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STUDIES ON THE TOXICITY OF AMMONIA, NITRATE AND THEIR MIXTURES TO THE COMMON GUPPY

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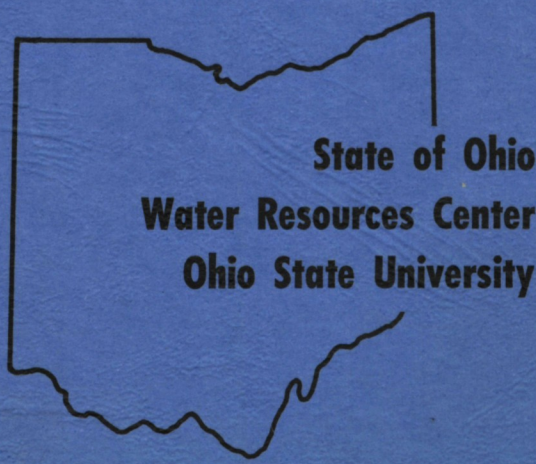

State of Ohio
Water Resources Center
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INTRODUCTION

Background

The survival of fish and other aquatic organisms is controlled by their environment and, thus, by changes induced by man's activities. The introduction of sewage and other wastes into a water course, even though they have been treated, can be expected to have an impact. Most massive fish kills have been related to rapid oxygen depletion or dumps of relatively large amounts of chemicals. More subtle are the effects of mixtures of substances at concentrations below their individual toxic limits. An example, and the subject of this report, is the additive toxicity of ammonia and nitrate, two common constituents of treated waste waters.

The traditional means of studying the effects of suspected toxicants is through short term (acute) bioassay techniques. Such procedures have been standardized to allow comparison of results (1). They are relatively easy to perform and require little in the way of time, materials or experience. The first problem is to select the test organism, and even if fish are decided upon, to select the species for testing. Any number of criteria may be used (25) and many lists of appropriate species are described in the literature. For example, Henderson and Pickering (12) recommend the common guppy (Poecilia reticulatus), mosquito fish (Gambusia affinis), goldfish (Carassius auratus), fathead minnow (Pimephales promelas), and bluegill (Lepomis macrochirus) as suitable standard test animals. More extensive lists are given by Kemp et al. (18). The species selected for this study was the common guppy.

In bioassay analysis it is necessary to take into consideration and try to control physical and chemical variables such as temperature, light, ionic strength, pH, and hardness. Undoubtedly they account for much of the variation in toxicity levels reported in the literature. A pertinent example is

the way pH controls the toxicity of ammonia by governing the forms present in solution. In particular, temperature must be controlled to permit meaningful interpretation of the results. Temperature governs system properties such as chemical reaction rate and the metabolic rate of the test organism. Raising the water temperature also decreases oxygen solubility in water and increases the organism's carbon dioxide production which itself raises the lowest oxygen content which the fish can tolerate. It has been generalized that at a given toxicant concentration, an increase in water temperature of 10°C halves the organism's survival time (2, 13). On the other hand, studies have shown that the toxicity of phenol and free ammonia increases with decreasing temperature (20). Brown et al. (3) attributed this effect to a decrease in the detoxication rate.

The median tolerance limit, TL_m, is usually used as the standard measure of acute toxicity. The TL_m is defined as the concentration at which fifty percent of the test animals are able to survive for a specified time of exposure, usually 24, 48, 72 or 96 hours. The value reported may be the exact concentration at which fifty percent survival actually was observed or it may be a value derived by straight-line graphical interpolation. The estimation of the TL_m by interpolation usually involves the plotting of the data on semi-logarithmic paper. A straight line is drawn between two points representing survival at the two successive concentrations that were lethal to more than half and to less than half of the fish. The concentration at which this line crosses the fifty percent survival line is the TL_m value (1). The median lethal concentration, LC50, is equivalent to the TL_m and is used in European literature (24).

The lethal threshold concentration, C_t, can also be used to characterize the toxicity of a substance. It can be defined as the highest toxicant

concentration that can be applied for an indefinite time before there are any deaths (4). The definition of the threshold concentration implies that it is impossible to measure it directly, so that empirical relationships or kinetic models of fish toxicity must be used to estimate its value.

The destruction or death of an organism when exposed to a lethal concentration of some poison is not instantaneous. The overall reaction involves many steps and it is believed that more than one of these may be rate limiting. Simple reaction kinetics are generally not adequate to describe the destruction of living organisms, but the overall rates of inactivation may be empirically represented by simple kinetic expressions. Lethal toxicity is analogous to disinfection so that some of its laws should also apply. This hypothesis will be examined.

Disinfection Laws and Models

The kinetics of disinfection are most frequently expressed as Chick's law (5):

$$-\frac{dN}{dt} = k'N \qquad \ldots.(1)$$

where the differential term is the rate of destruction, N is the concentration of organisms surviving at any given time t, and k' is a rate constant characteristic of the system. This relationship states that the rate of destruction is directly proportional to the number of living organisms remaining. Upon integration between the limits, $N = N_o$ at $t = 0$ and $N = N$ at $t = t$:

$$\log \frac{N}{N_o} = -k\,t \qquad \ldots.(2)$$

A plot of $\log N/N_o$ against time should be linear. However, applications of Chick's law are limited and deviations are commonly observed.

Chick's law does not include terms accounting for the effect of disin-

fectant concentration and thus it holds only for one specific constant concentration. An empirical concentration-kill time relationship is given by Watson's law (29):

$$C^n t = K \qquad \ldots.(3)$$

where C is the toxicant concentration, t is the time required to achieve a given percentage kill and n and K are constants. n is commonly referred to as the coefficient of dilution. The equation may be linearized by taking logarithms:

$$n \log C + \log t = \log K \qquad \ldots.(4)$$

Median survival time, T_m, can be used to express the value of t in Watson's law, where T_m is calculated from the geometric mean of each individual fish:

$$T_m = \text{Antilog} \left(\frac{\Sigma \log t_j}{N_o}\right) \qquad \ldots.(5)$$

where t_j is the survival time of each individual fish (14).

A general mathematical model that can be used to linearize disinfection data has been described by Hom (15):

$$-\frac{dN}{dt} = k N t^m C^n \qquad \ldots.(6)$$

Substituting $C^n = K'/t$ gives the differential equation:

$$-\frac{dN}{dt} = \frac{K'N t^m k}{t} \qquad \ldots.(7)$$

or

$$-\frac{dN}{dt} = K'' N t^{(m-1)}$$

Separating the variables and integrating between N, N_o and t, t_o:

$$\log \frac{N}{N_o} = -\frac{K t^m}{m} \qquad \ldots.(8)$$

where K is the reaction rate constant for logarithmic base of ten, and m is

the reaction kinetic constant for the reaction in which $m \neq 0$ and $n \neq 0$. According to Equation 8 the reaction may be verified by a linear relationship when a plot is made for log-log(N_o/N) against log t and the constant m can thus be determined from the slope of the line. Several different relationships can be obtained from Equation 6 under conditions such that $m = 0$ and $n = 0$ (Chick's Law), $m = 0$ and $n \neq 0$, and $m \neq 0$ and $n = 0$.

Chen and Selleck (4) observed that a linear relationship can be obtained when the logarithm of percent survival is plotted against exposure time and that the slopes of the curves are proportional to the toxicant concentration. The resulting differential equation can be written as:

$$\frac{dN}{dt} = - K C^n N + HN \qquad ....(9)$$

where N is the number of fish at any time t, K and H are rate coefficients and n is the order of the reaction. According to Chen and Selleck, the KC^nN term is equivalent to Watson's law and HN is the rate of detoxication. The concentration which makes the values of the two terms equal (i.e., at $dN/dt = 0$) is the threshold concentration, and is given by the relationship

$$C_t = (H/K)^{\frac{1}{n}} \qquad ....(10)$$

If the rate equation is integrated

$$\ln \frac{N}{N_o} = (-KC^n + H)\ (t-t_i) \qquad ....(11)$$

or

$$\log \frac{N}{N_o} = (-KC^n + H)\ t + T_c \qquad ....(12)$$

where N/N_o is the survival ratio, t_i is the induction period or time before the onset of death, and $T_c = t_i(KC^n-H)$, which is constant for a given concentration.

The coefficients n, H and K must be determined to evaluate the threshold concentration. Chen and Selleck obtained their values for zinc toxicity through an experimental design in which a series of assays were conducted at several different levels of concentration. Plotting $\log(N/N_o)$ against time for each concentration, the slopes of the lines produced could be viewed as the net mortality rate coefficient, $(-KC^n+H)$. Plotting these coefficients against concentration they obtained a straight line, which means that the order of the toxication reaction is unity and the value of the threshold concentration is equal to the line intersection with the concentration axis. Malcom et al. (21) in studies on the toxicity of cadmium to mammalian cells used the same procedure and found a non-linear relationship between net mortality rate coefficient and concentration. They came to the conclusion that the order of the inactivation reaction, the rate coefficients and the threshold concentration cannot be determined by this method.

Toxicity of Ammonia and Nitrate

"Ammonia" exists in aqueous solutions as either ammonium ion or as free molecular ammonia, depending on the pH of the solution in accordance with the equilibrium reaction

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^- \qquad ....(13)$$

Using the mass action law, the mathematical relationship between ammonia and ammonium ion is given by:

$$K_b = \frac{(NH_4^+)\ (OH^-)}{(NH_3)} \qquad ....(14)$$

where K_b has the value of 1.78×10^{-5} at 25°C. The expression indicates that as the pH is increased the equilibrium favors the formation of free ammonia.

It is well established that free ammonia is significantly more toxic to

fish than ammonium ion, although the toxicity of the latter has been demonstrated (26). Therefore, the great increase in toxicity upon increasing pH is due to the shift of the solution equilibrium towards the formation of free ammonia. The toxicity of the free ammonia has been attributed to the distinctive penetration properties of the molecule through cell membrane because of its lipid solubility and lack of charge (6, 10). Lloyd and Orr (20) found that exposure of rainbow trout to sublethal ammonia concentrations resulted in a considerable increase in urine flow rates and it was thought that this might be caused by an increase in the permeability of the fish to water. Flis (8, 9) demonstrated severe tissue damage after 35 days exposure to a free ammonia concentration of 0.11 mg/l.

The toxicity of nitrate depends greatly on the cationic composition of the solution. For example, sodium nitrate solutions are less toxic than potassium nitrate solutions (27). Jones (17) reported that the toxicity of different nitrate salts depends on the "solution pressure," that is the tendency of a solute in a solid phase to become hydrated upon going into solution. Solution pressure can also be interpreted as a measure of the tendency of the cation to give up its positive charge and enter into combination with other compounds. Apparently, it also has an effect on the permeability of living cells towards ions.

The literature on the toxicity of ammonia and nitrate to aquatic organisms has been summarized in several reports to the U.S. Environmental Protection Agency (18, 19, 22).

In solution, substances of different toxicities can interact biologically and chemically in various ways to increase or decrease the overall toxic effect. When the interaction decreases the toxicity of one of the substances, antagonism is said to occur. If the effect of two toxic substances is equal

to the sum of their individual toxicity, either the term summation or additive is applied. When the combined effect is more than additive, it may be called supra-additive or synergistic; potentiation is said to occur (25). Gaddum (11), Sprague (25), Warren (28) and Jones (16) have described graphical procedures to summarize the joint toxicity of multicomponent solutions. To our knowledge no such study has been reported in the literature on the joint toxicity of ammonia and potassium nitrate.

Purpose and Scope

The general purposes of the research described in this report were to develop procedures and facilities for determining toxicity and to investigate the toxicity of ammonia and nitrate ion to fish. Specifically, the study was directed towards achieving the following objectives:

1. Determination of the toxicity of free ammonia to fish as estimated by short term TL_m values.

2. Determination of potassium nitrate toxicity to fish as estimated by short term TL_m values.

3. Determination of the toxicity of mixtures of potassium nitrate and ammonia to fish.

4. To investigate the applicability of established kinetic models to the toxicity of ammonia and nitrate to fish.

The limitations of this study were that the TL_m values were determined at a single pH value using solutions of only reagent grade aqueous ammonia, ammonium chloride and potassium nitrate; other water quality parameters such as temperature, hardness, salinity and dissolved oxygen were not varied nor examined directly; and the only test organism examined was 6 to 11 mm long fry of a single species of fish, the common guppy (Poecilia reticulatus).

