

The Effects of Fluoride and Magnesium Ions  
on the Metabolism of the Blue Crab

A Thesis

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Faculty of the Department of Biology  
East Carolina University

In Partial Fulfillment of  
the Requirements for the Degree  
Master of Science in Biology

by

Patricia D. McClellan

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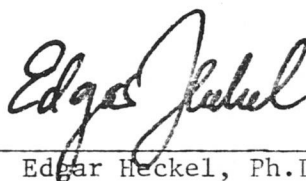
THE EFFECTS OF FLUORIDE AND MAGNESIUM IONS  
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Patricia D. McClellan

APPROVED BY:

CO-SUPERVISOR OF THESIS



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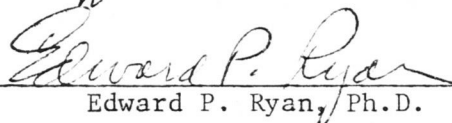
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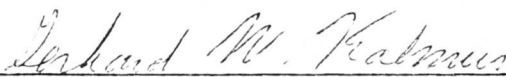
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### Abstract

Patricia D. McClellan. THE EFFECTS OF FLUORIDE AND MAGNESIUM IONS ON THE METABOLISM OF THE BLUE CRAB. (Under the direction of Edgar Heckel) Department of Biology, East Carolina University.

The content of fluoride in natural waters varies greatly in different areas depending on the source of the water and the geological formation of the region. Fluoride enrichment of waters may occur through several pathways--sewage treatment, steel production, fertilizers, pesticides, and the fluoridation of drinking water. The accumulation of fluorides by some marine or estuarine organisms has been discussed in previous studies. The effects of fluoride on the metabolism and the ion regulation of the blue crab are objectives of this study.

Crabs were collected and maintained in two identical aquarium systems. Each crab was weighed and measured then randomly placed in one of four different ion treatment levels of a salinity of 10‰. Level A had 0.3 ppm  $F^-$ , 118 ppm  $Ca^{++}$ , and 370 ppm  $Mg^{++}$ . Level B had 30 ppm  $F^-$ , 118 ppm  $Ca^{++}$ , and 370 ppm  $Mg^{++}$ . Level C had 30 ppm  $F^-$ , 414 ppm  $Ca^{++}$ , and 1295 ppm  $Mg^{++}$ . Level D had 0.3 ppm  $F^-$ , 118 ppm  $Ca^{++}$ , and 1296 ppm  $Mg^{++}$ . The crabs were then randomly sacrificed at 10 day intervals and six different portions removed for ion analysis.

Crabs maintained in fluoride concentration of 30 ppm were found to molt at a faster rate than those held in containers having 0.3 ppm  $F^-$ . Molting was shown to be retarded in levels containing high calcium concentrations, while high  $Mg^{++}$  containing levels had an increased molting rate.

A high fluoride content showed a reduced life expectancy for the crabs except in those levels having a higher magnesium content.

Hemolymph samples showed a converse relationship, the lower the fluoride, the greater the  $Mg^{++}$  increase and vice-versa. Tissue samples showed an increased  $Mg^{++}$  concentration in the higher magnesium containing levels. Fluoride content increased in tissue samples in those levels with the higher  $F^-$  and  $Mg^{++}$  concentrations. Exoskeleton samples showed an increase  $Mg^{++}$  content in those levels having the lowest fluoride concentration, whereas the fluoride content of these samples increased in those levels having the highest  $F^-$  and lowest  $Mg^{++}$  content.

## Acknowledgements

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## 1. Introduction

### 1.1 The Blue Crab, An Economic Resource

The blue crab, Callinectes sapidus, is a common inhabitant of the coastal waters of the eastern United States, and can tolerate a wide range of salinities and temperatures.<sup>1,2,3</sup> Because of the savory flavor of its meat and its relative abundance, commercial crabbing has become an important industry in many coastal states. Record catches of blue crab have been reported in recent years. In 1961, 40 million pounds were reported sold in the southeastern United States, and in 1975, North Carolina alone reported catches totaling 10.7 million pounds.<sup>4,5</sup>

As a result of the crabs economic importance, several studies have been conducted concerning its growth and metabolic control mechanism.<sup>6,7,8</sup> More recently, changes of environmental conditions in most of the regions inhabited by the crab have prompted a series of investigations with regard to the effects caused by toxic substances which are produced as by-products in manufacturing processes.<sup>9</sup> It is believed that these pollutants interfere with the control mechanisms which maintain the delicate balance of certain ionic concentrations in the crab's body.<sup>10</sup>

### 1.2 Mechanism of Ionic Regulation of the Blue Crab

The overall metabolic rate of the blue crab is directly related to the ability of the crab's gills and antennal glands to actively establish certain blood ionic levels. The loss of water and salts in excretions from the antennal glands is balanced by the uptake of water and ions through the gills. Excretions by the antennal glands are isosmotic with the blood, however they do contain relatively high concentrations of  $Mg^{++}$

and  $\text{SO}_4^{--}$  and lower concentrations of  $\text{K}^+$  and  $\text{Ca}^{++}$ .<sup>11</sup> The  $\text{Ca}^{++}/\text{Mg}^{++}$  ratio has been shown to affect the tonus and excitability of the crab.<sup>11, 12</sup> The hemolymph of less active crabs has been found to contain levels of  $\text{Mg}^{++}$  which are 81-99% of the equilibrium values, while the more active crabs had a  $\text{Mg}^{++}$  concentration that was less than half the equilibrium value of sea water.<sup>11</sup> Since the crab preferentially discharges  $\text{Mg}^{++}$  over  $\text{Ca}^{++}$ , the Ca:Mg ratio in the crab's body may vary with that of its environment. The  $\text{Ca}^{++}/\text{Mg}^{++}$  ratio is approximately 1 ppm : 3 ppm for sea water and the Pamlico River Estuary.<sup>13</sup>

### 1.3 Fluoride - An Increasing Pollutant of Natural Waters

Fluoride naturally occurs in the igneous and sedimentary rock of the earth's crust, and constitutes between 0.06% and 0.09% by weight of the upper layers of the lithosphere.<sup>14</sup> Fluorspar ( $\text{CaF}_2$ ), cryolite ( $\text{Na}_3\text{AlF}_6$ ), and fluorapatite ( $\text{Ca}_{10}\text{F}_1(\text{PO}_4)_6$ ) are the chief fluorine containing minerals. Cryolite has been mined from natural deposits in Greenland, and fluorapatite is mined chiefly in the United States for the manufacture of phosphate fertilizers.<sup>15</sup>

The content of fluoride in natural waters varies greatly in different areas depending on the source of the water and the geological formation of the region. In the northeastern United States, water supplies have a normal fluoride concentration ranging from 0.02 to 0.1 ppm, while in midwestern and southern states the range is often above 0.2 ppm.<sup>16</sup> In areas rich in fluoride containing minerals, concentrations of 1-7 ppm are not uncommon.<sup>17</sup>

Addition of fluoride to natural waters occurs in part through discharges from industrial sources. Manufacturing processes which

release fluorides in large quantities involve superphosphate fertilizers (9,700 tons/year), pesticides (3,000 tons/year), phosphoric acid (3,000 tons/year), aluminum (16,000 tons/year), steel (16,800 tons/year), brick and tile products (18,500 tons/year) and glass (2,700 tons/year).<sup>15</sup> Each of those processes is known to increase the overall fluoride content of the immediate surrounding area.

The fluoridation of water since 1945 has also contributed to the increase of the fluoride content of natural waters. Municipal water systems add appropriate amounts of sodium fluoride, sodium fluorosilicate, hydrogen fluoride, or fluorosilicic acid to drinking water to bring the total fluoride content to approximately 1 ppm.<sup>18</sup> This is the optimal concentration for reducing dental caries without causing mottling of tooth enamel.<sup>19</sup>

The fluoride concentration of unpolluted sea water is approximately 1.3 mg F<sup>-</sup>/kg.<sup>20,21</sup> The influx of fluorides through rivers and estuaries can cause a general increase of this content in localized areas. Moore<sup>22</sup> reported fluoride concentrations of 20 ppm in the Pamlico River Estuary, just offshore of a phosphate plant in Aurora, NC. Concentrations as high as 50 ppm have been observed under similar conditions in Tampa Bay, FL.<sup>23,24</sup>

#### 1.4 Uptake of Fluoride by Marine and Estuarine Organisms

The accumulation of fluorides by marine or estuarine organisms has been discussed in previous studies. Wright and Davison<sup>25</sup> reported considerable uptake of fluoride in the skeleton of both vertebrates and invertebrates. They demonstrated a dramatic increase of fluoride concentration in the exoskeletons of Portunus depurator and Carcinus maenas. After 12 weeks of exposure to 30 ppm F<sup>-</sup>, contents of fluoride in the exoskeletons were  $11.6 \pm 5.6$   $\mu\text{g/g}$  wet wt. and  $14.2 \pm 6$   $\mu\text{g/g}$  wet wt., respectively.

A lesser accumulation was noted in the gut and gills of these organisms.

Hemens and Warwick<sup>26</sup> have reported an increased mortality rate in the mud crab, Tylodiplax blephariskios, and the mullet, Mugil cephalus, when exposed to 52.0 ppm F<sup>-</sup> for 72 days. Moore<sup>27</sup> showed that oysters were unable to tolerate water fluoride concentrations above 32 ppm F<sup>-</sup> and that fluoride (as high as 100 ppm, dry wt. basis) was accumulated in the soft tissues of these animals. In 1971, Moore<sup>22</sup> performed a study on the uptake and concentration of fluoride by the blue crab. Exposure to 20 ppm fluoride was shown to affect the growth rate of the crab by a 4.7% reduction in size after one molt. Also at this concentration, an accumulation of 50 ppm dry weight fluoride was reached in crab muscles after a 90 day exposure period.

## 1.5 Analytical Methods

### 1.51 Fluoride Ion Analysis with the Fluoride Ion Sensitive Electrode

The physical and chemical diversity of animal tissues has resulted in the development of many different methods of fluoride analysis. However, use of the fluoride specific-ion electrode has been found to yield the most consistent results in a minimal amount of time. The fluoride ion electrode employs a lanthanum fluoride crystal which exhibits a preferential selectivity for fluoride ions.<sup>28</sup> The lower limit of detection is approximately 10<sup>-7</sup> M (0.002 ppm) fluoride ion.<sup>29</sup>

When the electrode is placed in a sample containing fluoride, the electrode potential observed is due to the fluoride ion activity of the sample solution. The potential is related to free fluoride concentration by:

$$E = E_a - S \log \gamma C_f$$

where  $E$  is the measured potential of the system,  $E_a$  is the contribution to the total potential resulting from internal and external reference solutions,  $S$  is the Nernst factor ( $2.3 RT/F$  where  $R$  is the ideal gas constant,  $T$  the absolute temperature and  $F$  the Faraday constant),  $\gamma$  is the free ion activity coefficient, and  $C_f$  is the free fluoride ion concentration.<sup>30</sup>

Because of the many parameters involved, the potentiometric analysis requires control over pH, ionic strength, and the presence of reagents which prevent complexation of fluoride or release  $F^-$  from metal ion complexes. In basic solutions, the electrode responds to hydroxide ions, as well as, to fluoride ions. Conversely, in acidic solutions hydrogen fluoride is formed which is not sensed by the electrode. Complexes with polyvalent cations such as  $Si^{+4}$ ,  $Al^{+3}$ , and  $Fe^{+3}$  may also be formed in strongly acidic solutions. The pH of the sample should be adjusted to between pH 5-6.<sup>29</sup> Use of Orion's TISAB II<sup>31</sup> (Orion Research Inc., Cambridge, Mass.), an ionic strength adjustor, containing CDTA (trans-1,2-diaminocyclohexane-N,N,N',N-tetraacetic acid monohydrate) preferentially complexes polyvalent cations ( $Si^{+4}$ ,  $Al^{+3}$ , or  $Fe^{+3}$ ), thereby releasing fluoride. In addition, it adjusts the ionic strength and the pH.

The fluoride ion sensitive electrode has been successfully employed on several occasions to determine fluoride in aqueous solutions and organic materials. Crosby, Dennis and Stevens<sup>32</sup> compared five spectrophotometric procedures for the determination of fluoride with the use of the Orion fluoride-ion electrode for fluoride determination. The electrode was shown to be less susceptible to interference from other ions in solution than the other methods. It also gave theoretical recoveries of fluoride

added to several drinking water supplies. Warner<sup>33</sup> showed the fluoride electrode to demonstrate accuracy and precision for measurements down to 0.02 mg F/kg in various sea water samples.

Ke et al.<sup>34</sup> employed the fluoride ion electrode to measure fluoride contents of fish protein concentrate and raw material used in preparation of the concentrate, prepared by a nonfusion distillation technique. The overall error involved with the use of the electrode was demonstrated to be less than 0.5%. Wright and Davison<sup>25</sup> determined the fluoride content of the exoskeleton, muscle, and gill portions of several estuarine organisms by use of the fluoride ion electrode. Employing a simple acid extraction procedure in conjunction with KOH fusion of the samples, they were able to accurately detect fluoride concentrations above 100 ppm.

The fluoride ion electrode was also employed by Hemens and Warwick<sup>26</sup> to determine the fluoride content of various estuarine organisms. Concentrations ranging from 0.0 to 7743.0  $\mu\text{g F/g}$  ash were detected. Spencer et al.<sup>35</sup> employed the fluoride ion electrode in their study of the effects of fluoride on the magnesium metabolism of man. Determinations were made of the amount of fluoride increase of urinary and fecal excretion from NaF fed male patients.

#### 1.52 Magnesium and Calcium Ion Analysis

Biological and water samples can be analyzed for their metal content by use of atomic absorption spectroscopy. Atomic absorption is metal specific and quantitative measurements can be carried out when the compounds containing a metal can be dissolved in a solvent. The atomic absorption measurements account for total metal content of a sample including the ions held in complexes such as  $\text{CaF}^+$  or  $\text{MgF}^+$ .

### 1.6 Objective of Study

The objective of this study is to determine the effects of fluoride on the metabolism of the blue crab, Callinectes sapidus, especially with regard to growth, mortality and ionic regulations. In addition, it should be determined whether the blue crab will exhibit selective uptake for fluoride and magnesium ions.

## 2. Materials and Methods

### 2.1 Collection, Transport, and Initial Maintenance of Crabs

The crabs were obtained from the Cape Fear and Pamlico Rivers. The size of the crabs ranged from an average of 6.0 cm to 10.0 cm; all were immature.

In order to reduce mortality, the crabs were transported from the field to the laboratory in three 25-liter tubs containing estuarine water. Several pumps (Whisper 300, Willinger Bros., Ft. Lee, NJ) supplied air to the tubs during the transport. They were powered by a 12-volt lead acid battery in conjunction with a D-C to A-C Power Inverter (Tripp Lite Model 350, Chicago, IL). Once the crabs reached the laboratory, they were placed in individual plastic freezer containers which had been perforated to permit maximum air and water circulation. These crabs were allowed to acclimate to their new surroundings in estuarine water, at a salinity of 10‰, for a period of fourteen days before being exposed to different ion treatment levels.

### 2.2 Measurements of Sample Crabs

Before being exposed to various solutions of different ionic levels, each crab was weighed and measured in width. The sex of the crabs was determined by direct observation of the abdomen; the males have a T-shaped abdomen with broad basal segments and narrow distal segments, while the immature female has a triangular or more rounded abdomen. Each crab was weighed to the nearest tenth of a gram after being towel dried, using a triple beam "Dial-O-Gram" balance (Ohaus, Florham, NJ). The width of each crab was measured from the tips of the lateral spines using vernier calipers. The crabs were weighed and measured again after each molting during the treatment period.

