DETERMINATION OF ALUMINUM IN FISH TISSUE BY INSTRUMENTAL NEUTRON ACTIVATION ANALYSIS

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ABSTRACT

An instrumental neutron activation analysis (INAA) method for the determination of aluminum (Al) in fish tissue was developed to aid in the investigation of Al-caused death in fish. Six treatments having pH values of 4.0, 4.4, 4.8, 5.2, 5.6, and 6.0 and each containing 1 mg Al l^{-1} were followed over a 14-day period and subsequent Al concentrations were determined. Bluegills (Lepomis macrochirus) and orangespotted sunfish (Lepomis humilis) subjected to the acid and Al treatments were removed when moribund, preserved in liquid nitrogen, and their gill samples analysed for Al by INAA. The gills from the pH treatments of 4.0, 4.4, and 6.0 demonstrated little uptake of Al (statistically similiar to the control). The gills from the pH treatments of 4.8, 5.2, and 5.6 evidenced an increase in mean Al concentration as the treatments were 2.3, 2.0, and 3.2 times larger, respectively, than the mean control concentration of Al. The pH 5.6 treatment, however, was the only ststistically significant treatment. The overall results demonstrate that Al can be determined at ppm levels in fish gills by the INAA method developed in this study.

Dedicated to Anonymous
who has done so much for
so little recognition

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

ABSTRACT

An instrumental neutron activation analysis (INAA) method for the determination of aluminum (Al) in fish tissue was developed to aid in the investigation of Al-caused death in fish. Six treatments having pH values of 4.0, 4.4, 4.8, 5.2, 5.6, and 6.0 and each containing 1 mg Al l^{-1} were followed over a 14-day period and subsequent Al concentrations were determined. Bluegills (Lepomis macrochirus) and orangespotted sunfish (Lepomis humilis) subjected to the acid and Al treatments were removed when moribund, preserved in liquid nitrogen, and their gill samples analysed for Al by INAA. The gills from the pH treatments of 4.0, 4.4, and 6.0 demonstrated little uptake of Al (statistically similiar to the control). The gills from the pH treatments of 4.8, 5.2, and 5.6 evidenced an increase in mean Al concentration as the treatments were 2.3, 2.0, and 3.2 times larger, respectively, than the mean control concentration of Al. The pH 5.6 treatment, however, was the only ststistically significant treatment. The overall results demonstrate that Al can be determined at ppm levels in fish gills by the INAA method developed in this study.

CHAPTER ONE

INTRODUCTION

In order to determine the mechanism of aluminum (Al) toxicity in teleost fishes, scientists must have an Al detection method which is specific, reproducible, and accurate. Elucidation of the mechanisms of metal toxins ideally involves the use of radiotracers, as they can be easily followed, quantifed, and isolated, establishing a direct relationship of causality. Unfortunately, there is no suitable radioisotope of Al which is appropriate for tracer studies. Therefore, an indirect method of determining Al concentrations in toxicity studies must be developed.

There are four shortcomings associated with indirect methods of Al toxicity evaluation currently in use: sensitivity of Al detection is low (50 ppb), the sample is destroyed during analysis, the sample preparations are often complicated, and there is uncertainity in determining the precise biological pathway of toxicity.

The present state-of-the-art method of Al determination is accurate to 50 ppb (50 ug l^{-1}) (Willard, et al., 1965) when no interferences are present. (In radiotracer studies 1 ppt (1 ng l^{-1}) levels are commonplace). Gross biological effects due to Al toxicity can be prevalent at the 50 ppb level in water and above (Baker and Scholfield, 1981). Due to the destruction of tissue subjected to these relatively low Al concentrations, the study of the toxic mechanism of

Al poisoning is severely hindered. Additionally, interferences in Al analysis in the sample arise due to the organic composition of tissues, which lowers the sensitivity of Al determination. To be accurate and useful, determination of the biological pathway and the toxic action of Al in an organism must start at pre-lesion stages.

In the present state-of-the-art analyses, the sample is consumed, precluding corroboration by another analytical method or reanalysis. In Al toxicological studies, obtaining and preparing samples is costly, and a method which utilizes a small-sized sample and derives as much information as possible is desirable.

Complicated and tedious sample preparation is associated with the present Al assay methods of atomic absorption flame spectrophotometry. Up to four steps are often necessary to remove analysis interferences from each sample introducing error at each step. Additional steps are necessary to convert the sample into the proper physical or chemical state for analysis. Concurrent with the time lost in sample preparation, is a loss of sensitivity owing to sample manipulation and aging (Willard et al., 1965).

Finally, the elucidation of the chemical forms of Al and each of their pathways is complicated by the fact that concentrations are often below presently detectable limits and, therefore, uptake and elimination studies of Al are not possible with the detection techniques now available. The Al chemistry of the pathway must be known to determine the series of biologically destructive events. Using present methods found in the literature, Al cannot be determined and followed through an organism, giving rise to uncertainity in the

toxic action of Al.

The purpose of this thesis is to investigate the feasibility of the use of instrumental neutron activation analysis (INAA) to measure elevated levels of Al in fish tissues with the ultimate aim of establishing a method to study Al-caused toxicity.

Acid Rain and Aluminum Toxicity - An Historical Perspective

Over the past two decades, the number and scope of fish kills in Eastern Canada and the New England states has increased at a steady rate (Quellet and Jones, 1983). The cause of these kills is believed to be atmospheric deposition of acidified liquids and particles. The acidic component most likely emanates from the combustion by-products of industry and transportation, combines with clouds, and is ultimately deposited downwind as precipitation.

This causal relationship of combustion by-product formation of acid rain has been supported by Quellet and Jones (1982) and Richter (1983). Not all researchers agree with this assesment of the source of the acid (Huber, 1984); however, studies using historical data have shown that the precipitation has become more acidic over the last twenty years (Harvey, 1975; Scholfield, 1976; Wright and Snekvik, 1978).

The present contention is that with the combustion of fossil fuels both the nitrogen oxides and the oxides of sulfur are liberated into the atmosphere and through interaction with water vapor form nitric and sulfuric acids. The liberation of NO, NO_2, and SO_2 gases has been shown to occur during combustion, but the resulting formation

of nitric and sulfuic acids in the atmosphere has not. However, the atmospheric chemistry which regulates their formation is favorable (Robinson and Stokes, 1959).

Rain typically has a pH of 5.6. In acid precipitation, a range of pH values from 4.9 to 2.8 has been recorded, representing a 5 to 600 fold increase in hydrogen ion concentration. The effects of this influx of acid into aquatic systems have been examined (Burton, et al., 1981; Driscoll, et al., 1980) and influences are seen upon ion and acid-base regulatory mechanisms. The acidic influence is manifested in tissue destruction of aquatic organisms and is evidenced in the increased production of mucous. Ionic imbalances are caused by disturbances to both the active transport and diffusional losses of Na^+ and Cl^- in the gills (McDonald, 1983). These effects of acid deposition have been seen in the laboratory as well as in relatively unbuffered lakes and streams which accumulate acid through precipitative run-off (Schofield, 1980); in these essentially unbuffered aquatic systems, subsequent kills have left the lakes and streams nearly devoid of fish and rendered those fish that do remain unable to reproduce.

With the acidification of susceptible lakes and streams, chemical elements in the sediment such as lead, cadmium, arsenic, iron, and zinc, which were previously innocous, can become soluble at the lower pH's and express their toxicity (Khalid, et al.,1977). One of the metals liberated by the acidification is Al. While generally non-toxic, chemical speciation of Al, and therefore, its toxicity varies with water hardness, turbidity, and pH. Recent studies have

shown Al to be the cause of mortality among several species of fishes during acid events (Baker and Scholfield, 1981; Brown, 1982a; Scholfield and Trojnar, 1980). Aluminum in a solution of pH between 5.2 and 5.5 has been shown to be toxic to embryos, larvae, and the postlarvae of brook trout (Salvelinus fontinalis) and white sucker (Castomus commersoni) at the 0.5 mg Al 1^{-1} level (Baker and Scholfield, 1981).

Calcuim (Ca) acts as a buffering agent when added to a system identical to the one previously described by Baker and Scholfield (1981). The prophylaxis of Ca can be seen at concentrations above 0.5 mg Ca 1^{-1}. The source of this protection is believed to be in the reduced net flux of H^+ across the gill membrane (McDonald, 1983). Calcium has been shown to reduce plasma ion loss in brown trout (Salmo truta), but at higher concentrations than expected (3-5 mg Ca 1^{-1}) (Muniz and Lievestad, 1980).

The study by Baker and Scholfield (1981) has shown that at a pH of 5.4, a concentration of 0.5 mg Al 1^{-1} can reduce brook trout survival to 25% after 14 days. If Ca is added at a concentration of 1.0 mg Ca 1^{-1} to a system containing water at pH 5.4 and 0.5 mg Al 1^{-1}, 50% of the trout population survives after 14 days. The immediate effects of Al toxicity also appear to be reversed when dissolved oxygen content and water hardness increase and acidity decreases (Muniz and Lievestad, 1980).

The time sequences of water chemistry during acid events need to be closely related to fish response and recovery before the significance of Al as a toxic agent can be determined. Central to the

understanding of Al toxicity is a need to comprehend the mechanism by which Al acts.

CHAPTER TWO

MATERIALS AND METHODS

Theoretically, the four problems associated with low level Al determination in an organic matrix, e.g., decreased sensitivity, destruction of the sample, complicated sample preparation, and inability to follow samples through a system, which were introduced in Chapter One, can be avoided by the use of instrumental neutron activation analysis (INAA). The INAA method utilizes stable tracer elements (non-radioactive) which can be safely introduced to water, air, or soil, then recovered at a later date and assayed by neutrons produced at a research nuclear reactor. In this way the movement and ultimate fate of materials in the environment can be studied (Guinn and Hoste, 1980).

Instrumental neutron activation analysis has a sensitivity of 1 pg Al g^{-1} when no interferences are present, and INAA is reproducible owing to nondestruction of the sample (Guinn and Hoste, 1980). Additionally, INAA can be specific for Al and can utilize a small sample size of 0.1 mg of tissue or water (Guinn and Hoste, 1980). The principle underlying INAA is that a sample consisting of stable elements is placed in a neutron flux in which neutrons impinge upon the sample and activate its elemental constituents. This neutron interaction causes the elements to become radioactive and the product atoms are said to be neutron activation products. The radioactivity of the sample's elemental constituents is strongly dependent upon the

original elemental composition as well as the intensity of the neutron flux. If a series of standards of known concentration of the element of interest is irradiated in conjunction with a sample of unknown concentration of the element, the concentration of the unknown can be determined.

In the specific case of Al, the reaction of interest is ^{27}Al (n,γ) ^{28}Al, where n represents the captured neutron, γ repesents a prompt gamma ray liberated as a result of neutron capture, and ^{28}Al the radioactive product formed. The radionuclide product of neutron activation, ^{28}Al, has a half-life of 2.3 min and will decay to a stable atom ^{28}Si. In the process of decay ^{28}Al liberates energy in the form of a beta particle and a gamma ray. Of these radiations, the gamma rays are the most important because their energies can be used to identify the radioactive product.

With the use of a suitable detection instrument, e.g. an intrinsic germanium or a lithium-drifted germanium detector, the gamma ray of decay is measured. From these gamma rays one can determine the concentration of an element in a sample. The total method of analysis is called INAA because the sample is not altered from its natural state either before or after neutron bombardment.

Experimental Criteria

Selection of Experimental Animals and Sample Sites

Past experimental work on the biological effects of acid and Al has been done with brown trout (Brown and Lynam, 1981; Brown,

1982a; 1982b; Jacobsen, 1977; Scholfield, 1980; and Trojnar, 1977;), flathead minnows (Mount, 1973), rock bass (Ryan and Harvey, 1980a), and yellow perch (Ryan and Harvey, 1980b). The reasons given for selection of these species generally were the economic importance and easy availability of these fishes.

Bluegills (Lepomis macrochirus) were selected for this study because the gills are large and allow for multiple sampling and cross sectional analyses. Additionally, they were of reasonable body size, easily obtained, hardy, and economically important to the sport fishing industry.

Samples were taken of each fish's gills as well as its liver and blood. Cross-sectional analysis of the gills of these fish was not feasible because contamination-free sectioning devices and methodologies were not available. These tissues were chosen to determine if Al was concentrated on the fish gill or if it was ubiquitously dispersed in the fish. If the Al passed through the gill membrane, it would be found in the blood, and as the liver cleanses the blood Al could be concentrated in the liver in the manner of zinc (Mount, 1967).

Treatments

The number and type of treatments were decided upon after a literature review. In Brown and Scholfield, (1981), the embryos, larvae, and post larvae of brook trout (Salvelinus fontinalis) and white sucker (Castomus commersoni) were exposed to Al^{3+} in concentrations of 0, 250, and 500 ug Al l^{-1} at pH's of 5.4, 5.1, 4.8,

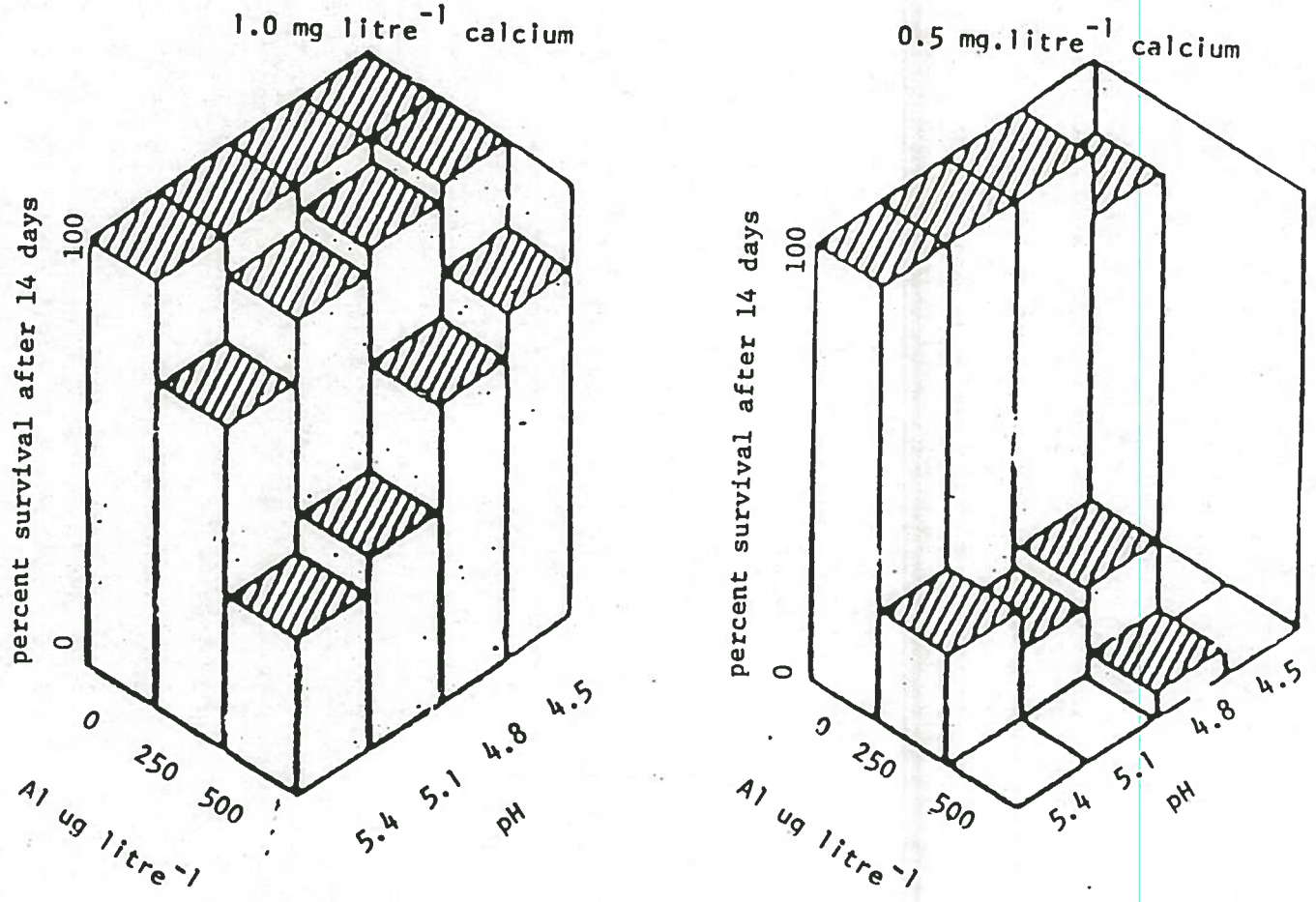


Figure 2.1 Results of Brown and Scholfield (1981), showing the combined effects of Al, Ca, and acid on survival rates.

From a preliminary statistical analysis, a 't' test was performed, and it was determined that each treatment required 26 members for statistical validity (Steel and Torrie, 1980). This estimate assumed "worst case," utilizing large variance, a small degree of difference between treatments, and a high degree of signifigance. With careful analysis, the number of animals needed could be reduced and still produce statistically significant results. Statistical validity with only 5 members in each treatment is attainable if the difference between the control and the treatments is large enough and the variance within a treatment is small. The relationship between variance(σ) and difference(d) is represented in the formula (Steel and Torrie, 1980):

$$n = \frac{t^2 \sigma^2}{d^2} \qquad (2.1)$$

where: n = the number of samples needed for validity

t = a measure of the distribution of the data

σ = the variance of the data

d = the difference level to be detected

As n approaches 30, t converges to 1.96. For first approximations where n is less than 30, an initial value of 2.0 is used for t.