EXPERIMENTAL METHODS AND MATERIALS

Test Fish and Facilities

The test organisms used in this study were common guppies (Poecilia reticulatus) which were grown, bred and maintained until testing in a 20-gallon stainless steel-glass aquaria. Each aquarium was equipped with under-gravel and outside filters, an airstone, and plants to help the fry in hiding from adults. The tanks were kept in an air-conditioned room and the water temperature was maintained with the heaters at 77° to 80°F. The aquaria were kept clean of unconsumed food and other debris by periodic siphoning and partial replacement of the water.

The fish were fed at least once but usually twice daily with dried commercial food (TetraMin or Biorell) or brine shrimp hatched from eggs obtained from Metaframe Corporation (San Francisco Brand). The fish were observed daily for the presence of diseases and dead specimens. The tanks were covered to prevent evaporation of the water, and fluorescent and incandescent bulbs were used to light the fish room continuously. Fish fry were harvested from the breeding tanks and set aside in 10-gallon all glass aquaria as needed just prior to testing. Sick fish were never used for testing except in one comparative study.

Columbus city tap water was used to maintain the fish and as experimental water. The tap water was vigorously aerated for at least one week and then was passed through a bed of activated carbon to remove residual chlorine. Finally the water was pumped through 8-micron Whatman filter tube to remove precipitated iron.

All tests were conducted in an isolated constant temperature room maintained at 77°F. The room was also used for aging the experimental water and preconditioning the fish. Five-gallon rectangular glass aquaria provided with

a loose fitting plastic cover were used as test tanks.

Stock buffer solution (pH 7.40 to 7.50) of 0.5 M potassium phosphate was prepared as described in "Standard Methods" (1). Ammonia stock solutions were prepared by titration of 2 M ammonium chloride solution with 30 percent aqueous ammonia to bring the stock solution to pH 7.40. The solution was stored in a tightly closed bottle at low temperature to prevent loss of the ammonia. Potassium nitrate stock solution of 2 M concentration were prepared from reagent grade chemicals, as with all the other solutions.

Experimental Procedure

Chemical and physical analyses of the experimental water including alkalinity, total hardness and total solids, were run at the beginning of each experiment. Total alkalinity was found by potentiometric titration with 0.02 N sulfuric acid. Total solids were determined by weighing the residue upon evaporation of 100 ml of the experimental water in a preweighed dish in an oven at 105°C. The hardness was determined by titration with 0.01 M EDTA solution at pH 10.0 using Eriochrome Black T as indicator (1).

The static (batch) bioassay technique described in "Standard Methods" was utilized in this study. Usually, six or eight 5-gallon aquaria were used in a test series. Each test tank held fourteen liters of water which was buffered with 28 ml of the stock buffer solution to bring the phosphate concentration to 10^{-3} M. Ten or twenty fish, 6 to 11 mm in length, were used in each tank. The fish were distributed to the test aquaria in the random fashion described by Sprague (24). They were acclimated to the test conditions for 48 hours during which time the tanks were aerated continuously. During the acclimation period, as well as the test period, the fish were not fed. The experimental room was continuously lighted during this period with fluorescent and incandescent bulbs.

To avoid the sudden increase in toxicant concentration, the usual procedure was to add toxicant solution in five equal and separate batches through a period of two hours to bring the experimental water to the desired final concentration. During experiments to investigate ammonia toxicity, aeration of the water was discontinued prior to the addition of the ammonia solution. The dissolved oxygen was measured and recorded daily during the testing period using a YSI model 54 oxygen meter. During the nitrate toxicity investigation aeration of the experimental water continued through the entire test period.

Ammonia concentration was determined at least twice during the test period using the standard Kjeldahl procedure (1). Twenty-five ml samples of the experimental water was distilled and the distillate was collected below the surface of a boric acid solution containing methylene blue and methyl red indicator solutions. This was titrated with standard 0.02 N sulfuric acid solution. Nitrate concentration was determined by direct ultraviolet spectroscopy at 220 nm in acid solution. The concentration-absorption relationship follows Beer's Law up to 11 mg/l-N. The procedure used was that described in "Standard Methods" except that the color removal step was eliminated.

The pH of the test water was measured daily during the test period using Sargent-Welch model LS pH meter. Sodium hydroxide or hydrochloric acid was added to the test water to adjust pH as needed.

The duration of the test was at least 96 hours. The number of dead fish in the test tanks were observed and recorded several times daily. With all tests the 24, 48, 72 and 96 hour observations were obtained and recorded. Dead fish were removed immediately with a small net.

EXPERIMENTAL RESULTS

Preliminary Studies

Preparatory studies were conducted to investigate the properties of Columbus tap water, to find suitable analytical methods to estimate the concentrations of ammonia and nitrate, and to investigate the reliability of the static bioassay technique in determining the toxicity of ammonia to fish by following the oxygen and ammonia concentrations during the test period.

The pH of the Columbus tap water was found to be 8.7 to 9.0 but with low buffer capacity, dropping rapidly to 7.7 or 7.8 after one day of aeration. The tap water also contained suspended iron hydroxide, most likely due to corrosion of the water pipes in the laboratory. The iron did not appear to be harmful to the fish but it caused a marked turbidity in the water. Aeration of the water for one week followed by filtration through Whatman 8-micron filter tubes was enough to remove the turbidity. The water hardness fluctuated between 108 and 170 mg/l as $CaCO_3$ and the total alkalinity of the water was always within the range of 25 to 43 mg/l as $CaCO_3$. Total solids after aeration and filtration was within the range of 136 to 362 mg/l.

The titrimetric procedure for the determination of ammonia as described in "Standard Methods" was satisfactory within the concentration range used in this study. It was found that the precision of the method varied between about $\pm$ 0.2 to 3 percent for an ammonia concentration range of 1.41 to 14.14 mg/l-N using a 50-ml sample. Determination of the ammonia using the phenate method (1) gave unreproducible results.

The ultraviolet spectrophotometric method described in "Standard Methods" was satisfactory for determining the nitrate concentrations within the range investigated in this study (30 to 500 mg/l-N). Another advantage of this method was the small volume, usually 5 ml, of sample required for the analysis.

It was observed that ammonia offers some interference to this method. The presence of 0.1 M NH_4Cl resulted in a +7.5 percent error in measuring a nitrate concentration of 200 mg/l-N, and the presence of 0.02 M NH_4Cl resulted in an error of +2.5 percent.

The static bioassay technique proved to be reliable for the determination of ammonia and nitrate toxicities. Table 1 shows the concentration of total ammonia in four test aquaria as determined during the four-day test period. In some of the tanks a slight increase in the total ammonia concentration was observed due to evaporation of water. In Tank 4, which showed the greatest change, the concentration increased by 5.52% by the end of the test period. Evaporation and change in ammonia concentration were minimized in later studies by using plastic covers on each tank. Measurements during testing indicated that a constant dissolved oxygen concentration was reached after 20 hours. This, in turn, suggests that the rate of oxygen uptake by the fish was equal to the rate of oxygen diffusion from the air to the water. In no tests did the dissolved oxygen concentration fall below 6 mg/l.

TABLE 1. CHANGE IN TOTAL AMMONIA CONCENTRATION WITH TIME

Time, Hrs.	Total Ammonia Concentration of Different Tanks, mg/l-N			
	Tank 1	Tank 2	Tank 3	Tank 4
2	91.4	107.0	127.6	154.2
24	90.5	108.8	126.2	157.1
48	91.0	111.0	127.5	161.0
72	89.5	--	129.0	162.0
96	--	--	132	163.0
Average	90.5	108.7	128.2	159.0

Phosphate buffer solution was used to attempt to hold the pH of the experimental water between 7.4 and 7.5. Initially, 1.0×10^{-3} phosphate concentration was used, but it failed to keep the pH constant. In one of the experiments to investigate the toxicity of ammonia to guppy fry, the pH dropped from 7.50 to 6.95 in 96 hours. In another experiment, in order to prevent the pH from changing, the phosphate buffer concentration was increased to 3×10^{-3} M. This resulted in the precipitation of a white solid, presumably calcium phosphate and the death of one-third of the fish in the control tank within 96 hours. In all subsequent tests, 1.0×10^{-3} M phosphate buffer was used and the pH was adjusted with acid or base as needed. It was observed during the ammonia studies that the pH of the experimental water decreased whereas it increased in case of investigating potassium nitrate toxicity.

Ammonia Toxicity

For tests with healthy guppy fry the number of fish surviving was counted as a function of time. Figure 1 shows typical plots of the data at different concentrations of total ammonia at pH 7.40 to 7.50. It is possible to divide each curve into three sections. The first in an induction period over which mortality is inhibited (i.e., $dN/dt = 0$). The length of this period decreased as the toxicant concentration increased. In the next section of the curves, the death rate increases sharply. The data indicate that the death rate and the length of this period also depended on the ammonia concentration. In the last section the slope of the curves decreases sharply and becomes parallel to the time axis indicating a significant decrease in the death rate. At the lowest ammonia concentration shown in the figure, 74.15 mg/l-N, only 4 fish died during the period between 24 to 96 hours. At this concentration no further deaths were observed during the period between 4 and 10 days. The

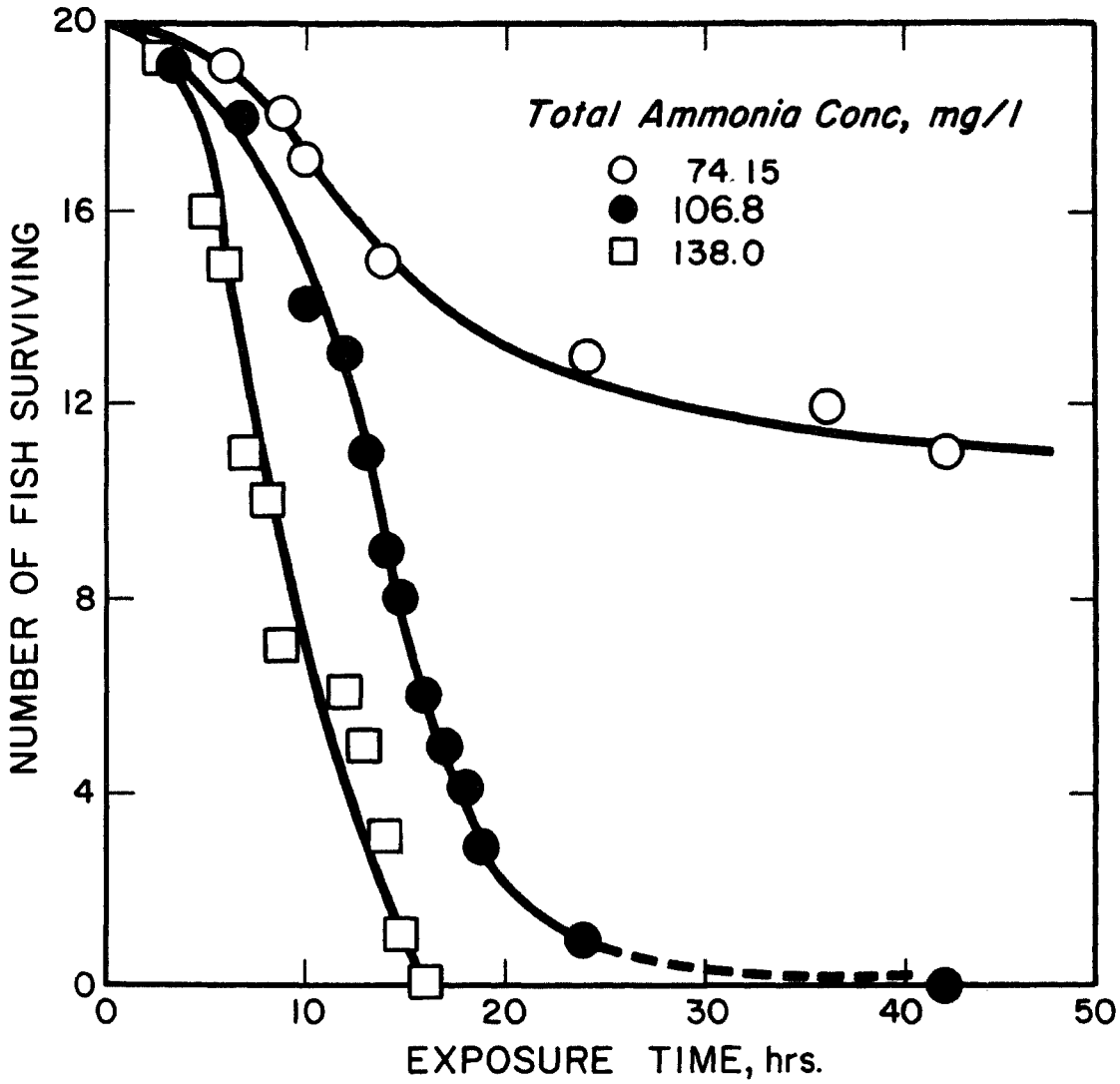

Figure 1. Typical Data Showing Fish Survival at Different Total Ammonia Concentrations at pH 7.4.

decrease in the death rate can be attributed to the fact that all of the weak fish die during the high death rate period while the stronger fish survive after this period. This decrease in the death rate can also be attributed to the biological ability of the surviving fish to develop a defense mechanism and thus tolerate the toxicant (4, 23). At the higher total ammonia concentration of 138 mg/l-N the last period is absent, in that all of the fish died in the first 15 hours.