### 2.3 Experimental Treatment of Crabs

Two identical aquarium systems, upright freezers modified for individual control of temperature, light, and air flow, were maintained in the laboratory. Each system held four 25-liter tubs. These tubs could accommodate a maximum of ten crabs housed in individual modified plastic containers. Charcoal filters, utilizing air from vibration air pumps and air stones were placed in each tub.

The temperature in each system was kept at a constant 21°C. Lighting was on a 12-hour on, 12-hour off cycle in conjunction with a thermo-safety switch which would act to decrease the length of the light period in case of overheating. A back-up system was connected to each tub via a three-way solenoid valve, which permitted an ample flow of oxygen from a tank containing compressed oxygen in case of power failure.

The crabs were fed frozen fish every third day, and any unconsumed portions were removed to prevent contamination of the levels.

After the initial period of acclimation, the crabs were randomly assigned to one of four experimental ion treatment levels. All levels were maintained at a salinity of 10‰. Each was prepared using artificial sea water made from a sea salt water mix (Aquarium Systems, Inc., Ohio) and CaCl<sub>2</sub>, MgCl<sub>2</sub>, or NaF solutions. Level A served as the control and had no extra fluoride, magnesium, or calcium ions added. All solutions were changed every ten days to maintain a constant ionic concentration.

The following list provides complete information on the chemical composition of each of the levels.

Level A contained 200 grams of sea salt and trace elements in 20 liters of distilled water.

Level B contained 200 grams of sea salt and 42 ml of 0.75 M NaF in 20 liters of distilled water.

Level C contained 92 grams of sea salt, 42 ml of 0.5 M NaF, 90 ml of 2 M CaCl<sub>2</sub>, and 406 ml of 2.28 M MgCl<sub>2</sub> in 20 liters of distilled water. Both the Ca<sup>++</sup> and Mg<sup>++</sup> content were identical to that of sea water of 35‰ salinity.

Level D contained 111.7 grams of sea salt, 0.18 ml of 0.75 M NaF, 13 ml of 2 M CaCl<sub>2</sub>, and 393.5 ml of 2.28 M MgCl<sub>2</sub> in 20 liters of distilled water. The Mg<sup>++</sup> content corresponded to that of sea water of 35‰ salinity.

The ionic concentrations of each level are shown in Table 2.1.

Table 2.1. Composition of Treatment Levels at 10‰ Salinity

Level	Fluoride ppm	Calcium ppm	Magnesium ppm
A	0.3	118	370
B	30	118	370
C	30	414	1295
D	0.3	118	1296

Calculation of the ionic composition of each level is shown for Level D by considering the following:

Table 2.2. Sea Water Ion Content

Ion	Salinity		$\Delta(\text{cation})$	$\Delta(\text{Cl}^-)$
	35‰	10‰		
	g/kg			
Mg <sup>++</sup>	1.295	0.368		
Ca <sup>++</sup>	0.118	0.118		
Cl <sup>-</sup>	19.353	5.50		

The ionic composition is attained by using solid  $\text{CaCl}_2$ ,  $\text{MgCl}_2$  and sea water mix. Since relatively large amounts of  $\text{CaCl}_2$  and  $\text{MgCl}_2$  are required, a correspondingly smaller quantity of sea salt water mix must be added to the water in order to maintain a  $10^\circ/\text{oo}$  salinity. Considering that the concentration of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  should be 0.118 and 1.295 g/kg, respectively,  $\Delta(\text{cation})$  and  $\Delta(\text{Cl}^-)$  relate to the quantities of these ions which must be added to a  $10^\circ/\text{oo}$  salinity solution made from sea salt mix, which are:

$$\Delta(\text{Mg}^{++}) = 1.295 - 0.368 * M/10$$

$$\Delta(\text{Ca}^{++}) = 0.118 - 0.118 * M/10$$

$$\begin{aligned} \Delta(\text{Cl}^-) &= (1.295 - 0.368 * M/10) \frac{2 * 35.45}{24.31} + (0.118 - 0.118 * M/10) \\ &\quad \frac{2 * 35.4}{40.08} \\ &= 3.986 - 0.128 * M \end{aligned}$$

M is the amount in g/l of salt mix to be added to distilled water in order to prepare a solution of  $10^\circ/\text{oo}$  salinity. The numerical values of 34.45, 40.08, and 24.31 in the equation  $\Delta(\text{Cl}^-)$  refers to the atomic masses of  $\text{Cl}^-$ ,  $\text{Ca}^{++}$ , and  $\text{Mg}^{++}$ , respectively. In a solution prepared from  $\text{CaCl}_2$  and  $\text{MgCl}_2$ , the factors  $M/10$  accounts for the additional  $\text{CaCl}_2$  and  $\text{MgCl}_2$  needed to compensate for  $\text{CaCl}_2$  and  $\text{MgCl}_2$  losses through reduction of salt mix in achieving an overall salinity of  $10^\circ/\text{oo}$ . Consequently, we obtain the following correlation.

$$\begin{aligned} M(\text{g/kg solution}) &= 10 - \Delta(\text{Ca}^{++}) - \Delta(\text{Mg}^{++}) - \Delta(\text{Cl}^-) \\ &= 10 - 1.295 + 0.0368 * M - 0.118 + 0.118 * M \\ &\quad - 3.986 - 0.1287 * M \end{aligned}$$

$$0.824 M = 4.601$$

$$M = 5.584^\circ/\text{oo}$$

$$\begin{aligned}\Delta(\text{Mg}^{++}) &= 1.295 - 0.368 * 5.584 \\ &= 1.090 \text{ g/kg } (\hat{=} 3.18 \text{ g/kg Cl}^{-}) \\ \Delta(\text{Ca}^{++}) &= 0.118 - 0.0118 * 5.584 \\ &= 0.052 \text{ g/kg } (\hat{=} 0.92 \text{ g/kg Cl}^{-}) \\ \Delta(\text{Cl}^{-}) &= 3.986 - 0.128 * 5.584 \\ &= 3.27 \text{ g/kg}\end{aligned}$$

The total ion content of Level D is shown in Table 2.3.

Table 2.3 Composition of Artificial Salt Water for Level D

Ion	Salinity		Cl, F, Ca, Mg Added	PPM Total
	35‰	5.58‰		
g/kg salt water				
Cl	19.353	3.085	3.27	6355
Na	10.765	1.716	0.13	1845
SO <sub>4</sub>	2.711	0.432		432
Mg	1.295	0.206	1.09	1296
Ca	0.414	0.066	0.052	118
K	0.387	0.062		62
HCO <sub>3</sub>	0.142	0.023		23
Br	0.066	0.011		11
Sr	0.008	1.28 x 10 <sup>-3</sup>		1.3
H <sub>3</sub> BO <sub>3</sub>	0.026	4.15 x 10 <sup>-3</sup>		4.2
F	0.001	1.59 x 10 <sup>-4</sup>	0.13	0.3

For a 20-liter solution, the following quantities of chemicals are required:

Sea Salt Mix	111.7 g
2 M CaCl <sub>2</sub>	13.0 ml
2.28 M MgCl <sub>2</sub>	393.5 ml
0.75 M NaF	0.18 ml

#### 2.4 Preparation of Samples for Analysis

Every ten days, intermolt stage crabs were randomly removed from each of the ionic treatment levels. At this time the hemolymph, ranging from 1 ml to 5 ml depending on the size of the crab, was extracted using a 5 cc syringe inserted at the joint of the fifth peripod. The entire crab was then frozen until dissection for sample analysis could be performed.

Equal parts of hemolymph, 35% perchloric acid ( $\text{HClO}_4$ ), and deionized water (prepared by distillation of an alkaline potassium permanganate solution) were refluxed in a 25 ml round bottomed flask until all material had dissolved. The column was rinsed with deionized water to ensure that no material was lost. The digested crab sample was then stored in an 18 ml polyethylene sample vial at  $5^\circ\text{C}$ . Analysis for fluoride, magnesium, and calcium concentration was performed subsequently.

Six separate samples were taken from each crab. These included the large proximal muscles of the fifth peripod, entire tissues of the merus region of the first peripod and of the cheliped, the pleurobranch gills of the second and third peripods, and the dorsal exoskeleton of the merus region of the first peripod and of the cheliped.

Tissue samples were rinsed in deionized water on Whatman Number One filter paper (W & R Balston Ltd., England) and drained under suction for two minutes. This effectively removed any hemolymph present on the tissues. Exoskeleton samples were scrubbed with a stiff bristle brush to remove any remaining tissue, rinsed in deionized water and then towel dried.

All samples, tissue and exoskeleton, were weighed to the nearest 0.001 gram using a Mettler H20T balance, oven dried at  $50^\circ\text{C}$  for 24 hours and then weighed again. Each sample was then refluxed in one ml of

35% HClO<sub>4</sub> until all material had dissolved. The column was rinsed and the digested crab sample diluted to a final volume of 10 ml and stored in an 18 ml polyethylene sample vial at 5°C until ion analysis could be performed.

## 2.5 Fluoride Analysis

Fluoride analysis was accomplished with the Orion Model 96-09 combination fluoride electrode. Electrode potentials were measured using the Orion Model 801 pH/mV meter and recorded using the Orion Model 751 digital printer. Because the electrode sensitivity depends on the ionic strength of a solution, Orion developed a buffer specially designed to alleviate this problem. TISAB II (Total Ionic Strength Adjustment Buffer) acts not only to adjust the pH but decomplexes fluoride from trivalent metal ions. The buffer was additionally saturated with potassium perchlorate (KClO<sub>4</sub>) to reproduce conditions which prevail during sample analysis by the presence of KOH neutralized perchloric acid, which was used in the digestion of the samples.

Seven fluoride standard stock solutions were prepared using Orion 0.1 M NaF standard and deionized water. Each standard was saturated with KClO<sub>4</sub>. The stock solutions ranged from  $8 \times 10^{-7}$  to  $8 \times 10^{-4}$  M NaF. They were stored in polyethylene bottles to prevent absorption of F<sup>-</sup> on the walls as observed during storage in glass bottles. Standards for fluoride analysis were prepared by combining 3 ml of TISAB II/KClO<sub>4</sub> with 3 ml of each of the fluoride standard stock solutions. Fifty microliters of 35% HClO<sub>4</sub> was added to each of the standards to adjust for the volume increase which does occur during pH adjustment of the samples. Subsequently, the pH was adjusted to 5.5 using a 5 M KOH solution. As a result, each set of standards ranged from  $4 \times 10^{-7}$  to  $4 \times 10^{-4}$  moles/liter.

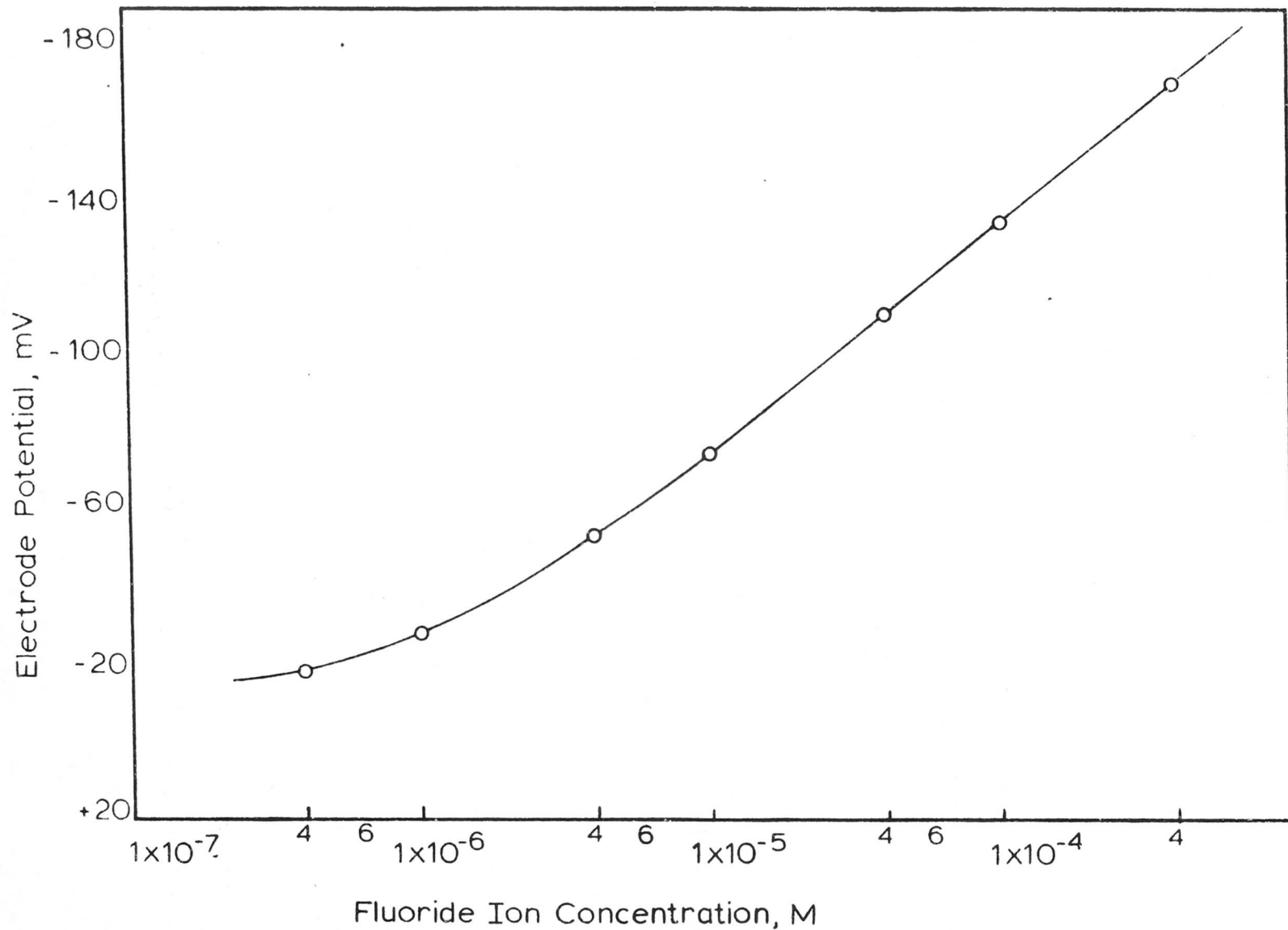


Figure 2.1 Typical Calibration Curve for the Fluoride Ion Sensitive Electrode

Crab samples were prepared for fluoride analysis by combining 3 ml TISAB II/ $\text{KClO}_4$ , 2 ml deionized water, and 1 ml of digested sample. The pH was then adjusted to 5.5 using 5 M KOH. All samples and standards were prepared in 30 ml polyethylene beakers. Samples and standards were measured for approximately ten minutes to allow the electrode to become stabilized. The electrode potentials were collected in one minute intervals with the digital printer. During this period, each solution was stirred using a teflon coated magnetic spin bar in conjunction with a magnetic stirrer. A cork pad was placed between the sample beaker and the stirrer to prevent transfer of heat. Between samples the electrode was rinsed with 0.1 M  $\text{La}(\text{NO}_3)_3$  and deionized water to prevent build-up of fluoride by the  $\text{LaF}_3$  crystal of the electrode.