Stabilization of pH and Determination of Aluminum Speciation

In preliminary studies, hydrochloric acid was added to aged Baton Rouge tap water and the pH monitored over four days. It was discovered that the pH fluctuated over a wide range, from 4.0 to 6.8 on a single day. Stabilization was attempted by checking the pH frequently during the day and adjusting it with buffers as needed. This method proved inadequate as alkalinity would rise overnight and the solution return to neutrality.

A buffer solution of pH 5.0 was prepared from Coleman Certified Buffer tablets and placed in a delivery bottle. A hose with an adjustable clamp was attached to the bottle and a stirbar placed in the bottle. The whole assembly was placed on a magnetic stirrer and a constant drip from the hose to the tank was attained (Figure 2.2). The method worked well initially, but airlock in the tubing prevented maintainance of the drip rate.

As an alternate method, the Coleman buffer solution was added to the tank several times daily, and the pH was determined between additions. This method produced the most stable ranges of pH with little to no variation over a 24-hour period. It was determined that different buffer solutions for each of the pH's would be needed. These solutions were found in Robinson and Stokes (1959) and are reprinted in Table 2.1. Two liters of buffer solution were made up for each of the pH values of 4.0, 4.4, 5.2, 5.6, and 6.0. The buffer solutions were standardized using a pH reference electrode while stirring the sample for uniformity. Additional hydrochloric acid (0.1 M) was added when pH levels were too high and sodium hydroxide (0.1 M)

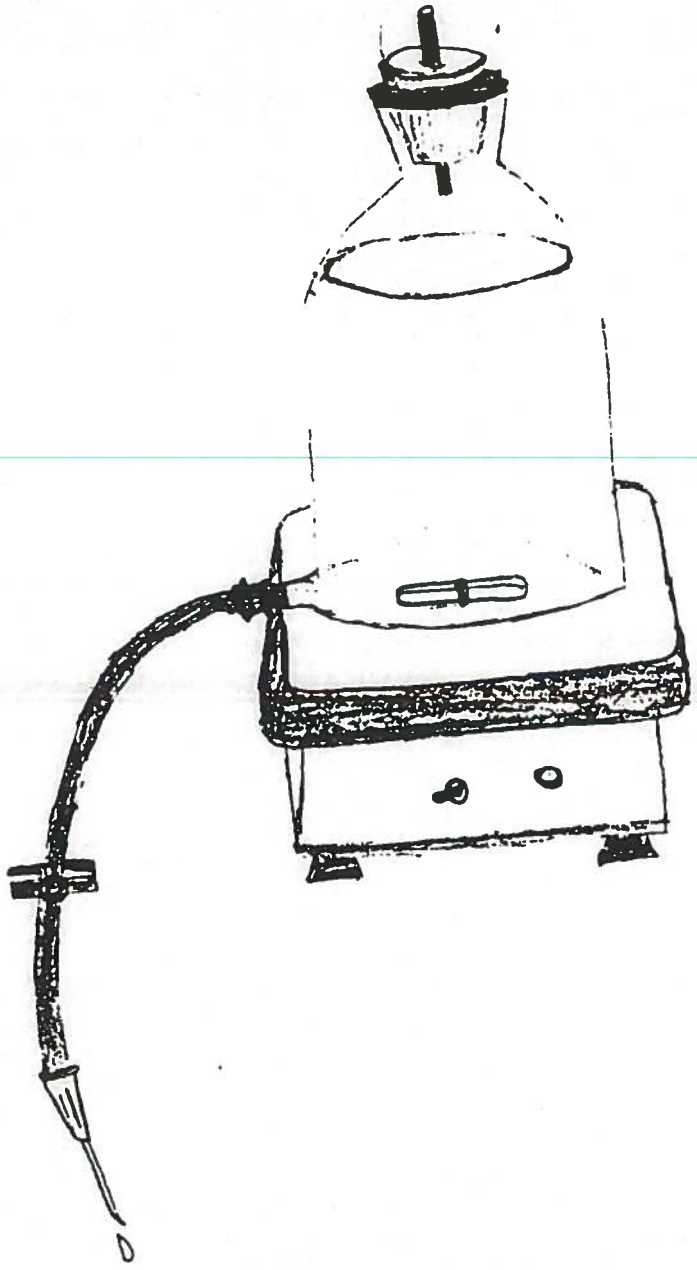


Figure 2.2 An I.V. style drip bottle system. This system developed airlock in the tubing even with the bottle top removed.

Table 2.1 A Table of buffer solutions listing how to make buffer solutions for pH values from 1.00 to 9.00. (Taken from Robinson and Stokes, 1959.)

A		*B*		*C*		*D*		*E*	
y	*x*	*y*	*x*	*y*	*x*	*y*	*x*	*y*	*x*
1·00	67·0	2·20	49·5	4·10	1·3	5·80	3·6	7·00	46·6
1·10	52·8	2·30	45·8	4·20	3·0	5·90	4·6	7·10	45·7
1·20	42·5	2·40	42·2	4·30	4·7	6·00	5·6	7·20	44·7
1·30	33·6	2·50	38·8	4·40	6·6	6·10	6·8	7·30	43·4
1·40	26·6	2·60	35·4	4·50	8·7	6·20	8·1	7·40	42·0
1·50	20·7	2·70	32·1	4·60	11·1	6·30	9·7	7·50	40·3
1·60	16·2	2·80	28·9	4·70	13·6	6·40	11·6	7·60	38·5
1·70	13·0	2·90	25·7	4·80	16·5	6·50	13·9	7·70	36·6
1·80	10·2	3·00	22·3	4·90	19·4	6·60	16·4	7·80	34·5
1·90	8·1	3·10	18·8	5·00	22·6	6·70	19·3	7·90	32·0
2·00	6·5	3·20	15·7	5·10	25·5	6·80	22·4	8·00	29·2
2·10	5·1	3·30	12·9	5·20	28·8	6·90	25·9	8·10	26·2
2·20	3·9	3·40	10·4	5·30	31·6	7·00	29·1	8·20	22·9
		3·50	8·2	5·40	34·1	7·10	32·1	8·30	19·9
		3·60	6·3	5·50	36·6	7·20	34·7	8·40	17·2
		3·70	4·5	5·60	38·8	7·30	37·0	8·50	14·7
		3·80	2·9	5·70	40·6	7·40	39·1	8·60	12·4
		3·90	1·4	5·80	42·3	7·50	40·9	8·70	10·3
		4·00	0·1	5·90	43·7	7·60	42·4	8·80	8·5
						7·70	43·5	8·90	7·0
						7·80	44·5	9·00	5·7
						7·90	45·3		
						8·00	46·1		

The buffer solutions are made by mixing two solutions and diluting to 100 ml. The solutions to be mixed are as follows:

A 25 ml 0·2 M — KCl + *x* ml 0·2 M — HCl
B 50 ml 0·1 M — Potassium hydrogen phthalate + *x* ml 0·1 M — HCl
C 50 ml 0·1 M — Potassium hydrogen phthalate + *x* ml 0·1 M — NaOH
D 50 ml 0·1 M — KH_2PO_4 + *x* ml 0·1 M — NaOH
E 50 ml 0·1 M — *tris* (Hydroxymethyl)aminomethane + *x* ml 0·1 M — HCl

added when levels were too low.

The pH of each treatment was checked several times each day to prevent any significant fluctuation since wide swings in pH values would affect the Al concentration and the Al speciation in that treatment. The pH prior to readjustment was checked and recorded with a Beckman model 730 pH meter. This meter allows for readings to the 5/100's of a unit without the introduction of parallax error. The meter was calibrated with pH 4.00 $\pm$ 0.01 standardized buffer for the 4.0, 4.4, and 4.8 readings and 7.000 $\pm$ 0.005 standardized buffer for the 5.2, 5.6, 6.0, and control readings.

The time of the readjustment of each treatment was recorded so that a nomograph of pH versus time could be constructed, and any relationship of acid- caused death and pH could be recorded. The mean of the pH value for each treatment was generated by Statistical Analysis Systems (SAS, 1982) on an International Business Machines computer, model 3081 (IBM 3081).

The study of Al speciation in ambient water samples is an area of intense research at present, owing to its hypothesized relationship with acid rain (Campbell et. al., 1983; Driscoll, 1984; Siep et al., 1984). A thorough review of the modern methods was preformed and their merits evaluated. The preferred methods require little sample manipulation and utilize instruments to determine Al concentration which are uncomplicated, accurate, and accessible. The methods of Al introduction, sample treatment, and speciation methods were chosen from the studies cited below.

In Scholfield (1980), Al was added to the system as $AlCl_3$. In Baker and Scholfield (1981), Al was introduced as the metal, dissolved in nitric acid. Both methods produce the monomeric species of Al in an inorganic form, but the metal-dissolved sample allows for immediate hydroxy complexing. Since hydroxy forms are the most highly suspect species of Al toxicity, metal-dissolved preparation is the preferred method. It should be pointed out, however, that the chloride salt will dissassociate in solution also and complexing will occur, but at a slower rate (Robinson, 1959).

The determination of Al-complexed species versus time and pH has been explored at some length by Smith (1971). "If the pH of the solution is above neutrality, it appears that the predominant species present is the anion $Al(OH)_4(H_2O)_2^-$. If the pH is below about 4, most authors agree that the hexaaquo-Al(III) ion $Al(H_2O)_6^{3+}$ dominates." Between pH 4 and 7, where highest Al toxicity is manifested, there is little agreement among authors as to the speciation of Al.

Smith (1971), in an attempt to determine Al speciation, set up eight solutions with initial sequential pH's between 4 and 7 and determined Al speciation and resultant pH's over a 250-day period. Three different classes of Al species were found such that:

$$Al = Al_a + Al_b + Al_c \qquad (2.2)$$

Where:

Al - total Al present

Al_a - fast reactive Al

Al_b - slow reactive Al

Al_c - solid material, Al

Smith (1971) postulated that since Al_a reacted almost instantly, it would seem reasonable that it consisted of only simple monomeric species: Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^{+}$, and $Al(OH)_4^{-}$. Since Al_c appears to be solid material, it is actually incorrect to think of Al_c in terms of concentration "in solution." The designation Al_c was used to help follow the rate of Al solid formation in the system as a function of time.

From reaction rates and electron photomicrographic sizing, Smith believed Al_b to be composed of coalesced, six-membered aluminum hydroxide rings consisting of 20-100 Al atoms. This contention was supported by Hem and Roberson (1967) as well as Hsu and Bates (1964). From his samples, Smith (1971) determined that Al_a and Al_b concentrations decreased with time and that Al_c increased. Additionally, the pH of the solutions increased with time until equlibrium pH was attained. In natural waters, however, considerations must be made for sulfate as it affects Al concentrations and speciation (Nordstrom, 1982).

Methodologies for chemically determining Al speciation have been reported by Barnes (1975), Davenport (1949), LaZerte (1984), Smith (1971), and Turner (1969). The older methods complex Al as a dye (ferron or 8-hydroxyquinoline) and measure the intensity of the dye formation by a spectrophotometer. Since the dye intensity is

proportional to concentration, the Al concentration can be determined when compared to standards. More recent methods incorporate atomic absorption spectrophotometry (AA) or flameless atomic absorpton spectrophotometry (FAAS) to measure the intensity in place of the the spectrophotometer.

The above methods require pretreatment of the samples into separate species before analysis. These methods are described in Driscoll (1980) and a summary is presented here. Each sample is split into three equal parts and assayed for total Al (samples acid-digested before analysis), total monomeric Al (samples analysed without acid digestion), and non-labile monomeric Al (Al separated by an ion exchange chromatography technique and analyzed as monomeric Al). From these methods we can determine Al speciation as such:

Organic Al = non-labile monomeric Al (2.3)

Inorganic Al = total monomeric Al minus non-labile monomeric Al (2.4)

Acid-Soluble Al = total Al minus total monomeric Al (2.5)

Organic Al is typically found complexed in large organic moieties. Inorganic Al is found as flouride, sulphide, hydroxide, and sulfate compounds of Al. Polymeric, colloidal, stable organic, and hydroxy organic complexes generally comprise the acid-soluble species.

To determine Al speciation, LaZerte's (1984) separation techniques were utilized and Turner's (1969) spectrophotometric methods (8-hydroxyquinoline) followed in this work. (See Appendix B for methods.) After the samples were analyzed for Al concentration, they were placed in cold storage to allow for reassay and comparison to spectrophotometric methods. Both Barnes (1975) and Lazerte (1984)

have shown that retaining the sample in cold storage for later analysis is an acceptable practice as it does not alter speciation or concentration.

In the present study Al was introduced to the pH treatments as the Al salt, $AlCl_3$, resulting in a concentration of 1 mg l^{-1}. (An attempt was made to present the Al as nitric acid-dissolved metal, however, the acid dissolved the metal so slowly and incompletely that this method was abandoned.) The water from each of the treatments was sampled daily throughout the experiment and dialysed Al, fast reactive Al, and total reactive Al determinations were performed. The dialysed Al determinations were undependable, however, due to inconsistent sample to volume ratios. Additionally, the refrigeration unit in which the samples were kept failed halfway through the sampling period causing the loss of two days' samples. Due to lack of alternate space, further sampling of the systems to determine dialysed Al concentration was deemed impractical.

Only generalized trends in Al speciation were possible with the failure of the dialysis systems. However, the Al speciation was secondary; the development of a method to determine Al in fish tissue was of prime importance ot this thesis. The results of the Al speciation study are therefore presented as Appendix C, as an aid to subsequent work in this field.

Equipment

The aquaria used present an inexpensive approach applicable for short-term toxicological studies which are not affected by plastic

interaction. Seven thick-walled plastic 32-gallon trash cans were used with their interiors washed with 2 N hydrochloric acid and rinsed with deionized distilled water. Approximately 30 gallons of Baton Rouge City tap water were added and allowed to age for one week before any chemical additions. During the aging period, air was bubbled through the water in each tank by an airstone. The airstones were attached in parallel by rubber tubing to a equal dispersion manifold and pump (Figures 2.3 and 2.4).

Lighting was provided by eight 75 Watt incandescent bulbs and a 48 inch flourescent bar attached to a wooden frame. This assembly was hung fron the ceiling approximately 150 centimeters from the water surface. By use of a timer, 12 hour day-night cycles were maintained. Similiar methods have been employed by botanists to mimic sunlight, and in combining the two types of light all necessary wave lengths are are assumed to be present.

Sample Preparation

Bluegills (Lepomis macrochirus) and orangespotted sunfish (Lepomis humilis) were removed from stocking ponds on LSU's Ben Hur Farm and were transferred to a holding tank at the Nuclear Science Center. Fifty fish, 5 to 8 cm in length, were selected from the population and placed in a 32 gallon plastic container of aged tap water. The fish remained in this tank for four days before any further movement so that they could become acclimated to the laboratory. At the end of four days the animals were evenly divided among the treatments.

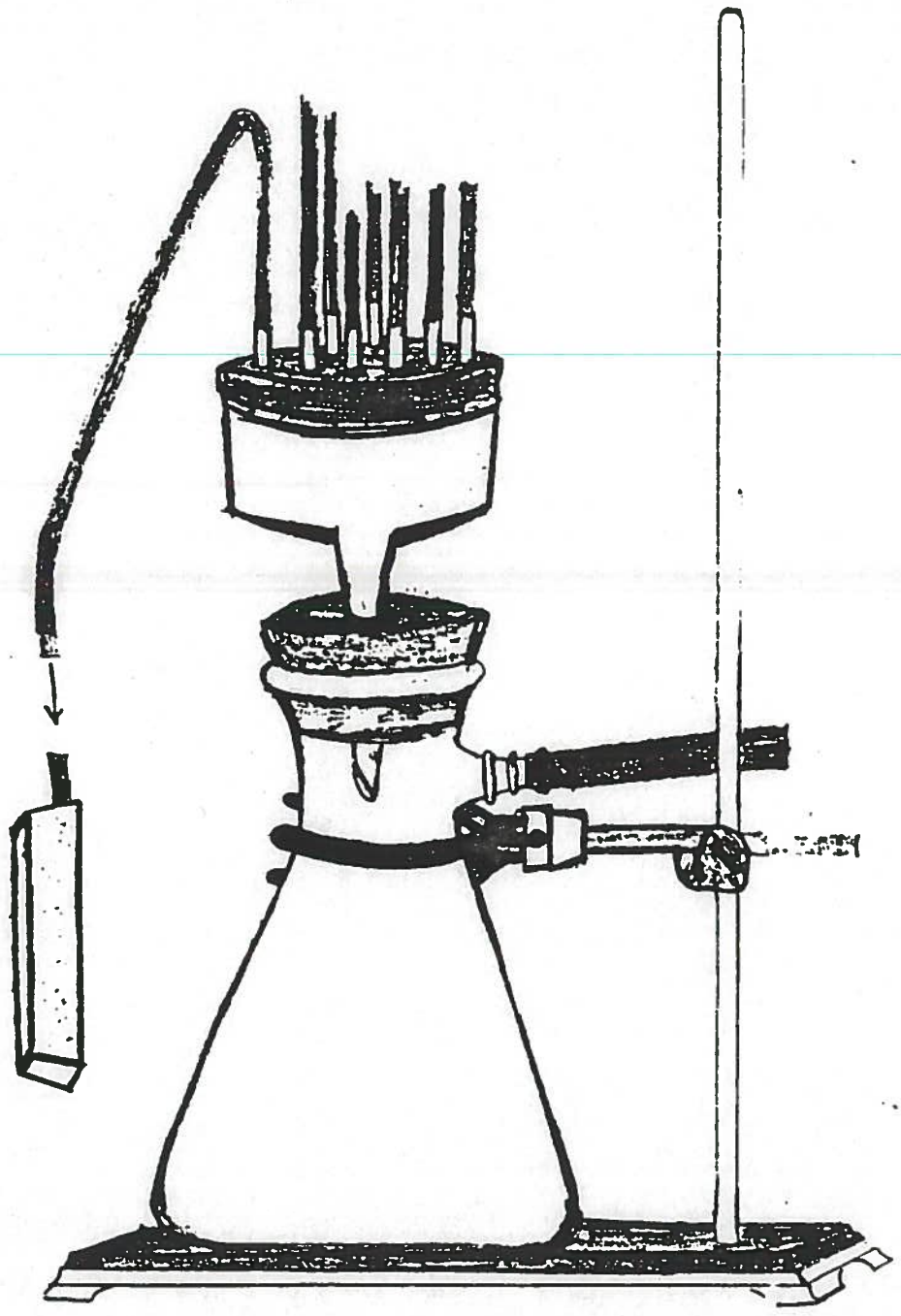


Figure 2.3. A drawing of the equal-pressure manifold and the airstone used.

