LABORATORY STUDIES OF THE EFFECTS OF ELEMENTAL PHOSPHORUS ON SELECTED MARINE ORGANISMS

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LABORATORY STUDIES OF THE EFFECTS OF ELEMENTAL PHOSPHORUS ON SELECTED MARINE ORGANISMS

by

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A Thesis

submitted in partial fulfilment
of the requirements for the degree of
Master of Science
Department of Biology
Memorial University of Newfoundland

St.John's Newfoundland

January 1971

ABSTRACT

Literature reviews of bio-assay methodology and phosphorus toxicology are presented as part of the introduction to the thesis. A temperature-controlled, serial dilution unit designed to provide eight successive 2:1 dilutions and its actual performance are described. Marine organisms were collected in waters free of pollutants and tested at the Marine Sciences Research Laboratory. The three-spined stickleback, Gasterosteus aculeatus, and the Atlantic cod, Gadus morhua, were assayed to determine median tolerance limits (TLm) and to compare the effects of two types of colloidal phosphorus formulations: phossy water from the Electric Reduction Company of Canada plant at Long Harbour and, colloidal dispersions of pure phosphorus (P_4) prepared in the laboratory. The 48 hour TLm values for stickleback and cod at 8.0°C are 190 ugm/L. and 27 ugm/L. respectively in ERCO phossy water; the 48 and 96 hour TLm's in pure P_4 dispersions at 8.0°C are 185 ugm/L. and 68 ugm/L. for stickleback and 28 ugm/L. and 16 ugm/L. P_4 for the cod. In bio-assays conducted on the winter flounder, Pseudopleuronectes americanus, the 48 and 96 hour TLm's are 70 ugm/L. and 25 ugm/L.P_4 respectively. Temperature affects the toxicity of yellow phosphorus to the three-spined stickleback, while particles of P_4 greater than 0.8microns have little or no effect on the same species. ERCO phossy water retards the righting response of the northern starfish, Asterias vulgaris, at concentrations lower than those required for

lethality. Dispersions of phosphorus affects the hematocrit of the cod and winter flounder as well as the white cell count of the cod. P_4 causes a depression of the acetyl cholinesterase activity of the three-spined stickleback, a sufficient amount to cause death.

" The fishers also shall mourn, and all
they that cast angle into the brooks
shall lament, and they that spread nets
upon the waters shall languish."

Isaiah, 19:8

ACKNOWLEDGEMENTS

First and foremost, I wish to thank Dr.Richard E. Warner for providing the opportunity to conduct this research, and for the guidance and encouragement he offered me during the research and writing of this thesis. He has become more than a supervisor, he has become a friend. Financial support for this work came from two grants to Dr.Warner: National Research Council grant no. A-5462, and a Vice-President's grant to the M.U.N. Coordinating Committee on Placentia Bay.

Thanks must be extended to Dr.F.A.Aldrich for allowing this potentially dangerous research to be conducted at the Marine Sciences Research Laboratory, as well as to the staff of the MSRL for all assistance during the research period. Dr.Aldrich's review of the draft before final typing was also much appreciated.

Miss Jennifer Sacrey and Mr.D.Eddy were instrumental in setting up the bio-assay unit and must be commended. I also am grateful for the kind help of Messrs. F.Hicks, B.LeDrew, M.Farwell, W.Bradley, W.Glasgow and R.A.Cote, as well as divers employed by the MSRL, who assisted me in securing the marine animals required for the research.

The work of Mr.G.McCurdy of the MSRL and especially Mr.R. Ficken, the department photographer, is very much appreciated. Many other people offered advice and support while the research was being done; Dr.G.Mct.Cowan, Dr.M.Newlands of the Chemistry

Department, Dr.R.Khan, to name but a few.

I would also like to thank Dr.G.Bennett for the loan of the microhematocrit centrifuge and the Department of Chemistry for the loan of the ultrasonic generator. The assistance of the of the Fisheries Research Board of Canada, Halifax Laboratory, whose staff conducted some phosphorus analyses for me and the FRBC St.John's Laboratory for the loan of the 30m seine used at Bryant's Cove is also acknowledged. Thanks also to the management of ERCO for providing samples of the phossy water from the Long Harbour plant.

A Fellowship from the Deparment of Biology of Memorial University allowed me to spend two very fruitful years here in Newfoundland and I am grateful for that opportunity.

Last but not least, Miss Chris Keddy made the writing of this thesis a more pleasant task. To all of these people and any others who assisted me in any way, I owe my sincere thanks.

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INTRODUCTION

The research for this thesis was motivated by a desire to obtain basic information on the effects of elemental phosphorus (P_4) on marine life. In order to accomplish this, it was necessary to develop a bio-assay unit capable of continuous serial dilution of toxicants, to a series of concentrations which could be quantified, and whose effects could be evaluated by observation and analysis.

The impetus for this work was the pollution incident at Long Harbour, Placentia Bay, during the winter of 1968-1969 which affected a large number of marine organisms of many species. As regards fish, Atlantic herring and Atlantic cod were the species most seriously afflicted by what has now been recorded as phosphorus poisoning (Idler, 1969).

Yellow phosphorus, as produced by the Electric Reduction Company of Canada Ltd. (ERCO) plant at Long Harbour, had not until this time attracted much attention as a pollutant in marine or fresh waters. The Fisheries Research Board of Canada, as a result of the incident, has been involved in determining the cause of the pollution and the subsequent fish kills which were of sufficient magnitude that they attracted the attention of fishermen and subsequently various government agencies. One of these agencies, the Fisheries Research Board, has now established that elemental phosphorus was the cause of the kills.

The Fisheries Research Board have conducted research on the toxicity of various forms of yellow phosphorus to the Atlantic herring, Clupea harengus, brook trout, Salvelinus fontinalis, lobster, Homarus americanus, smelt, Osmerus mordax, and the beach flea, Gammarus oceanicus (Zitco,1970; Fletcher,1970).

The series of experiments described in this thesis attempts to provide a more comprehensive look at phosphorus toxicology in marine situations. The research was conducted with greater attention paid to the control of variables with the intention of demonstrating some of the environmental conditions which affect the toxicity of yellow phosphorus and some of the physiological conditions which are affected by P_4. These bio-assays involved the following group of organisms: the three-spined stickleback, Gasterosteus aculeatus, the Atlantic cod, Gadus morhua, the winter flounder, Pseudopleuronectes americanus, and the northern starfish, Asterias vulgaris. Additional experiments were run to determine among other things, the effects of elemental phosphorus on the blood of the Atlantic cod and on the level of acetyl-cholinesterase in the brain of the three-spined stickleback.

The facilities of the Marine Sciences Research Laboratory (MSRL)of the Department of Biology were used because of the availability of pure sea water and holding tanks for specimens.

Review of Literature on Bio-assays

A bio-assay has been defined as the use of a living system to evaluate the effects of an environmental contaminant (Warner, 1965). Bio-assays are generally used to determine the kind of aberration induced in the biological system, the magnitude of this change, and the quantity of contaminant required to produce it. The basic bio-assay procedure consists of preparing different concentrations of an effluent or other test material,i.e. the pollutant, with a selected dilution water, adding the test specimens and observing their behavioral and physiological reactions over a definite period of time.

In the majority of cases, the bio-assay is used to assess the effect of a microchemical contaminant, one which is present in concentrations less than one part per million (ppm) or one milligram per liter (mgm/L.). These may be heavy metals, pesticides,oils or many other organic and inorganic chemicals. In order to have a measurable effect, the pollutant must be a toxicant, that is, producing an aberration in one or more life processes. The aberrations, in turn, are described as acute or chronic. Acute effects refer to those short term changes produced by a toxicant, generally within the first 100 hours of exposure. This time was suggested as rule of thumb by Warner (1965). Acute toxication is often characterized by nervous or enzymic disruption. Chronic refers to adverse effects recognized after 100 hours;

these may be hematological or other histological changes, or may involve kidney or liver malfunction, for example.

In 1945, Hart,Doudoroff and Greenbank described a procedure for bio-assays of industrial wastes using fish as tests animals. Subsequently, Doudoroff et al.(1951) published a paper on bio-assay methods which are now regarded as standard procedures. These methods, and their various modifications since then, have been widely used by scientists in government, industry and in universities for assessing the toxicity of various potentially dangerous materials. The basic method remains, but researchers have modified it to accomodate the many types of toxicants, test organisms, and parameters being examined.

Much information has accumulated in aquatic toxicology since the early 1950's (Cairns,1966;Sprague,1969;Warner,1965). It has been said that a revolution is taking place in bio-assay techniques, with many new developments. The major shift is toward the use of sublethal measurements but there is also an important change of emphasis from static assays to continuous flow systems. The latter point derives partly from the realization that acute response experiments are not sufficient and that death is not the only important parameter. Because death, defined as lack of response, provides no information on sublethal effects of contaminants, other responses are now being monitored so as to provide a more complete picture of the effects of these toxicants

(Warner,1965;Wilber,1962). An animal must be able to function adequately, if not optimally, if it is to survive. A significant disruption of normal life patterns (eg. reproductive behavior, resistance to heat or cold stress, etc...) is as likely to cause eventual death as a strongly lethal compound, even though it may not be as easily demonstrated in the laboratory (Fromm,1962). Effects of seemingly minor changes internally may cause major disruptions in the biology of the species.

Some of the bio-assay types now being utilized, which measure the response as a deviation from the norm, are: Behavioral change physiological change, biochemical change, ecological change, embryological change and, growth change. For example, effects which have been measured have dealt with feeding rate (Cairns,1966), reproduction (Johnson,1967) and, acetylcholinesterase inhibition (Weiss,1958).

There are many ways in which bio-assays can be used.Toxicity of final effluents can be determined as well as their likely effects on receiving waters. The effectiveness of treatment processes may be established. In the location of new plants, the quantity of dilution water necessary (if any material is to be released into the environment) or the degree of treatment of wastes may be found in advance of construction. This holds true whether the work involves pesticides on agricultural land which are finally transported by water, phosphorus reduction plants, oil refineries,

or pulp and paper mills.