To obtain the median tolerance limit the percent of test animals surviving at the different observation times was plotted against the logarithm of the ammonia concentration expressed as free ammonia or as total ammonia. The concentration at which 50 percent of the tested animals survived for a specific time was then interpolated from the graphs. Figures 2 and 3 show typical applications of this method to find the TL_m values. Figure 2 shows the 24- and 72-hour data for total ammonia. It is apparent that the 24-hour data are more highly scattered and that the TL_m value is higher than at 72 hours. Figure 3 shows the 72- and 96-hour results expressed as free ammonia. There was very little difference in the TL_m determined at either 72 or 96 hours. Table 2 shows the details of the experimental conditions in the test tanks. All the experiments were conducted at 25±1°C with a total phosphate of 1×10^{-3} M. Free ammonia concentration at each tank was calculated using the mass action law (Equation 14) and a time-weighted value of hydrogen ion concentration calculated by the following equation:

$$(H^+)_{ave} = \frac{1}{t_n} \Sigma\left[\left((H^+)_j + (H^+)_{j+1}\right)\left(\frac{t_{j+1} - t_j}{2}\right)\right] \quad \ldots.(15)$$

The terms $(H^+)_j$ and $(H^+)_{j+1}$ are the hydrogen ion concentrations in the test tank at time t_j and t_{j+1}, respectively. The value of t_n represents the total test period which is 96 hours in this study. The hydrogen ion concentration

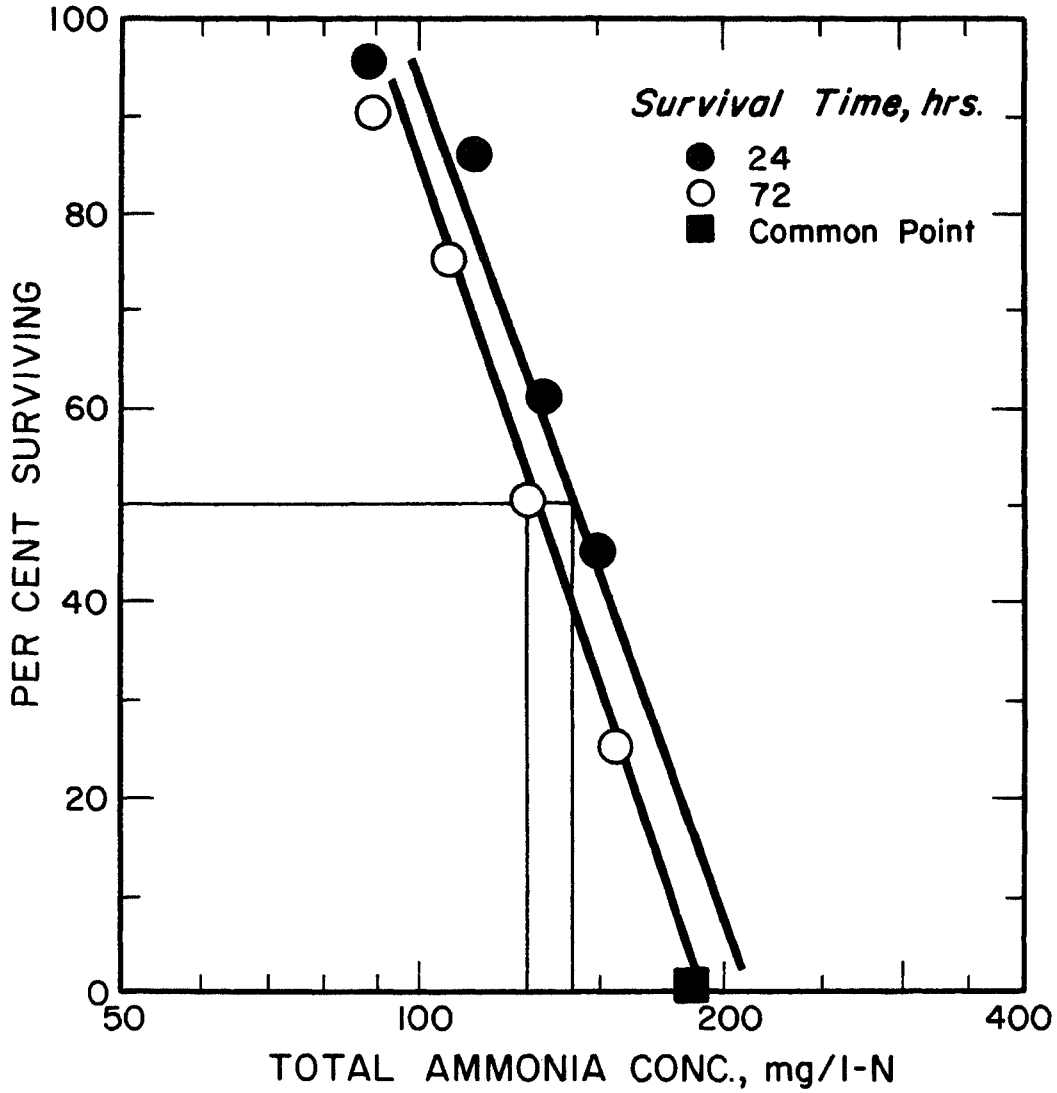

Figure 2. Estimation of Median Tolerance Limits of Total Ammonia for Guppy Fry at pH 6.95-7.5.

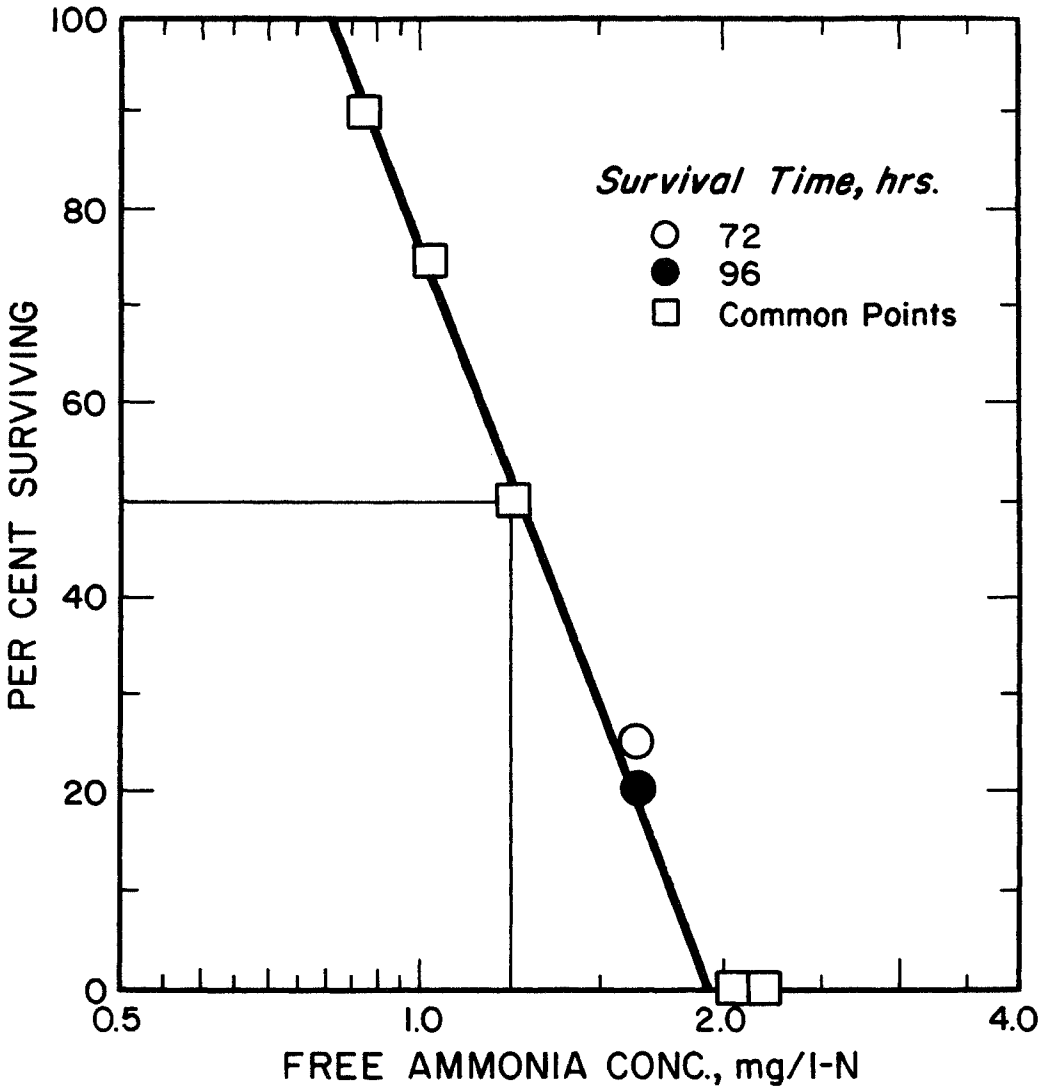

Figure 3. Estimation of Median Tolerance Limit of Free Ammonia for Guppy Fry.

TABLE 2.

DETAILS OF EXPERIMENTAL CONDITIONS IN TESTS TO DETERMINE THE TOXICITY OF AMMONIA AND POTASSIUM NITRATE TO GUPPY FRY

Test	Length of Fish mm	pH Range	Dissolved Oxygen mg/l	Total Solids mg/l	Hardness mg/l as $CaCO_3$	Alkalinity mg/l as $CaCO_3$
			AMMONIA TOXICITY STUDIES			
A_1	8.0 (7.1-11.0)	6.95-7.50	6.80-8.20	362	127.9	33.20
A_2	8.25 (6.3-11.0)	7.40-7.50	6.60-8.20	335	193.6	31.35
A_3	8.70 (6.8-10.6)	7.40-7.50	7.10-8.20	243	148.0	25.70
			NITRATE TOXICITY STUDIES			
B_1	7.81 (7.0-9.5)	7.41-7.60	8.30	148	125.9	43.80
B_2	8.53 (7.0-9.0)	7.40-7.58	8.30	136	122.2	30.75
B_3	8.50 (7.0-10.9)	7.48-7.68	8.30	142	123.1	39.40
B_4	7.95	7.40-7.65	8.30	188	116.8	25.15

was estimated from the solution pH as measured with the Sargent-Welch combination electrode and pH meter.

The average TL_m values expressed as free ammonia were found to be 1.47, 1.27, 1.26 and 1.24 mg/l-N for 24, 48, 72 and 96 hours, respectively. The TL_m values for total ammonia as well as free ammonia are summarized in Tables 3 and 4. The reproducibility of the results will be discussed later.

TABLE 3. RESULTS OF TESTS ON THE TOXICITY OF TOTAL AMMONIA TO GUPPY FRY

Test	pH Range	24-hr. TL_m	48-hr. TL_m	72-hr. TL_m	96-hr. TL_m
A_1	6.95-7.50	148.0	128.2	128.2	128.2
A_2	7.40-7.50	79.0	78.0	74.2	74.2
A_3	7.40-7.50	94.0	75.5	74.4	71.1

In an additional study, mature guppy males were used as the test animals at a free ammonia concentration of 0.14 to 1.30 mg/l - N and pH range of 7.10 to 7.50. Even at free ammonia concentrations exceeding the TL_m for guppy fry, no deaths were observed during the 96-hour test period. In another experiment, sick fish were used. It was observed that the 72-hr. TL_m value for free ammonia decreased from 1.26 mg/l - N to 0.78 mg/l - N.