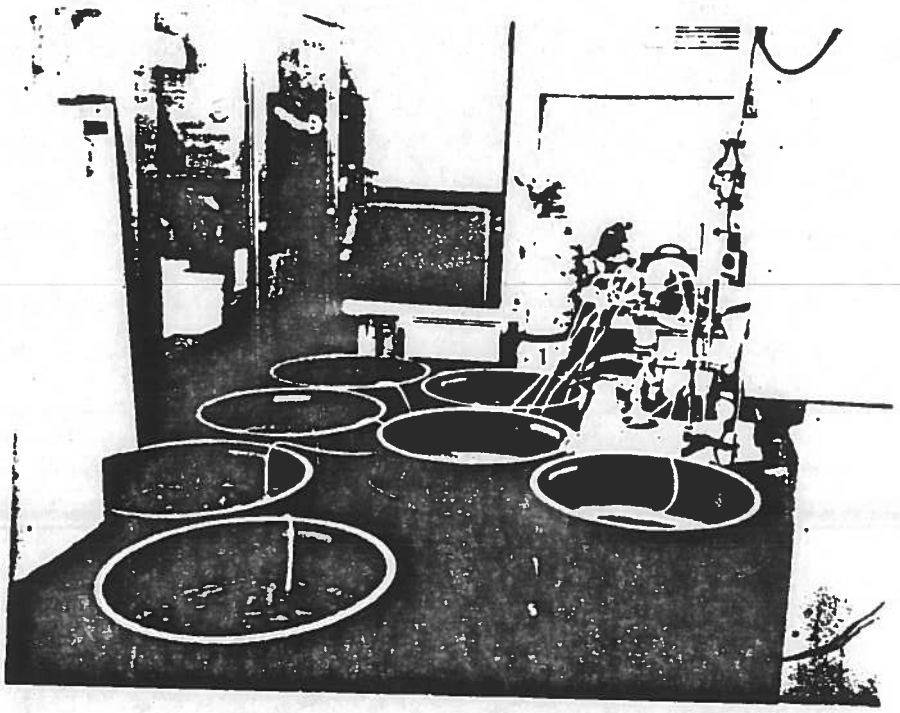


Figure 2.4 The seven laboratory aquaria with a vigorous bubling rate evidenced. The rapid bubbling rate insured sample homogenicity and provided adequate dissolved oxygen.

The treatments were checked several times daily for mortalities. As the experiment progressed, tanks with Al became murky from the fish's over production of mucous. It was noted that the control fish retained their natural timidity, while the Al-treated fish developed a demonstrated lack of fear when approached. The Al-stressed fish usually died within days from the onset of these behaviors.

As the fish died or became moribund, they were removed from the plastic containers, quickly frozen in liquid nitrogen and placed in cold storage until the experiment was completed. This quick-freeze method immobilized the fishs' fluids and preserved the interstitial water above the gill. After freezing, the fish were easily dissected and two gill samples as well as heart and liver tissues were taken from each.

These tissues were placed in acid-washed crucibles and dried in an oven at 65°C until constant weight was achieved. This method parallels the ones used for Zn, Cu, and Fe by Giesey and Wiener (1977), Mount (1967), and Saltes and Bailey (1984), and appeared to be the method of choice for Al. The dried material was weighed on a Sartorius model 1710 Automatic Tare ballance accurate to 0.1 $\pm$ 0.005 mg., the weight recorded, and the sample placed in a 2/27-dram polypropylene neutron activation vial. These polyvials were heat-sealed and placed in larger 2-dram vials. The outer 2-dram vial was also heat-sealed, thus doubly encapsulating the sample.

Neutron Activation

Instrumental neutron activation analysis was performed at the Oregon State University Radiation Center located in Corvallis Oregon. The neutron source used was a TRIGA Research Reactor (Figures 2.5, and 2.6). Operation at a peak power of one megawatt (thermal) produced a flux of $1.3*10^{13}$ neutrons cm^{-2} sec^{-1} in the in the reactor core region of the pneumatic transfer system.

Samples were placed in pneumatic transfer capsules (rabbits) and were loaded into the injection port of the transfer system. They were then blown by air pressure into the core region of the reactor, the time was recorded, and the samples were returned to the injection port after a pre-set irradiation time (Figure 2.7). Upon their return, samples were removed from the rabbits and then analyzed for total radioactivity with a Technical Associates "cutie pie" direct-reading R meter prior to release to the experimenter for gamma ray analysis. Samples reading less than 500 mrem/hr when in contact with the open window of the cutie pie were released and prepared for assay. The samples were moved to a non-contaminated hood, de-encapsulated, and transferred to a clean 2-dram polyvials.

The polyvial containing the irradiated sample was placed on a Ge(Li) detector which was linked to a multichannel analyzer to determine the the energy and intensity of the radioactive elements present (Figure 2.8). The start time was recorded and counting ensued until an elapsed livetime of five minutes was reached. The counts in the gamma-ray photopeak regions of interest were recorded, the net photopeak-area determined, and a corrected counts per second datum

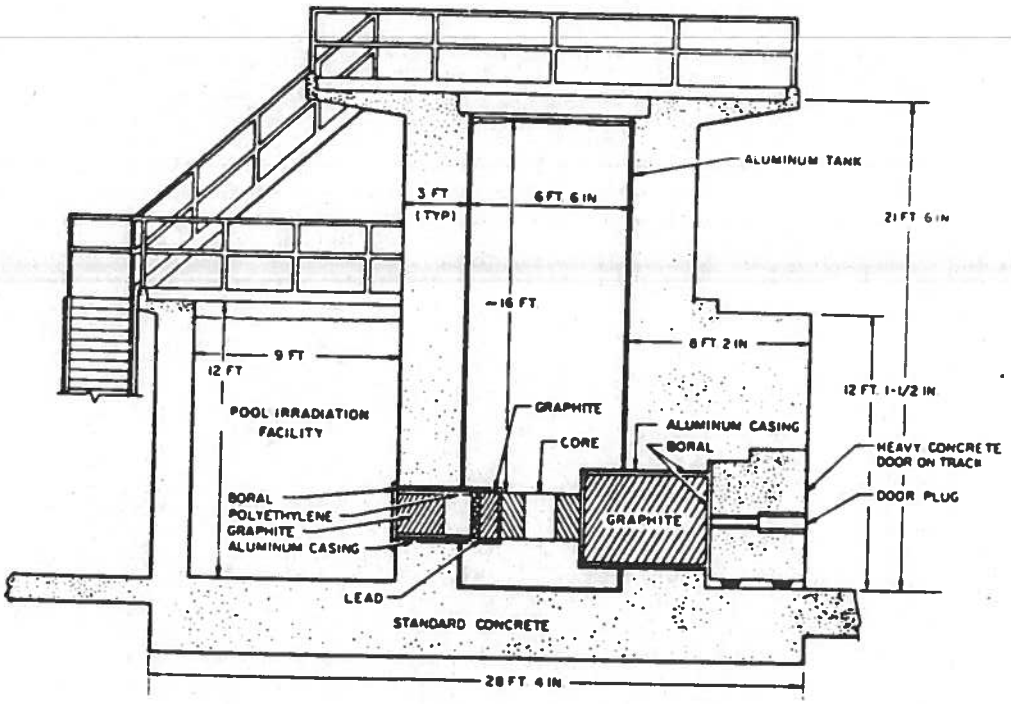


Figure 2.5 The OSU TRIGA Research Reactor (OSTRR).

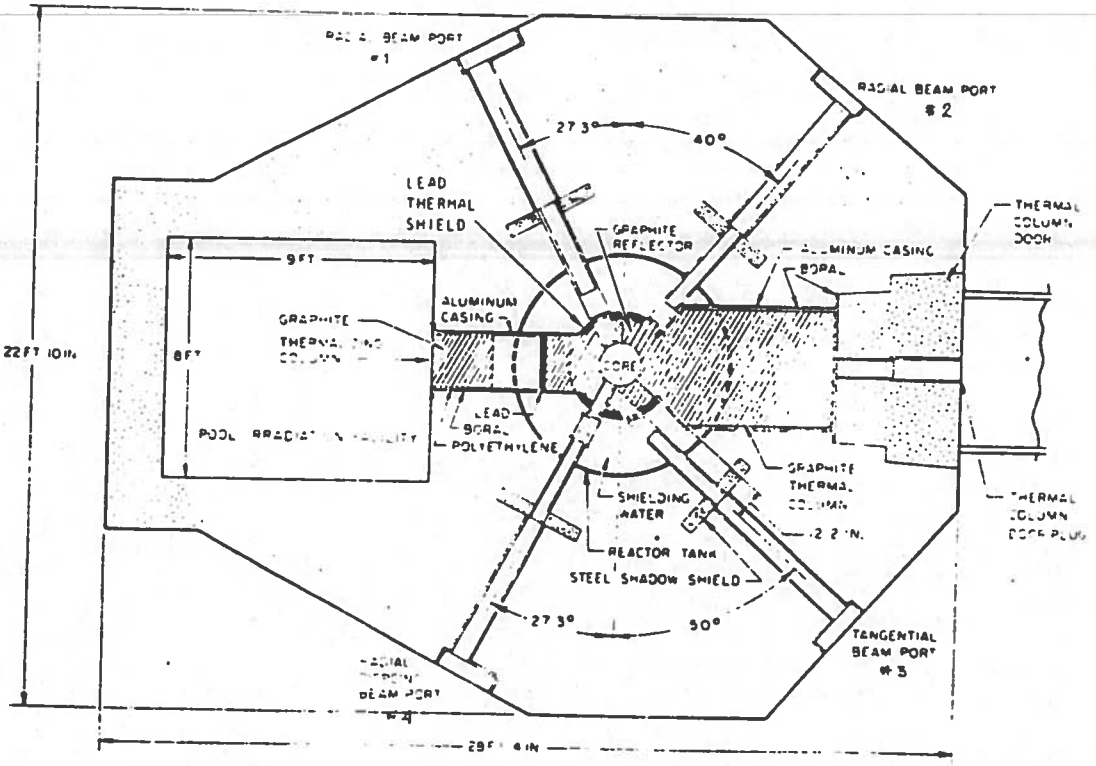
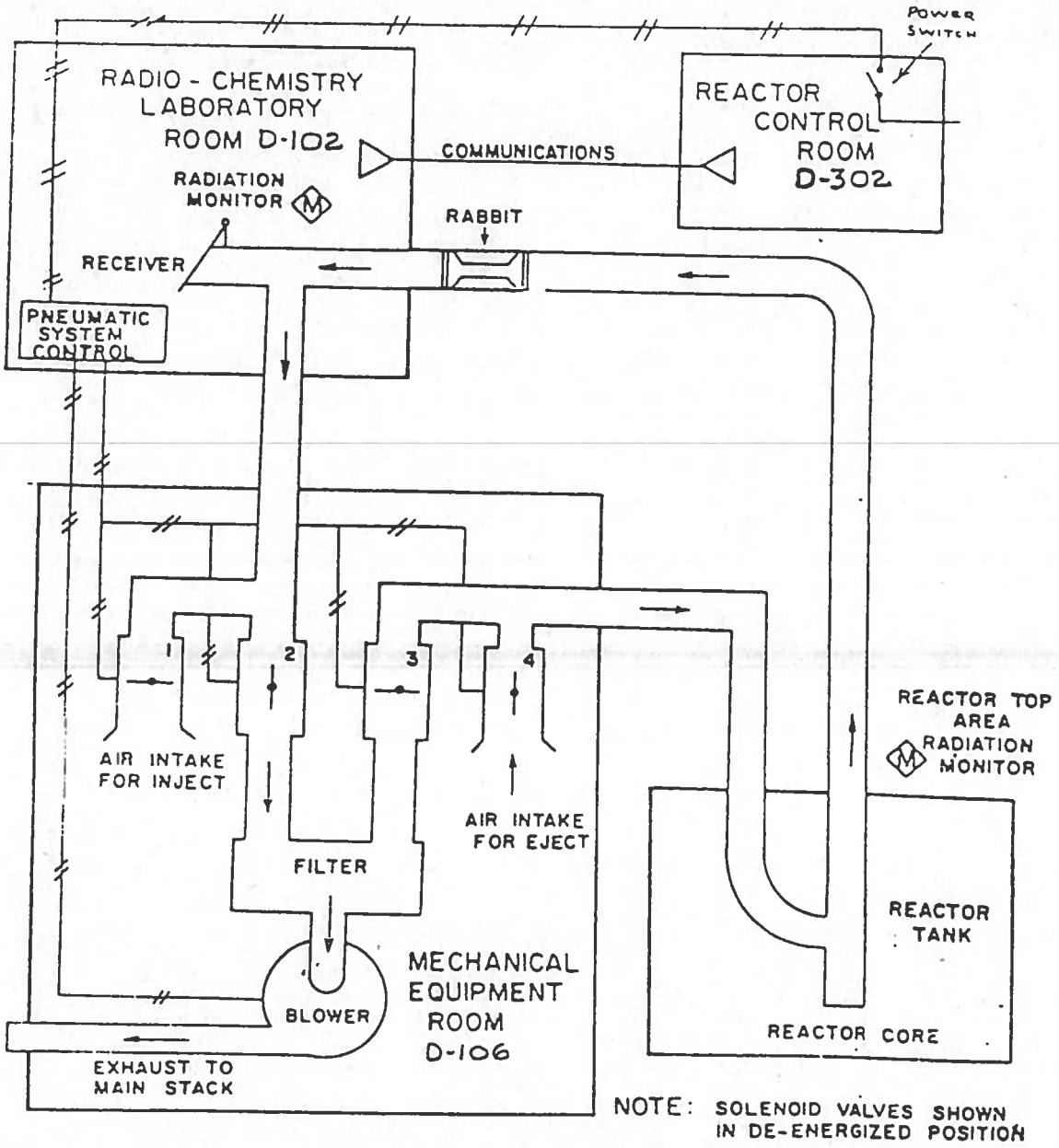


Figure 2.6 A view from the top of the OSTRR reactor with the core visible and the pneumatic transfer tube seen traversing the moderating water and traveling down to the core.



PNEUMATIC TRANSFER SYSTEM SCHEMATIC

Figure 2.7 The pneumatic transfer system at Oregon State University showing the the tube in which the rabbits travel and the air blowing mechanics.

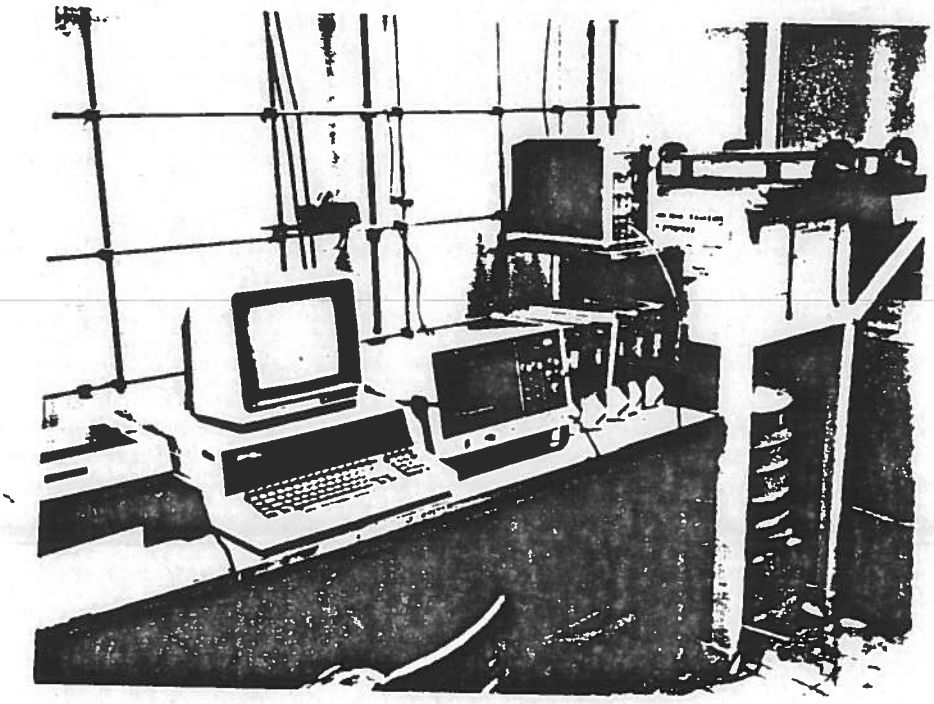


Figure 2.8 On the far right is a typical Ge(Li) detector housed in a lead-lined container. In the center of the photograph is the multichannel analyzer which is attached to the detector. On the left, a microcomputer used in photopeak analysis is shown.

reported for each region of interest. A representative spectrum of the samples is shown with the known photopeaks identified (Figure 2.9).