The electrode potential readings (mV) were evaluated by standard computer techniques on an SWTPC 6800 microcomputer. An infinite time extrapolation subprogram (subprogram EXTRAPOL of Appendix A) provided the EMF values for the true equilibrium potentials. The EMF measurements for the standards were then used to plot a calibration curve (as a visual check procedure), i.e., EMF (mV) versus fluoride ion concentration (moles/liter) on semilogarithmic paper. An EMF difference of approximately 59 mV was observed per decade of concentration, except in the lower concentration range ( $10^{-6}$ ). Calibration curves were made with each set of samples analyzed due to shifts in the electrode potential (Sample Calibration Curve, Figure 2.2). Also included in the computer program was a method of determining the best fit curve for the standards. This was accomplished by use of a modified third order polynomial algorithm. The EMF (mV) readings for the crab samples were thereby fitted to the fluoride standards and the fluoride content could directly be obtained for each sample (subprogram FLUORAN/3).

## 2.6 Magnesium and Calcium Analysis

Analysis of magnesium and calcium concentrations was accomplished using a Farrell-Ash atomic absorption flame emission spectrometer with a hollow cathode lamp for  $Mg^{++}$  and  $Ca^{++}$ . A calibration curve was first established for each set of samples run using a set of dual standards, ranging from 0.5 ppm  $Mg^{++}/Ca^{++}$  to 100 ppm  $Mg^{++}/Ca^{++}$ . Standards were made using solutions of  $MgCl_2$  and  $CaCl_2$  with deionized water. Each standard was additionally treated with 25 microliters of 35%  $HClO_4$ , per 10 ml of standard, to simulate conditions existing in the digested crab samples. The percent absorption data of each of the standards was evaluated by the CA-MG-AA subprogram (Appendix A) of the comprehensive computer program, and a calibration curve was also plotted.

Crab samples were prepared in the following manner: hemolymph samples were diluted 1:1, tissue samples 1:5, and exoskeleton samples 1:100 with deionized water. Each dilution factor was chosen to correspond with the standards. The percent absorption of each sample was then fitted to the calibration curve, according to subprogram 3 of the computer program, and the concentration and percent ion content of magnesium or calcium were directly determined. The wavelengths at which each ion, either  $Mg^{++}$  or  $Ca^{++}$ , was measured are 2852.1 and 4226.7, respectively (Sample Calibration Curve, Figure 2.2).

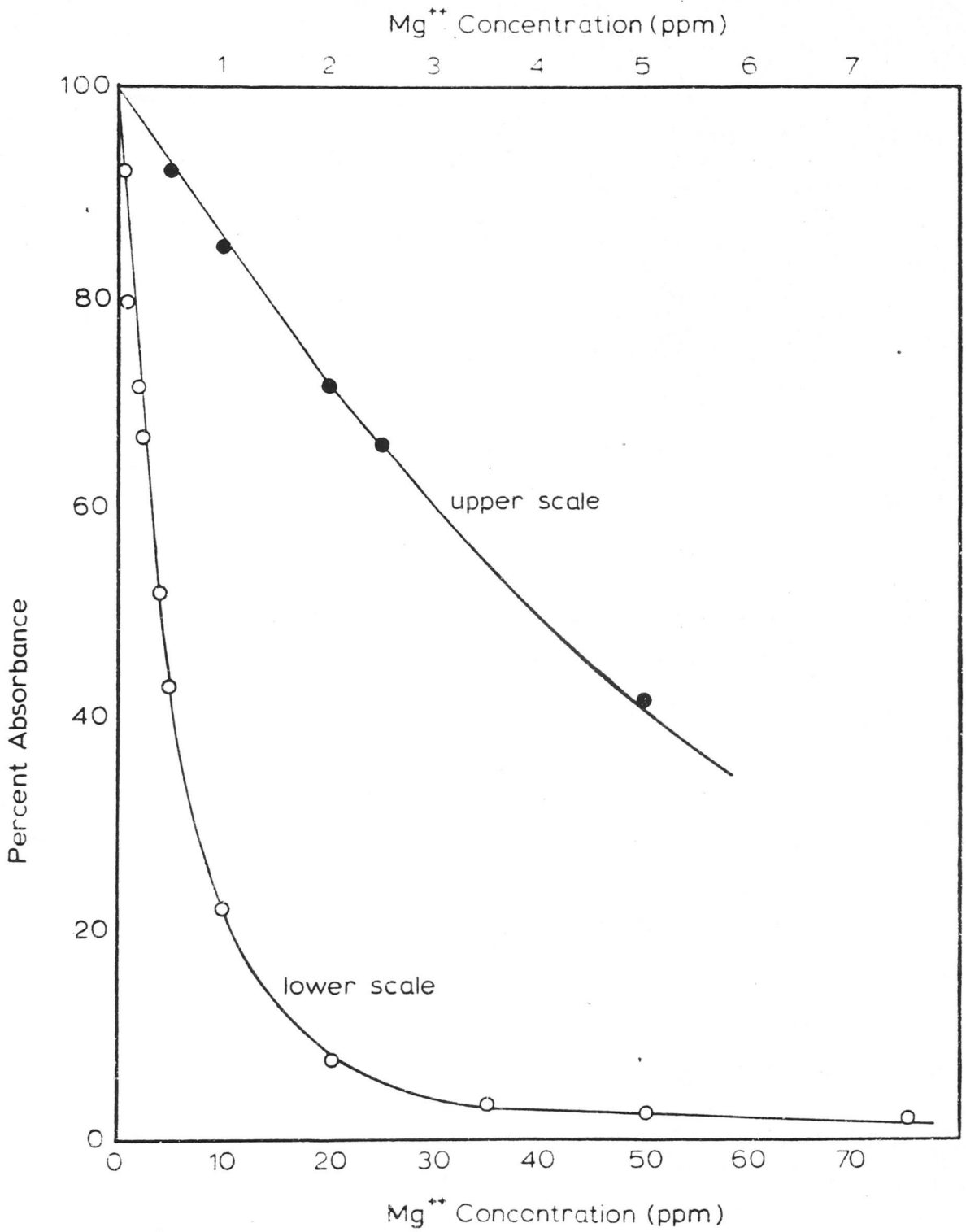


Figure 2.2 Typical Calibration Curve of Magnesium, using Atomic Absorption Spectroscopy

### 3. Results

#### 3.1 Growth Rates of Blue Crabs in the Ion Concentration Levels

A summary of the weight and size measurements of crabs molting during the treatment period is shown in detail in Table 3.1. Coding for the samples are as follows:  $C_1 - 6_f$ , where C refers to the individual treatment (subscripts are used to identify duplicate levels), 6 to the assigned number of the crab, and f (female) to the sex of the crab. All crabs were maintained in the levels as described in Section 2.3. Measurements of size and weight are reported only for those crabs which molted during the course of the experiment (see Section 2.2). Figure 3.1 shows the relationship between the length of exposure to the various ion solutions and the percentage of crabs molting in each level. The crabs had been randomly placed in each solution and the ecdysis stage at introduction was unknown. This data was obtained from an earlier portion of this study conducted in 1978.

#### 3.2 Mortality Statistics of Blue Crabs

Table 3.2 contains the survival length of any crabs which died, before sampling occurred, in Levels A to D, i.e., crabs investigated during 1979. A more complete statistical analysis of mortality rate was obtained from the study carried out in 1978, which is shown in Figure 3.2. All crabs in the latter study were maintained until death occurred due to natural causes. It should be noted that all levels were identical to the ionic compositions of the 1979 study with the exception of Level E. The composition of this level was as follows: 30 ppm  $F^-$ , 414 ppm  $Ca^{++}$ , and 337 ppm  $Mg^{++}$  ions.

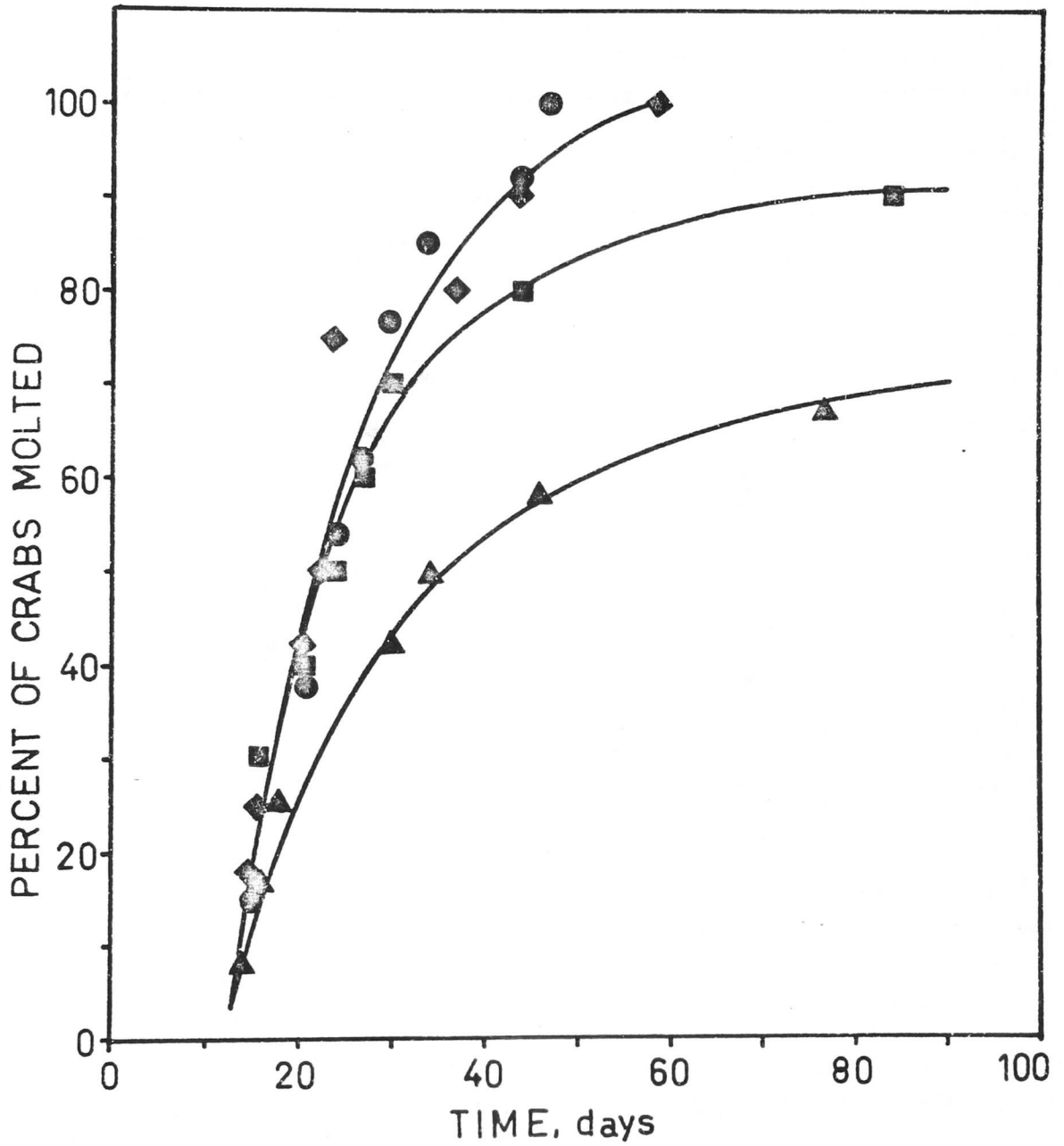


Figure 3.1 Percentage of Crabs Molting after Introduction to the Various Ion Levels. Data obtained from the 1978 study. (Level A ■ , Level B ● , Level C ◆ , Level E ▲ )

### 3.3 Fluoride, Magnesium, and Calcium Content of Samples

The data shown in Tables 3.3 to 3.9 summarizes the mean concentrations of fluoride, magnesium and calcium of each sample portion. The fluoride, magnesium, and calcium concentrations of tissue and exoskeleton samples are given in percent ion content per sample on a dry weight basis for each sample period. Hemolymph content is given on a wet weight basis for each sample period. A graphical presentation of the fluoride and magnesium concentrations of each portion is also shown in Figures 3.3 to 3.16. No graphical representation is given for the calcium content as this was not an objective of this investigation.

Straight lines, obtained by a Linear Least Squares Fit (Appendix B), were drawn through the data points. Those were found to present the most reasonable treatment of the data. Any other treatment of the data would not yield significant improvement of the interpretations. Unreasonable data were eliminated to obtain the best fit curve for the remaining sample points, on the basis that all samples would originate from a common intercept.

### 3.4 Fluoride, Magnesium and Calcium Content of Commercially Packaged Frozen Fish

Frozen fish were purchased at grocery stores and stored in a freezer until used for feeding the crabs. Small portions were sliced from the supply and thawed before feeding. An analysis of a typical fish sample showed the ion contents as follows:

	Fluoride	Magnesium ppm	Calcium
Wet Weight	2.5	152	45
Dry Weight	11.6	712	210

Table 3.1. Summary of Weight and Width Measurements of Crabs, Which Molted during the Treatment Process

Sample Code	Weight(g)			Size(mm)			Days Exposed Prior to Molting
	Initial	Final	%Increase	Initial	Final	%Increase	
A-1f	13.62	17.89	31.35	60.0	72.0	20.0	9
A-5m	23.12	33.25	43.82	66.0	86.0	30.30	9
B-1f	56.59	81.66	44.30	100.0	121.0	21.00	11
B-221	9.32	18.05	93.67	50.4	65.0	28.9	20
C <sub>1</sub> -6f	12.16	16.20	33.22	57.0	66.0	15.79	11
C <sub>1</sub> -7f	17.27	27.55	59.53	60.0	77.0	28.33	3
C <sub>2</sub> -2f	59.55	90.85	52.56	97.0	121.0	24.74	3
C <sub>2</sub> -3f	25.49	27.02	6.01	78.0	83.0	6.41	3
C <sub>2</sub> -5f	45.26	68.21	50.71	100.0	108.0	8.0	17
D <sub>1</sub> -1m	19.06	22.79	19.57	65.0	73.0	12.31	47
D <sub>1</sub> -3f	6.07	8.46	39.37	50.0	55.0	10.0	7
D <sub>1</sub> -5f	30.43	38.55	26.68	83.0	87.0	4.82	25
D <sub>2</sub> -3f	21.46	21.60	0.65	71.0	87.0	22.54	14
D-10f	16.93	27.55	62.73	69.0	82.0	18.84	5
D-12m	110.4	112.55	1.95	122.0	124.0	1.64	5
D-13m	19.29	29.54	53.14	68.0	81.0	19.12	42

Average % Increase:

Level

Weight

Size

A

38±6(51±6)\*

25±5(18±4)

B

70±25(52±5)

25±4(16±2)

C

40±19(53±4)

17±9(20±2)

D

29±22

13±7

\*Reference 37

Table 3.2. Summary of Survival Length of Crab in Individual Levels, Not Sampled at Regular Intervals

Sample Code	Days in Solution Prior to Death	Average Survival Length per Level
A Level	No Deaths	60
B-2m	6	10.5
B-6f	15	
C <sub>1</sub> -4f	52	35.6
C <sub>1</sub> -5f	12	
C <sub>2</sub> -1f	39	
C <sub>2</sub> -4f	62	
C <sub>2</sub> -7m	13	
D <sub>1</sub> -4m	13	30.3
D <sub>1</sub> -7m	62	
D <sub>2</sub> -2f	15	
D <sub>2</sub> -4f	33	
D <sub>2</sub> -7f	31	
D-16m	28	