Toxicity of Potassium Nitrate

The toxicity of nitrate ion to guppy fry was investigated using the potassium salt. Typical results showing the number of fish surviving as a function of exposure time at various concentrations of potassium nitrate are given in Figure 4. The curves are similar to those for ammonia having three stages: an induction period, a high death rate period, and a low death rate period. At a nitrate concentration of 200 mg/l - N, 50 percent of the fish

TABLE 4.

RESULTS OF TESTS ON THE TOXICITY OF FREE AMMONIA TO GUPPY FRY

	TL_m		TL_m		TL_m		TL_m	
Test	Conc. mg/l - N	Percent Deviation	Conc. mg/l - N	Percent Deviation	Conc. mg/l - N	Percent Deviation	Conc. mg/l - N	Percent Deviation
A_1	1.48	0.68	1.21	4.72	1.21	1.58	1.21	2.42
A_2	1.35	8.18	1.34	5.51	1.31	3.97	1.31	5.65
A_3	1.57	6.80	1.25	1.58	1.20	4.75	1.19	4.03
Average	1.47	±5.22	1.27	±3.94	1.24	±3.43	1.24	±4.03

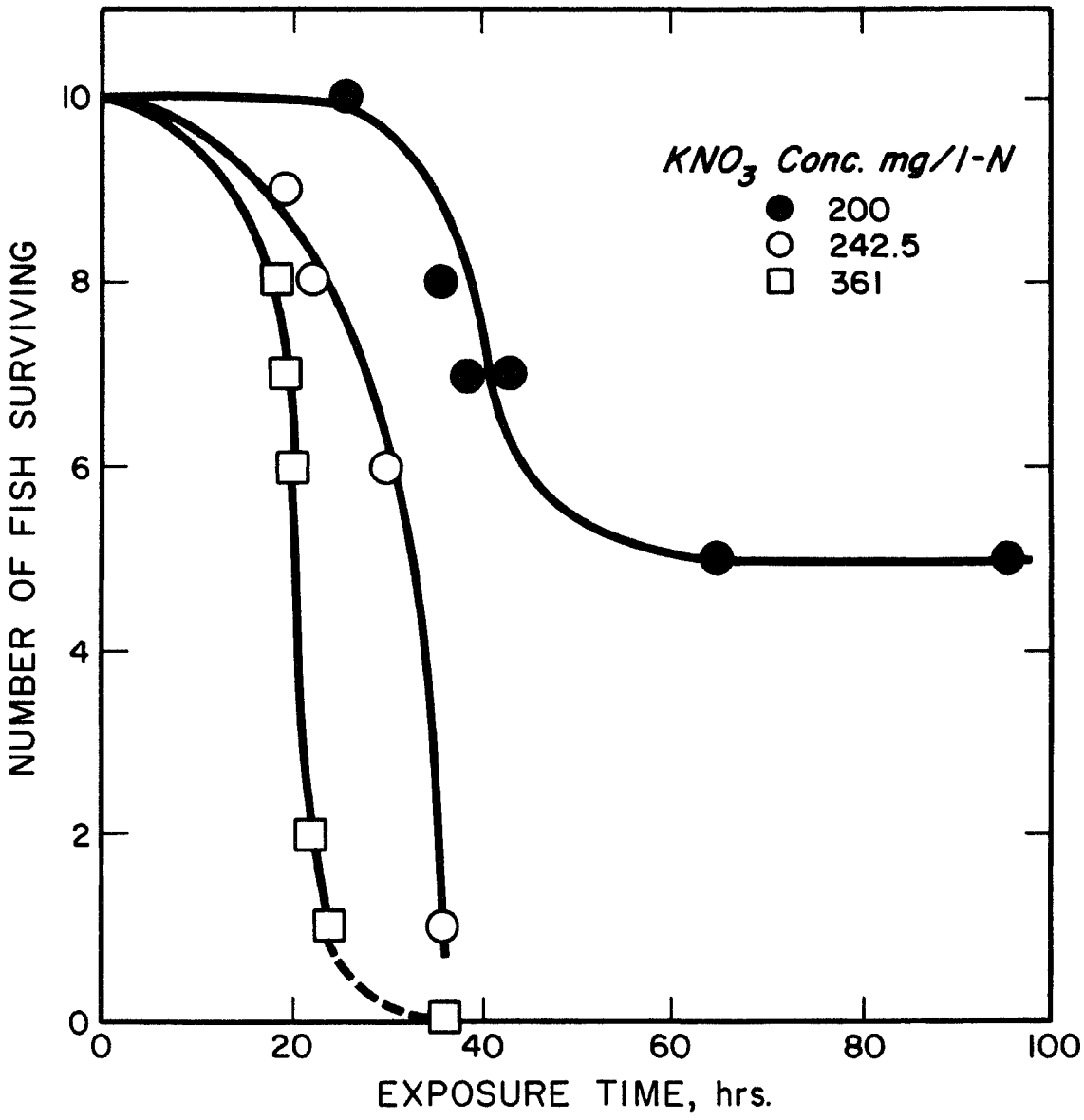

Figure 4. Typical Data Showing Fish Survival at Different Potassium Nitrate Concentrations at pH 7.4-7.5.

died in the first 70 hours. In the next 7 days no further deaths were observed. The absence of the third stage at concentrations above 242.5 mg/l - N can be seen in the figure. It was found that the induction period for an ammonia solution is less than that of a potassium nitrate solution having the same concentration/TL_m ratio. This suggests that the toxicity of the two substances occurs by different mechanisms.

Figure 5 shows the estimation of the 24 and 72-hr. TL_m values for the toxicity of potassium nitrate to guppy fry by straight line graphical interpolation. Similar straight lines were obtained for the 48 and 96-hour data. The experimental conditions and the results of the tests are summarized in Tables 2 and 5, respectively. All experiments were conducted at a temperature range of 24°C to 26°C and total phosphate buffer concentration of 1×10^{-3} M. The average values of the mean tolerance limits for potassium nitrate obtained from four different tests were determined to be 266.5, 218.8, 199 and 191.3 mg/l - N for 24, 48, 72 and 96 hours, respectively. The reproducibility of the results will be discussed in a following section.

Toxicity of Ammonia and Nitrate Mixtures

The toxicity of ammonia in the presence of potassium nitrate was also investigated. Figure 6 shows typical death-time curves for different concentration ratios of ammonia and potassium nitrate. The curves are similar to those for ammonia and potassium nitrate having the typical three states. The length of each and the corresponding death rate depended on the concentration of both ammonia and nitrate. The median tolerance limit was estimated as previously described at different ratios of ammonia and nitrate by interpolation as shown in Figure 7. Table 6 summarizes the details of the experimental conditions and the results of the tests. It is obvious that the presence of

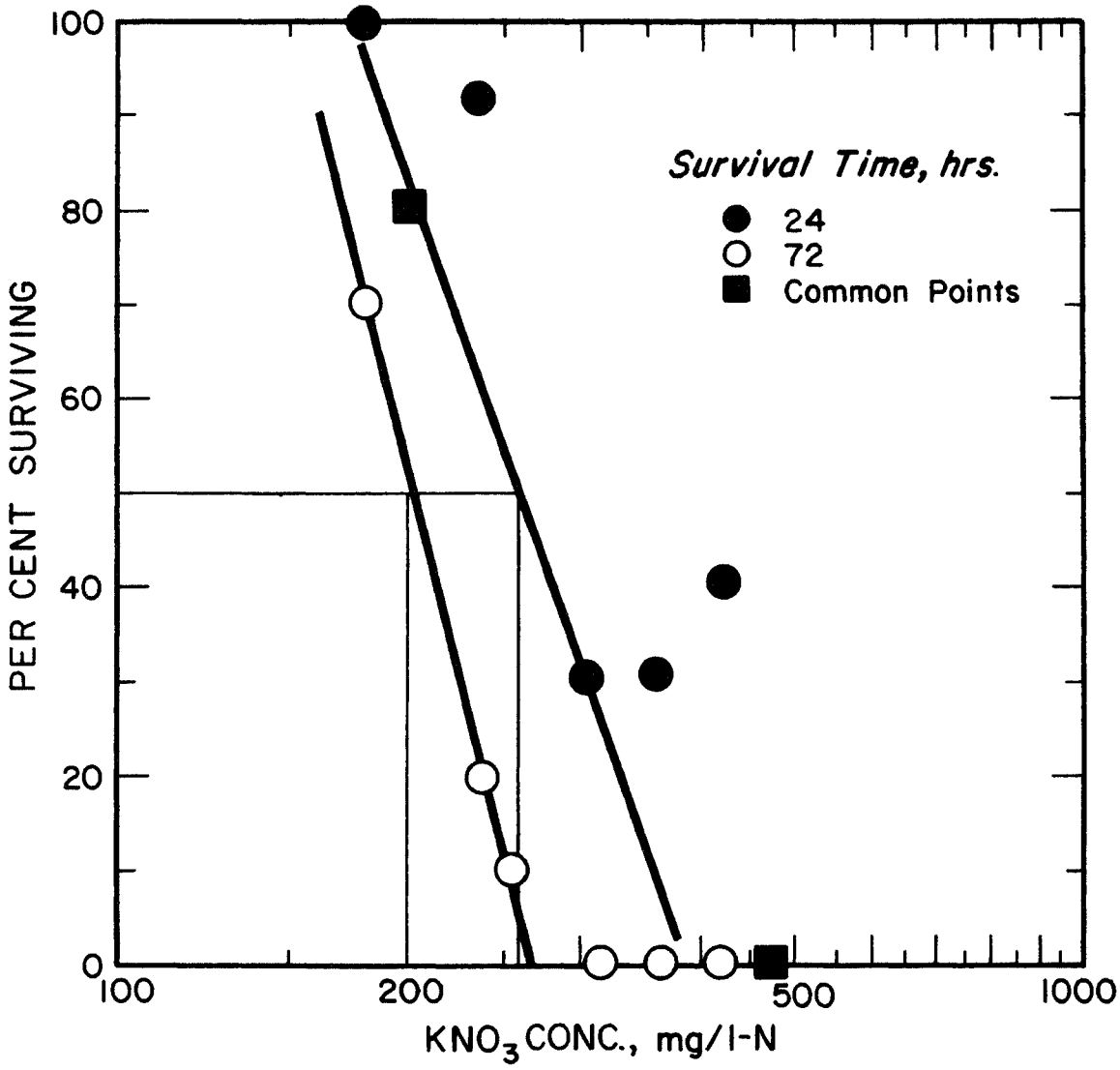

Figure 5. Estimation of Median Tolerance Limits of Potassium Nitrate for Guppy Fry.

TABLE 5.