It is also one of the good examples where the university community can relate to industry and government, as well as society as a whole.

Review of Literature on Elemental Phosphorus Toxicology

In reviewing the literature, limited information was found on industrial workers and experimental animals exposed to elemental phosphorus. Moreover, only meagre and sometimes contradictory results are available on the threshold of physiological tolerance to this material administered in relatively large amounts over short periods of time, or small amounts over prolonged periods (Heimann, 1946; Fleming et al.,1942).

Yellow phosphorus (P_4) is used in the manufacture of chemical smoke screens, incendiary bombs, as a constituent of rat poison and in metallurgy. Its use in the manufacture of matches and firecrackers, which had been a major cause of chronic phosphorus poisoning, was discontinued after the First World War. The development of the electrothermal process of phosphorus production, which is now used for the production of large quantities of elemental phosphorus for the phosphate fertilizer industry, rendered more urgent, basic investigations of the toxicity of the element if the experience of the match and fireworks was to be avoided.

It is apparent from the literature,however, that the symptoms of acute phosphorus poisoning differ from those of exposure over long periods of time to small amounts of the material (Heimann , 1946). Difficulty was experienced in distinguishing between those animal experiments which could be classified as short as opposed to long-term tests (Heimann,1946). It would appear that growing animals would react differently than adults to chronic poisoning, especially with regard to the osseous system, but no consideration was given to this point.

In acute poisoning, the predominant effect is the fatty degeneration of the liver (Cameron and Patrick,1963). Liver dysfunction is presumably tha cause of death (Cameron and Rentoul, 1960). As little as 1/8 grain (8mgm.) has been reported to cause acute toxicity and death of humans . The reported lethal dose of P_4 required for man also varies from author to author; 8 mgm. as reported by Heimann(1946) to 50 mgm. reported by Cameron(1960).

Other observations of acute poisoning of humans and dogs suggest at least two stages of toxication: The first stage is one of gastro-intestinal irritation, which within several hours is chracterized by thirst, nausea, vomiting, haematemesis and gastric discomfort. This normally lasts about twenty-four hours and is followed by a variable period of well-being which depends on the rate of absorption of the phosphorus by the tissues. The terminal phase is evidenced by nausea,vomiting,abdominal pain,

a tender liver, haemorrhages, peripheral circulatory collapse, coma and death which occurs from hepatic, renal and cardiac failure (Cameron and Rentoul,1960; Blumenthal and Lesser,1935).

In cases of chronic phosphorus poisoning of mammals, however, involvement of the osseous system is the principal characteristic. The most typical effect is necrosis of the jaw bones. The change in the bones is a generalized reaction of the periosteum producing a hyper-ostosis of the entire skeletal structure,a periosteal thickening of the bone and the laying down of more bone (Heimann,1946). It has been hypothesized that such extra deposition results in cutting down the blood supply to the Haversian canals (Heimann,1946;Hamilton,1925).

The level of ammonia nitrogen in the urine is reported to increase while urea nitrogen decreases, due to the general cellular damage and possibly more specifically to liver damage (Heimann,1946). There is no evidence at present suggesting that chronic toxication of mammals produces severe liver tissue destruction or physiological dysfunction.

Similarly, there is no reported evidence that chronic phosphorus exposure in man produces damage to the central nervous system, although this possibility cannot be ruled out.

An extensive literature search revealed very few papers on the effects of phosphorus to fish. Isom (1960) conducted tests

on Lepomis macrochirus, the bluegill sunfish, and his study was motivated by a large fish kill in a Tennessee river, downstream from a phosphorus reducing plant. The stated objective of his work was to determine whether elemental phosphorus was toxic at levels as low as its solubility, or if the toxicity was due to colloidal phosphorus or its oxidation products only.

Isom concluded that colloidal suspensions were more acutely toxic than solutions of dissolved phosphorus. His experiments involved testing the effects of colloidal suspensions having variously sized particles. A saturated solution of yellow phosphorus (solubility in fresh water: 0.3 gm/100 mls at $15^{o}C$) revealed no toxicity in water. Colloidal P_4 was found to be toxic in small quantities at pH's sufficiently high to rule out the complex acids of phosphorus as the toxicant. In Isom's studies, the bluegill was not appreciably affected by low concentrations of colloidal P_4 in the first 24 hours, but by 48 hours showed considerable mortality. The 48 hour TLm (median tolerance limit - concentration causing 50% mortality in 48 hours) was approximately 0.105mgm/L. and the 72 hour TLm was approximately 0.053mgm/L.

Due to the static bio-assay procedure employed, the fish and the aeration removed the suspended and dissolved P_4 in the course of one test. Thus no mortality occurred when new fish were introduced into the same waters.

Zitco (1970) has reported that in the Atlantic herring, Clupea harengus, the Atlantic salmon, Salmo salar, and the lobster,

Homarus americanus, the toxic effects of phosphorus are irreversible and probably cumulative. No clear indication of an incipient lethal level (the concentration at which the test animal is not able to survive for an indefinite period of time) was found for the herring as TLm values were obtained at a concentration of 2.5 ugm/L. Incipient lethal level of yellow P_4 for lobster was 40 ugm/L.: for Atlantic salmon, 18 ugm/L. and for the beach flea, 3 mgm/L.. Fish poisoned by elemental phosphorus, the Atlantic herring, for example, showed signs of extensive hemolysis and turned red.

Studies of the oxidation rates of diluted phossy water and yellow phosphorus in dispersions, showed that they are first order reactions with half-lives of ? and 7.5 hours respectively Zitco, Anderson and Tibbo, 1969).

Dyer(1970) conducted experiments on the uptake of yellow phosphorus by cod muscle and liver. In 16 hours exposure to 20 ugm/L. the liver concentrated P_4 up to 40 ugm/gm. while the concentration in the red muscle was approximately 1750 ngm/gm. of tissue. With the elemental P_4 in sea water of 21-83 ugm/L. liver concentrated the phosphorus some 880-2000 times. The distribution of P_4 between the white and red muscle, and between flesh and liver was found to be roughly proportional to the lipid content.

This background information suggested many avenues of research because so little is known about phosphorus toxicology especially in the marine environment.

MATERIALS AND METHODS

Serial Dilution Unit: Theory and Practice

Evaluation of the research aspects of phosphorus toxicology in sea water made it clear that a continuous flow bio-assay system would have to be designed and built. The system was planned in such a way as to eliminate many of the drawbacks of static tests, by providing a continuous flow of toxicant solution to the test containers rather than removing the animals to fresh solutions at regular intervals. The continuous flow procedure eliminated the need for aeration of the test water, which in this case would have caused undue loss of elemental phosphorus from the test system. Toxicant concentration fluctuations in test solutions due to absorption by test animals, is greatly reduced. This procedure also decreases the test error caused by the adsorption of the toxicant to the walls of the aquaria.

In the studies reported, the concentrations of toxicant were automatically maintained by a Serial Dilution Unit, thus eliminating the additional problem of human error in mixing solutions, When temperature control is applied to the system, the water in the tanks is more readily maintained at a constant temperature.

In order to preclude possible pollution of Logy Bay by test effluents, a 5000 gallon concrete tank was constructed to receive and hold toxic materials. The wastewater from the experimental work was removed periodically by sewage disposal tank trucks, the effluent being released onto a gravel bed at the city dump, some distance from the ocean.

A description of the bio-assay apparatus, which is based on a system developed by Warner (1965), follows.

A length of 3/4 inch Tygon tubing is attached at one end to a salt water tap while the other is fastened to a Hypur water filter unit capable of filtering out material down to 15 microns. The filter is required for two reasons 1) to remove as much plankton as possible and eliminate the problem which might arise if the test animals were to ingest large quantities of food already toxicated and 2) to remove large organisms and particles in the sea water which might clog the valves of the Serial Dilution Unit (S.D.U.), decreasing the flow rate and hence increasing the concentration of toxicant delivered to the test aquaria.

The water then flows into a temperature controlled bath which is described in section 2.

From the top of the bath, the water flows through 2.5 cms tubing into a horizontal plexiglass tube at the top of the S.D.U.. This tube has holes in its lower surface which serve to distribute the water evenly throughout the length of the sea water trough. If the water is allowed to enter at one end only, wave action results in the trough, causing irregularities in the flow rates. Another safety feature was built into the sea water trough: an overflow box running on a vertical track was fitted into the center of the upper trough; this maintains a constant head of water and allows for any changes in water pressure. (see fig. 1)

Figure 1.

Bio-assay unit - list of parts

A - Peristaltic pump control and speed selector.
B - Test aquarium.
C - Magnetic stirrer.
D - Insulated container for toxicant (4 liters).
E - Peristaltic pump and tubing.
F - Influent trough for sea water of S.D.U.
G - Influent trough overflow unit (on track).
H - Flow rate control valves.
I - Toxicant - sea water mixing trough.
J - Control section (sea water only).
K - Funnelling troughs.
L - Aquarium input tube.
M - Flow meter.
N - Filter apparatus.
O - Thermoregulator and relay controller.
P - Heaters (1000 watts).
Q - Water bath with input and output tubes (20 L.).
R - Relay unit for heaters.
S - Thermoregulator.