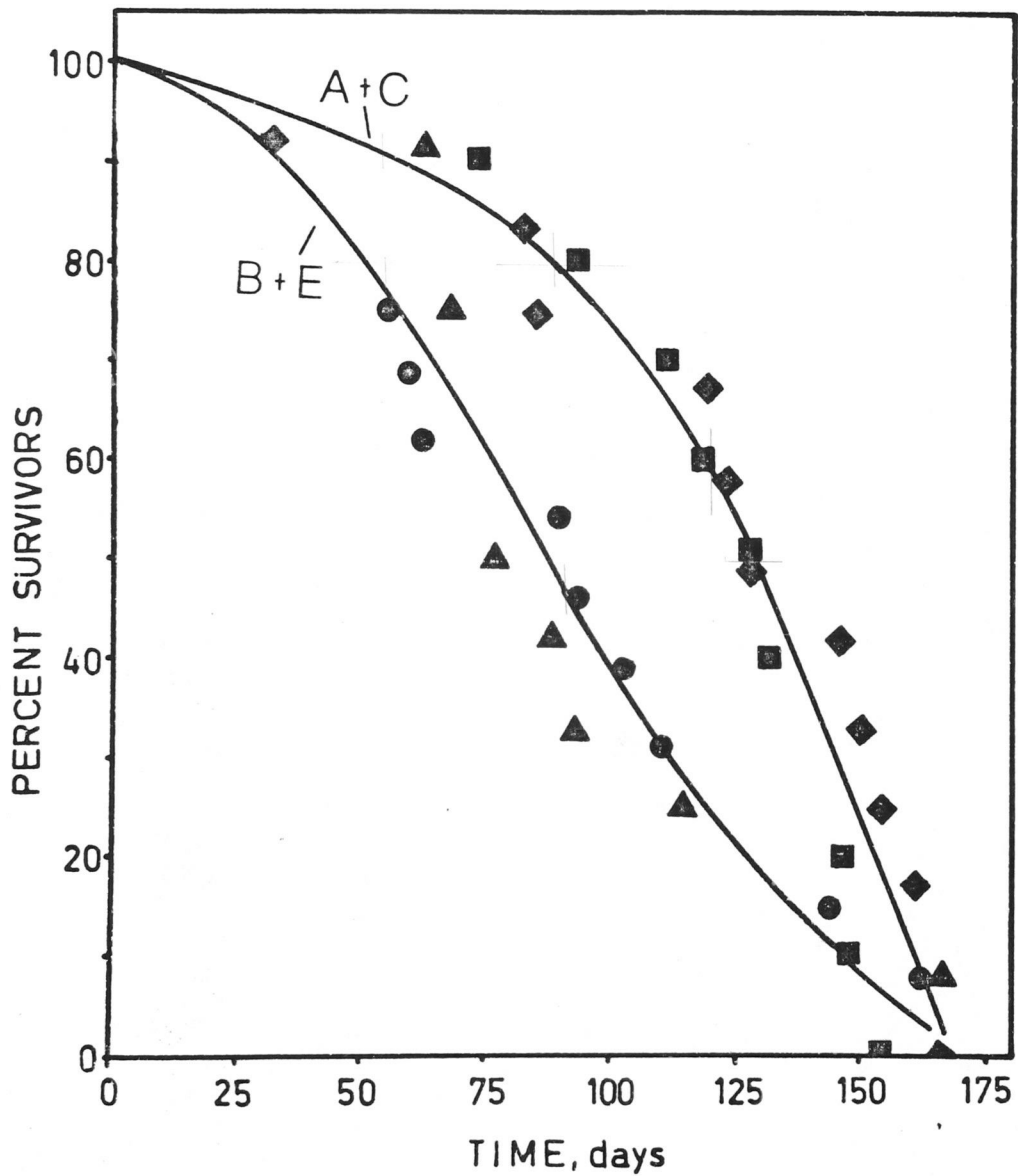


Figure 3.2 Percentage of Crabs Surviving after Exposure to the Various Ion Solutions. Data obtained from the 1978 study. (Level A  $\blacksquare$ , Level B  $\bullet$ , Level C  $\blacklozenge$ , Level E  $\blacktriangle$ )

Table 3.3. Summary of Percent Ion Contents for Hemolymph Samples on a Wet Weight Basis (Sampled One Crab in Levels A and B, Two Crabs in Level C and Three Crabs in Level D)

Treatment Period Days	Average Weights		% Ion Content (Wet Weight)		
	Wet (g)		Fluoride	Magnesium	Calcium
Level A					
10	1.0		$1.930 \times 10^{-5}$	$2.775 \times 10^{-3}$	$1.014 \times 10^{-3}$ *
20	1.0		$1.760 \times 10^{-5}$	$3.416 \times 10^{-3}$	0.0111
30	1.0		$3.167 \times 10^{-5}$	$1.058 \times 10^{-3}$	$7.718 \times 10^{-4}$
40	1.0		$1.543 \times 10^{-5}$	$1.995 \times 10^{-3}$	$1.372 \times 10^{-3}$
50	1.0		$1.116 \times 10^{-5}$	$1.452 \times 10^{-3}$	$7.950 \times 10^{-4}$
60	1.0		$3.978 \times 10^{-5}$	$3.735 \times 10^{-4}$	$1.434 \times 10^{-4}$
Level B					
10	1.0		$4.567 \times 10^{-5}$	$7.648 \times 10^{-3}$	$7.123 \times 10^{-3}$
20	1.0		$7.200 \times 10^{-5}$	$1.508 \times 10^{-3}$	$3.769 \times 10^{-3}$
30	1.0		--	$4.009 \times 10^{-3}$	$4.091 \times 10^{-3}$
40	1.0		$7.240 \times 10^{-5}$	$2.212 \times 10^{-4}$	$1.335 \times 10^{-3}$
50	1.0		$6.707 \times 10^{-5}$	$3.381 \times 10^{-3}$	$1.761 \times 10^{-3}$
60	1.0		$1.071 \times 10^{-4}$	$1.441 \times 10^{-3}$	$1.276 \times 10^{-3}$

\*Value inconsistent with overall standard deviation

Table 3.3 (Continued)

Treatment Period Days	Average Weights		% Ion Content (Wet Weight)		
	Wet(g)	Fluoride	Magnesium	Calcium	
		Level C			
10	1.0	--	--	--	
20	1.0	$6.101 \times 10^{-5}$	$1.711 \times 10^{-3}$	$6.225 \times 10^{-3}$	
30	1.0	$6.281 \times 10^{-5}$	$4.901 \times 10^{-3}$	0.0130	
40	1.0	$2.089 \times 10^{-5}$	$1.456 \times 10^{-3}$	$9.848 \times 10^{-4}$	
50	1.0	$3.515 \times 10^{-5}$	$3.021 \times 10^{-3}$	$3.171 \times 10^{-3}$	
60	1.0	$1.864 \times 10^{-5}$	$1.092 \times 10^{-3}$	$7.416 \times 10^{-3}$	
		Level D			
10	1.0	$2.489 \times 10^{-5}$	$3.172 \times 10^{-3}$	$3.835 \times 10^{-3}$	
20	1.0	$3.987 \times 10^{-5}$	$3.503 \times 10^{-3}$	$5.264 \times 10^{-3}$	
30	1.0	$3.578 \times 10^{-5}$	$3.746 \times 10^{-3}$	$6.499 \times 10^{-3}$	
40	1.0	$4.330 \times 10^{-5}$	$4.922 \times 10^{-3}$	$4.081 \times 10^{-3}$	
50	1.0	$2.318 \times 10^{-5}$	$3.986 \times 10^{-3}$	$1.983 \times 10^{-3}$	
60	1.0	$5.239 \times 10^{-6}$	$3.665 \times 10^{-3}$	$2.465 \times 10^{-3}$	

Table 3.4. Summary of Percent Ion Contents for the Proximal Muscles of the Fifth Peripod on a Dry Weight Basis (Sampled One Crab in Levels A and B, Two Crabs in Level C and Three Crabs in Level D)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level A				
10	--	--	--	--	--
20	0.2140	0.0430	$1.833 \times 10^{-3}$	0.1945	0.1444
30	0.1510	0.0252	$2.249 \times 10^{-3}$	0.1773	0.3056
40	0.1469	0.0293	$3.369 \times 10^{-3}$	0.3105	0.9500
50	0.3925	0.0360	$5.265 \times 10^{-3}$	0.2523	0.3488
60	0.2621	0.0369	$6.941 \times 10^{-4}$	0.1476	0.1753
	Level B				
10	0.9737	0.2073	$3.882 \times 10^{-4}$	0.2837	1.0749
20	0.2155	0.0519	$2.123 \times 10^{-3}$	0.1338	0.1309
30	0.3102	0.0506	$6.082 \times 10^{-3}$	0.0772	0.0562
40	0.1828	0.0277	$6.644 \times 10^{-3}$	0.0658	0.1064
50	0.5912	0.0576	$1.550 \times 10^{-3}$	0.1886	0.3902
60	0.1294	0.0222	$8.082 \times 10^{-3}$	0.2495	0.2723

Table 3.4 (Continued)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level C				
10	0.1622	0.0319	$2.377 \times 10^{-3}$	0.1761	0.0995
20	0.2802	0.0554	$8.386 \times 10^{-4}$	0.0800	0.0721
30	0.3372	0.0721	$1.330 \times 10^{-3}$	0.0832	0.0533
40	0.1420	0.0207	$4.162 \times 10^{-3}$	0.1862	0.1886
50	0.3450	0.0475	0.147	0.2082	0.3894
60	0.4422	0.0473	$2.916 \times 10^{-3}$	0.2321	0.1897
	Level D				
10	0.2608	0.0401	$2.786 \times 10^{-3}$	0.1601	0.3113
20	0.2134	0.0343	$1.184 \times 10^{-3}$	0.1453	0.1701
30	0.2319	0.0405	$4.006 \times 10^{-3}$	0.1503	0.0948
40	0.3617	0.0642	$2.901 \times 10^{-3}$	0.1800	0.1091
50	0.1952	0.0258	$4.331 \times 10^{-3}$	0.2963	0.2103
60	0.2187	0.0412	$2.221 \times 10^{-3}$	0.3062	0.2612

Table 3.5. Summary of the Percent Ion Contents for the Merus Tissues of the First Peripod on a Dry Weight Basis (Sampled One Crab in Levels A and B, Two Crabs in Level C, and Three Crabs in Level D)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level A				
10	--	--	--	--	--
20	0.1072	0.2064	$6.021 \times 10^{-3}$	0.2654	0.0564
30	0.0623	0.0115	$1.456 \times 10^{-3}$	0.1786	0.5182
40	0.0823	0.0148	$6.444 \times 10^{-3}$	0.1454	0.6565
50	0.1224	0.0193	$7.936 \times 10^{-3}$	0.2801	0.6973
60	0.0569	0.128	$1.830 \times 10^{-3}$	0.1469	0.4162
	Level B				
10	0.2390	0.0446	$7.978 \times 10^{-3}$	0.2953	0.7670
20	--	--	--	--	--
30	0.1575	0.0263	$5.203 \times 10^{-3}$	0.1925	0.3767
40	0.1091	0.0225	0.0877*	0.2939	0.4525
50	0.2272	0.0281	$5.971 \times 10^{-3}$	0.2909	0.6736
60	0.1065	0.0223	$5.365 \times 10^{-3}$	0.2115	1.0454

\*Value inconsistent with overall standard deviation

Table 3.5 (Continued)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level C				
10	0.0911	0.0218	0.0131	0.3437	0.3268
20	0.1905	0.0485	$2.267 \times 10^{-3}$	0.1198	0.0977
30	0.1993	0.0412	$7.880 \times 10^{-3}$	0.1508	0.1244
40	0.080	0.0181	$5.488 \times 10^{-3}$	0.2350	0.9702
50	0.0930	0.0175	0.0103	0.2325	0.8181
60	0.1764	0.0364	$9.636 \times 10^{-3}$	0.3744	0.5513
	Level D				
10	0.1935	0.0343	$6.598 \times 10^{-3}$	0.3073	0.7533
20	0.1319	0.0195	$1.643 \times 10^{-3}$	0.3107	0.5285
30	0.1874	0.0353	$4.609 \times 10^{-3}$	0.3142	0.2944
40	0.1539	0.0249	0.0143	0.2612	0.4877
50	0.1256	0.183	0.0150	0.3914	0.4725
60	0.0969	0.0195	$3.062 \times 10^{-3}$	0.3629	0.5528

Table 3.6. Summary of the Percent Ion Contents for the Merus Tissues of the Cheliped on a Dry Weight Basis (Sampled One Crab in Levels A and B, Two Crabs in Level C, and Three Crabs in Level D)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level A				
10	--	--	--	--	--
20	0.2281	0.0443	$1.940 \times 10^{-3}$	0.2357	0.1138
30	0.1964	0.0443	$1.748 \times 10^{-3}$	0.2307	0.6785
40	0.1820	0.0292	$3.823 \times 10^{-3}$	0.2352	0.7814
50	0.2235	0.0327	$4.022 \times 10^{-3}$	0.2732	0.6521
60	0.1511	0.0296	$7.300 \times 10^{-4}$	0.1447	0.6175
	Level B				
10	0.8136	0.1489	0.0433*	0.2548	0.4023
20	0.2698	0.0643	$5.188 \times 10^{-4}$	0.1396	0.1103
30	0.2965	0.0513	$4.555 \times 10^{-3}$	0.0578	0.1140
40	0.1467	0.0278	0.0235	0.3215	0.2641
50	0.5994	0.0670	$3.880 \times 10^{-3}$	0.235	0.6218
60	--	--	--	--	--

\*Value inconsistent with overall standard deviation

Table 3.6 (Continued)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level C				
10	0.1860	0.0399	$2.506 \times 10^{-3}$	0.3783	0.4276
20	0.3272	0.741	$6.713 \times 10^{-4}$	0.1134	0.0600
30	0.3862	0.0820	$8.626 \times 10^{-3}$	0.1287	0.0500
40	0.1847	0.0261	$9.355 \times 10^{-3}$	0.2389	0.7987
50	0.2166	0.0351	0.0151	0.2608	0.9263
60	0.3541	0.0559	0.0126	0.3270	0.6207
	Level D				
10	0.2803	0.0505	$1.908 \times 10^{-3}$	0.3534	0.8209
20	0.2695	0.0469	$1.609 \times 10^{-3}$	0.2339	0.3259
30	0.3624	0.0609	$1.431 \times 10^{-3}$	0.2916	0.3629
40	0.2774	0.0453	$2.827 \times 10^{-3}$	0.2636	0.4604
50	0.2286	0.0295	$8.849 \times 10^{-3}$	0.3799	0.3411
60	0.15234	0.02944	$2.779 \times 10^{-3}$	0.3386	0.9216