RESULTS OF TESTS ON THE TOXICITY OF POTASSIUM NITRATE TO GUPPY FRY

	TL_m		TL_m		TL_m		TL_m	
Test	Conc. mg/l - N	Percent Deviation	Conc. mg/l - N	Percent Deviation	Conc. mg/l - N	Percent Deviation	Conc. mg/l - N	Percent Deviation
B_1	--	--	215	1.72	200	0.50	200	4.58
B_2	--	--	230	5.14	188	5.52	188	1.70
B_3	268	1.56	215	1.72	208	4.52	180	5.88
B_4	265	0.56	215	1.72	200	0.50	197	3.00
Average	266.5	±0.56	218.75	±2.58	199	±2.76	191.25	±3.79

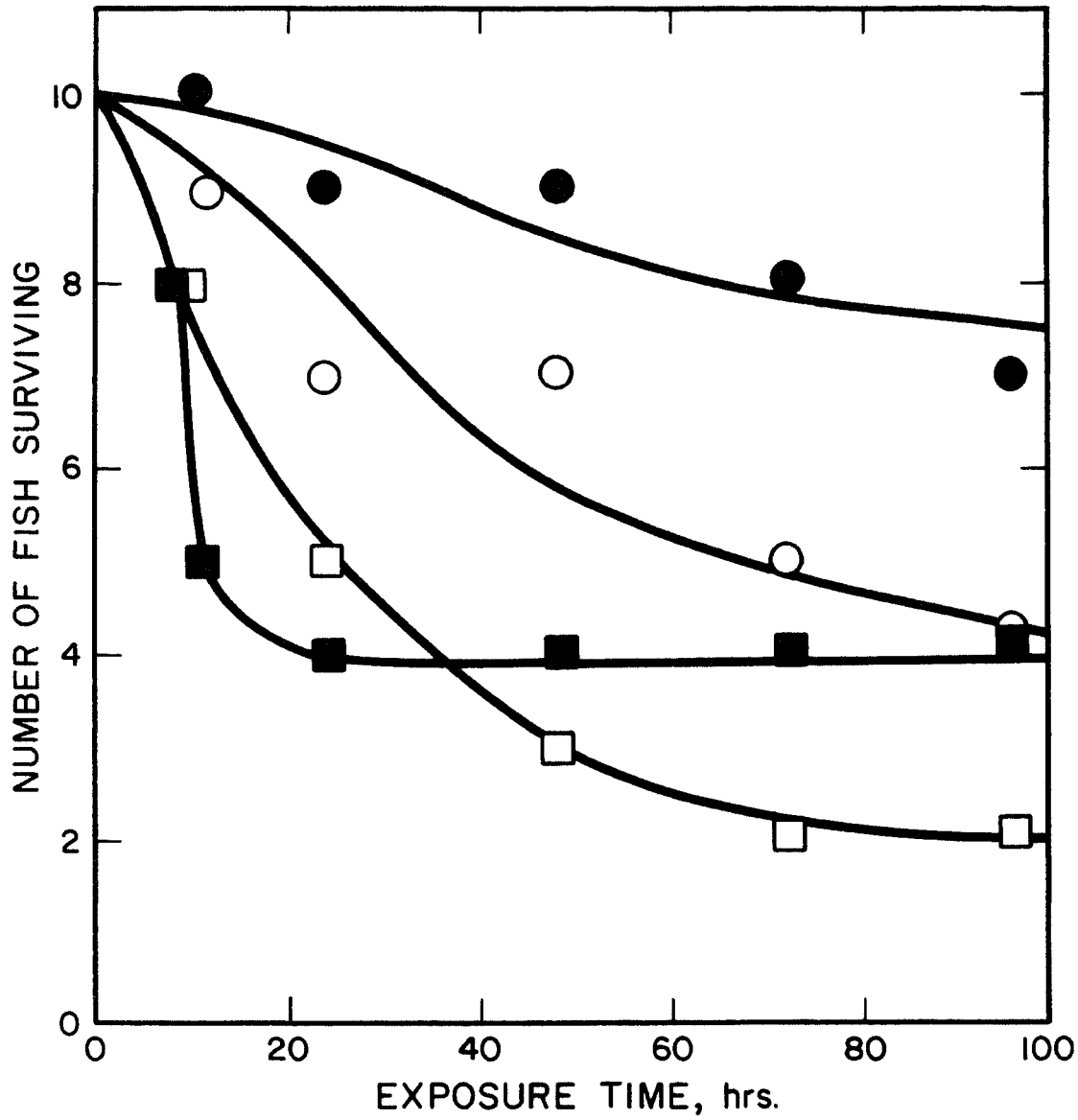

Figure 6. Typical Data Showing Fish Survival as a Function of Time for Mixtures. The respective total ammonia and potassium nitrate concentrations in mg/1 - N are 9.6 and 148 (blackened circles), 50 and 50 (open circles), 47.5 and 103 (open squares) and 38.14 and 100 (blackened squares).

nitrate can decrease the median tolerance limit for ammonia. For example, 50 and 102 mg/l - N of potassium nitrate results in the 72-hour median tolerance limit of free ammonia being lowered from 1.24 to 0.82 and 0.62 mg/l - N, respectively.

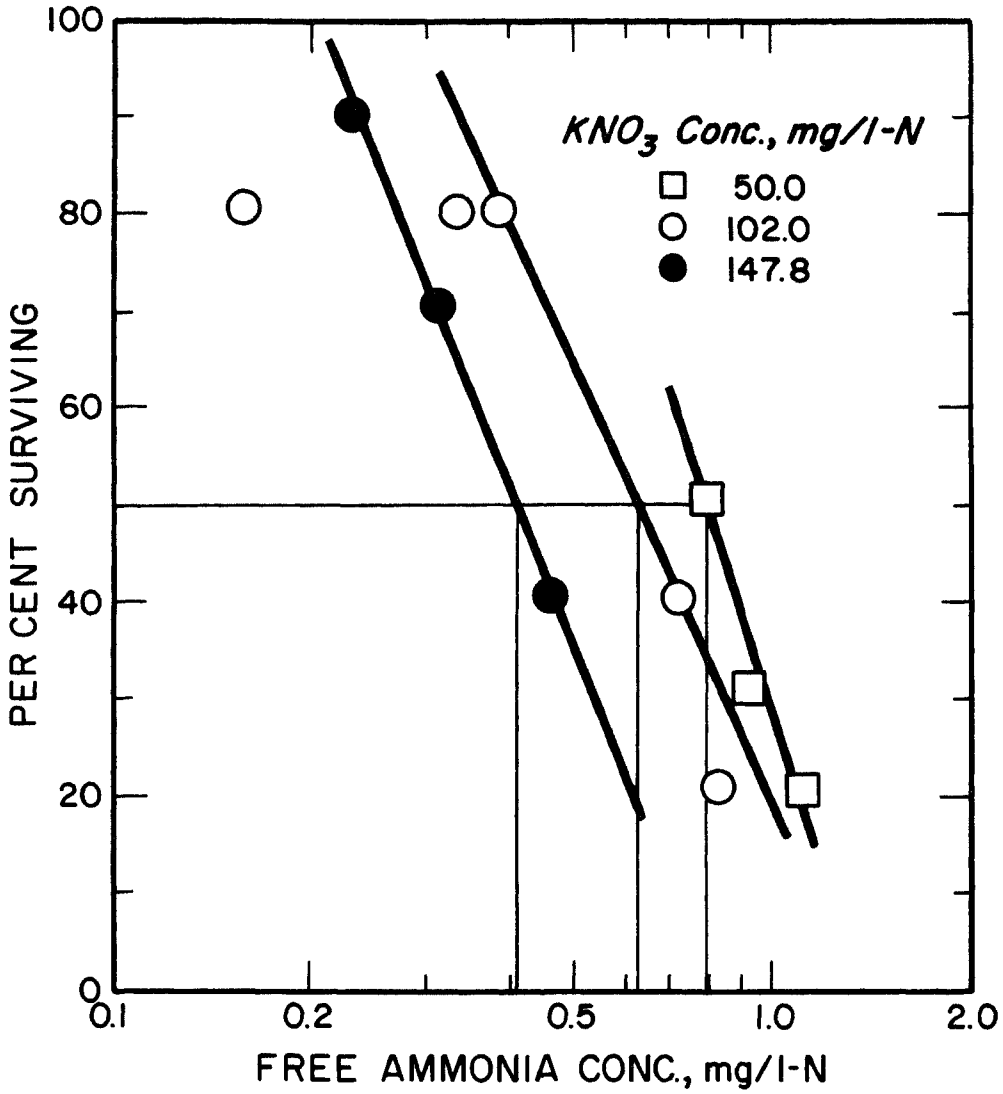

Figure 7. Estimation of 72-Hour TL_m of Ammonia in the Presence of Potassium Nitrate.

TABLE 6.

DETAILS OF EXPERIMENTAL CONDITIONS IN TESTS ON THE JOINT TOXICITY OF FREE AMMONIA AND POTASSIUM NITRATE TO GUPPY FRY

Length of Fish mm	pH Range	Dissolved Oxygen & Total Solids mg/l	Hardness & Alkalinity mg/l as $CaCO_3$	72-hr. TL_m, mg/l - N		Σ Toxicant Conc.*
				Free Amm.	KNO_3	
7.33 (6.1-8.1)	7.41-7.62	7.58-8.40 178	108.0 31.05	0.62	102.0	0.995
7.81 (6.0-9.5)	7.41-7.61	7.40-8.20 320	170 27.2	0.41	147.8	1.061
7.64 (6.7-9.9)	7.39-7.57	7.40-8.20 320	170 27.2	0.82	50.0	0.888
7.42 (613-10)	7.41-7.60	7.10-8.20 280	140 29.2	1.040	32.3	1.07
8.70 (6.7-10.5)	7.39-7.57	7.30-8.20 385	160 27.2 285	0.59	191	1.39
7.92 (6.8-11.0)	7.40-7.59	7.10-8.30 285	160 27.2	0.48	186.3	1.32
8.0 (7.1-10.8)	7.37-7.62	7.60-8.30 270	173	0.51	137.1	1.09

* The toxicant conc. was expressed as a fraction of its 72-hr. TL_m value.

DISCUSSION AND CONCLUSIONS

Toxicity of Ammonia and Nitrate

Numerous studies reported in the literature on the toxicity of ammonia and nitrates to fish have yielded varying results reflecting differences between species and environmental variables such as the level of free carbon dioxide, hardness, and temperature. In general, it has been reported in the literature that the toxicity of ammonia to several species of fish as determined by short term tests lies between 0.3 and 3 mg/l - N (22). As summarized in Table 4, the 72-hr. TL_m of free ammonia for guppy fry as determined in this study falls within this range, having an average value of 1.26 mg/l - N. The concentration range for the TL_m for nitrate is very strongly dependent on the cationic composition of the solution. For example, the literature reveals that solutions of potassium nitrate are much more toxic than sodium nitrate solutions (7, 27). The average 96-hr. TL_m value for potassium nitrate determined in this study is 191 mg/l - N and appears to be within the range reported in the literature. Comparison of the TL_m values for ammonia and nitrate shows that ammonia is more toxic than nitrate. This was expected, although potassium nitrate appears to be more toxic, at least to guppies, than is usually thought.

The TL_m values expressed as total ammonia varied widely depending on pH. In the experiment labeled Test A_1 in Table 3, the pH was 7.50 initially and gradually fell to 6.95 during the 96-hr. test period, while in the experiment labeled Test A_2 the pH was adjusted daily to keep it within the range of 7.40-7.50. The 72-hr. TL_m values expressed as total ammonia for the two experiments were 128.2 and 74.2 mg/l - N, respectively. Correcting for pH the 72-hr. TL_m values calculated for free ammonia were 1.21 and 1.31 mg/l - N for tests A_1 and A_2, respectively. The difference between the latter two values

is insignificant. It is important to notice that within the pH range studied, a change of only 0.10 unit results in a significant variation in the free ammonia concentration. For example, if the pH of a solution containing 74 mg/l - N ammonia is lowered from 7.44 to 7.34, the free ammonia concentration will decrease from 1.13 to 0.90 mg/l - N. The decrease in the free ammonia concentration, which is considered the most toxic component in the system, is about 23 percent in this example. The 24, 48, 72 and 96-hr. TL_m values reproduced very well as indicated by Table 4. The maximum deviation in the 72-hr. TL_m was 4.75 percent. The average value of the 72-hr. TL_m as free ammonia and total ammonia are 1.26 and 74 mg/l - N, respectively, in the pH range of 7.40 and 7.50. These values indicate that within this pH range the ammonia molecule is about 56 times more toxic to the guppy fry than the ammonium ion.

The maximum percent deviation in the 72-hr. TL_m values of potassium nitrate for guppies was 5.52 percent as shown in Table 5. Apparently the results were reproducible because the toxicity of potassium nitrate is not as sensitive to pH as ammonia. The 96-hr. TL_m value for guppy fry found in this study was 1380 mg/l as KNO_3. As reported by Dowden and Bennett (7), the 24-hr. TL_m values of $NaNO_3$ and KNO_3 for bluegills were 2110 and 761 mg/l - N, respectively.

It was observed with both ammonia and nitrate that the major changes in the TL_m value occurred during the early exposures, while the 48, 72 and 96-hr. TL_m values were about the same. For example, for free ammonia the average 24-hr. TL_m value was 1.47 mg/l - N, while the 48 and 96-hr. TL_m values were 1.27 and 1.24 mg/l - N, respectively.