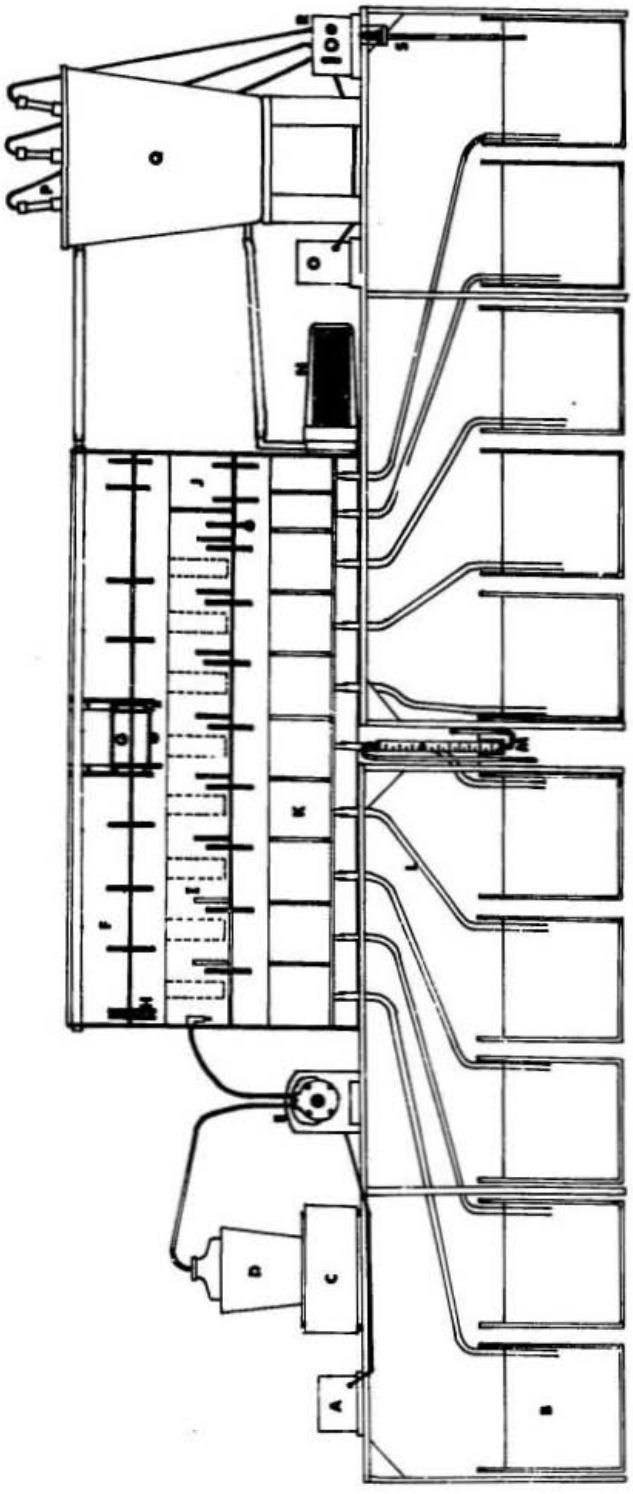


Figure 1.

Figure 2.

Serial Dilution Unit

In this case, a solution of neutral red dye was pumped through the mixing trough to demonstrate the effectiveness of the dilution mechanism.

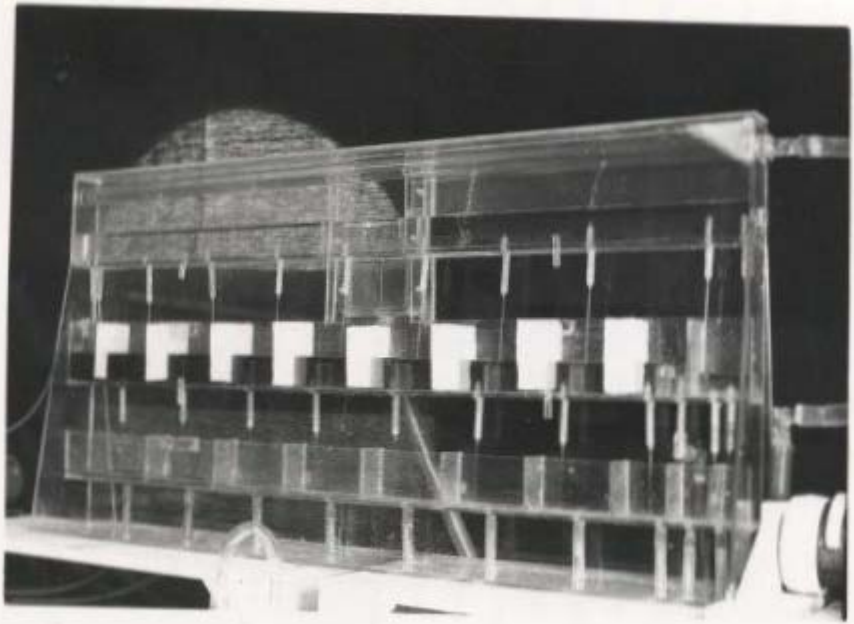


Figure 2.

The bottom of this trough is fitted with eleven threaded tubes which regulate the flow rate of water into the mixing trough. Of these valves, the first two are 2.5 cms apart while the remaining nine are placed at 12.7 cms intervals. The last two valves are control water outlets while the first nine are involved in the dilutions of the toxicant.

The part of the mixing trough involved in the dilution of the toxicant is divided into nine sections. The first two tubes flow into the first section (see fig.3); the seven tubes which follow in the upper trough, flow into seven sections on the mixing trough; a small section adjacent to the control section is used as a overflow for the toxicant and drains directly into the effluent trough (see fig. 2). Each section of the mixing trough is separated from the next by a baffle over which the water flows; at the midpoint of each section, a polystyrene block has been inserted approximately 5 mm. from the floor of the trough to aid the mixing of the toxicant (see fig. 3.).

A peristaltic pump, capable of delivering 1 - 1300 mls/min., pumped the concentrated toxicant into the first section of the mixing trough. The pump provided many variations in the available concentrations for bio-assays. Each section of the mixing trough is also fitted with a threaded tube to the right of the block, and in front of the baffle. The principle then, which provides the

Figure 3.

The theory of the Serial Dilution Unit

A diagram of the serial dilution mechanism, demonstrating the flow patterns.

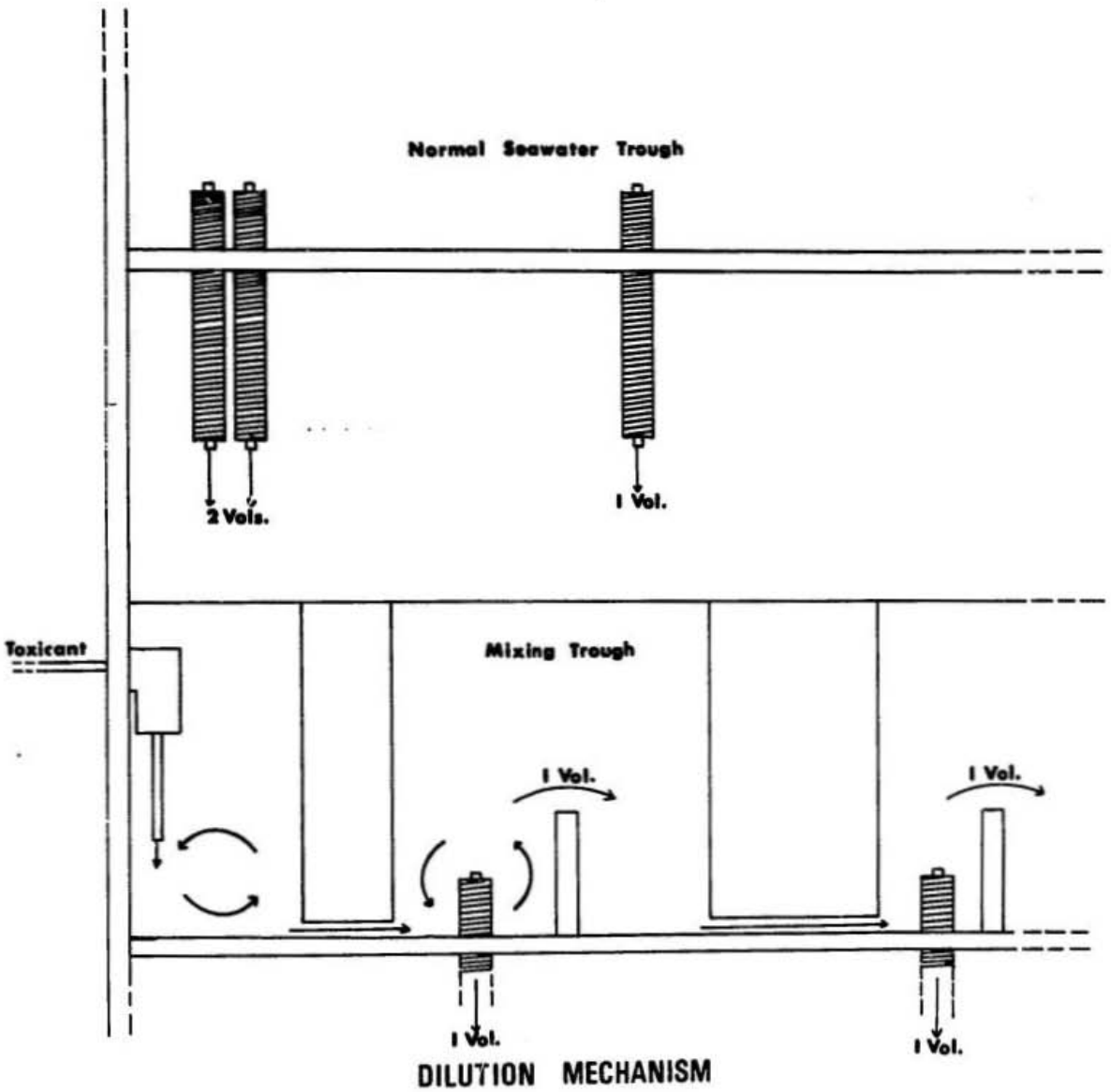
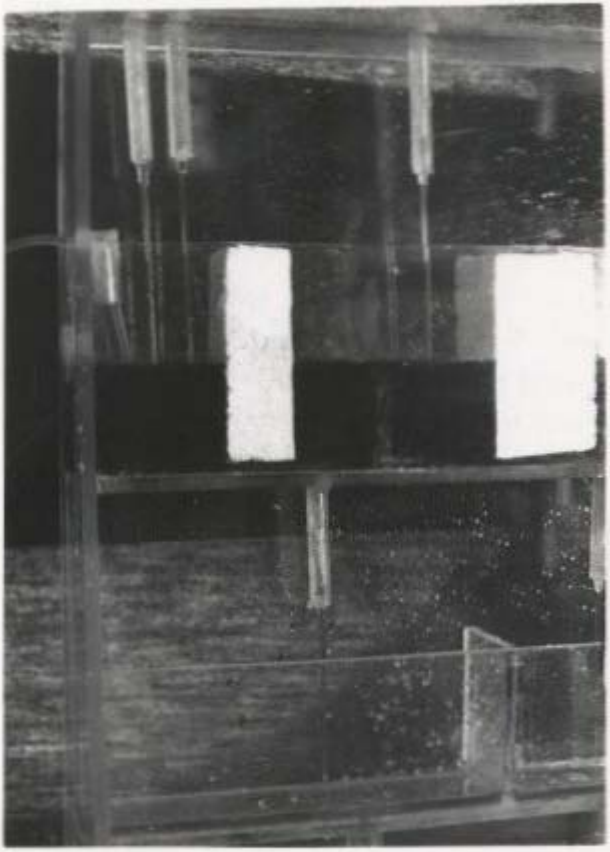

Normal Seawater Trough
2 Vols.
1 Vol.
Toxicant
Mixing Trough
1 Vol.
1 Vol.
1 Vol.
1 Vol.
DILUTION MECHANISM

Figure 3.