A standard of known Al content was prepared as a direct comparator; an Al metal turning weighing 11.5 ug was cut from 99.9999% pure Al wire, placed in a 2/27 dram polyvial, and activated for an identical amount of time as the unknowns, in the same region of the reactor. It was analysed by Ge(Li) spectroscopy and a direct comparison was made by means of the formula (De Soete et al., 1972):

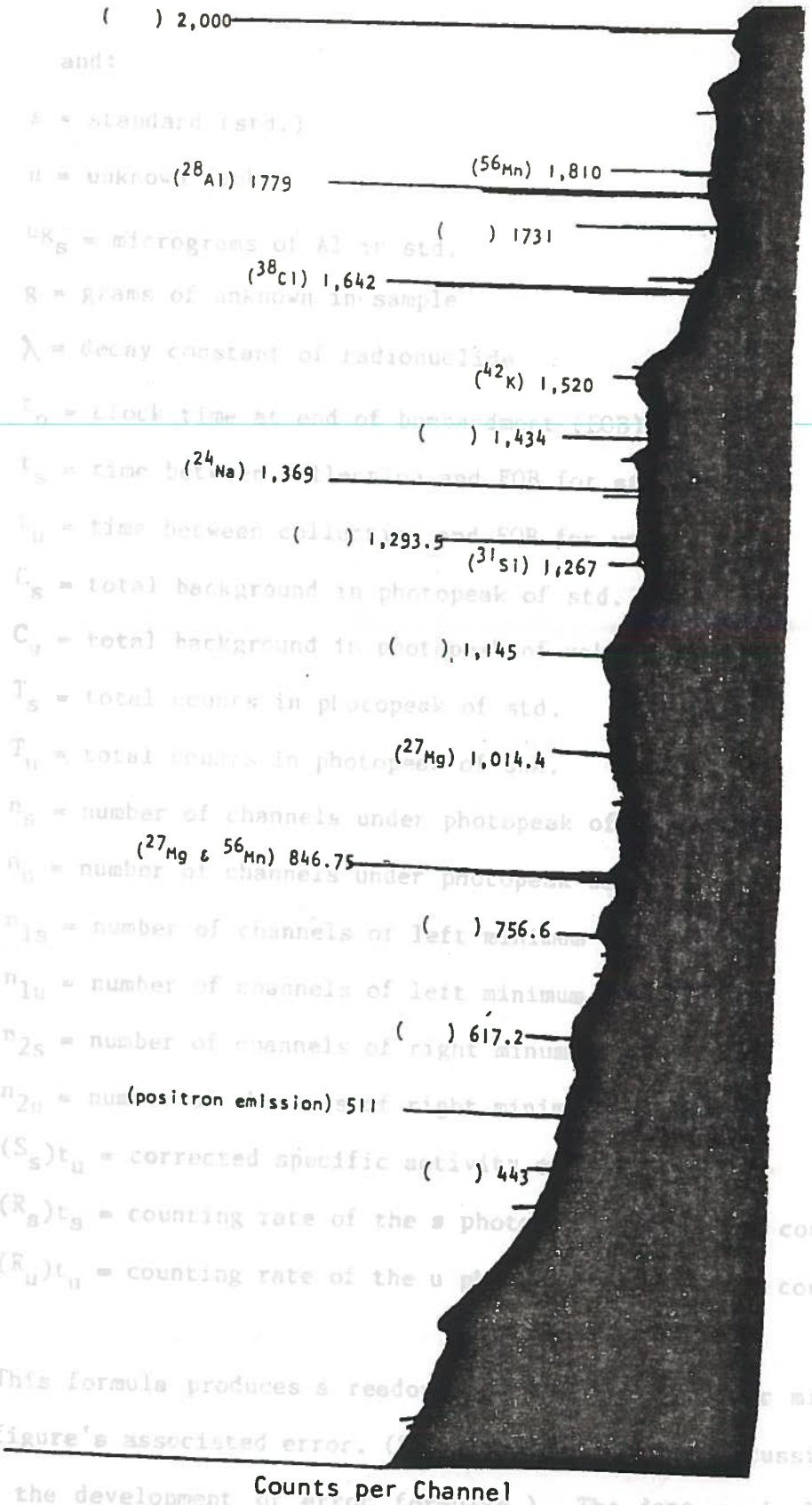
$$(S_s)_{t_o} = \frac{(R_s)_{t_s} \left(e^{\lambda(t_s - t_o)}\right)}{\mu g_s} \qquad (2.6)$$

Where:

$$\text{ppm} = \frac{(R_u)_{t_u} \left(e^{\lambda(t_u - t_o)}\right)}{(S_s)_{t_o} (g_u)} \pm \text{error} = \qquad (2.7)$$

$$\pm \frac{(R_u)_{t_u} \left(e^{\lambda(t_u - t_o)}\right)}{(S_s)_{t_o} (g_u)} \left[\frac{\left(T_s + C_s \left(\frac{n_s}{2n_{1s}}\right)\right)}{\left(T_s - C_s\right)^2} + \frac{\left(T_u + C_u \left(\frac{n_s}{2n_{1u}}\right)\right)}{\left(T_u - C_u\right)^2} \right]^{1/2}$$

Figure 2.9 The multichannel analyzer's output for a typical treated gill sample. (Fish sample## 21) For this sample, the ^{28}Al photopeak is the second largest on in the spectrum.


( ) 2,000
(^{28}Al) 1779
(^{56}Mn) 1,810
( ) 1731
(^{38}Cl) 1,642
(^{42}K) 1,520
( ) 1,434
(^{24}Na) 1,369
( ) 1,293.5
(^{31}Si) 1,267
( ) 1,145
(^{27}Mg) 1,014.4
(^{27}Mg & ^{56}Mn) 846.75
( ) 756.6
( ) 617.2
(positron emission) 511
( ) 443
Energy in KeV
Counts per Channel

flux reduces ϕ_f by slowing the neutrons to thermal energies. The moderators are composed of water, graphite, or paraffin and, as the neutrons collide with the molecules of the substance, they are slowed and a more thermalized spectra is produced (i.e., more neutrons at lower energies). Unfortunately, thermal columns were not available for short irradiations at either Oregon State University or Texas Agricultural and Mechanical University.

Since the ϕ_f could not be reduced, the feasibility of the $^{27}Al(n,p)^{27}Mg$ reaction was considered. The fast neutron capture cross section for this reaction is favorable, but problems arise due to the Mg naturally present in the sample. The Mg undergoes $^{26}Mg(n,\gamma)^{27}Mg$, and determination of the Al contribution is complicated.

Double irradiations of the samples with and without cadmium (Cd) were considered. The Cd shields the sample from thermal neutrons, acting a thermal neutron "sponge" and capturing the thermal neutrons due to the high thermal cross section of Cd. In the first irradiation total ^{28}Al would be determined. The same samples would then be Cd lined, neutron irradiated again, and the portion of ^{28}Al produced only from ^{31}P determined. The ^{27}Al contribution to the total ^{28}Al measured would then be determined by the difference in values from the two irradiations. This method was deemed an acceptable alternative if the P levels were too high to permit indirect measurement of Al. Some encouragement was gained from the fact that in human blood P would be only 40 times the Al concentration (not the 18,000 times reported in Bowen and Gibbons, 1972), and it was hoped that fish blood paralleled that of humans.

The experimental feasibility of the indirect method was determined by irradiating the control fish gills and comparing their average ^{28}Al concentration at end of neutron bombardment to that of the gills from the pH 5.2 treatment. The samples were neutron activated for 60 seconds and counted for 300 seconds.

It was estimated that the contribution from Al produced 2/3 of the response in the ^{28}Al gamma photopeak (1.7787 MeV), making the Al determination from unshielded irradiations only possible and uncomplicated. With cautious optimism, the nuetron irradiation time was increased to 240 seconds with a count time of 300 seconds. The sample geometry was kept constant by using identical polyvials and placing the samples at a constant 6 cm distance from the Ge(Li) crystal. This distance was determined to be ideal as it allowed for a high count rate with low dead times. The efficency was relatively high (1%) and the associated error kept minimal.

After the first day of neutron irradiations, it was determined that it would not be possible to assay all of the samples and that only 1/4 of them could be done in the time alloted for use of the reactor. The gill samples were determined to be the most important and were the only type sample assayed. Additionally, it was determined that there would not be enough time to double irradiate the samples for direct determination of P contribution to the ^{28}Al peak. Determination of the P contribution was calculated indirectly by comparison of values from the ^{28}Al channel response versus those of the control. A direct determination of the ^{27}Al contribution was performed for sample 20, since it had previously evidenced a large

^{28}Al photopeak. A control gill sample and sample number 20 were both irradiated without Cd and again encapsulated in Cd.

CHAPTER THREE

RESULTS

The Al data derived from the methods presented in Chapter Two was reduced by Statistical Analysis Systems (SAS, 1982) on a IBM model 3081 computer. The presentation of the data and results is in the order it was taken.

The pH values of each treatment were recorded several times daily throughout the 14-day course of the experiment and the mean pH value for each treatment was generated by SAS. These values with their associated standard deviations are recorded in Table 3.1. From Table 3.1 it is seen that the pH values were kept near the designed levels.

To determine if the means of the pH treatments were significantly different, an analysis of variance was performed (Table 3.2). An analysis of variance (ANOVA) determines if the differences in values within treatments are smaller than the differences in values among treatments by comparing the mean of the values for each treatment. If at least one of the means is significantly different from the others, the null hypothesis H_o is invalid (Steel and Torrie, 1975).

$$H_o : u_1 = u_2 \ldots = u_i \qquad (3.1)$$

Where: u_1 = mean of the first treatment
u_2 = mean of the second treatment
u_i = mean of the i th treatment

Table 3.1. The mean pH values of each of the different acid treatments are listed. 'Label' refers to the treatment and the number of samples is denoted by 'n'.

OUTPUT FROM PH MEANS PROCEEDURE

VARIABLE	LABEL	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
A	4.0	20	4.05312500	0.10480833	3.90000000	4.2750000
B	4.4	20	4.46290000	0.11064067	4.27500000	4.6750000
C	4.8	20	4.80765000	0.22385599	4.60000000	5.3000000
D	5.2	20	5.22125000	0.10074558	4.95000000	5.5000000
E	5.6	20	5.56125000	0.10146136	5.40000000	5.7000000
F	6.0	20	6.00750000	0.19451356	5.60000000	6.3000000
G	CONTROL	20	7.48250000	0.20760223	7.22500000	7.9000000

Table 3.2. An analysis of variance which determines if pH of treatments differs significantly among themselves. The degree of surety associated with this determination is equal to 1 minus the probability of a greater f (0.0001 - from above) or 99.99%.

ANALYSIS OF VARIANCE PROCEDURE

CLASS LEVEL INFORMATION

CLASS	LEVELS	VALUES
TRT	7	4 6 4.4 4.8 5.2 5.6 7.3

NUMBER OF OBSERVATIONS IN DATA SET = 140
SAS

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: PH

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	6	156.01975752	26.00329292	1041.82	0.0001	0.979166	2.9415
ERROR	133	3.31960279	0.02495942				
CORRECTED TOTAL	139	159.33936031					

ROOT MSE	PH MEAN
0.15798550	5.37088214

SOURCE	DF	ANOVA SS	F VALUE	PR > F
TRT	6	156.01975752	1041.82	0.0001

The magnitude of these differences is reported as an f value. In general, greater degrees of significance accompany larger f values. In Table 3.2, an f value of 1,041 was generated from the comparison of pH treatments and the null hypothesis was not supported.

Additional comparisons of the means of treatments must be performed to determine which means differ significantly. The simplest approach to multiple comparisons is to do a t test on each possible pair combination of the means (SAS, 1982). Table 3.3 lists the values for the paired t tests, showing that all of the pH treatments are distinctly significant. Variations of the t test have been developed to control either comparisonwise or experimentwise error rate.

A graph of fish mortality within each treatment versus time was generated by SAS and is presented as Figure 3.1. Figure 3.1 shows the correlation between time and death within treatments. Marked, spiked peaks are seen as the toxicity of each treatment manifests itself. The treatment of pH 5.2 demonstrates this correlation well, as all members of the treatment died within a 24-hour period.

The net area in the ^{28}Al photopeak for each sample of fish gill, as determined by the indirect method (see Chapter Two, page 35), was related to Al concentration by formulas 2.6 and 2.7. SAS performed the manipulation of this data and produced readout as uncorrected ppm Al per sample $\pm$ error. Table 3.4 contains these SAS-generated values of uncorrected ppm Al per sample as well as sample weights and the sample treatments. The data in Table 3.4 is ordered chronologically, begining with the first fish to die. Gill samples 24 and 25, from pH treatment 5.6, have very high Al

Table 3.3. Results of paired t tests for pH. Under the heading of grouping seven different letters are reported denoting significant differences among all of the pH treatments.

T TESTS (LSD) FOR VARIABLE: PH
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE, NOT THE EXPERIMENTWISE ERROR RATE.
ALPHA=0.05 DF=133 MSE=.0249594
CRITICAL VALUE OF T=1.97796
LEAST SIGNIFICANT DIFFERENCE=.0988178
MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

T	GROUPING	MEAN	N	TRT
	A	7.4825	20	7.3
	B	6.0075	20	6
	C	5.5612	20	5.6
	D	5.2212	20	5.2
	E	4.8076	20	4.8
	F	4.4629	20	4.4
	G	4.0531	20	4

Figure. 3.1. A correlation of fish death within treatment by date. 'Num' denotes the number of fish that died on that particular day. The actual dates during which the experiment was performed are denoted by 'date'.

TREATMENTS WITHIN DATES

BAR CHART OF NUM

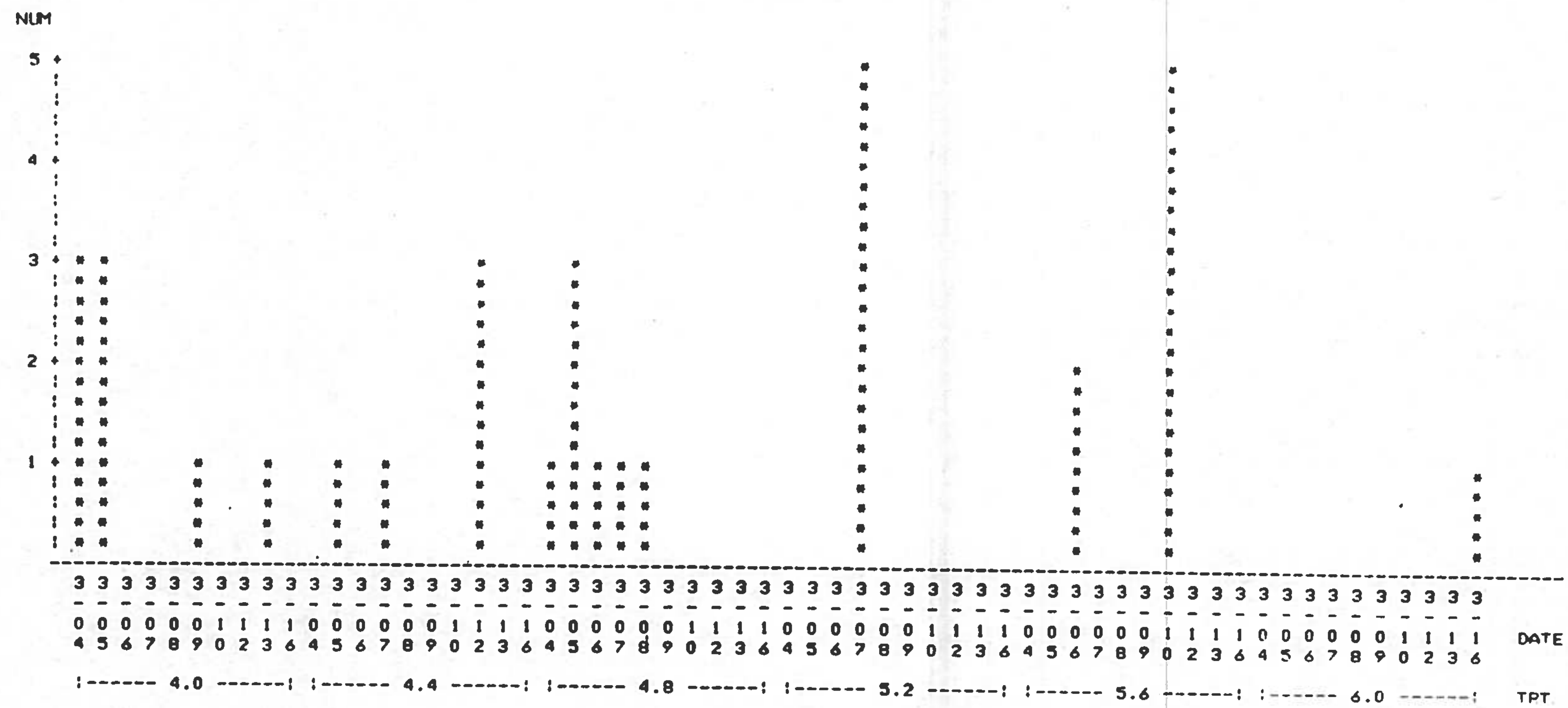


Table 3.4. A listing of Al concentrations for each gill sample as ppm Al (m) and the error associated with the ppm reading (P) as ± ppm as determined by the indirect method. Also 'wt.' denotes sample weight in grams and 'trt' is the sample treatment.