The simultaneous effect of free ammonia and potassium nitrate was tested to determine their joint toxicity. The results are summarized in Table 6 and represented graphically in Figure 8. The free ammonia 72-hr. TL_m fractions

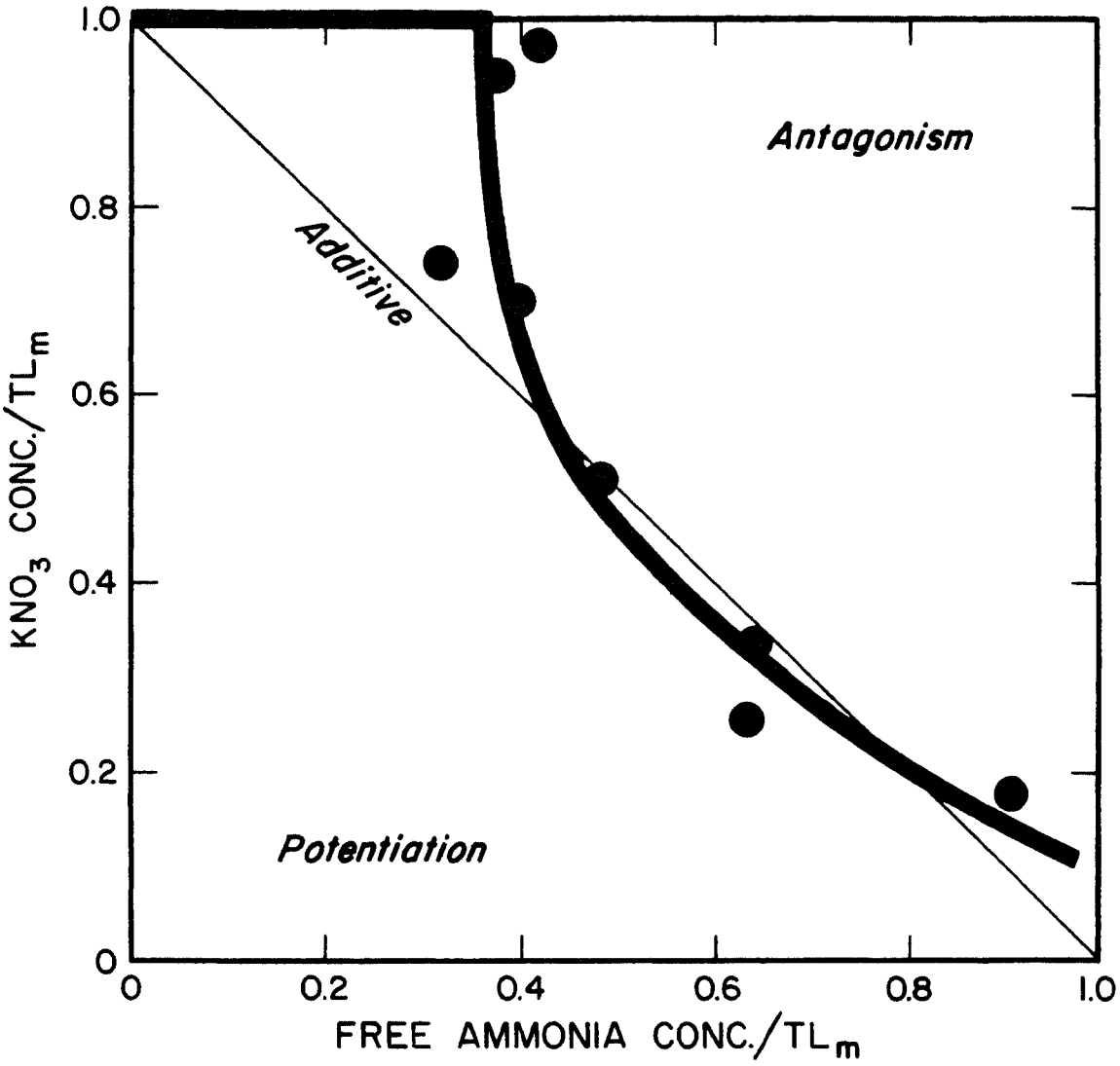

Figure 8. Joint Toxicity of Free Ammonia and Potassium Nitrate to Guppy Fry. The points represent the 72-hr. TL_m values of the free ammonia and potassium nitrate mixtures, expressed as fractions of their individual TL_m values.

were plotted against the potassium nitrate 72-hr. TL_m fractions. The TL_m fraction of a specific toxicant can be defined as the ratio of the TL_m value for that toxicant in the mixture to the TL_m for that toxicant by itself. The resulting curve shown in the figure represents all the possible combinations of ammonia and potassium nitrate concentrations at pH 7.37-7.62 which would kill 50 percent of the fish at 72 hours of exposure. The area above and to the right of the experimental curve represents the toxicant mixture concentrations which would be expected to kill more than 50 percent of the fish at 72 hours, while the area below represents the combinations which would kill less than 50 percent of the fish at that time.

The points located on the 45° diagonal line in Figure 8 occurred at 72-hr. TL_m fractions of ammonia and potassium nitrate greater than 0.35 and 0.1, respectively. With these points the sum of the TL_m fractions of ammonia and potassium nitrate in the mixture were approximately one, meaning that the two substances have an additive response. If the 72-hr. TL_m fraction of free ammonia or potassium nitrate is outside the above limits, the sum of the TL_m fractions is always more than one, and the joint toxicity of the mixture is represented by points above the diagonal straight line. This means that the joint toxicity of the mixture is less than additive. For such a mixture, the actual toxicity would be less than that predicted by adding the concentration fractions of all the toxicants. This observation agrees with Sprague (25), that when the concentration of a toxicant in the mixture is very low, its share in the mixture toxicity will be less than additive. If the points fell below the diagonal line this would indicate potentiation of the toxicity. It can be concluded that the joint action of NH_3 and KNO_3 is not more than additive.

Applicability of Chick's and Watson's Laws

The contact time is one of the most important variables in the disinfection process. The kill-time relationship was formalized in the literature in Chick's law (Equation 2). This equation indicates that the relationship between log N/N_o and t should be represented by a straight line. In most cases the fit of the fish survival data was not linear and the curves did not go through zero at zero time. It can be concluded for both the ammonia and potassium nitrate studies, that Chick's law is not applicable.

The coefficient of dilution, which is a measure of the order of the toxicity reaction, was calculated for both ammonia and potassium nitrate. Watson's equation, where the logarithm of time is plotted against log C, is represented by a straight line with a slope of -n and intersection of log K. A typical plot is shown in Figure 9. The median survival time, T_m, was used to express the value of t in Watson's Law being calculated from the geometric mean of the survival time for each individual fish using Equation 5. The values of the coefficient of dilution and log K as computed by the method of least squares were 1.94 and 1.57 for free ammonia, and 1.35 and 4.77 for potassium nitrate, respectively. The applicability of Watson's Law for potassium nitrate will be discussed further in the next section.

The General Equation

Studies on the kinetics of the toxicity of ammonia and potassium nitrate for guppies fry were conducted to test the general mathematical equation described by Hom (Equation 9). Plots of the equation for the toxicity of the free ammonia to guppy fry were not linear and the slope of the curve depended on the toxicant concentration, indicating that the data does not fit the m-order and n-order reaction model.

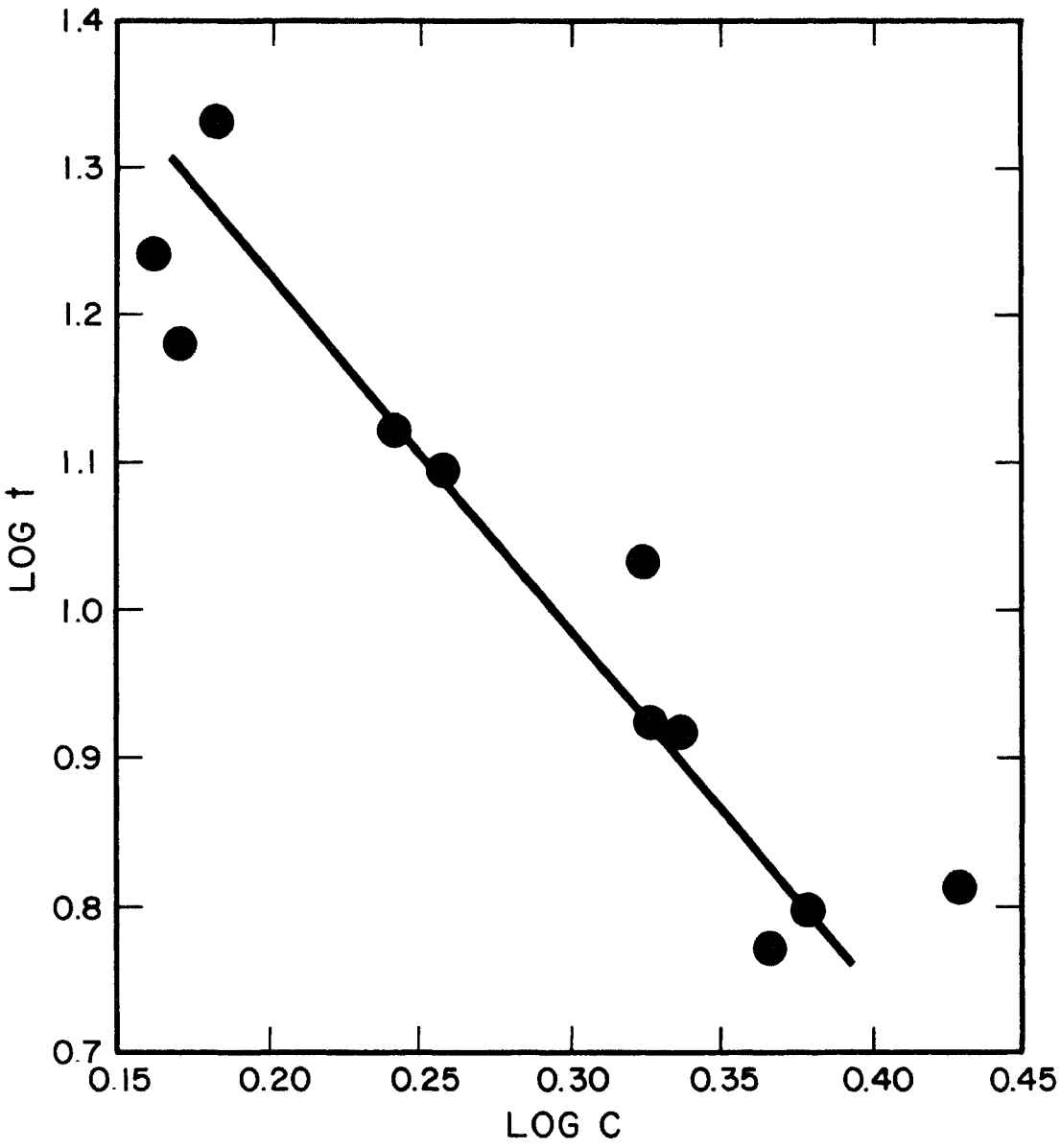

Figure 9. Typical Relationship Between Contact Time and Free Ammonia Concentration Suggested by Watson's Law

Studies to investigate the applicability of the general equation to the toxicity of potassium nitrate for guppy fry indicate that the relationship between log-log N_o/N and log t is linear as shown in Figure 10. Except for the concentration of 237.5 mg/l - N, the slopes of these lines do not appear to be dependent on the toxicant concentration. The results of this study are also summarized in Table 7. In the table the value of m for five different potassium nitrate concentrations is about the same, having an average of 4.43. The value of m at a concentration of 237.5 mg/l - N was not included in the calculation; the average deviation of the value of m was $\pm$ 0.24.

Using the average value of m and the intersection which were determined experimentally, the theoretical relationship between N/N_o and t can be calculated using Equation 8. Figure 11 shows the graphical representation of the calculated relationship between N/N_o and t (solid line) and the deviation of the experimental points (circles) from the theoretical curve. The figure shows that the experimental points are very close to the calculated curve except in the induction period where a clear deviation between the experimental points and the theoretical curve can be observed. The time required to kill 50 percent of the fish at each concentration, T_m, can be obtained by using Equation 8 and the average value of m. The value of the Watson's Law constant, K, can be determined by using the calculated T_m and the average value of n. Table 7 shows that K is essentially constant at all concentrations. The average value of log K is 4.75 $\pm$ 0.05. This value is nearly identical to the log K of 4.77 which was obtained by direct application of Watson's Law. This is evidence that both Watson's Law and the general equation may have some value for representing the toxicity of potassium nitrate to guppy fry.

