0.19 ± 0.03
6.5	1.8	3.86	9.4 ± 0.42	6.5	58.42 ± 1.91	–	58.36 ± 1.81	0.21 ± 0.08
	3.2	4.10	9.46 ± 0.46	6.5	60.18 ± 2.13	–	58.54 ± 1.28	0.19 ± 0.06
	5.6	9.60	9.44 ± 0.57	6.5	60.14 ± 1.58	–	57.30 ± 1.64	0.18 ± 0.07
	10.0	13.75	9.53 ± 0.50	6.5	60.17 ± 1.05	–	58.07 ± 2.0	0.15 ± 0.09
	18.0	20.70	9.57 ± 0.47	6.5	59.65 ± 2.19	–	56.35 ± 3.89	0.18 ± 0.02
	control	–	9.58 ± 0.56	6.5	59.90 ± 1.57	–	56.32 ± 1.56	0.20 ± 0.03
	Blank	2.54 ± 0.47	9.48 ± 0.43	6.45	58.94 ± 0.76	–	19.42 ± 1.01	0.20 ± 0.03