Table 3.7. Summary of the Percent Ion Contents for the Gills on a Dry Weight Basis  
(Sampled One Crab in Levels A and B, Two Crabs in Level C, and Three Crabs  
in Level D)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level A				
10	--	--	--	--	--
20	--	--	--	--	--
30	0.0182	0.0021	0.0159	0.2440	0.5914
40	0.1395	0.0120	$9.152 \times 10^{-3}$	0.2318	0.3258
50	0.2294	0.0187	0.0102	0.2811	0.3121
60	0.1241	0.0145	$2.574 \times 10^{-4}$	0.1650	0.2307
	Level B				
10	--	--	--	--	--
20	0.0842	0.0146	$3.940 \times 10^{-3}$	0.2790	0.1621
30	0.2570	0.0286	$1.429 \times 10^{-3}$	0.0873	0.1858
40	0.0862	0.0122	0.0512	0.2262	0.1624
50	0.5245	0.0433	0.0182	0.2988	0.3898
60	0.1192	0.0099	0.0467	0.2864	0.4268

Table 3.7 Continued)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level C				
10	--	--	--	--	--
20	0.2043	0.0293	0.0377	0.3501	0.2628
30	0.1781	0.0325	$2.364 \times 10^{-3}$	0.2934	0.1204
40	0.0879	0.0108	0.5097*	0.3876	0.3637
50	0.1618	0.0157	0.2279	0.3856	0.4523
60	0.3870	0.0403	0.0294	0.3241	0.2591
	Level D				
10	0.2038	0.0214	0.0139	0.3812	0.3257
20	0.1933	0.0195	0.0145	0.3403	0.2950
30	0.2400	0.0344	$8.282 \times 10^{-3}$	0.3337	0.1672
40	0.1570	0.0168	$9.241 \times 10^{-3}$	0.4180	0.3054
50	0.1672	0.0148	0.0204	0.5222	0.3706
60	0.1452	0.0146	0.0163	0.4236	0.2299

\*Value inconsistent with overall standard deviation

Table 3.8. Summary of the Percent Ion Contents for the Dorsal Merus Exoskeleton of the First Peripod on a Dry Weight Basis (Sampled One Crab in Levels A and B, Two Crabs in Level C and Three Crabs in Level D)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level A				
10	--	--	--	--	--
20	--	0.0120	$5.490 \times 10^{-3}$	0.6942	9.8666
30	0.0190	0.0188	$9.693 \times 10^{-3}$	0.1152	5.7818
40	0.0251	0.0136	$2.682 \times 10^{-3}$	0.3560	6.5227
50	0.0305	0.01555	$3.569 \times 10^{-4}$	0.5670	6.4381
60	0.0115	0.0059	$8.193 \times 10^{-3}$	0.000	4.7517
	Level B				
10	--	0.0451	0.0591	1.4721	20.4344
20	--	0.0222	0.0346	0.6636	9.7607
30	0.0229	0.0163	0.0876	0.2174	6.1984
40	0.0182	0.0136	0.0877	0.3578	3.7304
50	0.0561	0.0286	0.0437	0.5903	5.0620
60	0.0258	0.0145	0.1135	0.5250	8.2166

Table 3.8 (Continued)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level C				
10	--	0.0160	0.0637	1.3668	8.4012
20	--	0.0168	0.0490	0.6095	9.4740
30	0.0425	0.0302	0.0418	0.2035	5.5608
40	0.0152	0.0098	0.0138	0.2204	6.0324
50	0.0275	0.0149	0.0497	0.4543	6.5538
60	0.0624	0.0326	0.0515	0.7483	3.8254
	Level D				
10	0.0520	0.0269	0.0163	0.7192	11.3541
20	0.0332	0.0229	0.0266	0.6450	13.4887
30	0.0429	0.0278	0.0351	0.4724	8.4709
40	0.0332	0.0193	0.0149	0.6775	6.8073
50	0.0330	0.0133	0.0253	0.8166	8.3932
60	0.0296	0.0150	$5.346 \times 10^{-3}$	0.7202	4.4708

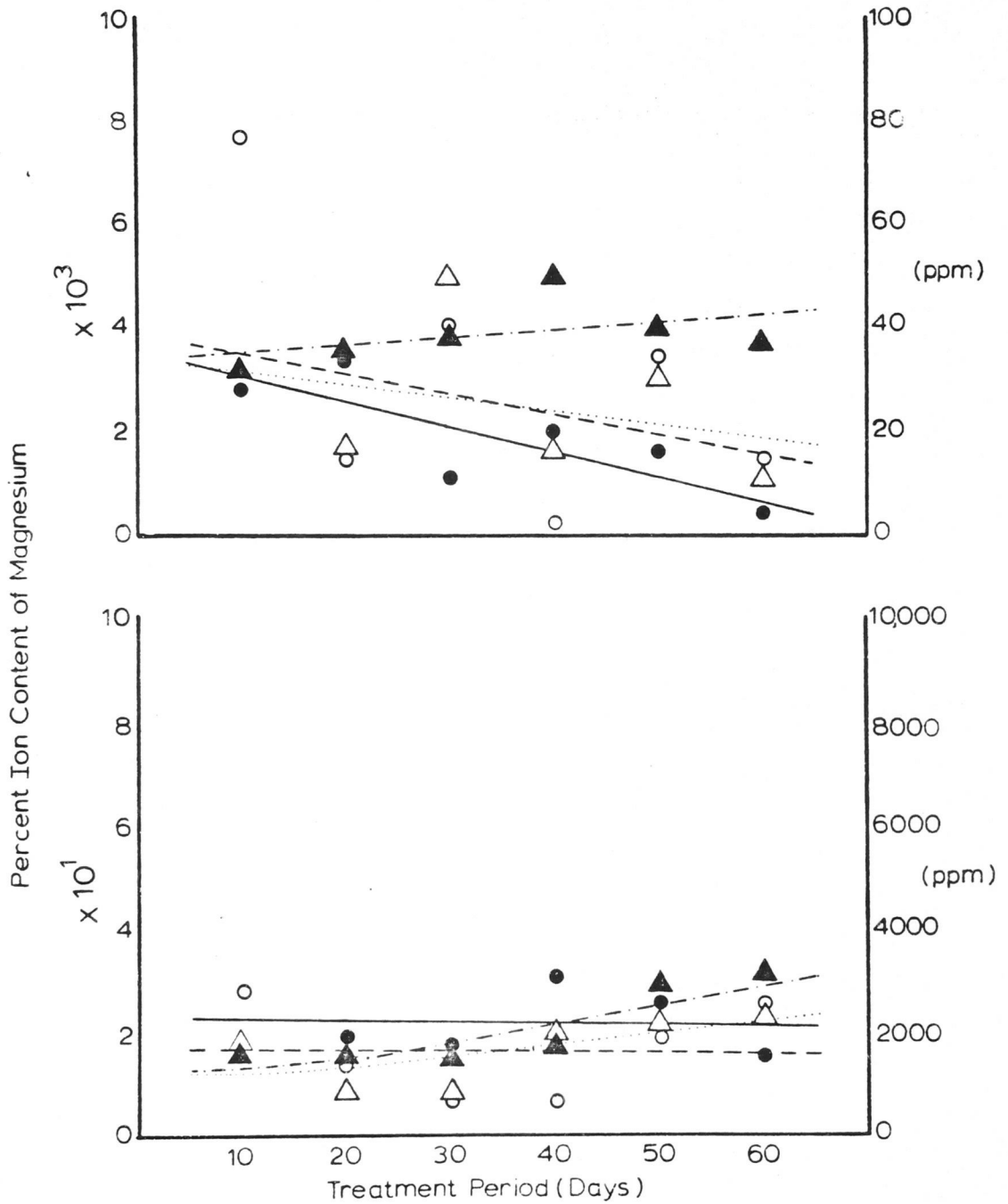
Table 3.9. Summary of the Percent Ion Contents for the Dorsal Merus Exoskeleton of the Cheliped on a Dry Weight Basis (Sampled One Crab in Levels A and B, Two Crabs in Level C, and Three Crabs in Level D)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level A				
10	--	--	--	--	--
20	--	0.0502	0.0326	1.4545	34.2979
30	0.0362	0.0253	0.0181	0.4698	--
40	0.0218	0.0172	$1.602 \times 10^{-3}$	0.5910	13.6593
50	0.0424	0.0288	$2.162 \times 10^{-3}$	1.0375	12.7022
60	0.0199	0.0146	$6.218 \times 10^{-3}$	0.7850	10.2688
	Level B				
10	--	0.0827	0.0503	2.2352	48.2110
20	--	0.0593	0.0719	1.0812	22.4750
30	0.1128	0.0931	0.1025	0.4821	13.2334
40	0.0374	0.0333	0.0810	1.1688	19.2114
50	0.1204	0.0849	0.0506	1.1002	15.4359
60	0.0312	0.0219	0.1341	0.8363	14.3748

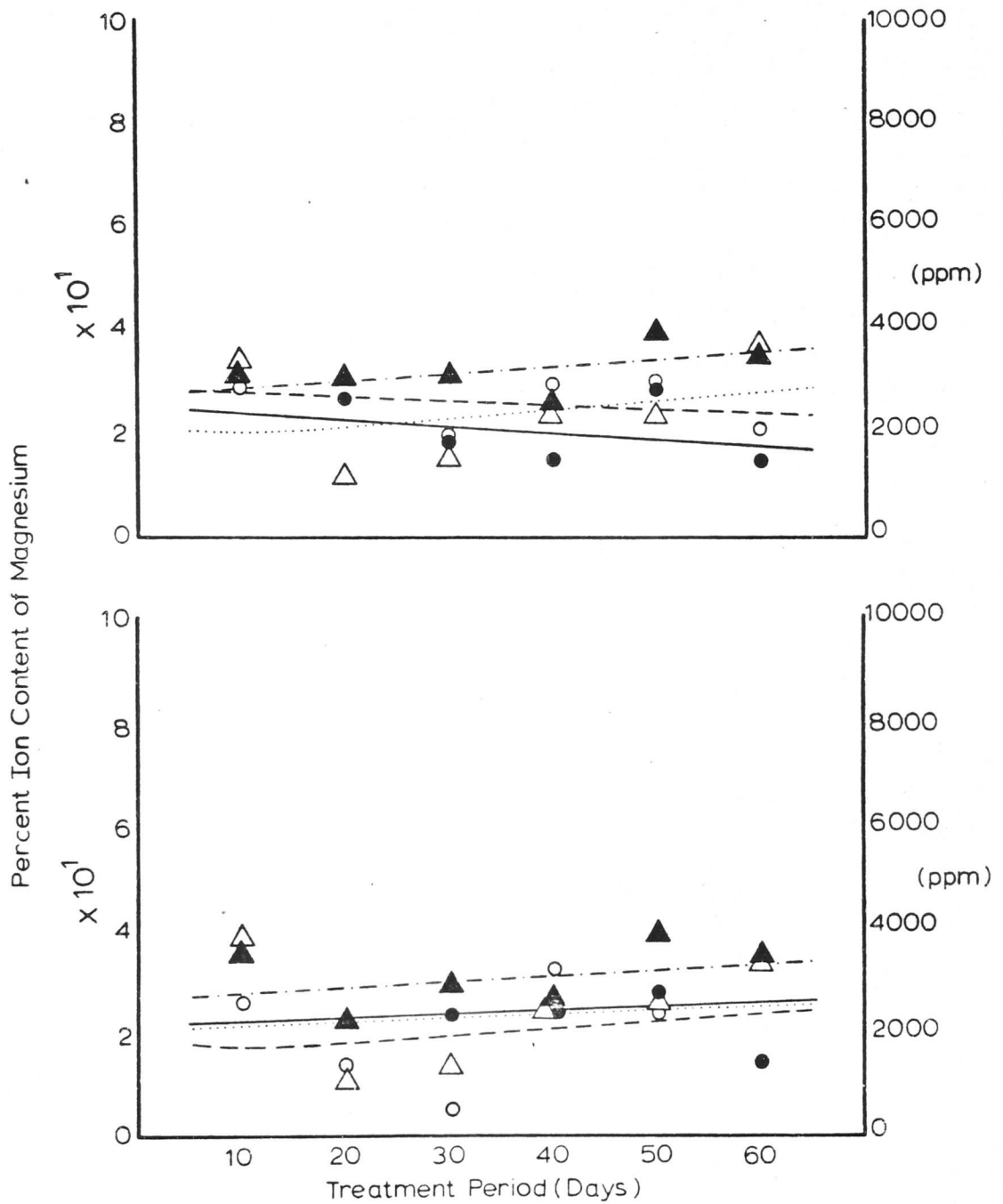
Table 3.9 (Continued)

Treatment Period Days	Average Weights		% Ion Content (Dry Weight)		
	Wet(g)	Dry(g)	Fluoride	Magnesium	Calcium
	Level C				
10	--	0.0504	0.0522	1.4711	28.2221
20	--	0.0781	0.0798	1.1440	22.1212
30	0.1741	0.1431	--	--	--
40	0.0456	0.0378	0.0113	0.9662	0.4915*
50	0.0503	0.0344	0.0419	0.9100	12.4557
60	0.07148	0.0415	0.0887	0.9308	6.0230
	Level D				
10	--	0.0327	0.0248	1.8549	34.4232
20	0.0678	0.0586	0.0246	1.1774	16.6872
30	0.1064	0.0879	0.0276	0.8628	17.4398
40	0.0622	0.0484	0.0315	1.3332	15.2880
50	0.0703	0.0538	0.0156	1.9164	13.1471
60	0.0397	0.0266	6.378 x 10 <sup>-3</sup>	1.4121	9.3868

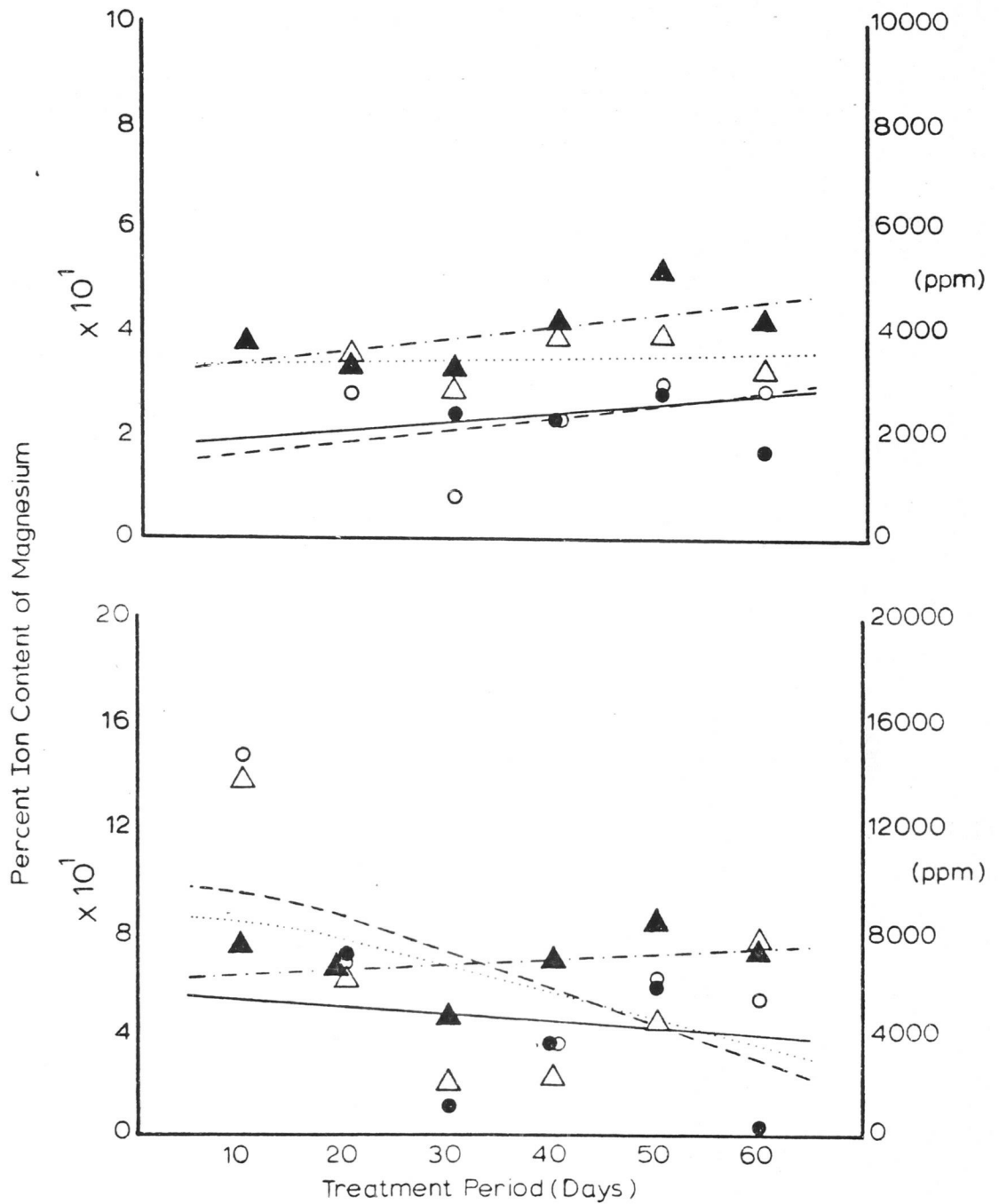
\*Value inconsistent with Overall Standard Deviation