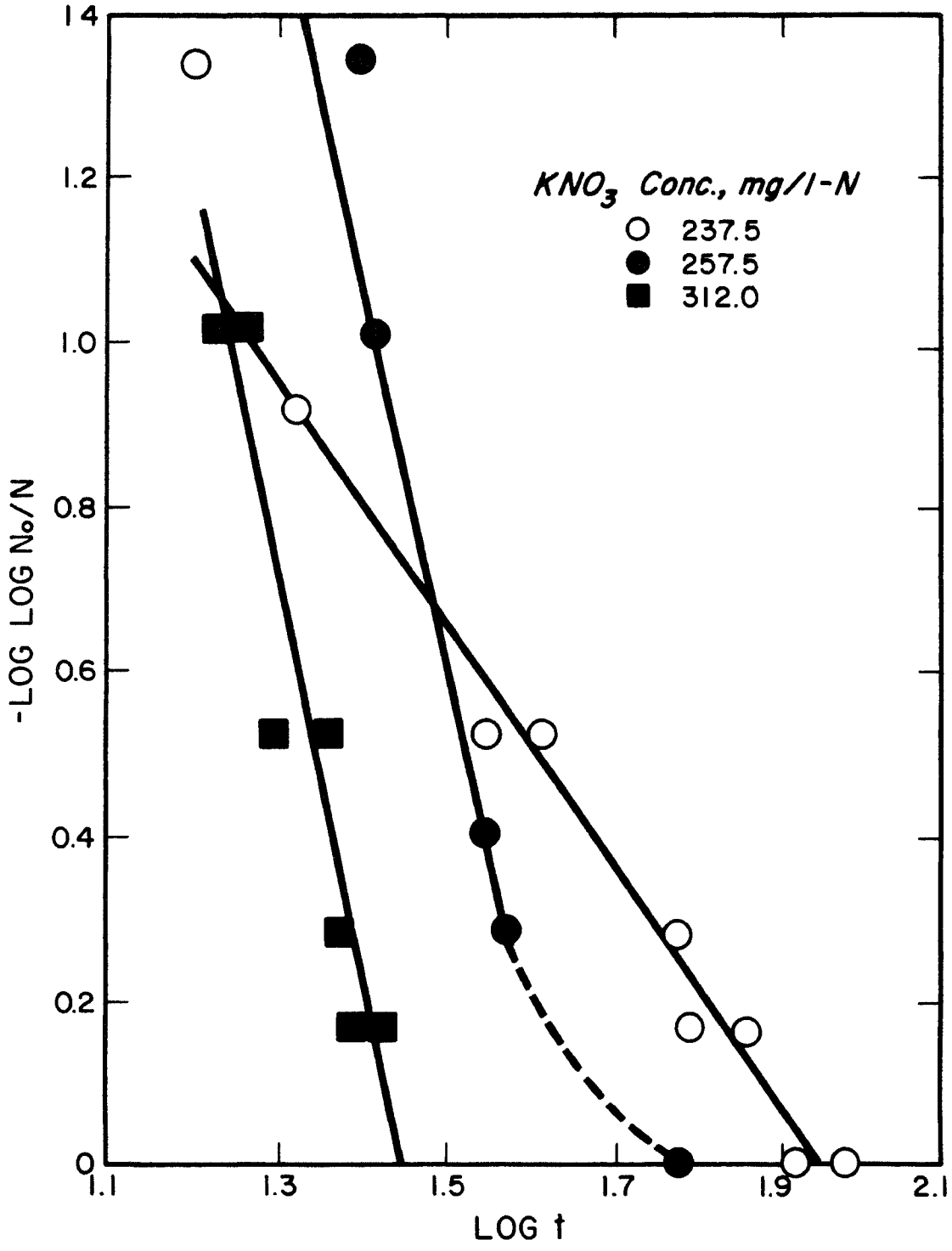

Figure 10. Typical Application of the General Equation to the Toxicity of Potassium Nitrate to Guppy Fry.

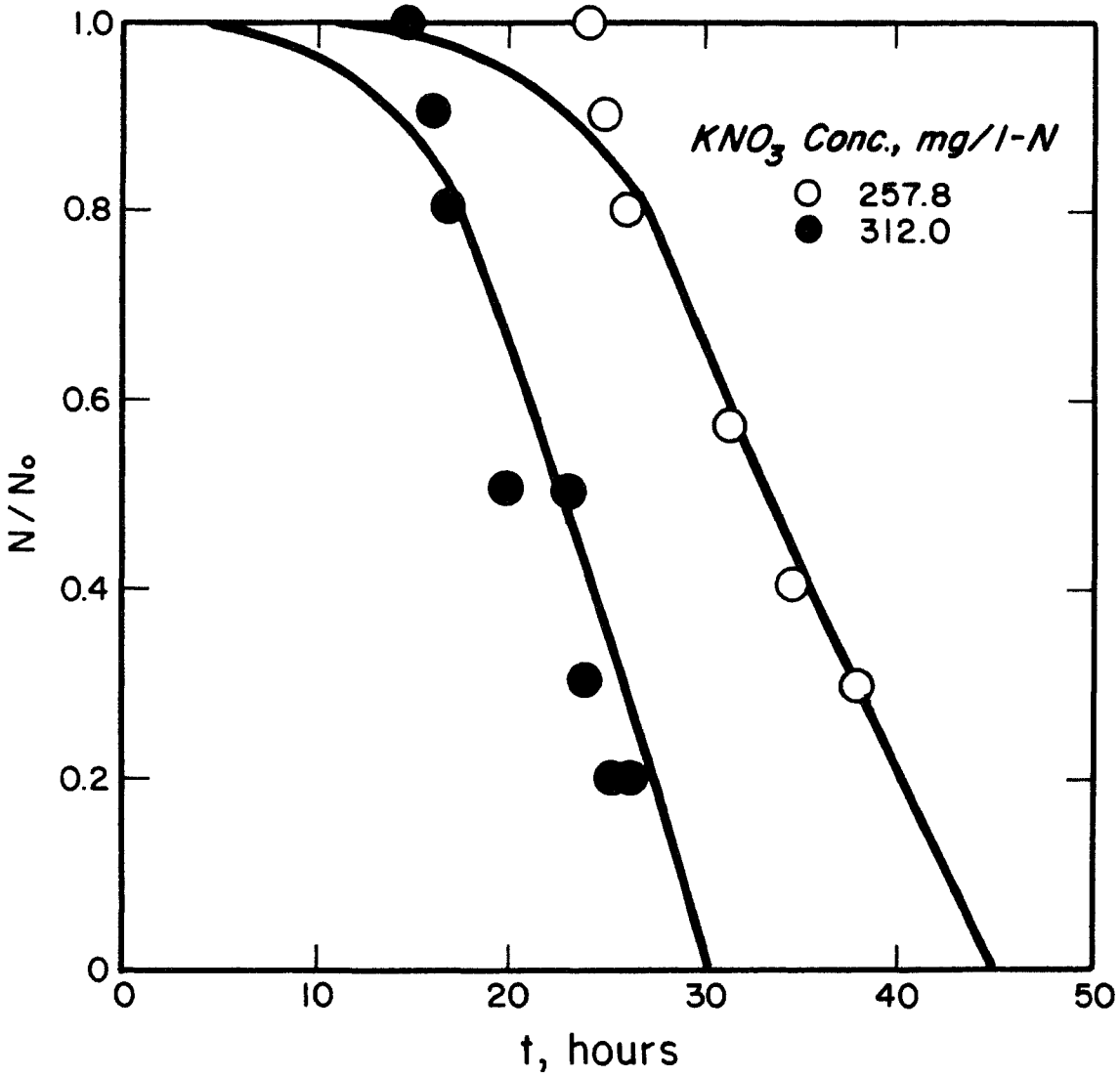

Figure 11. Comparison of Experimental Data with the Line Calculated from the Parameters Determined for the General Equation.

TABLE 7. APPLICABILITY OF HOM'S MODEL AND WATSON"S LAW TO POTASSIUM NITRATE TOXICITY TO GUPPY FRY

KNO_3 Conc., mg/l - N	Slope, m	Intercept	t_{50}, hrs.	Log K(a)
237.5	1.45	-2.84	39.8	4.800
257.5	4.55	-7.28	33.7	4.860
312	4.78	-6.95	22.5	4.700
362	4.10	-5.90	20.4	4.750
410	4.16	-5.75	18.0	4.775
465.5	4.54	-5.41	12.0	4.668
Average (b)	4.43 $\pm$ 0.24			4.75 $\pm$ 0.05

(a) K calculated using n=1.347 and t_{50} (cal. from Hom's Model)

(b) Results for first row not included in averages; $\pm$ average deviation.

Chen and Sellect Kinetic Model

The Chen and Sellect Model was tested for its applicability to the toxicity of ammonia and potassium nitrate to guppy fry. The concentration ranges investigated were 0.61 to 2.12 mg/l - N for free ammonia and 150 to 450 mg/l - N for potassium nitrate. The logarithm of the survival ratio was plotted against the exposure time, t, as shown in Figure 12 to represent Equation 12. The coefficients ($-KC^n+H$) were obtained from the slopes of the lines of data plotted as shown in the figure and represent the correlation between log N/N_o and t at different toxicant concentrations.

For potassium nitrate, plotting this coefficient against C yielded a straight line as shown in Figure 13. This indicates that the value of n must be equal to one and that K and H may be obtained from the slope and intercept. The least squares method was used to calculate the values of H and K. These values then were used to calculate the threshold concentration, C_t, by appli-

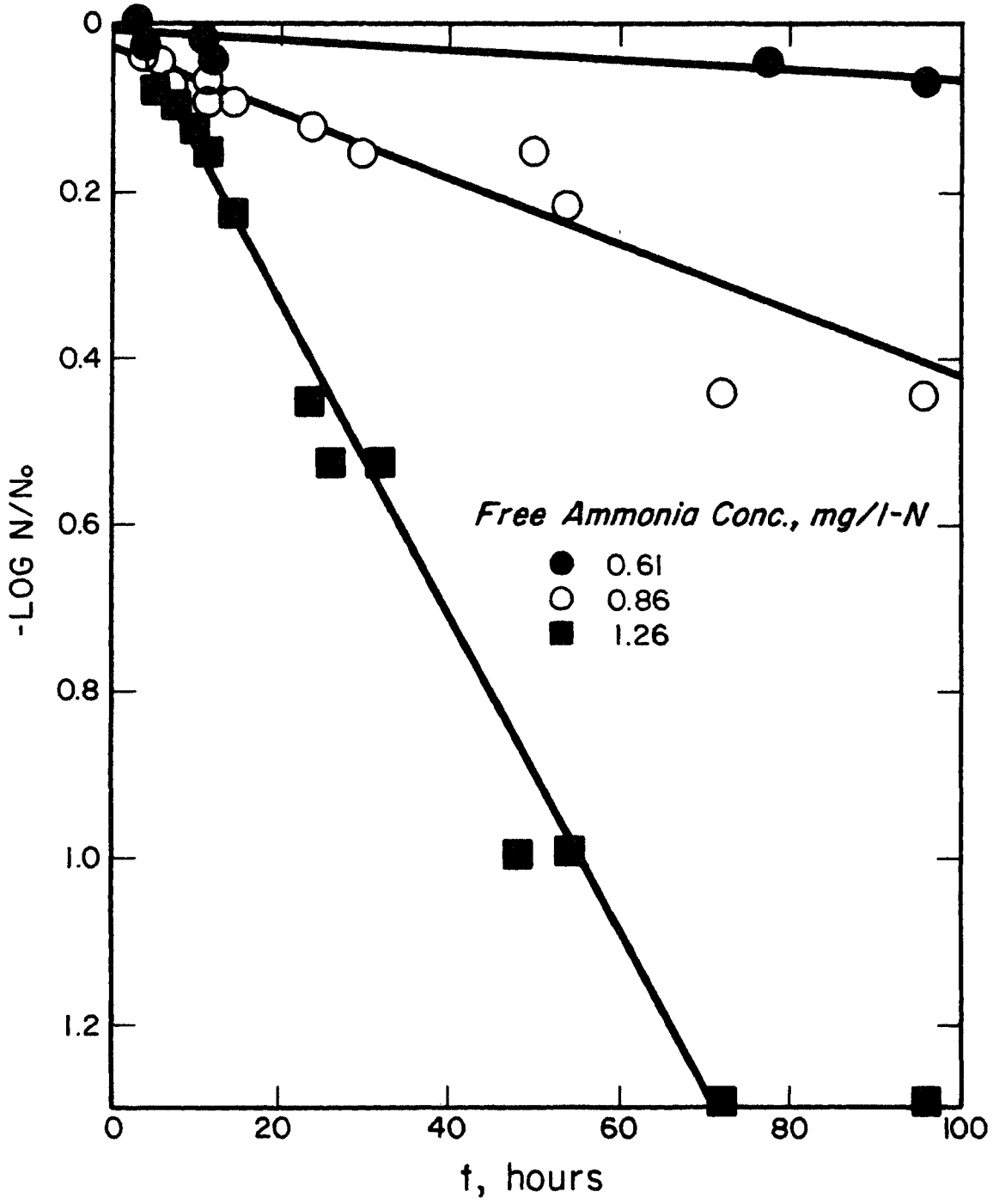

Figure 12. Semilog Plots of Survival Data at Different Free Ammonia Concentrations.