Figure 4.

The working parts of the dilution mechanism of the Serial Dilution Unit.



50% dilution from one aquarium to the next is: For every 2 volumes of water running into the first section of the mixing trough, 1 volume is drained out; at a constant flow rate of water, the remaining 1 volume flows into the next section where it is diluted by 1 volume of natural sea water from the sea water trough. This mechanism holds true throughout the length of the mixing trough which is involved with the dilution of the toxicant (see figs. 3 and4).

The control outlets of the sea water trough run into a section of the mixing trough which is completely separated from the diluted concentrations of the pollutant (see fig.2). All valves from the second trough empty into a funnelling trough and thence through tubing running into the bottom of the test aquaria. These solutions or suspensions are held to a level of 32 liters and from that point run through overflow tubes into an effluent trough situated at the front of the set-up (see figs 1 and 5). The outlet for this unit is connected by a pipe running through the building to the large effluent tank already referred to.

The complete system, that is, any part of it involved directly with sea water and the toxicant, is constructed of plexiglass, polyvinyl chloride (P.V.C.) or glass.

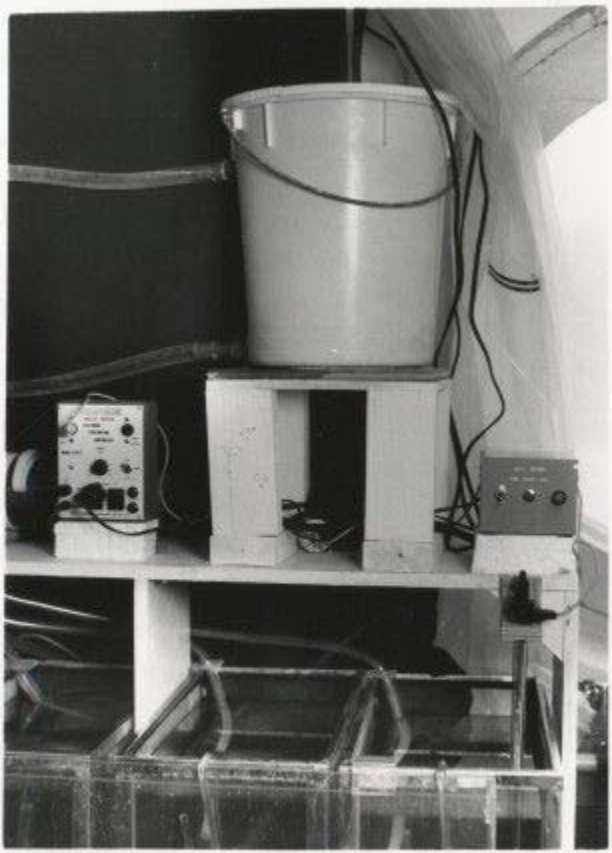
Temperature Control Component

The apparatus pictured in figure 5 is a modified sand

Figure 5.

The temperature control component

The temperature control unit has three heaters inserted into the water bath which were able to raise the water temperature from $0^{o}C$ to $15^{o}C$ within a few minutes. The unit has been used for periods of 10 days without failure of any of its parts.



filter which had originally been constructed for use with the S.D.U. but which had since been replaced by the Hypur filter.

Extra holes were cut into the pressure cover and into the wall of the 20 liters polyethylene container. Three 1000 watt immersion heaters were installed into the cover and caulked into place so that the elements were situated 2.5 cms from the inlet tube. This provided for optimum heating of the sea water which flows into the S.D.U.. The tube built into the bottom of the container is the inlet tube; the tube built into the wall of the container near the top, serves as the outlet and leads through 1.9 cms tubing to the inlet pipe of the S.D.U.(see figs.1 and 5).

The water heater was located such that the outlet from the bath and the S.D.U. inlet were at the same height, alleviating excessive pressure problems. The pressure provided by the salt water tap, reduced to 0.63 cm is sufficient to run water into the Serial Dilution Unit.

Temperature is controlled by the use of an intricate thermoregulator (see fig.6). This is divided into two sections: the lower one is constructed as a mercury thermometer with the exception of an electrical connection made to the lowest part of the mercury column. As temperature increases, the mercury column rises to a point where it touches the contact wire within

Figure 6.

The thermoregulator of the temperature control component.

the capillary, at which point, contact is made and the heaters are turned off automatically. Located in the upper portion of the thermoregulator, is a setting scale, contact wire and a follower nut which moves the contact up or down, depending on the rotation of the magnetic setting cap and subsequent rotation of the threaded spindle.

This thermoregulator is in turn connected to a controller which serves as a relay. Since the controller could only handle 600 watts, another relay capable of accepting 3000 watts was attached, in parallel, to the controller and the heaters. Thus the circuit is completed.

Choice and Preparation of Experimental Animals

1. Capture of Specimens.

Three-spined stickleback - the marine adapted form of the three-spined stickleback, Gasterosteus aculeatus, was captured in two ways. At Portugal Cove, a small number were taken from tide pools. The fish were caught by dip-net and returned to the MSRL where they were placed in a large holding tank of 150 L. capacity with a constant flowthrough of sea water for acclimation. During the holding period, sick or dead fish were removed when observed. The second method which provided 90% of the stickleback

Figure 7.

The Avalon Peninsula of the Province of Newfoundland.

1. St.John's.
2. Logy Bay.
3. Portugal Cove.
4. Bryant's Cove.
5. Bellevue.
6. Long Harbour

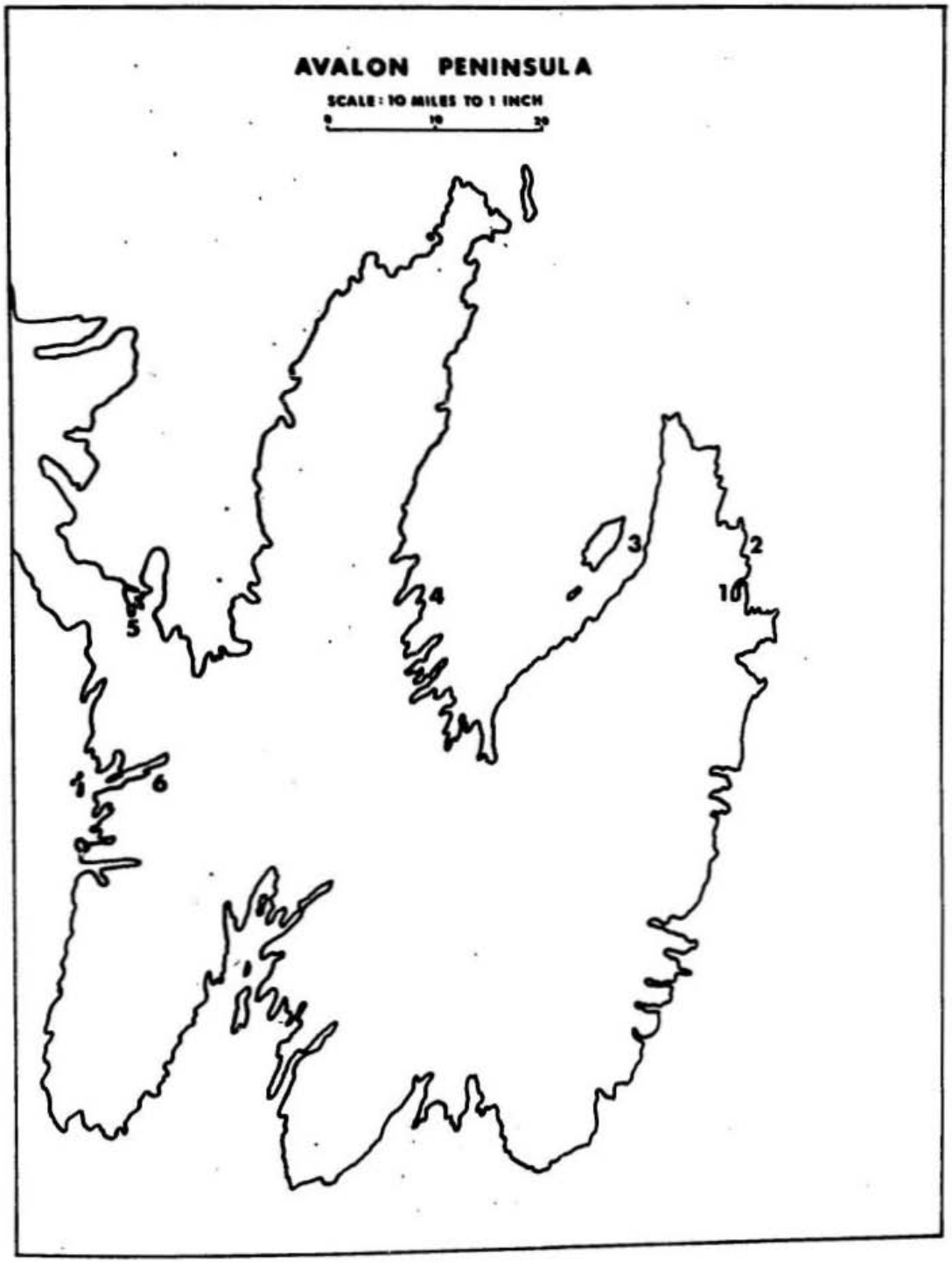


Figure 7.

used in the experiments, involved the use of a 10 meter nylon seine. The seining for these stickleback was done in Broad Lake at Bellevue, Trinity Bay.