GROSS CONCENTRATION OF ALUMINUM IN GILLS

SAMPLE	WT	TRT	M	P
1	0.0150	4.0	90.73	1.38820
2	0.0145	4.0	137.89	2.02703
3	0.0056	4.0	86.83	2.91751
4	0.0155	4.8	323.43	5.01320
5	0.0068	4.0	262.25	3.33062
6	0.0049	4.0	120.45	3.08352
7	0.0086	4.0	199.80	2.81720
8	0.0045	4.4	178.58	3.39305
9	0.0146	4.8	209.22	2.09224
10	0.0181	4.8	167.97	1.67966
11	0.0082	4.8	385.92	3.70486
12	0.0059	4.8	546.26	5.13482
13	0.0064	5.6	502.23	4.72098
14	0.0033	5.6	388.62	5.86813
15	0.0109	4.4	159.07	2.27477
16	0.0132	4.8	240.57	2.42971
17	0.0098	5.2	295.36	4.34183
18	0.0015	5.2	255.20	7.91113
19	0.0071	5.2	231.15	3.67526
20	0.0099	5.2	156.35	2.12630
21	0.0088	5.2	460.74	3.87024
22	0.0123	4.8	393.26	2.91011
23	0.0044	4.0	204.22	3.77808
24	0.0033	5.6	1392.33	9.46785
25	0.0048	5.6	975.69	6.73229
26	0.0060	5.6	146.45	2.84109
27	0.0051	5.6	146.11	3.00983
28	0.0172	5.6	135.66	1.61431
29	0.0053	5.6	129.53	2.83660
30	0.0087	4.4	150.21	2.32831
31	0.0163	4.4	112.42	1.49524
32	0.0057	4.4	109.63	3.32165
33	0.0121	4.0	140.19	1.82252
34	0.0035	6.0	92.33	3.73011
35	0.0189	6.0	79.06	1.48640
36	0.0074	6.0	98.22	2.30823
37	0.0136	6.0	76.52	1.62230
38	0.0062	6.0	117.32	2.87435
39	0.0112	6.0	156.94	2.33842
40	0.0129	7.3	192.58	2.83091
41	0.0096	7.3	121.36	2.96117
42	0.0098	7.3	71.80	2.08216
43	0.0136	7.3	220.22	2.42241
44	0.0081	7.3	93.30	2.80836

concentrations, 1,392 ppm and 976 ppm, respectively.

An ANOVA was also performed to determine if the differences in Al concentrations among treatments were significant. The ANOVA output is presented as Table 3.5 where it can be seen that the null hypothesis that all Al treatments are alike is not supported because the f value is too large. To determine which Al treatments differed significantly from each other, a paired t test was performed (Table 3.6). The paired t test determined that only the 5.6 pH treatment differed significantly from the control. Additional paired t tests were performed at lowered surety levels (larger alpha values) to determine additional treatments which were statistically significant at lowered confidence levels. An alpha value of 0.10 was utilized in Table 3.7 and an alpha of 0.15 used in Table 3.8. The 5.6 pH treatment, however, was still the only treatment which was statistically different when compared to the control.

Because the number of animals within each treatment (cell) was not consistent, a paired t test which takes into consideration unequal cell size (Tukey test) was utilize to determine any significant differences between Al treatments. Tukey's test utilized very tight parameters (requiring larger degrees of difference between treatments) to compensate for the unequal cell sizes and therefore none of the treatments was shown to be significantly different from the control (Table 3.9).

The amount of ^{28}Al produced by the $^{31}P(n,\alpha)^{28}Al$ reaction was directly determined for both a control gill sample and a treated gill sample by irradiation of the samples both without cadmium (Cd)

Table 3.5. An analysis of variance to which determine if Al concentrations among treatment differ significantly. The degree of surety associated with this determination is 97.25%. A general linear models ANOVA is used to accommodate the unbalanced cell numbers.

GENERAL LINEAR MODELS PROCEDURE

CLASS LEVEL INFORMATION

CLASS	LEVELS	VALUES
TRT	7	4.0 4.4 4.8 5.2 5.6 6.0 7.3

NUMBER OF OBSERVATIONS IN DATA SET = 44
DATA SORTED BY TREATMENT

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: M

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	6	773391.87857035	128898.64642839	2.72	0.0275	0.305807	89.1248
ERROR	37	1755629.87984891	47449.45621213		ROOT MSE		M MEAN
CORRECTED TOTAL	43	2529021.75841926			217.82896091		244.40886136

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE III SS	F VALUE	PR > F
TRT	6	773391.87857035	2.72	0.0275	6	773391.87857035	2.72	0.0275

Table 3.6. A paired t test used to determine which acid treatments differ significantly from each other. The comparison of greatest importance is that of the control (7.3) to the others. This paired t test utilizes an alpha value of 0.05 and demonstrates the stepwise comparison of treatments.

T TESTS (LSD) FOR VARIABLE: M
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE.
ALPHA=0.05 CONFIDENCE=0.95 DF=37 MSE=47449.5
CRITICAL VALUE OF T=2.02619
COMPARISONS SIGNIFICANT AT THE 0.05 LEVEL ARE INDICATED BY '***'

TRT COMPARISON	LOWER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	UPPER CONFIDENCE LIMIT	
5.6 - 4.8	-75.15	153.27	381.70	
5.6 - 5.2	-54.30	197.32	448.93	
5.6 - 4.0	101.10	321.78	542.46	***
5.6 - 4.4	83.48	335.09	586.71	***
5.6 - 7.3	85.61	337.23	588.84	***
5.6 - 6.0	135.31	373.68	612.04	***
4.8 - 5.6	-381.70	-153.27	75.15	
4.8 - 5.2	-214.39	44.04	302.48	
4.8 - 4.0	-59.92	168.51	396.93	
4.8 - 4.4	-76.62	181.82	440.26	
4.8 - 7.3	-74.48	183.95	442.39	
4.8 - 6.0	-25.15	220.40	465.96	
5.2 - 5.6	-448.93	-197.32	54.30	
5.2 - 4.8	-302.48	-44.04	214.39	
5.2 - 4.0	-127.15	124.46	376.08	
5.2 - 4.4	-141.37	137.78	416.92	
5.2 - 7.3	-139.23	139.91	419.05	
5.2 - 6.0	-90.90	176.36	443.62	

Table 3.6. (continued)

TRT COMPARISON	LOWER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	UPPER CONFIDENCE LIMIT	
6.0 - 5.6	-612.04	-373.68	-135.31	***
6.0 - 4.8	-465.96	-220.40	25.15	
6.0 - 5.2	-443.62	-176.36	90.90	
6.0 - 4.0	-290.26	-51.90	186.47	
6.0 - 4.4	-305.84	-38.58	228.68	
6.0 - 7.3	-303.71	-36.45	230.81	
4.0 - 5.6	-542.46	-321.78	-101.10	***
4.0 - 4.8	-396.93	-168.51	59.92	
4.0 - 5.2	-376.08	-124.46	127.15	
4.0 - 4.4	-238.30	13.31	264.93	
4.0 - 7.3	-236.17	15.45	267.06	
4.0 - 6.0	-186.47	51.90	290.26	
4.4 - 5.6	-586.71	-335.09	-83.48	***
4.4 - 4.8	-440.26	-181.82	76.62	
4.4 - 5.2	-416.92	-137.78	141.37	
4.4 - 4.0	-264.93	-13.31	238.30	
4.4 - 7.3	-277.01	2.13	281.28	
4.4 - 6.0	-228.68	38.58	305.84	
7.3 - 5.6	-588.84	-337.23	-85.61	***
7.3 - 4.8	-442.39	-183.95	74.48	
7.3 - 5.2	-419.05	-139.91	139.23	
7.3 - 4.0	-267.06	-15.45	236.17	
7.3 - 4.4	-281.28	-2.13	277.01	
7.3 - 6.0	-230.81	36.45	303.71	

T TESTS (LSD) FOR VARIABLE: M
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE.
ALPHA=0.1 CONFIDENCE=0.9 DF=37 MSE=47449.5
CRITICAL VALUE OF T=1.68709
COMPARISONS SIGNIFICANT AT THE 0.1 LEVEL ARE INDICATED BY '***'

TRT COMPARISON	LOWER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	UPPER CONFIDENCE LIMIT	
5.6 - 4.8	-36.93	153.27	343.47	
5.6 - 5.2	-12.19	197.32	406.82	
5.6 - 4.0	138.03	321.78	505.53	***
5.6 - 4.4	125.59	335.09	544.60	***
5.6 - 7.3	127.72	337.23	546.73	***
5.6 - 6.0	175.21	373.68	572.15	***
4.8 - 5.6	-343.47	-153.27	36.93	
4.8 - 5.2	-171.14	44.04	259.23	
4.8 - 4.0	-21.69	168.51	358.71	
4.8 - 4.4	-33.36	181.82	397.00	
4.8 - 7.3	-31.23	183.95	399.14	
4.8 - 6.0	15.95	220.40	424.86	***
5.2 - 5.6	-406.82	-197.32	12.19	
5.2 - 4.8	-259.23	-44.04	171.14	
5.2 - 4.0	-85.04	124.46	333.97	
5.2 - 4.4	-94.65	137.78	370.20	
5.2 - 7.3	-92.52	139.91	372.33	
5.2 - 6.0	-46.17	176.36	398.89	

Table 3.7. (continued)

TRT COMPARISON	LOWER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	UPPER CONFIDENCE LIMIT	
6.0 - 5.6	-572.15	-373.68	-175.21	***
6.0 - 4.8	-424.86	-220.40	-15.95	***
6.0 - 5.2	-398.89	-176.36	46.17	
6.0	[illegible]	-51.[illegible]	146.57	
6.0 - 4.4	-261.11	-38.58	183.95	
6.0 - 7.3	-258.98	-36.45	186.08	
4.0 - 5.6	-505.53	-321.78	-138.03	***
4.0 - 4.8	-358.71	-168.51	21.69	
4.0 - 5.2	-333.97	-124.46	85.04	
4.0 - 4.4	-196.19	13.31	222.82	
4.0 - 7.3	-194.06	15.45	224.95	
4.0 - 6.0	-146.57	51.90	250.37	
4.4 - 5.6	-544.60	-335.09	-125.59	***
4.4 - 4.8	-397.00	-181.82	33.36	
4.4 - 5.2	-370.20	-137.78	94.65	
4.4 - 4.0	-222.82	-13.31	196.19	
4.4 - 7.3	-230.29	2.13	234.56	
4.4 - 6.0	-183.95	38.58	261.11	
7.3 - 5.6	-546.73	-337.23	-127.72	***
7.3 - 4.8	-399.14	-183.95	31.23	
7.3 - 5.2	-372.33	-139.91	92.52	
7.3 - 4.0	-224.95	-15.45	194.06	
7.3 - 4.4	-234.56	-2.13	230.29	
7.3 - 6.0	-186.08	36.45	258.98	

Table 3.8. A paired t to determine which Al treatments differ significantly from each other at an alpha value of 0.15. As before, the comparison of greatest significance is that of the control to the other Al treatments.

T TESTS (LSD) FOR VARIABLE: M
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE.
ALPHA=0.15 CONFIDENCE=0.85 DF=37 MSE=47449.5
CRITICAL VALUE OF T=1.47005
COMPARISONS SIGNIFICANT AT THE 0.15 LEVEL ARE INDICATED BY '***'

TRT COMPARISON	LOWER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	UPPER CONFIDENCE LIMIT	
5.6 - 4.8	-12.46	153.27	319.00	
5.6 - 5.2	14.76	197.32	379.87	***
5.6 - 4.0	161.67	321.78	481.89	***
5.6 - 4.4	152.54	335.09	517.65	***
5.6 - 7.3	154.67	337.23	519.78	***
5.6 - 6.0	200.74	373.68	546.62	***
4.8 - 5.6	-319.00	-153.27	12.46	
4.8 - 5.2	-143.46	44.04	231.55	
4.8 - 4.0	2.78	168.51	334.24	***
4.8 - 4.4	-5.68	181.82	369.32	
4.8 - 7.3	-3.55	183.95	371.45	
4.8 - 6.0	42.25	220.40	398.56	***
5.2 - 5.6	-379.87	-197.32	-14.76	***
5.2 - 4.8	-231.55	-44.04	143.46	
5.2 - 4.0	-58.09	124.46	307.02	
5.2 - 4.4	-64.75	137.78	340.30	
5.2 - 7.3	-62.62	139.91	342.43	
5.2 - 6.0	-17.54	176.36	370.26	

Table 3.8, (continued)

TRT COMPARISON	LOWER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	UPPER CONFIDENCE LIMIT	
6.0 - 5.6	-546.62	-373.68	-200.74	***
6.0 - 4.8	-398.56	-220.40	-42.25	***
6.0 - 5.2	-370.26	-176.36	17.54	
6.0 - 4.0	-224.84	-51.90	121.04	
6.0 - 4.4	-232.49	-38.58	155.32	
6.0 - 7.3	-230.35	-36.45	157.45	
4.0 - 5.6	-481.89	-321.78	-161.67	***
4.0 - 4.8	-334.24	-168.51	-2.78	***
4.0 - 5.2	-307.02	-124.46	58.09	
4.0 - 4.4	-169.24	13.31	195.87	
4.0 - 7.3	-167.11	15.45	198.00	
4.0 - 6.0	-121.04	51.90	224.84	
4.4 - 5.6	-517.65	-335.09	-152.54	***
4.4 - 4.8	-369.32	-181.82	5.68	
4.4 - 5.2	-340.30	-137.78	64.75	
4.4 - 4.0	-195.87	-13.31	169.24	
4.4 - 7.3	-200.39	2.13	204.66	
4.4 - 6.0	-155.32	38.58	232.49	
7.3 - 5.6	-519.78	-337.23	-154.67	***
7.3 - 4.8	-371.45	-183.95	3.55	
7.3 - 5.2	-342.43	-139.91	62.62	
7.3 - 4.0	-198.00	-15.45	167.11	
7.3 - 4.4	-204.66	-2.13	200.39	
7.3 - 6.0	-157.45	36.45	230.35	

TUKEY'S STUDENTIZED RANGE (HSD) TEST FOR VARIABLE: M

NOTE: THIS TEST CONTROLS THE TYPE I EXPERIMENTWISE ERROR RATE

ALPHA=0.05 CONFIDENCE=0.95 DF=37 MSE=47449.5

CRITICAL VALUE OF STUDENTIZED RANGE=4.407

COMPARISONS SIGNIFICANT AT THE 0.05 LEVEL ARE INDICATED BY '***'

TRT COMPARISON	SIMULTANEOUS LOWER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	SIMULTANEOUS UPPER CONFIDENCE LIMIT	
5.6 - 4.0	-17.40	321.78	661.14	
5.6 - 4.4	-51.86	335.09	722.05	
5.6 - 7.3	-49.73	337.23	724.19	
5.6 - 6.0	7.10	373.68	740.25	***
4.8 - 5.6	-504.57	-153.27	198.02	
4.8 - 5.2	-353.40	44.04	441.49	
4.8 - 4.0	-182.79	160.51	519.80	
4.8 - 4.4	-215.62	181.02	579.26	
4.8 - 7.3	-213.49	183.16	581.40	
4.8 - 6.0	-157.22	220.60	598.03	
5.2 - 5.6	-584.29	-197.32	189.64	
5.2 - 4.8	-441.49	-48.04	353.40	
5.2 - 4.0	-268.49	124.46	517.42	
5.2 - 4.4	-291.51	137.78	567.06	
5.2 - 7.3	-289.38	139.91	569.20	
5.2 - 6.0	-234.65	176.36	587.37	

Table 3.9. **Tukey's paired t test to determine which Al treatments differ significantly from each other. This test has the ability to handle unbalanced cell sizes.**

Table 3.9. (continued)

TRT COMPARISON	SIMULTANEOUS LOWER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	SIMULTANEOUS UPPER CONFIDENCE LIMIT	
6.0 - 5.6	-740.25	-373.68	-7.10	***
6.0 - 4.8	-598.03	-220.40	157.23	
6.0 - 5.2	-587.37	-176.36	234.65	
6.0 - 4.0	-418.47	-51.90	314.68	
6.0 - 4.4	-449.60	-38.58	372.43	
6.0 - 7.3	-447.46	-36.45	374.56	
4.0 - 5.6	-661.16	-321.78	17.60	
4.0 - 4.8	-519.80	-168.51	182.79	
4.0 - 5.2	-511.42	-124.46	262.49	
4.0 - 4.4	-373.64	13.31	400.27	
4.0 - 7.3	-371.51	15.45	402.40	
4.0 - 6.0	-314.68	51.90	418.47	
4.4 - 5.6	-722.05	-335.09	51.86	
4.4 - 4.8	-579.26	-181.82	215.62	
4.4 - 5.2	-567.06	-137.78	291.51	
4.4 - 4.0	-400.27	-13.31	373.64	
4.4 - 7.3	-427.16	2.13	431.42	
4.4 - 6.0	-372.43	38.58	449.60	
7.3 - 5.6	-724.18	-337.23	49.73	
7.3 - 4.8	-581.40	-183.95	213.49	
7.3 - 5.2	-569.20	-139.91	289.38	
7.3 - 4.0	-402.40	-15.45	371.51	
7.3 - 4.4	-431.42	-2.13	427.16	
7.3 - 6.0	-374.56	36.45	447.46	

CHAPTER FOUR

DISCUSSION

In the Introduction Section of this thesis, the pressing need for a sensitive Al detection method was underscored because of the assumed relationship between acid rain and Al toxicity in the environment. Additionally, Al has been implicated as a possible causative agent in the manifestation of Alshiemer's disease (Elinder and Brusewitz, 1982). The conventional methods of Al determination, i.e., atomic adsorption flame photometry and conventional spectrophotometry, were examined and their limitations described in the Introduction Section. The contention was made that INAA would be better suited for determination of Al in fish tissue, a mixed organic matrix, than the other methodologies.