Figures 3.3 (top) and 3.4. Magnesium Content of Hemolymph (top) and the Proximal Muscles of the Fifth Peripod (bottom) (Level A—●, Level B—○, Level C—△, Level D—▲)



Figures 3.5 (top) and 3.6. Magnesium Content of the Muscle Tissues of the First Peripod (top) and the Cheliped (bottom) (Level A —●, Level B —○, Level C .....△, Level D ——▲)



Figures 3.7 (top) and 3.8. Magnesium Content of the Gills (top) and of the Dorsal Merus Exoskeleton of the First Peripod (bottom) (Level A—●, Level B—○, Level C—△, Level D—▲)

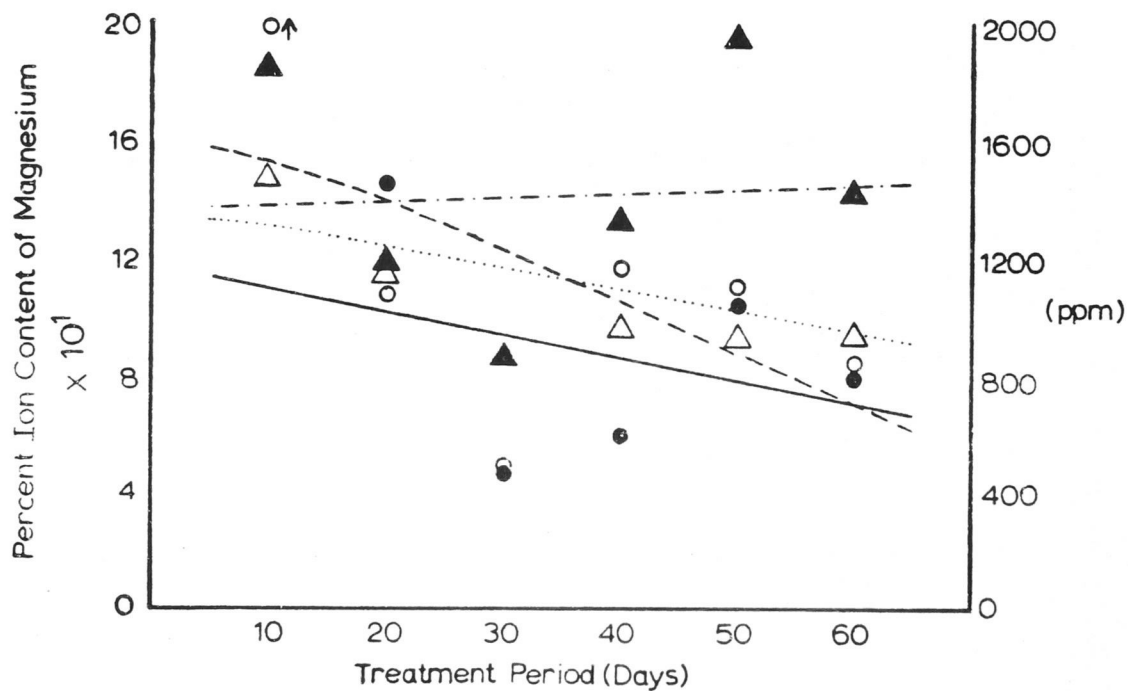
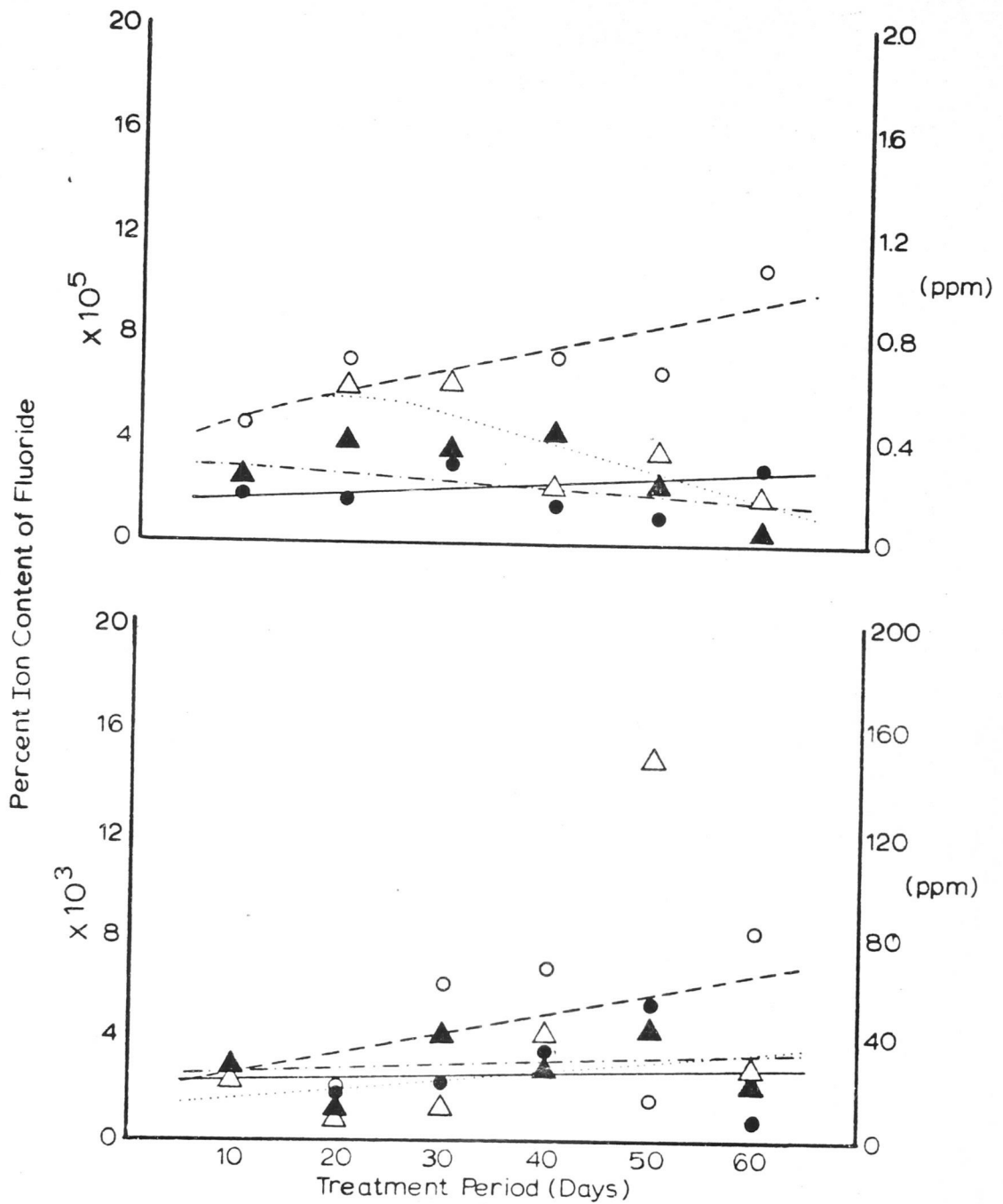
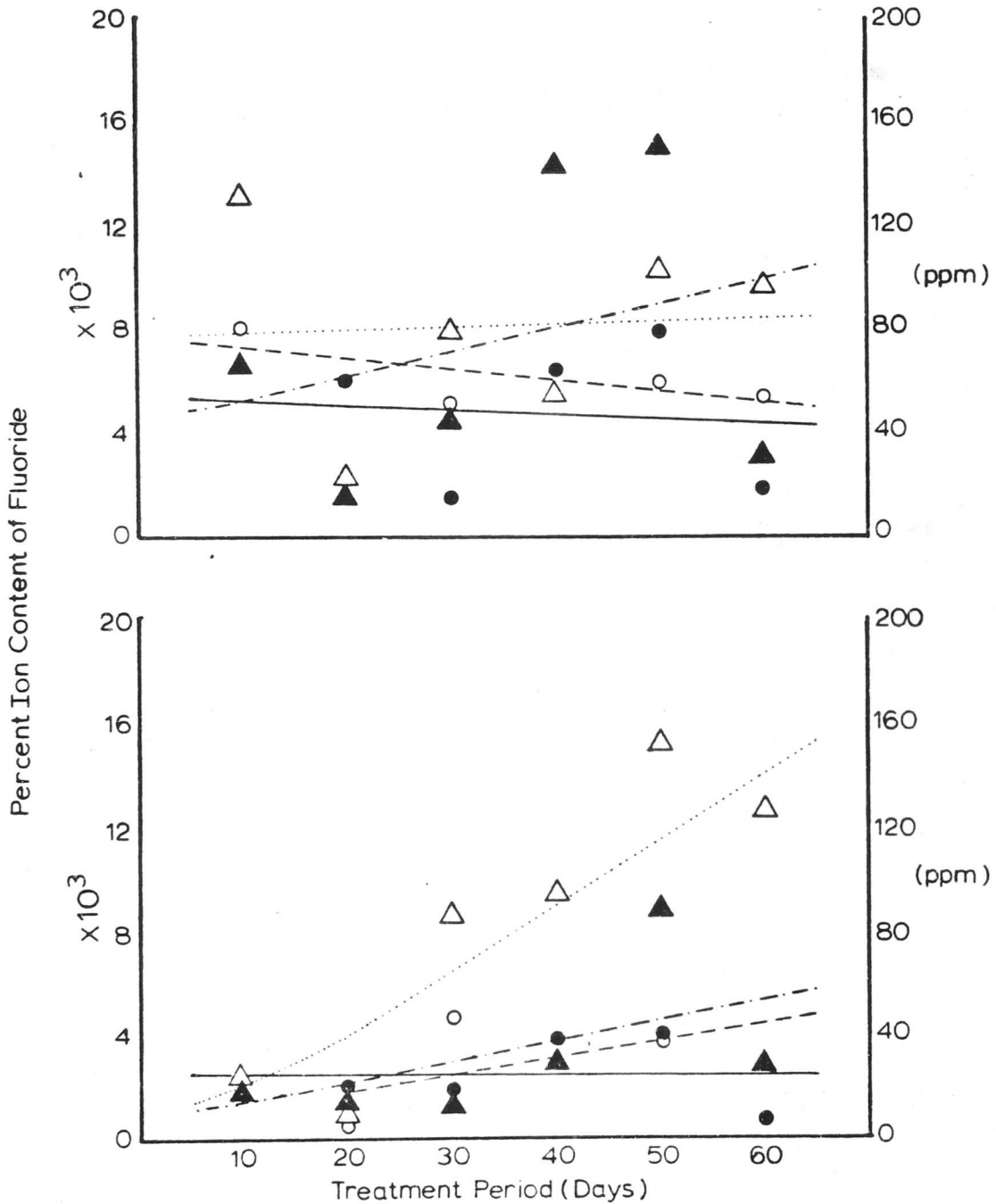


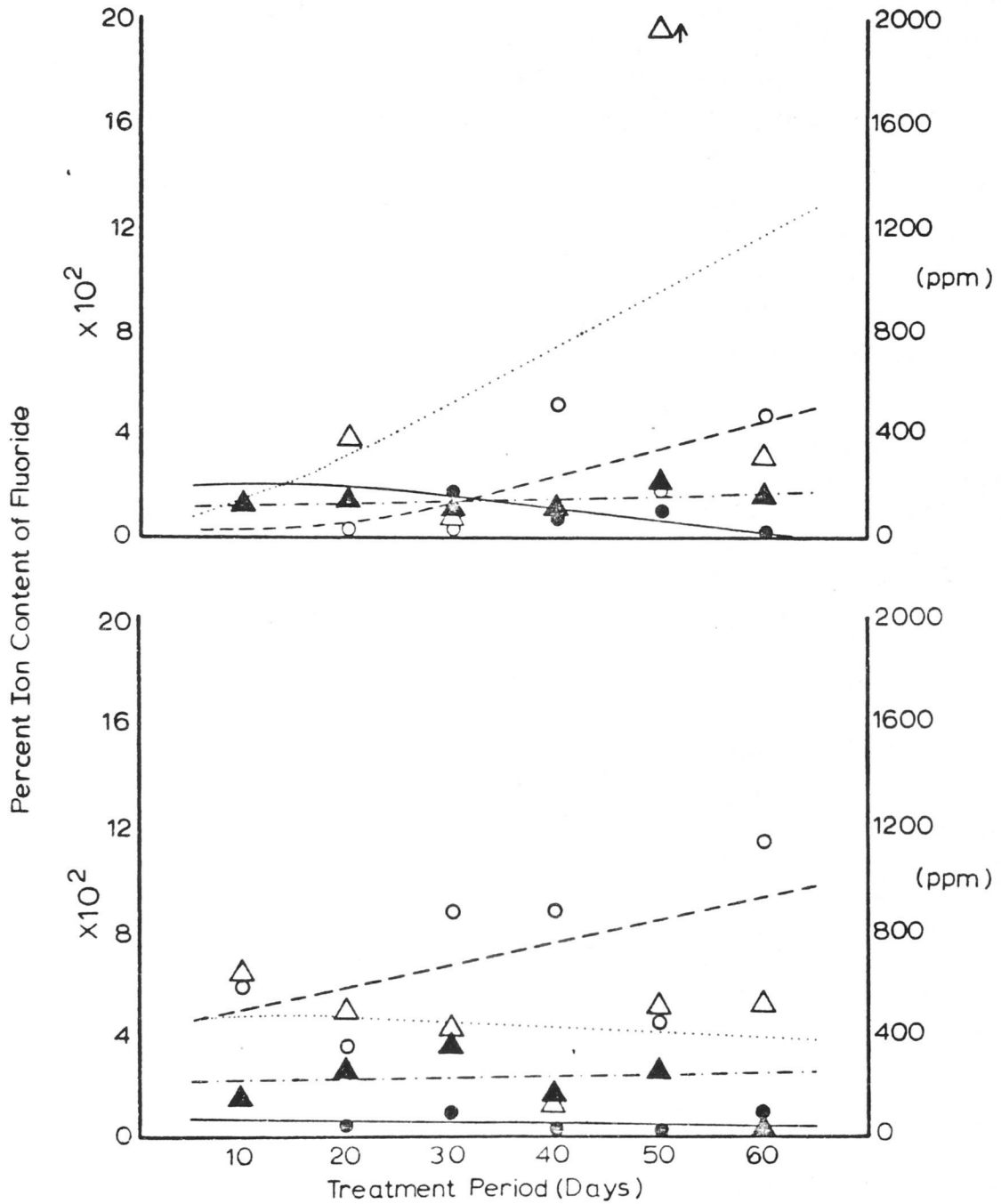
Figure 3.9 Magnesium Content of the Dorsal Merus Exoskeleton of the Cheliped (Level A—●, Level B—○, Level C—△, Level D—▲)