Atlantic cod - The number of Atlantic cod, Gadus morhua, required for the phosphorus experiments was more difficult to obtain and various capture methods were utilized.

a) A 10 meter seine was used at Bellevue, usually on the incoming night tide. 75% of the cod were taken in this way.
b) A 30 m. seine borrowed from the Fisheries Research Board of Canada was used at Bryant's Cove, Conception Bay,but few were taken.
c) The squid trap set by the MSRL in Logy Bay and Portugal Cove provided a small number of cod.
d) Steel mesh traps were built and set around the wharf at Portugal Cove. These were constructed with a funnel at one end and a removable screen at the other, and baited with capelin. This method was abandoned after catching a few cod as the traps were continuously being tampered with.

Starfish - The northern starfish, Asterias vulgaris were collected by divers employed by the MSRL, in Logy Bay and Portugal Cove.

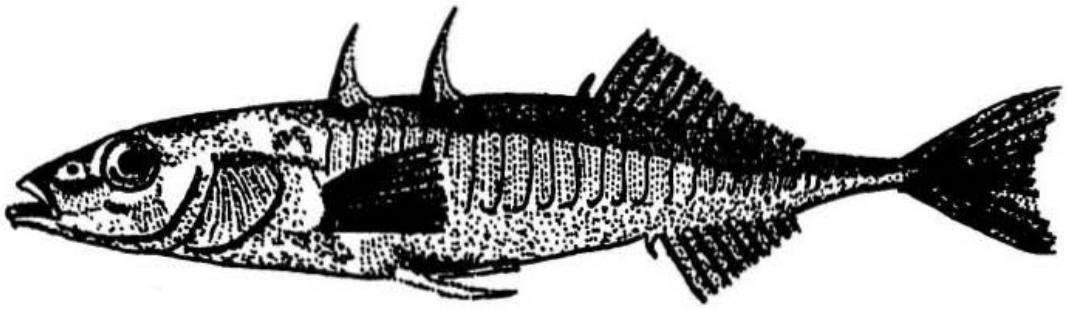
Flounder - Approximately 50 winter flounder, Pseudopleuronectes americanus, were collected in the 10 m. seine at Bellevue.

Figure 8.

The three-spined stickleback, Gasterosteus aculeatus Linnaeus 1758.

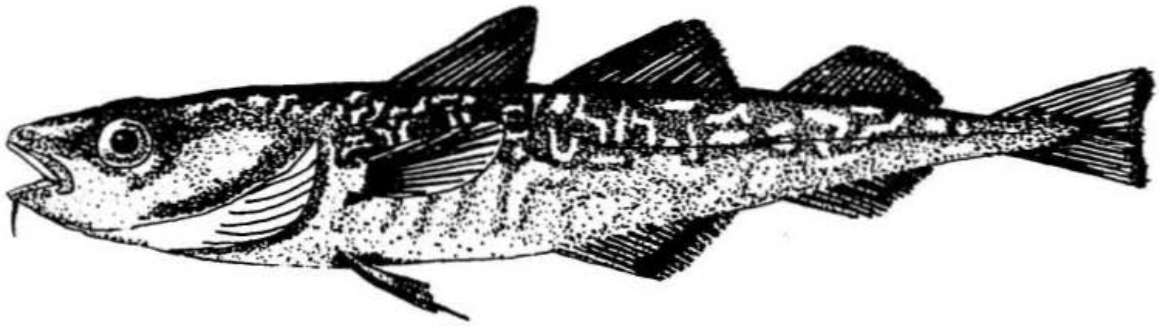
Figure 9.

The Atlantic cod, Gadus morhua, Linnaeus 1758.



Gasterosteus aculeatus

Figure 8.



Gadus morhua L.

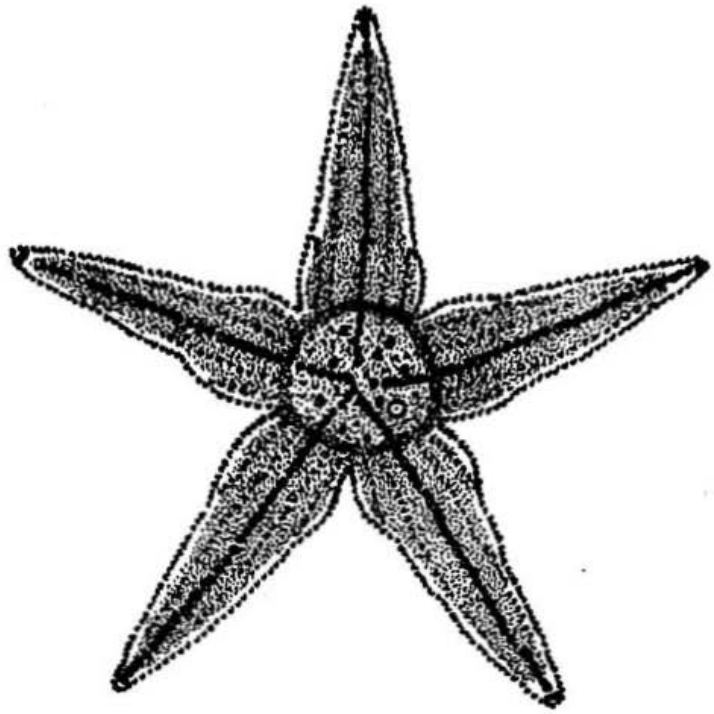
Figure 9.

Figure 10.

The northern starfish, Asterias vulgaris.

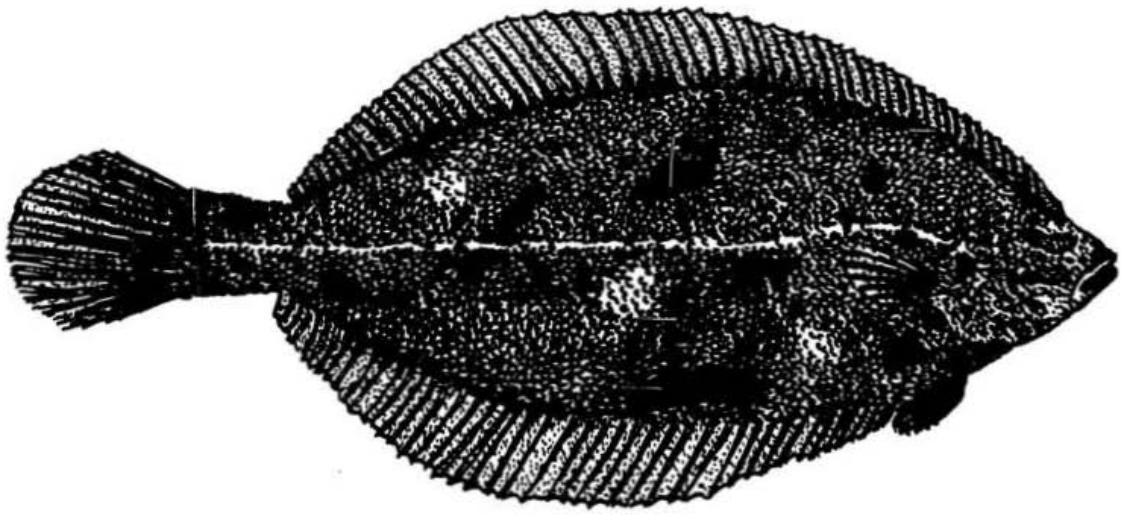
Figure 11.

The winter flounder,
Pseudopleuronectes americanus
(Walbaum) 1792.



Asterias vulgaris

Figure 10.



Pseudopleuronectes americanus

Figure 11.

2. Holding of Test Animals.

The holding tank for the various species was a fiberglass tank of 150L. measuring 48 in.X 32 in. X 12 in.. When a large number of cod were taken, some of these were placed in a cement tank 36 in.X 24 in.X 24 in. in the holding tank room of the MSRL The temperature of the water in both tanks varied with incoming water at the MSRL, and thus was related to the water temperature of the young cod's habitat.

Test subjects were held for at least ten days prior to the initiation of an experiment. Any fish which were not in good condition, due to noticeable physical disabilities, were removed from the holding tank. All specimens were fed daily on capelin during the holding period; feeding was stopped 48 hours prior to the experiment, as suggested by a number of authors. The percentage of cod dying or becoming seriously disabled during the holding period was less than 10% as was the case with flounder; in the case of stickleback and starfish, the figure was less than 1%.

All areas from which specimens were taken were free of industrial pollutants but could have been mildly polluted by sewage. The water from Logy Bay, free of both types, was pumped through the MSRL system which consists of fiberglass-lined P.V.C. pipes, fed by ceramic-lined pumps.

Phossy Water: Nature and Formulation

The dispersions of colloidal phosphorus used in the experimental work, were obtained by three different methods.

1. ERCO Phossy Water.

For the first series of experiments, the phossy water was procured from the Electric Reduction Company of Canada plant at Long Harbour , Placentia Bay. This water was taken from condensers and P_4 holding tanks and as such contained some dissolved phosphorus and variable amounts of colloidally-dispersed particles of yellow phosphorus (see individual experiments and appendices). The dispersions were not pure but contained particles of coke dust and silica. This resulted in a grey color and greater turbidity. The ERCO samples also contained some SO_2 and a reported few ugm/L. of cyanide (Idler,1969). Since ammonia was used to maintain a pH of 7 in the plant process, ammonium phosphate, ammonium silicon fluoride and ammonium fluoride were also present in undetermined quantities.