Six experimental treatments having an Al concentration of 1 mg Al 1^{-1} and pH values of 4.0, 4.4, 4.8, 5.2, 5.6, and 6.0 were tracked over the course of 12 days and examined for effects of Al toxicity. The gross effects exhibited by the fish in these treatments were mucous overproduction, loss of timidity, and death. The presence of Al in the gills of the fish in these treatments was determined by INAA methodologies.

Concurrent with change in Al speciation, i.e., polymeric, monomeric, etc., is a rise in pH (Smith, 1971) and, consequently, the six treatment levels required frequent checking to regulate the pH and at the desired pH level. The pH levels of the treatments were

sucessfully kept near the desired levels of 4.0, 4.4, 4.8, 5.2, 5.6, and 6.0 throughout the course of the experiment as evidenced by the small standard deviation associated with each mean (Table 3.1). An ANOVA and a paired t test comparing these pH treatments determined that each was statistically distinct (Table 3.2 and 3.3).

It had been hoped at the outset of the experiment that by direct correlations among Al speciation, death rates of the fish, and Al concentrations in the gill that the causative agent of Al toxicity could be determined. With the failure of the dialysis membranes to perform consistently and the unavailability of selective detection systems for chloride and fluoride ions, only general trends in Al speciation were noted (Appendix C).

From Appendix C in Figures C.1 to C.6, it is seen that each treatment had a distinct Al speciation pattern and the only obvious difference between these treatments was the pH at which the systems were maintained. The data in Tables C.1 to C.6 demonstrate that the Al speciation was a direct function of the pH. The observation that pH and Al speciation are interdependent was also noted in Smith,(1971). Logically then, the difference in death rates between the pH treatments was due to the different proportions of Al species as well as to the pH.

In the recent literature (discussed in Chapter One, page 3, Acid Rain), acid effects on fishes were believed to be more prominent than Al effects at pH's below 4.8 and above 6.0. However, since gill tissues from these studies were not assayed for Al and effects were not quantified, the effects of pH on Al toxicity was not proved. In

this study the Al concentration data on the gills from fish in the 4.0, 4.4, and 6.0 pH treatments presented in Table 3.5 do not differ greatly from the Al concentrations found in the gills of the control fish. Data from the gills of fish in the 4.8, 5.2, and 5.6 treatments, also in Table 3.5, generally evidence Al concentrations higher than that of the gills from control animals. Gill sample numbers 24 and 25, with ppm of Al of 1392 and 975, respectively, are 13 times and 8 times larger than minimal Al values found in control gills. These results support the contention that Al is the responsible agent of death when median pH values of 4.8 to 5.6 are present.

Are the results shown in Table 3.5 statistically significant? Tests of statistical significance were performed on the Al concentration values (ppm) (Tables 3.6, 3.7, 3.8, and 3.9). The differences between the mean ppm Al of the control group and the mean ppm Al of the 4.8, 5.2, and 5.6 treatments are large. In fact, 2.3, 2.0, and 3.4 times larger for pH 4.8, 5.2, and 5.6, respectively, but statistical significance is not attained in the 4.8 or 5.2 pH treatments due to the small sample number used (5 fish in each of the six treatments). However, the ability to determine small differences in Al concentration has been made possible in this study by the INAA method and statistical validity would be easily attainable if a larger number of fish samples were utilized (see Formula 2.1 for a description).

Although a neutron moderator was not available for this study, better statistics could be generated through its use. The

neutron moderator would reduce the uncertainity caused by the contribution of P (through the $^{31}P(n,\alpha)^{28}Al$ reaction) to the ^{28}Al photopeak (see Chapter Two, Interferences Associated with INAA Determination of Aluminum). Therefore, double neutron irradiation of the samples utilizing Cd containers was the method used for the direct determination of Al (Table 3.10).

In Table 3.10, the P-produced ^{28}Al appeared to be accurate to $\pm$ 10%, as the P contribution to the ^{28}Al photopeak represents only 6.9% of the total response for sample 20, in Table 3.10. The 6.9% contribution was determined by dividing the Al concentration in ppm for the unencapsulated Cd sample by the Al concentration in ppm for the encapsulated Cd sample. The 6.9% figure is considerably lower than the 33% figure generally used as "background" in the indirect method (In Chapter Two, Interferences Associated with INAA Determination of Aluminum, it was noted that roughly 2/3 of the ^{28}Al photopeak was due to Al and that P contributed 1/3 of the total ^{28}Al photopeak response).

The large difference between percent contribution in the direct and indirect methods is due to the presence of endogenous levels of Al in the fish. It must be noted that this conclusion is drawn from one fish, and these ideas concerning ambient Al require further experimentation. The 33% contribution figure from the indirect method, as mentioned above, represents both the P ($^{31}P(n,\alpha)^{28}Al$) and background Al contributions to the ^{28}Al photopeak. The difference in Al concentration of the gill of a control fish (Sample #40, Table 3.10) without Cd encapsulation as compared to the

gill of the control fish with Cd encapsulation is due to the presence of background Al. The INAA method of determining Al in fish gills appears to be sensitive enough to allow for quantification of background Al levels. If this belief holds true, the increasing body burden of Al in a fish population can be quantified over a period of years allowing researchers to determine at an early stage if a lake or stream is being Al-stressed. It is possible to achieve a sensitivity of 10 parts per million in such studies.

INAA is an expedient method for determining Al concentrations in fish gills that could contribute to the understanding of Al-caused mortalities. Sample preparation is simple, as only drying and weighing of the sample are necessary. The INAA methodology allows for reassay of the sample without signifigantly altering it. It appears to be within the realm of possibility that a high Al sensitivity can be determined by INAA to levels of ppb Al in fresh water fish gills.

INAA methodology for Al determination can be utilized by experimenters not familiar with its theory as INAA does not require that the experimenter have detailed knowledge of how the method operates. Samples can be sent to a research nuclear reactor and analyzed for Al concentration. The results of the Al assay are returned to the experimenter in any format desired. This ability to determine Al at low ppm levels may allow further study of the mechanisms and manifestations of Al toxicity in wild fish. It might also be possible to expand these methods to incorporate human tissues and determine Al toxicity in humans. With the development of the INAA technique, broad, new areas of Al toxicology studies are possible.

Appendix A

Quantitative Analysis of Aluminum Concentration

by Instrumental Neutron Activation Analysis

(From Lyon, 1972)

The radioactivity induced in a sample assayed by instrumental neutron activation analysis is dependent upon the amount of target element present, the cross section of the target nuclide, the irradiation flux, the irradiation time, and the decay characteristics of the element formed.

The cross section (σ) of a nuclide is a simple way of expressing the probability that the nuclide will undergo a reaction with the bombarding particles. The value of the cross section is energy dependent; if thermal neutrons are the bombarding particles, then the thermal-neutron cross sections are used in the calculations of induced activity. The unit of cross section is the barn where 1 barn = 10^{-24} cm^2.

The irradiation flux (ϕ) expresses the area-time density of the bombarding particles in units such as neutrons per cm^2 per second. The higher the flux, the greater is the induced activity for a given element and irradiation time.

The time (t_i) of an irradiation must be known in order to calculate the magnitude of the induced activity. However the time function is not linear since, during an irradiation , not only is the radionuclide being formed, but the induced activity of the

radionuclide is decaying at a rate proportional to the decay constant of the radionuclide product.

At some time (t_s), the rates of formation and decay will be exactly equal, and the sample is said to have reached saturation. The saturation activity is the highest activity that can be produced in a sample with a given irradiation flux. Figure A.1 illustrates these concepts.

All of the factors influencing the final activity in a sample due to the activation of a given nuclide can be expressed by the following activation equations:

$$A = N\sigma\phi(1 - e^{-\lambda t_i})\ e^{-\lambda t_d} \qquad (A.1)$$

and since

$$\lambda = \frac{0.693}{T_{1/2}} \qquad (A.2)$$

where A = induced activity present at end of irradiation, dis/sec

N = number of target atoms present

σ = cross section, cm^2

ϕ = irradiation flux, neutrons cm^{-2} sec^{-1}

t_i = irradiation time

t_d = decay time

$T_{1/2}$ = half life of product nuclide

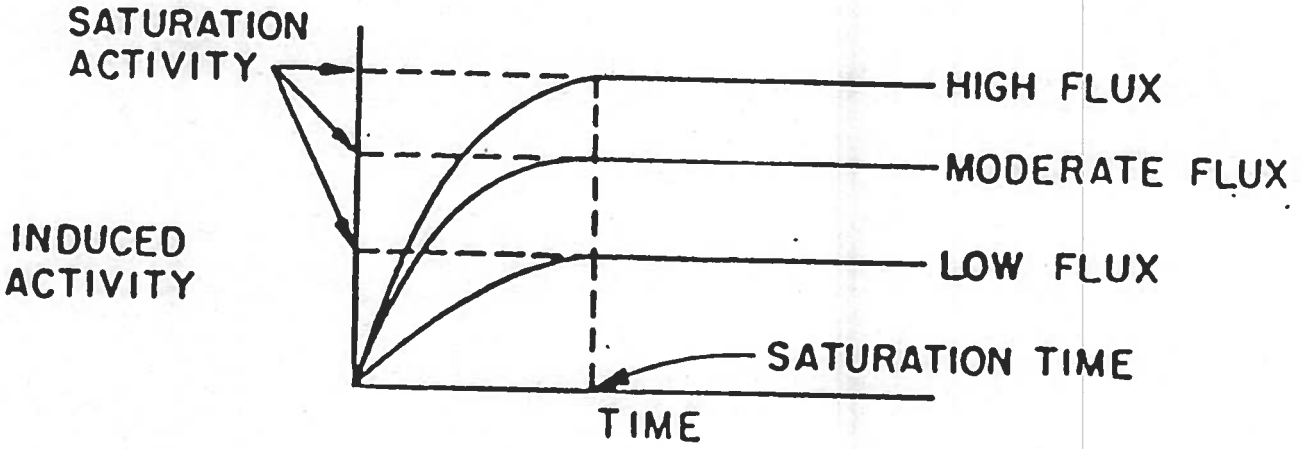


Figure A.1 This figure shows the relationship between flux, time, and saturation during irradiation. A higher flux allows for a higher saturation activity.

λ = decay constant of product nuclide

The term $(1-e^{-\lambda t_i})$ is sometimes called the saturation factor, S. As the irradiation time (t_i) becomes large compared to half life of the product ($T\frac{1}{2}$), the saturation factor approaches unity. Therefore at the saturation time

$$A = N\sigma\phi \qquad (A.3)$$

N can be calculated by means of the following relation.

$$N = \frac{N_{Av.}wk}{At.Wt.} \qquad (A.4)$$

where $N_{Av.}$ = Avogadro's number, $6.02 * 10^{23}$, atoms/mole

w = weight of element g

k = fractional isotopic abundance of given target nuclide

At. Wt. = atomic weight of element

When this technique is used, it is difficult to get accuracy of better than $\pm$ 20 per cent with out extreme care and effort in determinations with most samples.

The majority of activation analyses done today utilize the comparator technique. In this method, a pure sample (of known weight) containing the sought element and the unknown sample are irradiated simultaneously for the same time in the same flux. Under ideal conditions, the specific activites (disintegration rate/weight of element) of both standard and unknown are the same. Therefore, one

can count the standard and unknown under identical conditions (counting efficencies ϵ_s and ϵ_x equal) and use the relation:

$$W_x = \frac{W_s A_x}{A_s} \qquad \text{(A.5)}$$

also

$$R_x = A_x \epsilon_x \text{ and} \qquad \text{(A.6)}$$

$$R_s = A_s \epsilon_s \qquad \text{(A.7)}$$

$$W_x = \frac{W_s R_x}{R_s} \qquad \text{(A.8)}$$

where W_x = weight of element x in unknown

W_s = weight of element x in standard

A_x = disintegration rate of unknown

A_s = disintegration rate of standard

R_x = count rate of unknown

R_s = count rate of standard

ϵ_x = counting efficency of unknown

ϵ_s = counting efficency of standard

This formula is the same one presented as equations 2.6 and 2.7. The comparison technique eliminates many of the uncertainities in the absolute proceedure.

The choice of a standard is of great concern and is influenced by many factors:

1. The standard should be of the highest purity.

2. The standard should have only 1 activable species.

3. The standard should be easily soluble in conventional solvents.

4. The standard should be nonhygroscopic and easily weighed.

5. The standard should resist radiation and thermal decomposition.

For the comparison used in this thesis, Al wire, 99.9999 percent pure, was used as it fulfilled these five criteria.

Appendix B

Aluminum Speciation Methodology

As noted in Chapter Two, Al speciation methods combined the methods of Turner (1969) and LaZerte (1984). The following solutions are used in analysis by the 8-quinolinolate extraction method:

1. 8-quinolinol solution - dissolve 2.0 g 8-quinolinol in 5 ml glacial acetic acid and dilute to 200 ml with deionized distilled water.

2. Sodium acetate - dissolve 136 g in 1 liter for a 1 M solution.

3. Ammonium acetate - 223 ml of 10 M metal-free ammonium hydroxide and 115 ml of glacial acetic acid are added to approximately 500 ml of water, the pH adjusted to 8.8, and the solution diluted to 1 liter.

4. Distilled deionized water.

5. Nanograde chloroform.

6. Aluminum chloride stock solution - dissolve 9.11 g of $AlCl_3 6H_2O$ in 1 liter of water for 1 g Al $liter^{-1}$ solution. From this make working solutions of 1, 5, 10, 25, and 50 ug Al ml^{-1}.

7. Dye preparation - pour 250 ml of deionized distilled water, 50 ml of the 8-quinolinol solution, and 50 ml of the sodium acetate solution into a storage bottle.

Extraction Methods of Aluminum

Total Aluminum

Place 1 ml of each of the standards of 0, 1, 5, 10, 25, and 50 ug Al ml^{-1} into individual 250 ml separatory funnels. In a second set of 250 ml separtory funnels, deliver enough sample from each unknown to insure that the Al concentration of the whole sample is between 1 to 50 ug. (Determination of the proper amount of sample needed is done in preliminary investigations and volume of sample used kept constant throughout the experiment.) Vigorously add 5 ml of ammonium acetate buffer to all funnels. Add 15 ml of dye preparation to all funnels and allow the Al and the dye to interact for six hours, at a temperature of 25°C. At the end of the six hours, add 10 ml of chloroform to each funnel, shake the funnels vigorously for 15 seconds, and extract the chloroform, containing the reacted dye, through glass wool. Determine the Al extracted in the chloroform colormetrically; set the spectrophotometer at a wavelength of 390 nm, record the percent transmission of the standards and generate a standard curve to determine the Al concentrations of the unknown samples. Report the total Al concentration as ug Al ml^{-1}.

Fast Aluminum

Deliver enough sample of an unknown solution to a 250 ml separatory funnel to insure that the concentration of the fast reactive Al to be extracted is between 1 and 50 ug. Add 5 ml of ammonium acetate buffer and 10 ml of chloroform to the solution. Add 15 ml of dye preparation to the funnel, shake for 15 seconds and

extract the chloroform. Determine the Al concentration in the chloroform by using the standard curve, generated above, to determine the fast reactive Al concentration. Report fast reactive Al concentration as ug Al ml^{-1}.

Dialyzed Aluminum

A Spectrapor-6 dialysis membrane having a 1,000 molecular weight cut-off and a diameter of 11.5 millimeters is prepared for dialysis in the following manner:

1. Inspect tubing for uniformity of size and discard tubing having an inconsistent diameter.

2. Cut a 15 cm length of tubing, rinse it thoroughly in ultrapure water, and place the tubing in a beaker containing 1 N nitric acid for 24 hours.

3. Remove the tubing from the nitric acid and rinse it thoroughly with ultrapure water. Place the tubing in ultra pure water for 24 hours.

4. Pour a measured amount of ultrapure water (10-20 ml) into the tubing and clamp the ends, trapping the water in as small a space as possible to insure consistent sample to volume ratios.

The dialysis tubing, prepared as described above, is placed in a beaker containing 500 ml of sample and is allowed to interact for 48 hours at 5°C. The dialysate is removed and assayed for dialysed Al in the exact same manner as described for fast reactive Al. The concentration of dialysed Al (inorganic monomeric Al) is determined as ug Al ml^{-1}.

Appendix C

Aluminum Speciation Results

It had been hoped at the outset of the experiment that by direct correlations between Al speciation, death rates of the fish, and Al concentrations in the gill that Al could be suggested as the causative agent of toxicity. With the failure of the dialysis membranes to perform consistently and the unavailability of selective detection systems for chloride and fluoride ions, only general trends in Al speciation can be noted. It is for these reasons that the data from the Al speciation experiment is presented as Appendix C.

The water of the 7 plastic 32-gallon containers was sampled daily for dialysed Al, fast reactive Al, and for total reactive Al. A table of results and graphs of Al concentrations vs. time were produced for each treatment by SAS and are presented as Tables C.1-C.6 and as Figures C.1-C.6, respectively. Tables C.1-C.6 and Figures C.1-C.6 are grouped together by pH treatment. As such, Figure C.1 follows Table C.1, etc. On the tables charting Al concentration over time, Al is reported as part of the larger groupings of dialysed Al, fast reactive Al, and total Al. Additionally, the smaller subgroupings of polymeric Al, inorganic monomeric Al, organic monomeric Al, and crystaline Al have been determined by the use of formulae 2.2, 2.3, and 2.4. These calculations however, are unreliable due to failure of the dialysis membranes to perform.