Figures 3.10 (top) and 3.11. Fluoride Content of Hemolymph (top) and the Proximal Muscles of the Fifth Peripod (bottom) (Level A—●, Level B—○, Level C—△, Level D—▲)



Figures 3.12 (top) and 3.13. Fluoride Content of the Muscle Tissues of the First Peripod (top) and the Cheliped (bottom) (Level A —●, Level B —○, Level C .....△, Level D —▲)



Figures 3.14 (top) and 3.15. Fluoride Content of the Gills (top) and of the Dorsal Exoskeleton of the First Peripod (bottom) (Level A—●, Level B—○, Level C—△, Level D—▲)

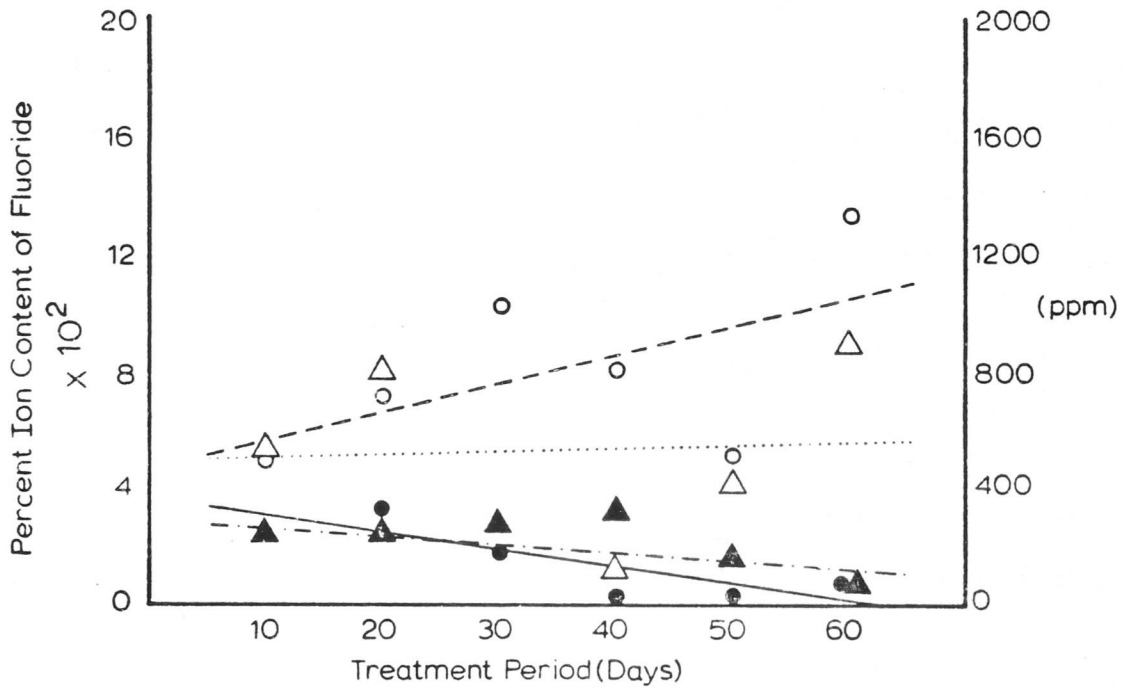


Figure 3.16 Fluoride Content of the Dorsal Merus Exoskeleton of the Cheliped (Level A —●, Level B —○, Level C .....△, Level D —▲)

#### 4. Discussion

##### 4.1 Growth Rates and Mortality Statistics

In 1979, the water temperature of the Pamlico and Cape Fear Rivers was relatively low during the months of February and early March. Crabs could not be collected until the first week of March, and then they were obtained by trawling along the mouths of the estuaries. Consequently, most of the crabs studied in this investigation came from these early catches. In contrast to a previous investigation in this laboratory<sup>37</sup>, most of these crabs molted during the acclimation period (Section 2.1). In addition, the crabs were sacrificed at regular intervals (Section 2.4), therefore a study of molting behavior could not be carried out and the data provided in Table 3.1 are not indicative of a general population of crabs. Consequently, the data on the average weight and size measurements in the same table also have only limited statistical value.

It is interesting to note as shown in Figure 3.1 (1978 study), that the crabs molted at a faster rate in the 30 ppm fluoride solution (B) than in the reference system (A). Molting was shown to be significantly delayed in the levels where the calcium ion content was raised from 118 ppm to 414 ppm (Level E). Apparently large  $\text{Ca}^{++}$  concentrations act to retard the molting of the crabs.

The increase of the molting rate exhibited in Level C and Level B suggests that  $\text{MgF}^+$  most likely promotes molting. Level B contained sufficient concentrations of  $\text{F}^-$  and  $\text{Mg}^{++}$  ions to yield  $\text{MgF}^+$  in such quantities that molting was accelerated in comparison to Level A.

Although the initial molting stage of each crab was unknown, the random placement of the crabs into the various levels allows for comparison of subsequent molting frequency.

The results of the 1979 study on the mortality rates of blue crabs are given in Table 3.2. Because the crabs in this study were sampled at regular intervals, these values do not represent a statistical average of the life expectancy of crabs. Results shown in Figure 3.2, for the 1978 study, offers a better representation for the expected mortality rates of the crabs. Crabs obtained from the Cape Fear or Pamlico River during the month of March of 1978 showed a much greater life expectancy in the laboratory than crabs obtained during the Fall of 1978.

The data obtained for the 1978 crabs show a drastic reduction of the life expectancy in water of 30 ppm fluoride. After about 2 months of treatment, the crabs in Level B died approximately twice as fast as in Level A. An increase in the Ca ion concentration had no bearing on the mortality rate (therefore one solid curve for B and E). However, the addition of magnesium offset the detrimental effects of fluoride, possible through formation of  $MgF^+$ , and consequently the mortality rates of Level A and C crabs were observed to be identical within the limits of statistical error.

#### 4.2 Magnesium Concentrations in Selected Portions of the Blue Crab

The variation of the magnesium content in the organisms with relation to the length of exposure to the different ionic solutions, as shown in Figures 3.3 to 3.9, reveal three significantly different patterns, namely for hemolymph, tissues and exoskeletons.

Magnesium ions increased in the hemolymph when the crabs were exposed to Level D solutions, i.e., to low fluoride and high magnesium concentrations. Obviously, the higher magnesium ion concentration of the water caused this increase of the magnesium level in the crab. The data indicate an isosmotic ion level of approximately  $3.5 \times 10^{-3}$  percent ion content or 35 ppm which is observed for the beginning of the treatment (0 days). This compares with 44 ppm of  $Mg^{++}$  which was determined in blue crabs obtained from New York Harbor. The decrease of  $Mg^{++}$  (Levels A, B, and C) with prolonged treatment apparently results from an active excretion of the ion by the crab. This activity may be stimulated by the presence of fluoride (Levels B and C) or low magnesium concentration in the water (Levels A and B).

It is interesting to note that all tissue samples exhibit a nearly identical pattern. All four tissues (Figures 3.4 - 3.7) reveal an isosmotic magnesium ion level between 0.2 and 0.3 percent ion content. In all cases Levels C and D show increases with prolonged exposure to the respective ionic solutions. The magnesium contents of Levels A and B vary identically, i.e., a slight increase is observed in the case of the gills, a decrease for the muscle tissues of the first peripod, a constancy for the muscle tissues of the cheliped and the proximal muscles of the fifth peripod. It appears that the magnesium concentration dominates the uptake of the ion in all cases. This explains the magnesium content increase in Levels C and D, but reductions were observed for most of the other samples. The exceptions seen in the gill tissues may be due to their constant exposure to the water and their active role in the ionic regulation of the crab. It should be mentioned

that all changes are of a relative large magnitude since the isosmotic concentrations are consistently high for these samples.

The exoskeleton samples (Figures 3.8 and 3.9) also exhibited an increase of the magnesium content with increasing length of exposure. The isosmotic levels are significantly higher in the exoskeleton in comparison with the tissues and hemolymph, but the data reflect a greater deviation from a common value. This would indicate that the exoskeleton samples have a lesser degree of homogeneity than the other samples. A general tendency to decrease in percent ion content is observed in levels A, B, and C with respect to exposure time.

It was observed in numerous cases, that after prolonged exposure to high levels of fluoride a softening of the exoskeleton occurred. The calcium concentration of the exoskeleton did not vary to any significant degree whether exposed to high or low levels of fluoride as reflected by the  $\text{Ca}^{++}$  data of Tables 3.8 and 3.9. This would indicate that the calcium ions remain held in the calcified portions of the epicuticle and endocuticle and are not removed. However, in the presence of fluoride, the entire structure is weakened and, apparently, a portion of the magnesium ions diffuse, in the form of  $\text{MgF}^+$  complex ions, into the bulk of the surrounding solution.

#### 4.3 Fluoride Concentrations in Selected Portions of the Blue Crab

The fluoride data, as shown in Figures 3.10 to 3.16, exhibit particular patterns for hemolymph and exoskeleton samples as well as for the tissues.

Both hemolymph and exoskeleton samples show an increase of fluoride with an extended treatment period in Level B. However, the fluoride content of the crab samples obtained from the other levels either does

not change or a slight decrease is observed. This observation can be traced to the relative concentrations of fluoride, calcium, and magnesium ions in the treatment levels. The increase of the B level fluoride content is apparently a result of high fluoride and relatively low magnesium concentrations, thus permitting a rather large portion of the free fluoride of the solution to be incorporated into the crab material. A low fluoride concentration as in Levels A and D does not promote fluoride uptake, either by the hemolymph or the exoskeleton since the solution equilibrium is shifted in favor of the formation of free ion rather than chemical complexes, such as  $\text{MgF}^+$ . Naturally a portion of the fluoride is incorporated in the calcified layers of the exoskeleton. Variations of the calcium content of these layers have no significant effect on the degree of fluoride incorporation. Since the amount of calcium of the solid (exoskeleton layers) always represent such a large quantity that a quasi-equilibrium can be considered where the uptake of  $\text{F}^-$  would be highly favored. This explains why the fluoride content of the exoskeleton is approximately 1000 times greater than that of the hemolymph.

The variation of the fluoride in the hemolymph for Level C samples, which exhibits a maximum value after 25 days of treatment, can be explained by two effects. As shown in Table 3.3, both the  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  concentration of hemolymph samples decrease over the time of the treatment period. In general, the  $\text{Ca}^{++}$  levels are approximately three times greater than the  $\text{Mg}^{++}$  concentrations. Each of the two ionic species is associated to fluoride, most likely as  $\text{CaF}^+$  and  $\text{MgF}^+$  complexes. The formation of  $\text{CaF}_2$  is not favored. As the  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  concentrations

became smaller, less fluoride is found in hemolymph samples. Therefore, the curve in Figure 3.10 representing Level C is a composite curve of the two different rates of ion release from the hemolymph.

An increase of fluoride is also observed for the proximal muscles of the fifth peripod of crabs treated in Level B, as shown in Figure 3.11. Interestingly, the magnesium content of these swimming muscles does not vary and the  $\text{Ca}^{++}$  content decreases (see Table 3.4), i.e., the fluoride uptake cannot be associated with the presence of either metal ion as in the previously discussed cases. It appears that another mechanism might be responsible for the fluoride increase from about 2 ppm to 7 ppm on a wet weight basis, however, no clues pertaining to this effect could be found due to the limitations of the experiment.

The fluoride uptake by the gills and the muscle tissues of the cheliped follows the general pattern of changes found for calcium and magnesium ions (Table 3.6). The uptake in 30 ppm fluoride containing water reaches a maximum of 120 ppm for gills and about 15 ppm (all wet weights) for the tissues of the cheliped. The data obtained for the tissues of the first peripod do not qualify for the meaningful discussion because of apparent experimental error.

The uptake of fluoride by feeding (see Section 3.4) can be neglected due to the small amounts involved.

In summary, the ionic composition of the various treatment levels had a significant effect on the growth and the mortality of the blue crabs. The effects exhibited by the ionic forms of fluorine, calcium, and magnesium on the metabolism of the blue crab could be grouped in three general patterns which involve hemolymph, tissues, and exoskeleton.

This study is the first comprehensive investigation of the effect of fluoride on growth, mortality, and ionic regulation of the crab under controlled conditions (salinity, temperature, light) for the wide range of ionic composition of simulated seawater.