The water samples were delivered in 20 or 40 liters containers on request.

Experiments were run on the northern starfish, the three-spined stickleback and the Atlantic cod, using the ERCO phossy water to provide the pollutant. The results of these bio-assays are found in the relevant sections of the manuscript.

2.Ultrasonic Preparation.

The second method involved an ultrasonic generator for the preparation of a colloidal dispersion of phosphorus. On advice from Dr.M.Newlands, who had done some phosphorus oxidation studies for the M.U.N.Coordinating Committee on Placentia Bay, immediately after the ERCO pollution incident, 30mgm of P_4 was sonicated in 1 liter of distilled water at $45^{o}C$ for 25 min. (The instrument was a Blackstone generator, Model BP - 2) The phossy water produced was poured into an insulated container, placed on a magnetic stirrer to keep the particles in suspension and pumped into the mixing trough of the S.D.U.. Only one experiment was run with this method.

3. Dissolution in Alcohol.

The last method used was that followed by the Fisheries Research Board In Halifax, in preparing dispersions of phosphorus for their investigations into the pollution incident. Yellow phosphorus was first dissolved in 100% ethanol, then precipitated as colloidal particles by adding de-oxygenated water at $45^{o}C$. The latter was prepared by bubbling N_2 through water for a few minutes. This produced the required dispersion. For the bio-assays with the pure dispersion, 0.08 gms. of yellow phosphorus were precipitated in 1 liter of alcohol-water (100 mls.ethanol - 900 mls. H_2O) to give 80 mgm/L. This method proved to be more practical than the ultrasonic technique.

Volume and Replacement Time of Test Water

The American Public Health Association (1965) has recommended that there should at least be 1 liter of water per gram of fish in static tests. Though these are continuous flow experiments, the nature of the phosphorus and the desire to maintain the fish in a system where other variables such as oxygen concentration and their effects can be minimized, suggested these calculations would be useful.

In the case of 3 tests with Gasterosteus aculeatus, the mean weight of a sample of 300 fish was 1.57 gms (see Table 1). Since there are 32 liters of suspension in each aquarium, each gram of fish had 2.1 liters available at all times. These figures are based on 10 fish per tank. According to Alasbaster and Abram (1965), this is satisfactory.

Table 1. Comparison of the weights of stickleback per tank in the first three ERCO phossy water bio-assays.

Tank Number	Expt.1.	Expt.2.	Expt.3.
0	19.0 gms.	16.8 gms.	16.4 gms.
1	15.8	18.4	16.9
2	17.0	11.3	17.3
3	17.3	13.5	15.1
4	16.9	9.0	15.7
5	16.9	17.2	21.8
6	15.9	17.3	14.0
7	17.2	12.8	14.0
8	16.7	12.2	14.6
9	17.9	13.9	16.5
Average weight of 10 fish in 10 aquaria	17.0 gms.	14.2 gms.	16.2 gms.
Average weight of 1 fish	1.7 gms.	1.4 gms.	1.6 gms.

Since the test apparatus was built for continuous flow bio-assays and a colloidal dispersion was used as the pollutant, it was important to calculate the replacement time of water in the aquaria. Heusner, as reported by Sprague (1969) devised a method to estimate this factor. Using a graph developed by Heusner, and presented in Sprague (1969), I found that 90% replacement of the test suspension takes approximately 6 hours. Sprague considers this more than adequate. This also reasonable when one remembers that the half-life of yellow phosphorus in diluted phossy water is 7.5 hours (Zitko, et al., 1969). If 100% replacement is necessary, this may not be adequate.

Therefore 90% replacement of the 32 liters of suspension which contains an average of 15 gms. of stickleback takes 6 hours; this provides a value of 7.7 L./gm./day. Over a 24 hour period, it would appear that the replacement time is adequate to provide for respiration of the fish and accomodate any oxidation of P_4 in the aquaria.

Experimental Procedure for Bio-assays

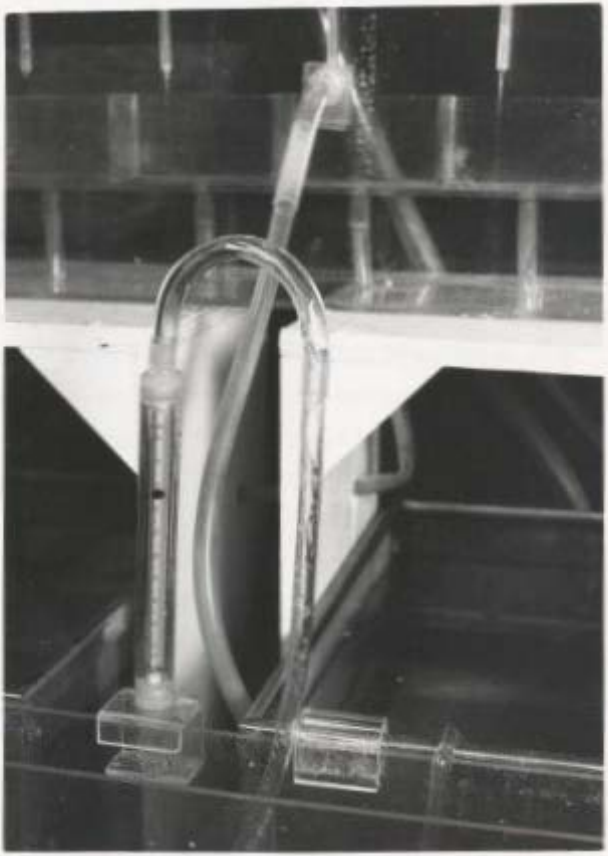
Readings of certain environmental variables were taken at regular intervals throughout the test period. Other techniques relating to the experimental work are also described in this section.

a) Temperature was monitored hourly during the experiments conducted before the control unit was installed. A temperature recorder

Figure 12.

The Gilmont Flowmeter, No. 2.

The flowmeter was installed to facilitate the monitoring of the valves in the S.D.U.



had also been used for two bio-assays. Since temperature dependency work was to be done, and abrupt changes in water temperature were known to occur at the MSRL, monitoring was a necessity to explain the possible effects of a drastic change in temperature in a confined area such as an aquarium, on the toxicity of P_4.

b) pH readings were taken during the tests conducted with the phossy water obtained from ERCO. These were recorded accurately with the ORION Ion Analyzer,with its expanded scale, which was obtained in the latter part of the work with ERCO phossy water.

c) Readings of the flow rates of the valves in the S.D.U. were taken every hour with the Gilmont Flowmeter No.2. (see fig.12). This was to determine if any of the valves were becoming clogged with matter which had not been filtered out. (It was found that algae settled in the troughs and grew rapidly even in diluted phossy water.)

d) During the fall and winter when most of the bio-assays were run, the dissolved oxygen concentration of the sea water at Logy Bay increases markedly at times due to the cooling of the water and the generally rough seas which prevail. The design of the S.D.U. is such that it provides for a loss of some of the excess oxygen as the seawater runs from trough to trough. This is evidenced by the fact that air bubbles were often

seen (when D.O. increased above 8 ppm) on animals in the A-frame trays whereas no bubbles have been noticed on the animals in the test aquaria. These observations on dissolved oxygen , however, were not substantiated by D.O. measurements by colorimetric titration or with an oxygen meter. Direct aeration of the test aquaria was avoided, as it would have increased the oxidation of the phosphorus.

e) Selected specimens were preserved for later study. The mortality time of every fish and starfish was recorded as well as the no. of the aquarium in which it died, and the length of the animal. The weight was also included for two stickleback experiments to obtain an indication of possible toxicity weight relationships. Those animals which were collected for possible histological study at a later date, were fixed and preserved as follows: Vertebrates were placed in Dietrich's solution for 10 minutes and stored in neutral formalin. Invertebrates were fixed in a modified Zenker's solution (Yevich - personal communication) before storage in neutral formalin. The period of fixation was 12 hours for the starfish.

The sticklebacks were preserved in individually labelled bottles while the cod were tagged and stored in a sealed plastic tray. Livers of the cod and flounder were fixed and preserved as the fish became moribund in the final 10 day bio-assays on these species. The livers were placed in labelled vials.

f) For the majority of bio-assays, ten animals were used to provide

a reasonable basis for interpretation and ease of computation. If a smaller number of animals was used, it was due to the difficulty of obtaining the required number. Though the cod were larger than the stickleback, the species used for the above computations on replacement etc..., the cod demonstrated no ill effects in the control aquaria.

g) In order to eliminate the possibility of bias in specimen handling, organisms were distributed on a random basis to the test and control aquaria, after being taken from the A-frame holding tank. For this purpose, the table of random numbers in Downie and Heath (1965) was used.

h) Morphological and behavioral changes from the norm were recorded as descriptively as possible in the log book. These are described in the relevant sections of the thesis.

Hematological Procedures

When work on the blood was contemplated, hematocrits were an obvious choice because of the work of Fletcher(1970) and Ackman(1970). Only white cell counts were done (as far as counts are concerned) because of difficulties encountered due to the rapid clotting of fish blood.

Two methods recommended in the literature (Snieszko,1960) were tried to ascertain the most practical one for obtaining the samples. The first involved dissection to reach the heart; it was then found

that the pericardial cavity was difficult to keep dry and generally more difficult to remove the blood from a particular spot. The second method consisted of removing the caudal fin at the peduncle and inserting a capillary tube at the open end of the dorsal aorta. At this time a drop of blood was put on each of three slides for the counts. The latter method proved simpler and more efficient.

1. Hematocrit.

Hematocrit refers to the relative volume of blood corpuscles in a given volume of whole blood. The red cell volume is the prime consideration in this case.

The capillary tubes used to take up the blood were heparinized, 75 mm. in length and 1.4-1.6 mm in diameter. Duplicate samples were taken from each specimen and centrifuged on a Clay-Adams centrifuge for 5 minutes at 12,500 rpm. The capillary tube was placed along a millimeter ruler and the relative percentage volume is calculated directly from the lengths.