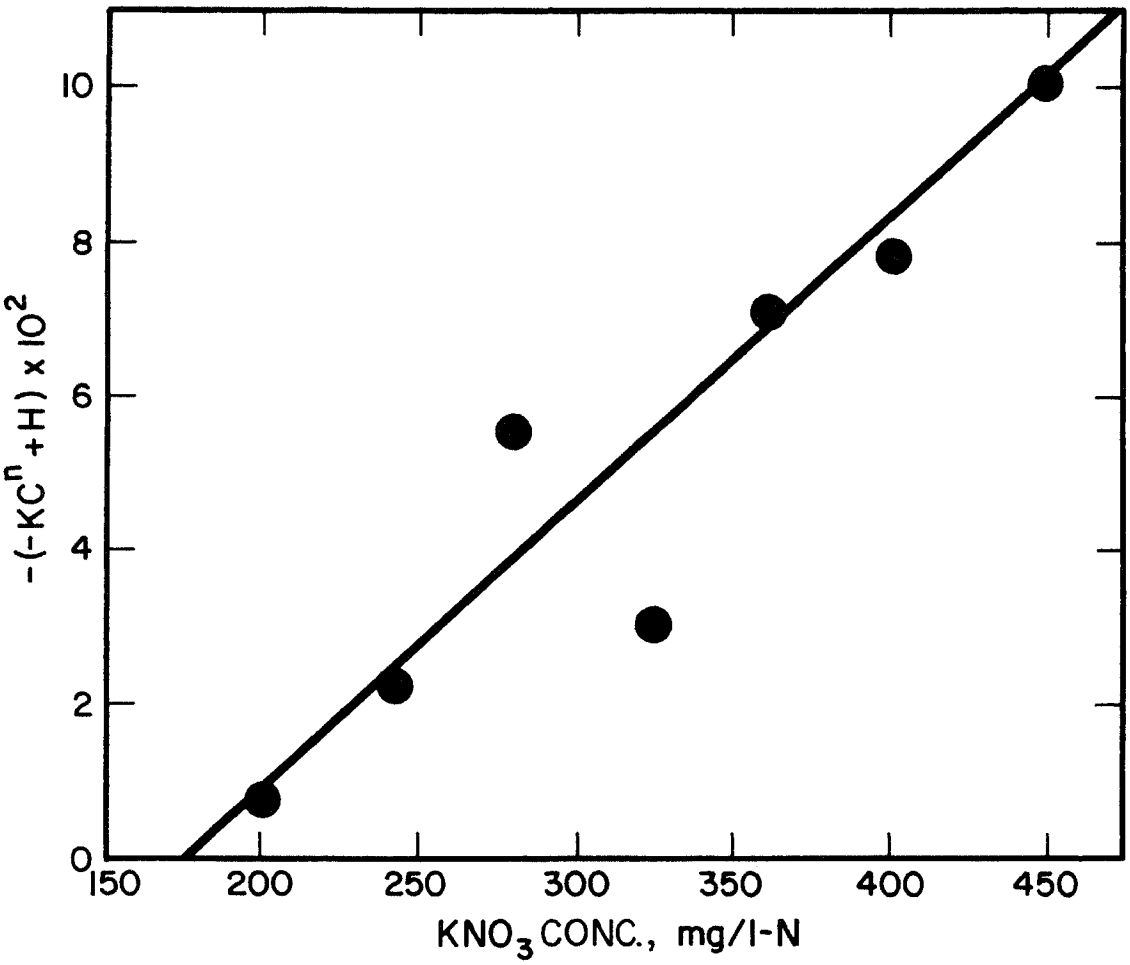

Figure 13. Relationship Between Net Mortality Rate Coefficient and Potassium Nitrate Concentration.

cation of Equation 10. The values of H, K and C_t for potassium nitrate were 6.1×10^{-2}, 3.5×10^{-4}, and 174 mg/l - N, respectively. This value of the threshold concentration is about 85 percent of the 72-hr. TL_m.

For ammonia, plotting $(-KC^n+H)$ against the free ammonia concentration, a nonlinear relationship was obtained as shown in Figure 14. This indicates that the value of n is not equal to one. The curve is similar in shape to the one found by Malcolm et al. (21). A computer program was developed to find the value of n numerically by solving the following equations:

$$S_i = KC_i^n + H \qquad ....(16)$$

$$S_j = -KC_j^n + H \qquad ....(17)$$

$$S_k = -KC_k^n + H \qquad ....(18)$$

where S_i, S_j and S_k are the slopes of the lines representing the relationship between log N/N_o and t at different concentrations C_i, C_j and C_k respectively. Subtracting Equation 17 from 16 and Equation 18 from 17 and dividing results in

$$\frac{S_i - S_j}{S_j - S_k} = \frac{C_i^n - C_j^n}{C_n^n - C_k^n} = S' \qquad ....(19)$$

then

$$F = (S' + 1)\, C_j^n - S'C_k^n - C_i^n \qquad ....(20)$$

Using three different concentrations and assuming different values for n between zero and seven then solving Equation 20, the value of n which makes F as near as possible to zero is the order of the toxicity reaction. Then by using Equations 16 and 17, the values of K and H can be calculated. Using Equation 10 the value of the threshold concentration, C_t, can also be esti-

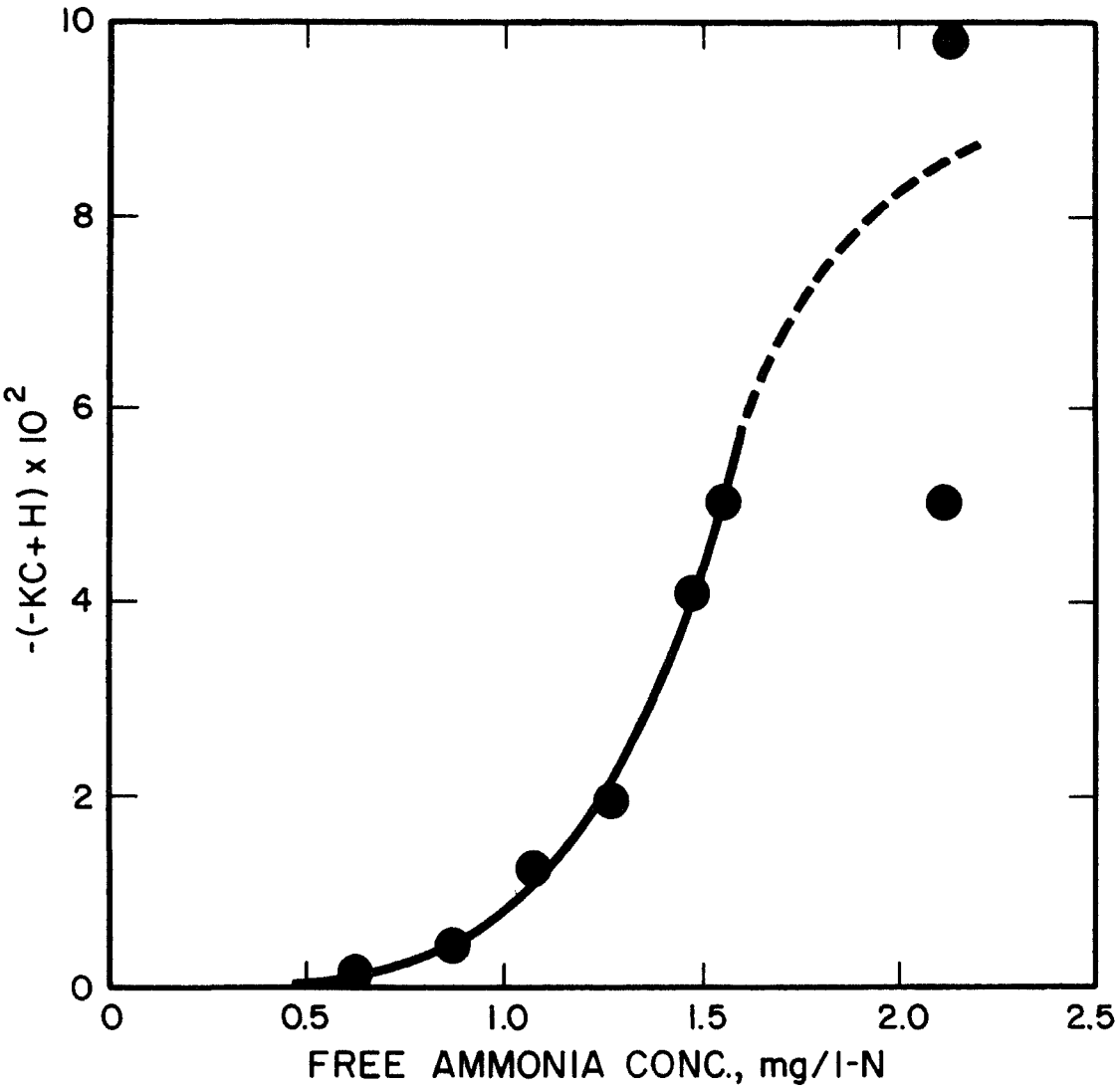

Figure 14. Relationship Between Net Mortality Rate Coefficient and Free Ammonia Concentration.

mated. The program chooses all the possible combinations between any three different concentrations in the data, and then goes through the previous procedure to find the n, H, K and C_t values for each combination. Finally, the average values of n, H, K and C_t for all combinations are calculated.

Running this program to test the model for ammonia, the values of n and C_t fluctuated from one combination to another. For example, the value of n ranged between 0.7 and 4.3 the value of C_t fluctuated between 0.41 and 1.20 mg/l - N. The average values for n and C_t were 2.77 ± 0.62 and 0.67 ± 0.24 mg/l - N, respectively. The reason for this fluctuation in the values of n and C_t is that the exponential function tends to amplify the experimental error. It may be necessary to neglect the points which are positioned far from the curve which represents the relationship between $(-KC^n+H)$ and C.

The calculated threshold concentration is not to be taken at face value for safe concentrations in receiving waters. To estimate such concentration, experimental water and test animals must be selected properly and a safety factor allowance must be made (4).

Summary of Conclusions

On the basis of the experimental results, the following conclusions are indicated:

1. The static bioassay test proved to be a reliable technique for determining the toxicity of ammonia and potassium nitrate to guppy fry. The 72-hr. TL_m values for free ammonia and potassium nitrate solutions for guppy fry were found to be 1.26 and 199 mg/l - N, respectively. The corresponding 96-hr. values were 1.24 and 191 mg/l - N, respectively. All values were reproducible within about 5 percent.

2. The simultaneous effect of ammonia and potassium nitrate mixtures to guppy fry, based on 72-hr. TL_m data, is additive when the concentrations of the two are more than approximately 0.5 and 30 mg/l - N, respectively. If the concentration of either of the toxicants is below these values the response will be less than additive.

3. Watson's Law is applicable for both ammonia and potassium nitrate, whereas Chick's Law does not apply to either. Hom's disinfection rate model appears to be applicable to the potassium nitrate toxicity to guppy fry while it is not applicable to the toxicity of ammonia to these organisms.

4. The Chen and Sellect toxicity model is of limited value for representing the ammonia data, but can be used to obtain the threshold concentration of potassium nitrate to guppy fry; this concentration was found to be 174 mg/l - N.

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