APPENDIX A

## FLUORAN/3 - EXTRAPOL - CAMGAA

```

0100 REM FLUORAN/3 & EXTRAPOL & CAMGAA
0101 REM=====
0300 DIM X(100),Y(100),A(4,4),B(100),D(100),E(100),M(100),P(100)
0301 DIM V(100),W(100),Z(100)
0400 PRINT "F=1, EXTRAP.=2, CA-MG=3"
0401 INPUT V4
0420 ON V4 GO TO 500,4000,5000

```

## FLUORAN/3

```

1140 PRINT #7," FLUORIDE "
1150 PRINT
1160 PRINT "NO . OF STANDARDS"
1170 INPUT NO
1171 LET N=NO+3
1172 FOR I=1 TO N
1174 READ M(I),X(I)
1176 IF I=1 THEN LET X(2)=X(1):LET M(2)=M(1):LET I=2
1178 IF I=2 THEN LET X(3)=X(1):LET M(3)=M(1):LET I=3
1180 IF I=6 THEN LET X(7)=X(6):LET M(7)=M(6):LET I=7
1185 NEXT I
1190 FOR I=1 TO N
1200 LET Y(I)=LOG(M(I))
1210 NEXT I
1220 GO SUB 3000
1260 LET X1=-C(3)/(3*C(4))
1270 FOR I=1 TO 4
1280 LET R(I)=C(I)
1290 NEXT I
1300 PRINT #7,"X1",.7*X1
1315 RESTORE
1320 FOR I=1 TO NO
1324 READ M(I),X(I)
1326 NEXT I
1330 LET G=NO+1:N=5
1335 FOR I=1 TO 5
1340 LET M(I)=M(G-I):X(G-I)
1350 LET Y(I)=LOG(M(I))
1360 NEXT I
1380 GO SUB 3000
1390 FOR I=1 TO 4
1400 LET U(I)=C(I)
1410 NEXT I
1450 PRINT "FOR STANDARD DISPLAY=1"
1460 INPUT M3
1470 IF M3<>1 THEN 1875
1475 PRINT "ENTER LEAST & MOST NEGATIVE VALUE OF DISPLAY"
1480 INPUT W1,W2
1490 LET W3=W1/5:W4=W2/5*W5=W3-W4
1495 LET N5=W5+1
1500 FOR N6=1 TO N5:X(N6)=W1-5*N6+5
1510 NEXT N6

```

```

1560 PRINT "HARDCOPY=1"
1570 INPUT H1
1580 IF H1<>1 THEN 1710
1590 PRINT #7
1600 PRINT #7,TAB(1);"EMF";TAB(10);"PPM";TAB(25);"[F]", M"
1700 GOTO 1740
1710 PRINT TAB(1);"EMF";TAB(10);"PPM";TAB(25);"[F]", M"
1740 FOR N6=1 TO N5
1750 IF X(N6)<.7*X1 THEN 1775
1755 LET W7=R(1)+R(2)*X(N6)
1760 LET Z(N6)=W7+R(3)*X(N6)*X(N6)+R(4)*X(N6)*X(N6)*X(N6)
1765 LET Z1=Z(N6)
1770 GOTO 1790
1775 LET W8=U(1)+U(2)*X(N6)
1780 LET Z(N6)=W8+U(3)*X(N6)*X(N6)+U(4)*X(N6)*X(N6)*X(N6)
1785 LET Z1=Z(N6)
1790 IF ABS(Z1)>80 THEN 1820
1800 LET S5=EXP(Z1)
1810 GOTO 1830
1820 LET S5=0
1830 IF H1=1 THEN 1870
1840 DIGITS= 2:PRINT TAB(1);X(N6);
1845 DIGITS= 3
1850 PRINT TAB(10);19000*S5;TAB(25);S5
1860 GOTO 1874
1870 DIGITS= 2:PRINT #7,TAB(1);X(N6);
1872 DIGITS= 3:PRINT #7,TAB(10);19000*S5;TAB(25);S5
1874 NEXT N6
1875 PRINT "NO. OF SAMPLES":INPUT N5
1920 PRINT #7:PRINT #7
1930 PRINT #7,"FLUORIDE      DATE OF ANALYSIS....."
1950 PRINT "INPUT: EMF, MASS, VOL., DILUT."
1960 FOR N6=1 TO N5
1970 INPUT X(N6),W(N6),V(N6),D(N6)
1980 NEXT N6
1990 PRINT #7
2005 PRINT #7,TAB(1);"NO.";TAB(5);"EMF";
2006 PRINT #7,TAB(15);"[F] TEST S.";TAB(28);"%ION CONT."
2010 FOR N6=1 TO N5
2020 IF X(N6)<.7*X1 THEN 2045
2025 LET W7=R(1)+R(2)*X(N6)
2030 LET Z(N6)=W7+R(3)*X(N6)*X(N6)+R(4)*X(N6)*X(N6)*X(N6)
2040 GOTO 2060
2045 LET W8=U(1)+U(2)*X(N6)
2050 LET Z(N6)=W8+U(3)*X(N6)*X(N6)+U(4)*X(N6)*X(N6)*X(N6)
2060 LET S6=EXP(Z(N6))
2070 LET D=1.9*S6*V(N6)*D(N6)/W(N6)
2080 DIGITS= 0:PRINT #7,TAB(1);N6;
2085 DIGITS= 4:PRINT #7,TAB(5);X(N6);TAB(15);S6;TAB(28);D
2090 NEXT N6
2100 PRINT "MORE SAMPLES=1;  #SAMPLES"
2110 INPUT N7,N5
2120 IF N7=1 THEN 1950
2130 END

```

```

3000 REM POLYNOMIAL APPROXIMATION OF 3RD DEGREE
3010 LET N2=6
3020 LET M=N-1
3030 FOR I=1 TO N2
3040 LET P(I)=0
3050 FOR J=1 TO M
3052 LET B=ABS(X(J)):LET W=1
3054 IF X(J)<0 THEN IF((I/2)-INT(I/2))>0 THEN LET W=-1
3060 LET P(I)=P(I)+(B*I)*W
3070 NEXT J
3080 NEXT I
3090 LET N1=4
3100 FOR I=1 TO N1
3110 FOR J=1 TO N1
3120 LET K=I+J-2
3130 IF K<= 0 THEN 3160
3140 LET A(I,J)=P(K)
3150 GOTO 3170
3160 LET A(1,1)=M
3170 NEXT J
3180 NEXT I
3190 LET B(1)=0
3200 FOR J=1 TO M
3210 LET B(1)=B(1)+Y(J)
3220 NEXT J
3230 FOR I=2 TO N1
3240 LET B(I)=0
3250 FOR J=1 TO M
3252 LET B=ABS(X(J)):LET W=1
3254 IF X(J)<0 THEN IF((I-1)/2-INT((I-1)/2))>0 THEN LET W=-1
3260 LET B(I)=B(I)+Y(J)*(B*(I-1))*W
3270 NEXT J
3280 NEXT I
3290 FOR K=1 TO 3
3300 LET K1=K+1
3310 LET L=K
3320 FOR I=K1 TO N1
3330 IF ABS(A(I,K)) <= ABS(A(L,K)) THEN 3350
3340 LET L=I
3350 NEXT I
3360 IF L<=K THEN 3450
3370 FOR J=K TO N1
3380 LET T=A(K,J)
3390 LET A(K,J)=A(L,J)
3400 LET A(L,J)=T
3410 NEXT J
3420 LET T=B(K)
3430 LET P(K)=B(L)
3440 LET B(L)=T
3450 FOR I=K1 TO N1
3460 LET F=A(I,K)/A(K,K)
3470 LET A(I,K)=0

```

```

3480 FOR J=K1 TO N1
3490 LET A(I,J)=A(I,J)-F*A(K,J)
3500 NEXT J
3510 LET B(I)=B(I)-F*B(K)
3520 NEXT I
3530 NEXT K
3540 LET C(N1)=B(N1)/A(N1,N1)
3550 LET I=3
3560 LET I1=I+1
3570 LET S=0
3580 FOR J=I1 TO N1
3590 LET S=S+A(I,J)*C(J)
3600 NEXT J
3610 LET C(I)=(B(I)-S)/A(I,I)
3620 LET I=I-1
3630 IF I>0 THEN 3560
3640 RETURN
3650 GO TO 400
3670 END

```

#### EXTRAPOLATION ROUTINE

```

4000 REM EXTRAPOLATION ROUTINE
4010 INPUT "INPUT THE NO. OF RUNS",N
4020 PRINT "CONCENTRATION"
4030 LET B=1
4040 INPUT C
4050 LET Z=6
4060 IF N=0 THEN 4570
4070 PRINT "TIME>2 MIN, EMF=10"
4080 FOR I=1 TO Z: INPUT K(I),M(I): NEXT I
4090 FOR I=1 TO Z
4100 LET X(I)=1/K(I)
4110 LET Y(I)=10*(M(I)/10000)
4120 NEXT I
4130 LET S1=0
4140 FOR I=1 TO Z
4150 LET S2=X(I)*Y(I)
4160 LET S1=S1+S2
4170 NEXT I
4180 LET S3=0
4190 FOR I=1 TO Z
4200 FOR J=1 TO Z
4210 LET S4=X(I)*Y(I)
4220 LET S3=S3+S4
4230 NEXT J
4240 NEXT I
4250 LET P1=0
4260 FOR I=1 TO Z
4270 FOR J=1 TO Z
4280 LET P2=X(I)*X(J)

```

```

4290 LET P1=P2+P1
4300 NEXT J
4310 NEXT I
4320 LET S5=0
4330 FOR I=1 TO Z
4340 LET S6=X(I)*X(I)
4350 LET S5=S5+S6
4360 NEXT I
4370 LET B=(S1-(1/Z)*S3)/(S5-(1/Z)*P1)
4380 LET S7=0
4400 FOR I=1 TO Z
4410 LET S8=X(I)
4420 LET S7=S7+S8
4440 LET S9=0
4450 FOR I=1 TO Z
4460 LET T1=Y(I)
4470 LET S9=T1+S9
4480 NEXT I
4490 LET A=((-B)*S7+S9)/Z
4500 LET O=434.3*LOG(A)
4510 PRINT #7:DIGITS=3
4520 PRINT #7,"EMF, MV(T-->00)=";O:PRINT #7, "CONC.=";C
4530 PRINT #7
4540 LET N=N-1
4550 IF N=0 THEN 4570
4560 GO TO 4020
4570 GO TO 400
4580 END

```

#### CALCIUM - MAGNESIUM

```

5000 REM CA-MG-AA
5100 DATA 0,100,0,100,0,100
5110 DATA .5,93.5,.5,95.2,1,87.6,1,91.2,2,79.4,2,84.3
5120 DATA 5,58,5,67
5200 PRINT #7,"CA & MG ", "DATE OF ANALYSIS....."
5210 PRINT #7
5215 LINE = 80
5220 PRINT "5100/5120 DATA=STANDARDS (ENTER PPM(LOW TO HIGH))";
5230 PRINT ",%A]"
5240 INPUT "ENTER # STANDARDS",N
5250 REM E(I)=ORDINATE,M(I)=ABSCISSA;PPM, %A
5270 FOR I=1 TO N
5280 READ E(I),M(I)
5290 LET Y(I)= E(I)
5300 LET O8=LOG(M(I)):LET X(I)=LOG(O8)
5310 NEXT I

```

```

5320 GOSUB 3000
5330 GOSUB 5670
5340 PRINT "# SAMPLES"
5350 INPUT N5
5360 PRINT "FOR % ION CONTENT IN SAMPLE=1"
5370 INPUT N8
5380 IF NR=1 THEN 5550
5390 PRINT "INPUT: %A"
5400 FOR N6=1 TO N5
5410 INPUT L(N6)
5420 NEXT N6
5440 PRINT #7, "%A", "PPM"
5450 FOR N6=1 TO N5: LET O9=LOG(L(N6))
5460 LET X(N6)=LOG(O9): LET O4=C(1)+C(2)*X(N6)
5470 LET Z(N6)=O4+C(3)*X(N6)*X(N6)+C(4)*X(N6)*X(N6)*X(N6)
5480 PRINT #7, L(N6), Z(N6)
5490 NEXT N6
5500 PRINT "MORE SAMPLES=1; # SAMPLES"
5510 INPUT N7, N5
5520 IF N7=1 THEN 5360
5530 END
5550 PRINT "INPUT: %A, MASS, VOL., DILUT."
5560 FOR N6=1 TO N5
5570 INPUT M(N6), W(N6), V(N6), D(N6)
5580 NEXT N6
5585 PRINT #7
5590 PRINT #7, TAB(1); "%A"; TAB(6); "DIL"; TAB(12); "MASS";
5595 PRINT #7, TAB(21); "PPM"; TAB(30); "%ION CONT."
5600 FOR N6=1 TO N5
5610 LET X(N6)=LOG(LOG(M(N6))): LET O5=C(1)+C(2)*X(N6)
5620 LET Z(N6)=O5+C(3)*X(N6)*X(N6)+C(4)*X(N6)*X(N6)*X(N6)
5630 LET D=Z(N6)*D(N6)*V(N6)/(10000*W(N6))
5640 DIGITS= 1: PRINT #7, TAB(1); M(N6);
5642 DIGITS= 0: PRINT #7, TAB(6); D(N6);
5645 DIGITS= 3: PRINT #7, TAB(10); W(N6); TAB(20); Z(N6);
5648 DIGITS= 4: PRINT #7, TAB(30); D
5650 NEXT N6
5660 GOTO 5500
5670 LET U4=-C(3)/(3*C(4))
5680 LET U5=C(1)+C(2)*U4+C(3)*U4*U4+C(4)*U4*U4*U4
5690 PRINT #7, TAB(1); "%A"; TAB(10); "PPM";
5700 PRINT #7, TAB(25); "PPM COMPUTED"
5710 FOR I=1 TO N
5720 LET O6=C(1)+C(2)*X(I)
5730 LET Z(I)=O6+C(3)*X(I)*X(I)+C(4)*X(I)*X(I)*X(I)
5740 PRINT #7, TAB(1); M(I); TAB(10); Y(I); TAB(25); Z(I)
5750 NEXT I
5760 RETURN
5770 END
5800 GOTO 400
9999 END

```

APPENDIX B

## LINEAR LEAST SQUARES FIT

```

0005 PRINT #7
0006 PRINT #7,"FMT A,B,C,D
0008 PRINT #7
0009 GO TO 30
0010 PRINT #7,"LINEAR LEAST SQUARES FIT"
0020 PRINT #7,"INTERCEPT IS NOT FIXED": PRINT #7
0030 DIM X(150),Y(150),W(150),Z(150),M(150)
0050 PRINT "# OF SAMPLES"
0060 INPUT N
0070 FOR I=1 TO N
0080 READ X(I),Y(I)
0090 NEXT I
0100 GO SUB 1000: DIGITS=3
0105 GO TO 125
0110 PRINT #7,"INTERCEPT",A0: PRINT #7,"STD. DEV.",D0
0120 PRINT #7:PRINT #7,"SLOPE",A1: PRINT #7,"STD. DEV.",D1:PRINT #7
0125 PRINT #7,TAB(5);"OVERALL STD. DEV.";TAB(25);D2: PRINT #7
0130 PRINT #7,TAB(1);"X(I)"*TAB(12);"Y(I)";TAB(25);"Y(I) CORR."
0140 FOR I=1 TO N
0145 LET Z(I)=A0+A1*X(I)
0150 DIGITS= 3: PRINT #7,TAB(1);X(I);TAB(12);Y(I);TAB(25);Z(I)
0160 NEXT I
0170 END
0600 DATA 10,7.98E-3,10,13.1E-3,10,6.59E-3,20,2.27E-3,20,6.02E-3
0601 DATA 20,1.64E-3,30,7.88E-3,30,5.2E-3,30,4.61E-3
0602 DATA 40,5.49E-3,40,6.44E-3
0603 DATA 40,14.9E-3,50,7.94E-3,50,5.97E-3,50,10.3E-3,50,15.1E-3
0604 DATA 60,3.06E-3,60,9.64E-3,60,5.36E-3,60,1.83E-3
1000 REM LINEAR LEAST SQUARES SUBROUTINE
1010 Z0=0: Z1=0: Z2=0: Z3=0: Z4=0
1020 FOR I=1 TO N
1030 LET W(I)=1
1040 LET Z0=Z0+1
1050 LET Z1=Z1+W(I)*X(I)
1060 LET Z2=Z2+W(I)*Y(I)
1070 LET Z3=Z3+W(I)*X(I)*Y(I)
1080 LET Z4=Z4+W(I)*X(I)*X(I)
1090 NEXT I
1100 LET D=Z0*Z4-Z1*Z1
1110 LET A0=(Z4*Z2-Z1*Z3)/D
1120 LET A1=(Z0*Z3-Z1*Z2)/D
1130 LET Q=0
1140 FOR I=1 TO N: Q1=Y(I)-A0-A1*X(I): Q=Q+W(I)*Q1*Q1: NEXT I
1150 LET S2=Q/(N-2)
1160 LET D0=SQR(S2*Z4/D)
1170 LET D1=SQR(S2*Z0/D)
1180 LET D2=SQR(S2)
1190 RETURN
9999 END

```

### Biography

Born November 20, 1954, the author grew up in Concord, NC, where she graduated from Concord Senior High School in 1973. Upon entering East Carolina University in September of the same year, she decided to pursue a degree in science. During her undergraduate years, she participated in many research efforts, including the reception of a National Science Foundation Grant for 1976. In 1977, having graduated with a Bachelor's of Science Degree in Biology, she re-entered East Carolina University to obtain her Master's of Science Degree in Biology. This was accomplished in May of 1980.

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