2. Differential White Cell Count.

Blood was spread on a slide in the regular fashion. When the smear was dry, it was immersed in ethanol for fixing. The slide was then placed in a dish containing a Giemsa staining solution (99:1) for forty-five minutes. It was then washed under running water for 1 minute and finally placed in a slide box for drying.

Counts of 100 white cells comprising lymphocytes, monocytes, and thrombocytes were made on each slide. Cells from two slides were counted and the percentage of each type on each slide was averaged.

These counts were made on control and toxicated specimens.

Preparation of Toxicant Samples for Analysis

A quantity of water was withdrawn from the test aquaria, and 100 mls were measured in a graduated cylinder. This sample was poured into a 150 mls separatory funnel. When 50 mls of benzene were added, air was forced out so that oxidation problems were minimized. The funnel was then shaken. The layers were allowed to separate, and the heavier fraction , water, was drained off. The solvent was drawn off into a 100 mls. bottle, and labelled.

The samples were held at -10°C until ready for shipping, packed in dry ice and airfreighted to the Fisheries Research Board Laboratory, Halifax where they were stored at -40°C until analyzed.

This procedure was discontinued, however, as of January,1970 because of the difficulty in preparing samples and the erratic results obtained by analyses. This is not a reflection on those conducting the analyses, but rather on difficulties inherent in dealing with elemental phosphorus. For this reason, then,concentrations of yellow phosphorus were calculated from amounts added to the system.

Test for Anti- Acetyl Cholinesterase Activity of P_4

Static tests were run in four fiberglass aquaria, each one containing 10 sticklebacks. Two tanks were used as controls while the other two contained a dispersion of 3 mgm/L. of colloidal

phosphorus prepared by the alcohol dissolution method. In all cases, the toxicated specimens were removed just before death and placed in a solution of M.S.222, 0.1% by weight, (Tricaine methanesulfonate; Sandoz Pharmaceuticals Ltd.). The same procedure was followed with the control fish and these were removed at the same time as the moribund fish. M.S.222 provided for a quick death. The analytical procedure as described by Weiss (1958) follows.

1. The brain was dissected out (all parts from and including the medulla oblongata forward) and wet-weighed;
2. The brain was homogenized in a phosphate buffer containing 0.2M NaCl, 0.02M $MgCl_2$, 0.19M K_2HPO_4, 0.006M KH_2PO_4, with the final pH adjusted to 8.2;
3. The brei (homogenized brain tissue) produced was then diluted to 3.5 mgm/ml. with buffer to the nearest 0.1 ml. Weiss demonstrated that the greatest enzyme activity occurred at a concentration of 3.5 mgm/ml;
4. The diluted brei was left for 4 hours at $25^{o}C$;
5. One ml. of the diluted brei was pipetted into a test tube and incubated for 20 minutes at $25^{o}C$ with 1.0 ml. of 0.004M acetylcholine chloride prepared in 0.001M sodium acetate at pH of 4.5;
6. Two mls. of alkaline $NH_4OH \cdot HCl$ (NH_4OH : HCl, 1:1) were then added to 1.0 ml. of the solution to be analyzed;
7. After 1 minute, the pH was brought to 1.2 ± 0.2 with 1.0 ml. of 50% HCl and finally 1.0 ml. of 0.37M $FeCl_2$ was added;

8. The residual acetylcholine was then determined by reaction with hydroxylamine and the resulting color was read on a Spectronic 20 photometer at 540 mu.

Tests of the Actual Dilution Ratio of the S.D.U.

1. The first test was run after the Unit was completed. A neutral red dye solution was made and pumped into the mixing trough at a set rate. Samples were taken from the test aquaria and forwarded to the Department of Chemistry for reading on a spectrophotometer. Originally, the values were erratic. It was concluded that styrofoam blocks could be placed in the individual sections of the mixing trough to compress the sections, thereby increasing the mixing. These blocks were positioned so that the solution or suspension would be forced under each one to further increase the mixing. A new dye solution was then run. Results can be found in the next section of the thesis.
2. Upon further modifications of the blocks, new calibration tests were run. These involved salinity titrations using fresh water as the diluent. The method employed was that described in Kolthoff and Sandell (1965): An indicator solution of 5% K_2CrO_4 in water was made up. Also required was a 0.1N $AgNO_3$ solution. Using a saturated solution of NaCl (35gms/100mls),

the system was allowed to equilibrate as the solution was pumped in, and samples were taken from the test aquaria. One ml. of indicator solution was added to a 25 mls. sample of the diluted NaCl solution. $AgNO_3$ was then titrated until the first permanent color change from the yellow of the suspension was obtained. This was a change from yellow to a reddish color. A standard was made up for comparison with an indicator blank in a control sample.

RESULTS

Performance of the Serial Dilution Unit

The S.D.U. was developed to provide eight dilutions of a toxicant, each concentration being 50% lower than the previous one. To find out whether the actual concentration agreed with the theoretical design, the unit was calibrated by two methods already referred to, after modifications were made to the mixing trough.

The values obtained by colorimetric analyses after the dye test represent the average of two readings and are as follows.

Table 2. Ratios obtained with the neutral red dye test.

Tank Number	Ratio*
0	-
1	-
2	1.86: 1
3	1.86: 1
4	1.56: 1
5	1.96: 1
6	1.93: 1
7	1.60: 1
8	control
9	control

* each number is regarded as 1 when establishing the ratio of the following number.

In the case of the test with the sodium chloride, two completes series of samples were titrated and the resulting mean ratios are as listed in table 3.

Table 3. Ratios obtained in test with NaCl solution.

Tank Number	Ratio
0	1
1	1.94: 1
2	2.02: 1
3	2.01: 1
4	2.06: 1
5	2.00: 1
6	1.92: 1
7	1.87: 1
8	control
9	control

A chi-square test was done on the latter values for goodness of fit and it can be said that deviations from the mean, that is 2.00, in all seven cases are not significant at the 99% confidence level.

pH Values

The Orion Ion Analyzer (Model No. 407) provided the precise metering required for measuring differences in the pH of test water in the individual aquaria during the phossy water experiments. The reason for this was probably the expanded scale of the meter which provides sufficient sensitivity to successfully monitor the small pH shifts. The Corning pH meter (Model No. 7) had not recorded the marked variations more easily recognized on the expanded scale.

Readings were not taken for each experiment, however, values were obtained for the second and third bio-assays (see Appendices II and III). These were conducted with phossy water from ERCO rather than colloidal dispersions prepared in the laboratory.

The heterogeneous nature of the phossy water from ERCO probably accounts for the variable readings listed in tables 4 and 5. There is definitely a gradation of pH values from lowest at the stronger concentrations to highest in the control aquaria.

Table 4. pH values for bio-assay with ERCO phossy water at atemperature of 8.0°C (see Appendix II)

Tank Number	pH series 1	pH series 2
0	6.48	6.92
1	7.30	7.22
2	7.52	7.40
3	7.71	7.76
4	7.78	7.82
5	7.79	7.90
6	7.75.	7.94
7	7.90	7.96
8	7.95	8.05

Table 5. pH values for bio-assay with ERCO phossy water at a temperature of 3.5°C (see Appendix III).

Tank Number	pH series 1	pH series 2
0	6.10	6.28
1	6.70	6.78
2	7.28	7.14
3	7.48	7.48
4	7.64	7.76
5	7.92	7.90
6	8.0	7.96
7	8.0	8.0
8	8.0	8.0

Reproducibility of Bio-assay Results

Two bio-assays were run to compare the results of experiments run under similar conditions. Both bio-assays were conducted at 8.0°C. A sample of 100 three-spined stickleback was employed in each case and these were essentially the only variables. Figure 13 demonstrates that even when all physical parameters are kept constant for two consecutive experiments, the results obtained will not be perfectly reproducible; the closeness of the lines suggest however that data from one sample can be extrapolated to another similar sample. Figure 14 on the other hand compares mortalities in individual tanks as well as demonstrating the variability in response of the fish in any one aquaria. Sprague (1969) suggests that this analytical procedure may be of use in determining whether there are different modes of toxicity demonstrated by changes in slope, for example.

Comparative Toxicity of Various P_4 Particle Sizes.

An experiment was conducted using a colloidal dispersion prepared with the ultrasonic generator, but which failed to produce any deaths among the sticklebacks during the 48-hour bio-assay. This was all the more baffling, because an experiment run the following week, under similar conditions using the alcohol dissolution method had caused the desired effect, in this case, death of the fish. An effort was therefore made to determine the reason for this occurrence.

Figure 13.

Toxicity curves of two bio-assays run under the same conditions.

A comparison of two bio-assays conducted under the same conditions using two samples from the same stock of Gasterosteus aculeatus.