The Figures C.1-C.6 chart the fluctuation of the larger groupings of dialysed Al, fast reactive Al, and total Al. The graphic representation of the dialysed Al is incomplete owing to the loss of

Table C.1 Data from the pH 4.0 treatment. 'Time1' represents the time in hours at which the total Al concentration was determined. 'Time2' represents the time in hours at which the fast Al concentration was determined. 'Total' represents total Al concentration, 'Fast' is the fast reactive Al, 'dial' is the dialyzed Al portion, 'A' is the polymeric Al, 'B' is the inorganic monomeric Al and 'C' is the crystalline Al fraction. Al fractions are in terms of ug ml^{-1}.

PH 4.0 DATA

OBS	TIME1	TOTAL	TIME2	FAST	DIAL	A	B	C
1	29.00	1.425	24.00	0.975	0.0000	0.450	0.9750	0.025
2	48.00	1.413	53.00	0.800	0.4725	0.613	0.3275	0.037
3	67.50	1.350	68.50	0.575	0.0000	0.775	0.5750	0.100
4	92.50	1.317	94.50	0.450	0.3266	0.867	0.1234	0.133
5	114.25	1.250	122.50	0.445	0.0000	0.805	0.4450	0.200
6	140.50	1.175	146.75	0.735	0.0000	0.440	0.7350	0.275
7	163.00	1.267	169.50	1.000	0.0000	0.267	1.0000	0.183
8	189.50	1.175	196.00	0.886	0.0000	0.289	0.8860	0.275
9	209.50	1.225	210.00	0.900	0.0000	0.325	0.9000	0.225

Figure C.1 Graphic representation of the fast Al, total Al, and dialyzed Al concentrations of ug ml^{-1} over time, in hours for pH 4.0 (From Table C.1).

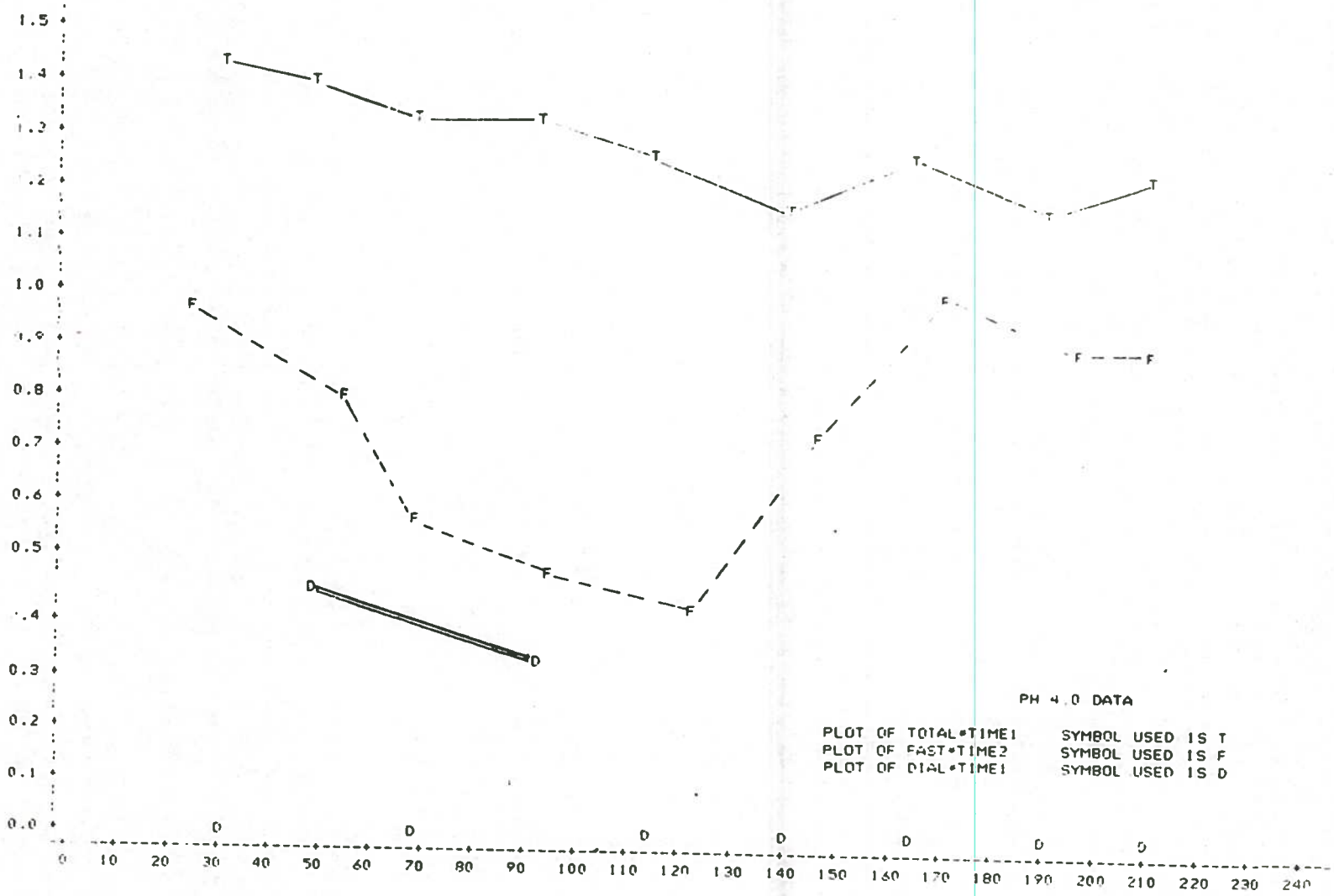

PH 4.0 DATA
PLOT OF TOTAL*TIME1 SYMBOL USED IS T
PLOT OF FAST*TIME2 SYMBOL USED IS F
PLOT OF DIAL*TIME1 SYMBOL USED IS D

Table C.2 Data from the pH 4.4 treatment. 'Time1' represents the time in hours at which the total Al concentration was determined. 'Time2' represents the time in hours at which the fast Al concentration was determined. 'Total' represents total Al concentration, 'Fast' is the fast reactive Al, 'dial' is the dialyzed Al portion, 'A' is the polymeric Al, 'B' is the inorganic monomeric Al and 'C' is the crystalline Al fraction. Al fractions are in terms of ug ml^{-1}.

PH 4.4 DATA

OBS	TIME1	TOTAL	TIME2	FAST	DIAL	A	B	C
1	29.00	1.[illegible]	[illegible]	.0875	0.0000	0.3875	1.0875	0.025
2	48.00	1.413	53.00	0.6375	0.4650	0.7755	0.1725	0.087
3	67.50	1.363	68.50	0.3630	0.0000	1.0000	0.3630	0.137
4	92.50	1.150	94.50	0.3875	0.2666	0.7625	0.1209	0.350
5	114.25	1.125	122.50	0.1250	0.0000	1.0000	0.1250	0.375
6	140.50	1.067	146.75	0.5650	0.0000	0.5020	0.5650	0.433
7	163.00	1.150	169.50	0.9450	0.0000	0.2050	0.9450	0.350
8	189.50	1.225	196.00	0.4666	0.0000	0.7584	0.4666	0.275
9	209.50	1.138	210.00	0.6600	0.0000	0.4780	0.6600	0.362

Figure C.2 Graphic representation of the fast Al, total Al, and dialyzed Al concentrations of ug ml^{-1} over time, in hours for pH 4.4 (From Table C.2).

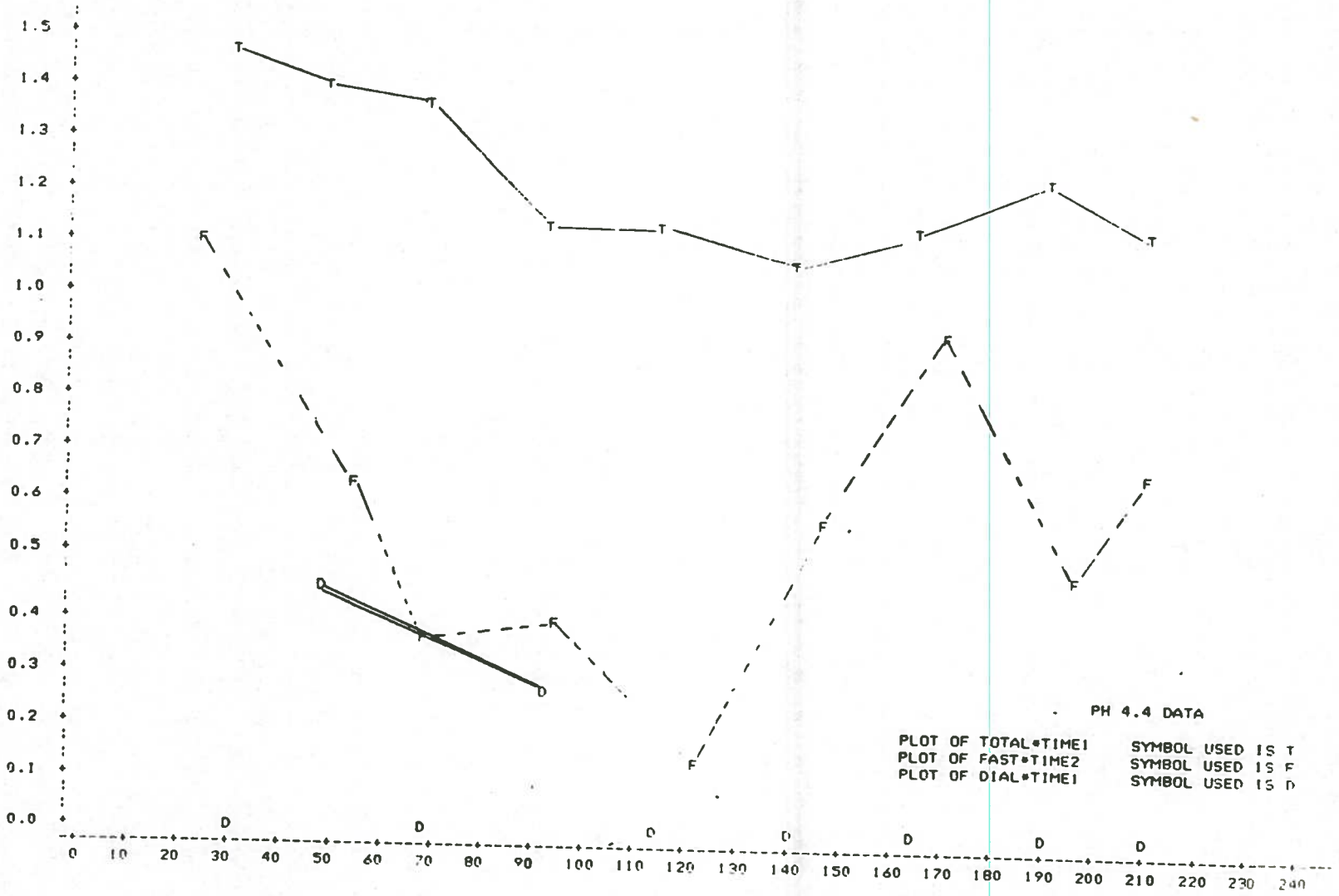

1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240
PH 4.4 DATA
PLOT OF TOTAL*TIME1 SYMBOL USED IS T
PLOT OF FAST*TIME2 SYMBOL USED IS F
PLOT OF DIAL*TIME1 SYMBOL USED IS D

Table C.3 Data from the pH 4.8 treatment. 'Time1' represents the time in hours at which the total Al concentration was determined. 'Time2' represents the time in hours at which the fast Al concentration was determined. 'Total' represents total Al concentration, 'Fast' is the fast reactive Al, 'dial' is the dialyzed Al portion, 'A' is the polymeric Al, 'B' is the inorganic monomeric Al and 'C' is the crystalline Al fraction. Al fractions are in terms of ug ml^{-1}.

PH 4.8 DATA

OBS	TIME1	TOTAL	TIME2	FAST	DIAL	A	B	C
1	29.00	1.325	24.00	0.7665	0.000	0.5585	0.7665	0.025
2	48.00	1.063	53.00	0.6500	0.315	0.4130	0.3350	0.287
3	67.50	0.850	68.50	0.4000	0.000	0.4500	0.4000	0.500
4	92.50	0.500	94.50	0.1625	0.010	0.3375	0.1525	0.850
5	114.25	0.488	122.50	0.0300	0.000	0.4580	0.0300	0.862
6	140.50	0.085	146.75	0.2550	0.000	-0.1700	0.2550	1.265
7	163.00	0.400	169.50	0.4050	0.000	-0.0050	0.4050	0.950
8	189.50	0.466	196.00	0.2800	0.000	0.1860	0.2800	0.884
9	209.50	0.493	210.00	0.3600	0.000	0.1330	0.3600	0.857

Figure C.3 Graphic representation of the fast Al, total Al, and dialyzed Al concentrations of ug ml^{-1} over time, in hours for pH 4.8 (From Table C.3).

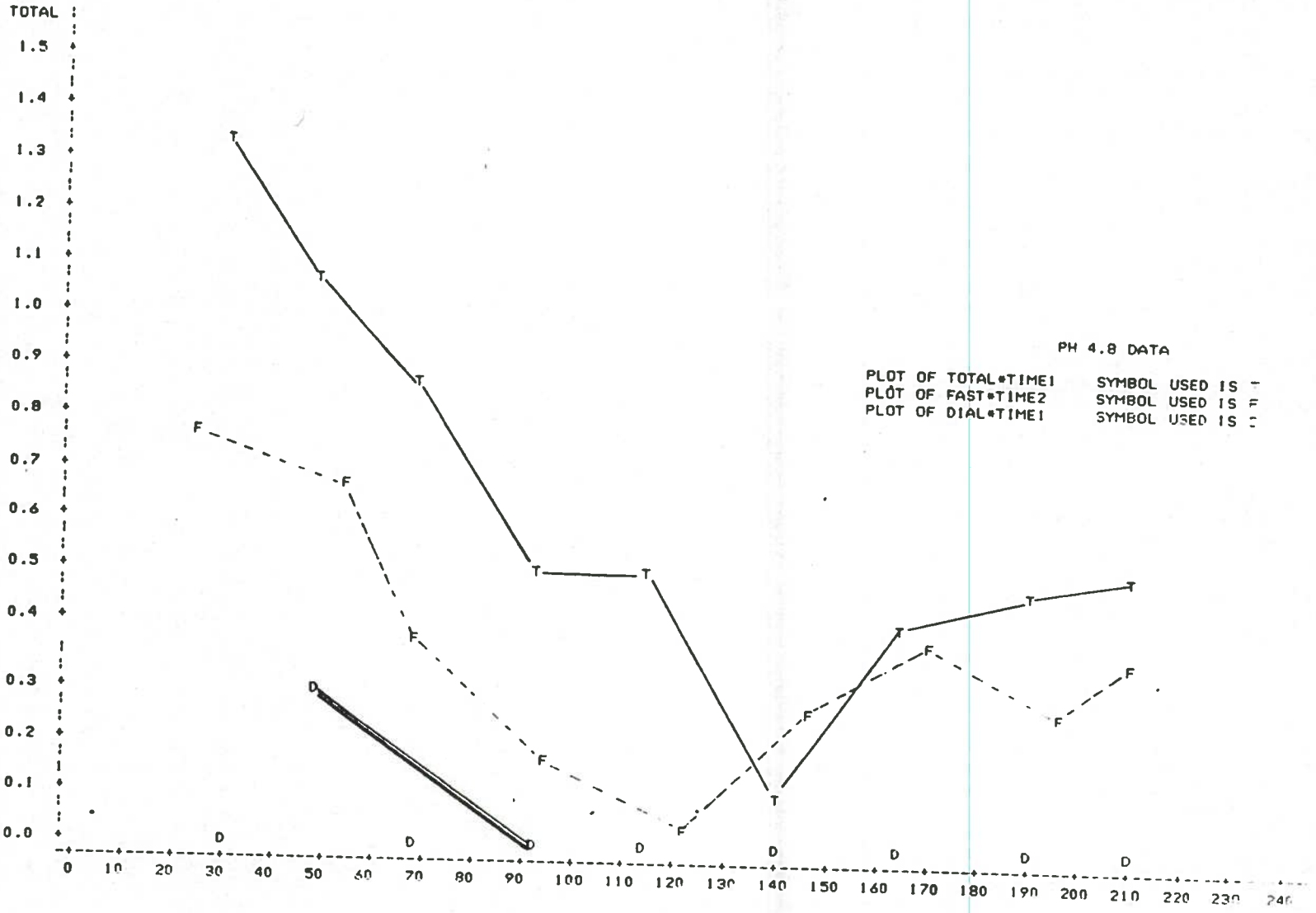

TOTAL
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240
PH 4.8 DATA
PLOT OF TOTAL*TIME1 SYMBOL USED IS T
PLOT OF FAST*TIME2 SYMBOL USED IS F
PLOT OF DIAL*TIME1 SYMBOL USED IS D

Table C.4 Data from the pH 5.2 treatment. 'Time1' represents the time in hours at which the total Al concentration was determined. 'Time2' represents the time in hours at which the fast Al concentration was determined. 'Total' represents total Al concentration, 'Fast' is the fast reactive Al, 'dial' is the dialyzed Al portion, 'A' is the polymeric Al, 'B' is the inorganic monomeric Al and 'C' is the crystalline Al fraction. Al fractions are in terms of ug ml^{-1}.

PH 5.2 DATA

OBS	TIME1	TOTAL	TIME2	FAST	DIAL	A	B	C
1	29.00	0.7750	24.00	0.8750	0.0000	-0.1000	0.8750	0.0250
2	48.00	0.8630	53.00	0.4250	0.2537	0.4380	0.1713	-0.0630
3	67.50	0.6000	68.50	0.2130	0.0000	0.3870	0.2130	0.2000
4	92.50	0.4630	94.50	0.4325	0.0350	0.0305	0.3975	0.3370
5	114.25	0.4500	122.50	0.0025	0.0000	0.4475	0.0025	0.3500
6	140.25	0.0350	146.75	0.1750	0.0000	-0.1400	0.1750	0.7650
7	163.00	0.2800	169.50	0.2200	0.0000	0.0600	0.2200	0.5200
8	189.50	0.1732	196.00	0.0000	0.0000	0.1732	0.0000	0.6268
9	209.50	0.1300	210.00	0.1200	0.0000	0.0100	0.1200	0.6700

Figure C.4 Graphic representation of the fast Al, total Al, and dialyzed Al concentrations of ug ml^{-1} over time, in hours for pH 5.2 (From Table C.4).

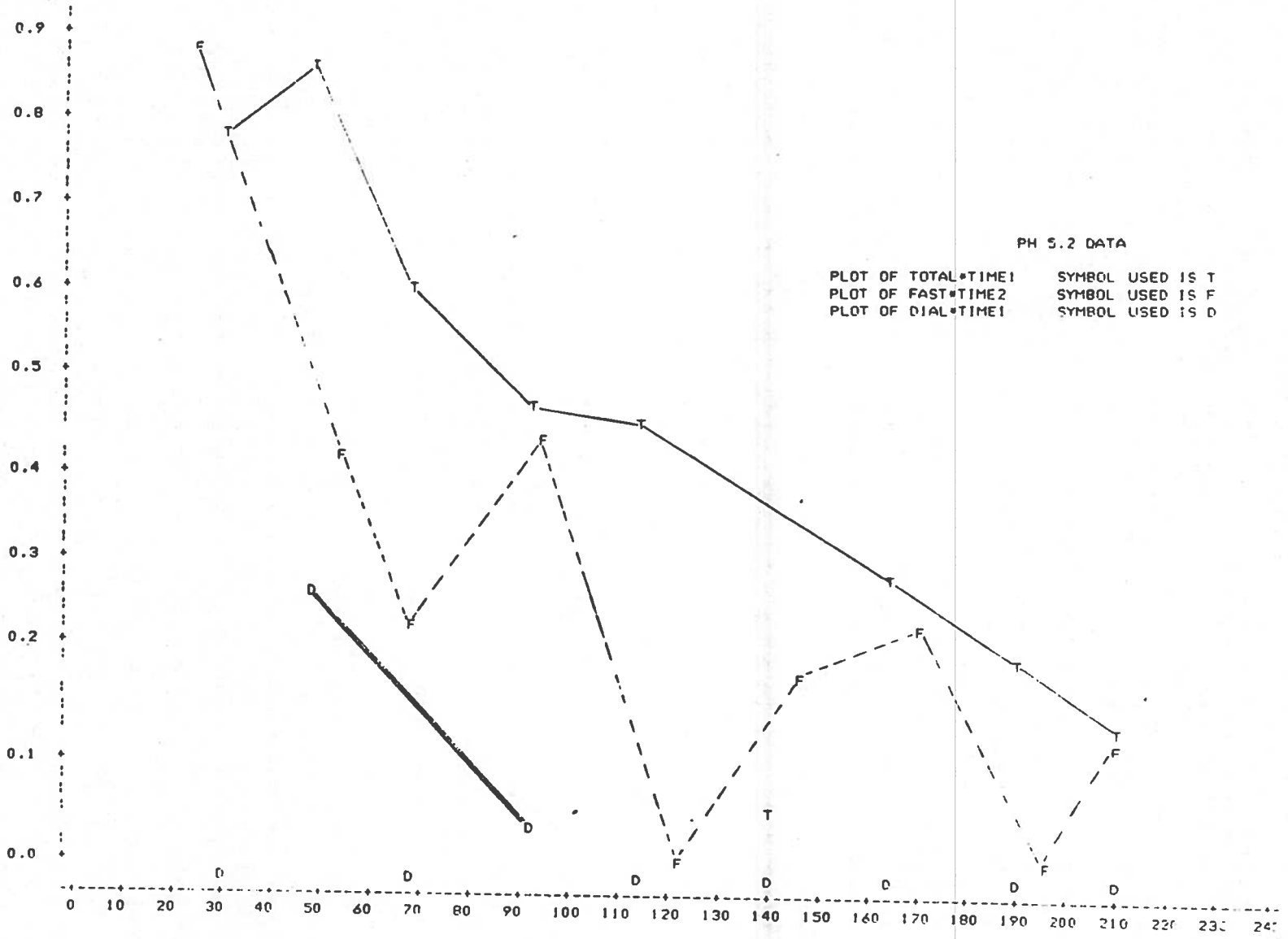

PH 5.2 DATA
PLOT OF TOTAL*TIME1 SYMBOL USED IS T
PLOT OF FAST*TIME2 SYMBOL USED IS F
PLOT OF DIAL*TIME1 SYMBOL USED IS D
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210

Table C.5 Data from the pH 5.6 treatment. 'Time1' represents the time in hours at which the total Al concentration was determined. 'Time2' represents the time in hours at which the fast Al concentration was determined. 'Total' represents total Al concentration, 'Fast' is the fast reactive Al, 'dial' is the dialyzed Al portion, 'A' is the polymeric Al, 'B' is the inorganic monomeric Al and 'C' is the crystalline Al fraction. Al fractions are in terms of ug ml^{-1}.

PH 5.6 DATA

OBS	TIME1	TOTAL	TIME2	FAST	DIAL	A	B	C
1	29.00	0.475	24.00	0.3000	0.0000	0.1750	0.3000	0.800
2	48.00	1.263	53.00	0.2875	0.0000	0.9755	0.2875	0.012
3	67.50	0.675	68.50	0.1630	0.0000	0.5120	0.1630	0.600
4	92.50	0.475	94.50	0.1625	0.0066	0.3125	0.1559	0.800
5	114.25	0.000	122.50	0.0332	0.0000	-0.0332	0.0332	1.275
6	140.50	0.035	146.75	0.0825	0.0000	-0.0475	0.0825	1.240
7	163.00	0.107	169.50	0.1600	0.0000	-0.0530	0.1600	1.168
8	189.50	0.740	196.00	0.1650.	0.0000	0.5750	0.1650	0.535
9	209.50	0.045	210.00	0.0150	0.0000	0.0300	0.0150	1.230

Figure C.5 Graphic representation of the fast Al, total Al, and dialyzed Al concentrations of ug ml^{-1} over time, in hours for pH 5.6 (From Table C.5).

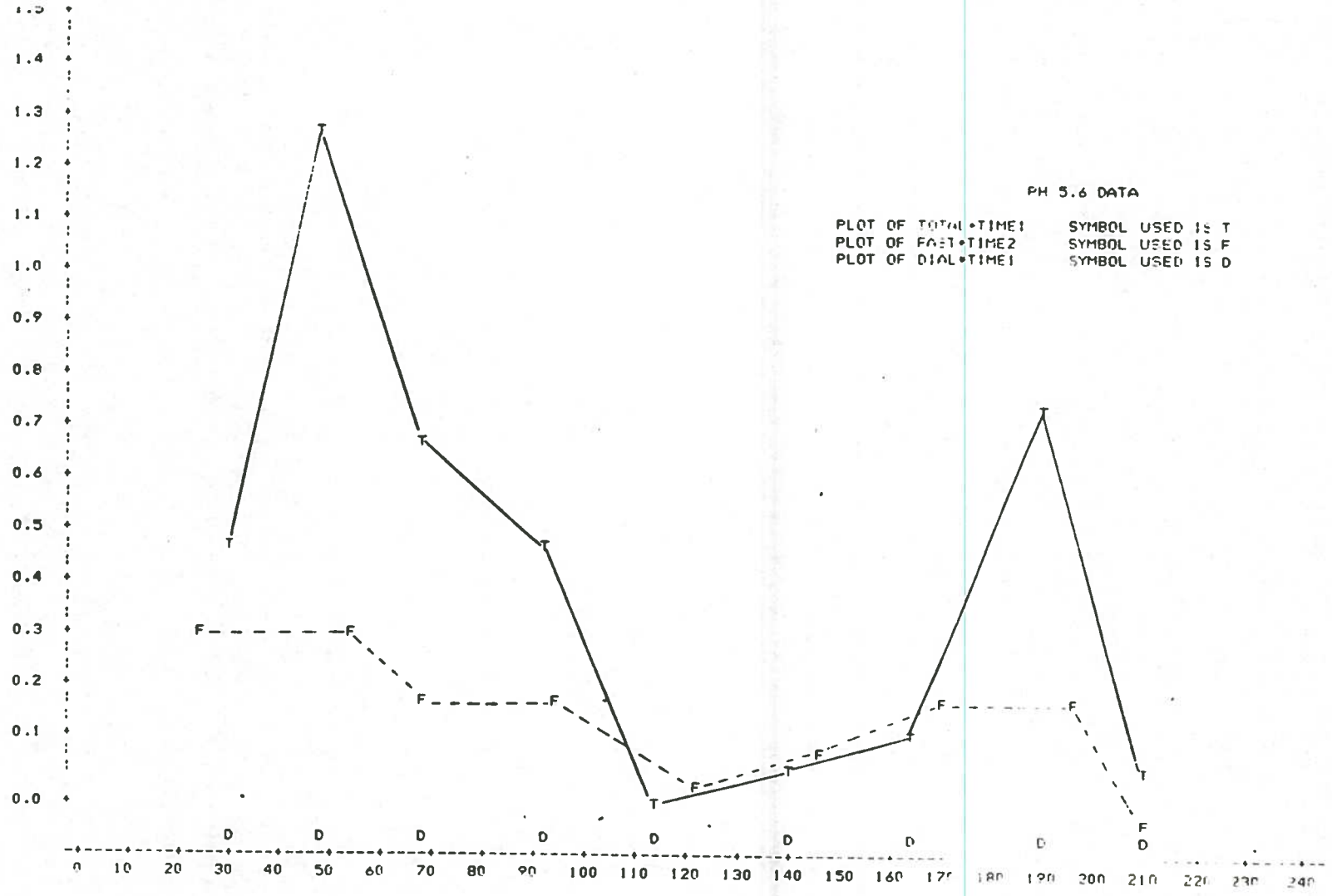
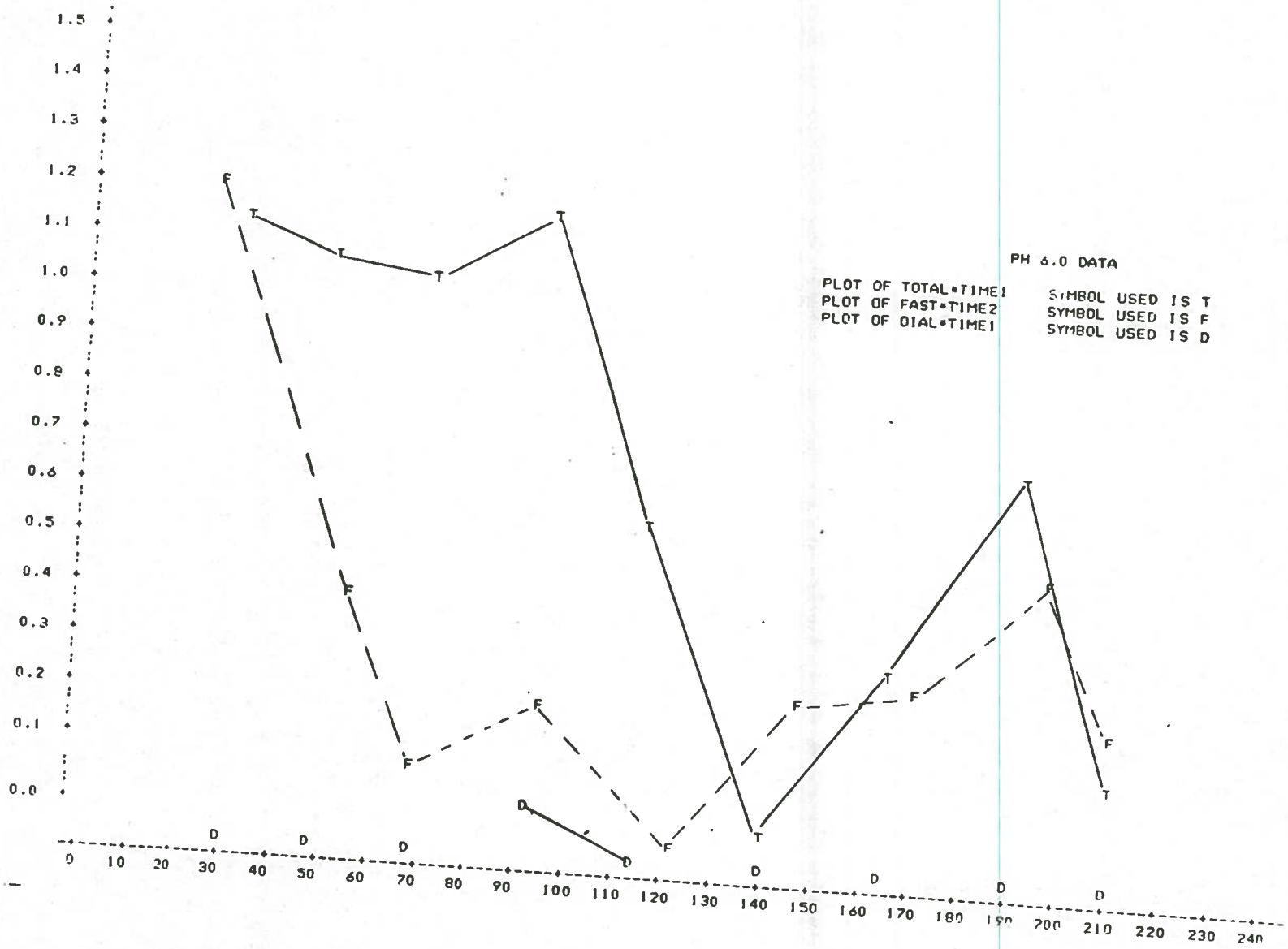

PH 5.6 DATA
PLOT OF TOTAL*TIME1 SYMBOL USED IS T
PLOT OF FAST*TIME2 SYMBOL USED IS F
PLOT OF DIAL*TIME1 SYMBOL USED IS D
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 180 190 200 210 220 230 240

Table C.6 Data from the pH 6.0 treatment. 'Time1' represents the time in hours at which the total Al concentration was determined. 'Time2' represents the time in hours at which the fast Al concentration was determined. 'Total' represents total Al concentration, 'Fast' is the fast reactive Al, 'dial' is the dialyzed Al portion, 'A' is the polymeric Al, 'B' is the inorganic monomeric Al and 'C' is the crystalline Al fraction. Al fractions are in terms of ug ml^{-1}.

PH 6.0 DATA

OBS	TIME1	TOTAL	TIME2	FAST	DIAL	A	B	C
1	29.00	1.150	24.00	1.1875	0.000	-0.0375	1.1875	0.025
2	48.00	1.063	53.00	0.4000	0.000	0.6630	0.4000	0.112
3	67.50	1.050	68.50	0.1130	0.000	0.9370	0.1130	0.125
4	92.50	1.175	94.50	0.2000	0.025	0.9750	0.1750	0.000
5	114.25	0.575	122.50	0.0000	0.000	0.5750	0.0000	0.600
6	140.50	0.010	146.75	0.2400	0.000	-0.2300	0.2400	1.165
7	163.00	0.287	169.50	0.2550	0.000	0.0320	0.2550	0.888
8	189.50	0.687	196.00	0.5000	0.000	0.1870	0.5000	0.488
9	209.50	0.100	210.00	0.1870	0.000	-0.0870	0.1870	1.075

Figure C.6 Graphic representation of the fast Al, total Al, and dialyzed Al concentrations of ug ml^{-1} over time, in hours for pH 6.0 (From Table C.6).


PH 6.0 DATA
PLOT OF TOTAL*TIME1 SYMBOL USED IS T
PLOT OF FAST*TIME2 SYMBOL USED IS F
PLOT OF DIAL*TIME1 SYMBOL USED IS D
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240

samples and the subsequent abandonment of method.

From the data plotted in Figures C.1- C.6, Al concentration can be seen to decrease after a 24-hour equlibration period. The total, fast, and dialysed Al in solution decreases at a steady rate in all the treatments. This rate approaches a 2/3 order reaction as reported by Smith (1971). After 140 hours, the depletion rate of total Al approaches zero, and the total Al concentration begins to rise. This behavior was noted in all the treatments except pH 5.2, where there was a constant decrease in total Al concentration throughout the experiment.

For the fast reactive Al component, a steady depletion rate is seen up to the 120 hour reading for all treatments (Figures C.1-C.6). Thereafter, there is a steady increase in the fast reactive Al until a maximum is reached at the 180 hour reading. At the 180 hour point, the fast reactive Al begins a second decrease in concentration.

In general, the data in Figures C.1-C.6 demonstrate that each pH treatment has a distinct Al speciation pattern and concentration and, therefore, each treatment differed in the way Al was made available to the fish.

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