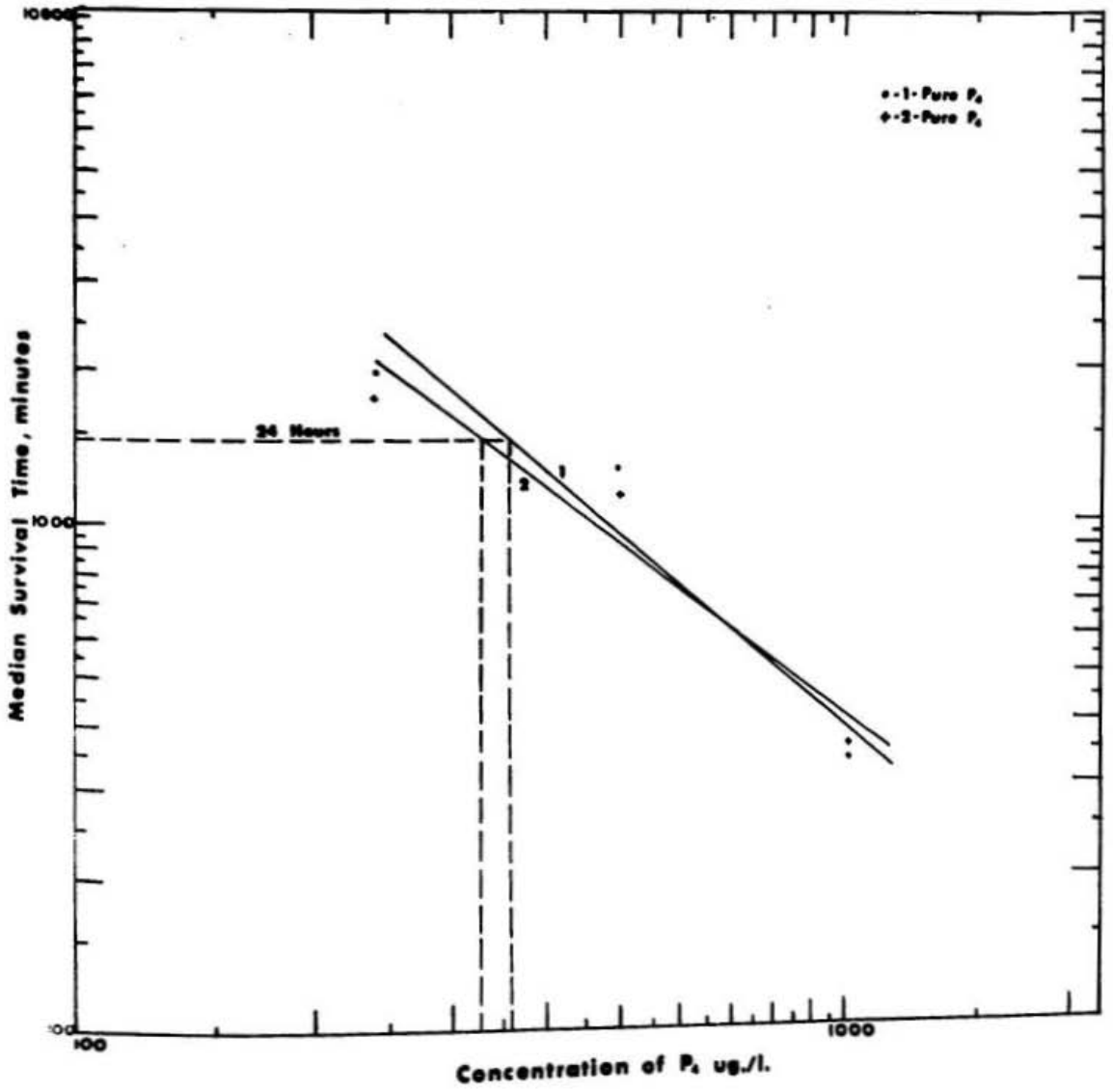

Median Survival Time, minutes
Concentration of P4 ug./l.
24 Hours
1
2
100
1000

Figure 13.

Figure 14.

Mortalities in individual aquaria for each of two similar bio-assays

A comparison of mortalities in the individual test aquaria for each bio-assay on stickleback. A point (·) denotes the first test while a cross (+) denotes the second experiment.

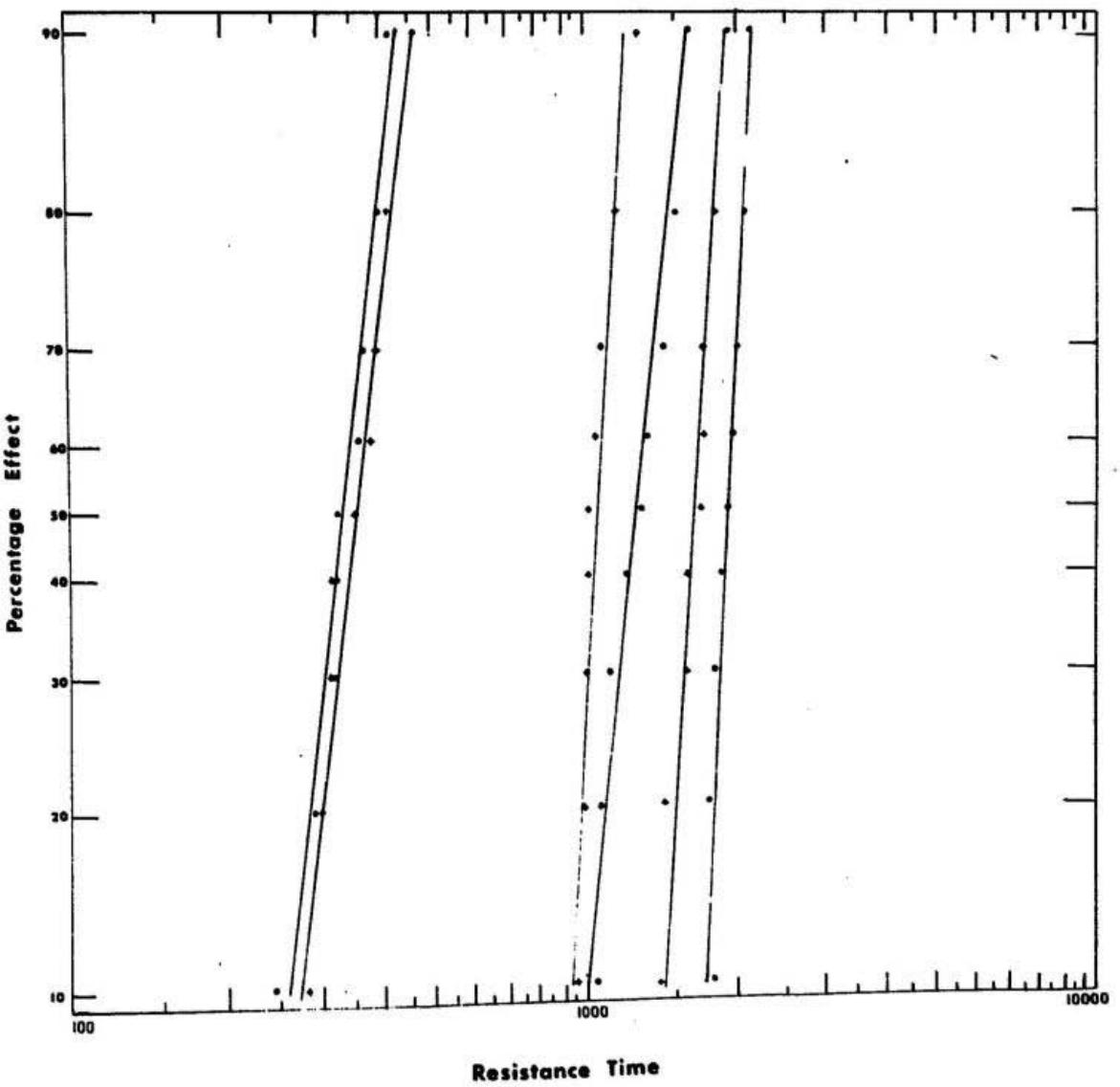

90
80
70
60
50
40
30
20
10
Percentage Effect
100
1000
10000
Resistance Time

Figure 14.

The first thought was that excessive oxidation was taking place. The second hypothesis suggested that the particles of phosphorus from the ultrasonic generation method were larger than those from the precipitation of dissolved phosphorus from alcohol.

Observation of P_4 particles produced by the two methods, using phase microscopy and oil immersion objectives demonstrated size differences of the majority of the particles in each case. The system used for observation comprised a slide to which had been applied, with silicone grease, a fiberglass ring divided into two parts so that both types of dispersion could be seen on the one slide. This made comparison much simpler. Drops of phossy water, prepared by the two methods (see pages 36 and 37), were added to fill the enclosures and a cover slip was pressed into place.

Table 6. Particle sizes of the dissolution in alcohol method and the ultrasonic generation method.

Type	Range of sizes (mm.)
Dissolution in alcohol	
99% distribution	5.7×10^{-4}mm. to 9.5×10^{-4}mm.
75% distribution	5.7×10^{-4}mm. to 7.8×10^{-4}mm.
Ultrasonic generation	
90% distribution	8.0×10^{-4}mm. to 1.5×10^{-3}mm.

The findings listed in table 6 indicated that additional work on particle size was necessary. For this reason, a series of tests was begun to compare the effects of various kinds of phosphorus dispersions. Seven 20 L. fiberglass aquaria were set up with 10 L. of water containing elemental phosphorus in the following preparations.

Aquarium 1. 30 mgm of P_4 were dissolved in 1 liter of water at at 30°C by shaking until no P_4 could be detected by eye and then diluted to 10L. with seawater. This provided a concentration of 3 mgm/L.. This concentration was chosen arbitrarily however bio-assays had shown that it was strong enough to cause death in a short time;

Aquarium 2. 30 mgm. of P_4 were added to 10 liters of seawater as a single piece of phosphorus. The container was not shaken;

Aquarium 3. 30 mgm. of phosphorus were dissolved in 100 mls of ethanol and poured into 900 mls of deoxygenated water at 45°C, to form a colloidal dispersion. This was then diluted to 10 liters;

Aquarium 4. As no. 3 except that the dispersion was filtered through 2 pieces of Whatman filter paper (size no.1);

Aquarium 5. As no. 3 except that the phosphorus dispersion was filtered through a millipore filter capable of removing particles larger than 0.45u;

Aquarium 6. As no. 3 except that the P_4 dispersion was filtered through a millipore filter capable of removing particles larger than 0.22u;

Aquarium 7. Seawater only serving as a control.

In each case, 10 sticklebacks were taken from the A-frame trays and placed in the test aquaria. Critical observation of the time of death of each fish was not a necessity and only time range for the mortalities was recorded for each aquarium.

Table 7. Range of mortality times for the 7 aquaria containing the different preparations.

Aquarium Number	Range for time of death
1	12 + hours (50% mortality after 15 hours)
2	24 + hours (similar to the control fish)
3	3 - 5 hours
4	3 - 5.5. hours
5	6 - 10 hours
6	8 - 13 hours
7	20 + hours (40% mortality after 30 hours)

Toxicity of Pure P_4 Dispersion vs ERCO Phossy Water

Two bio-assays were conducted to determine whether there was a marked antagonistic or synergistic effect which could be

Figure 15.

A comparison of the toxicity of a pure P_4 dispersion vs the toxicity of ERCO phossy water.

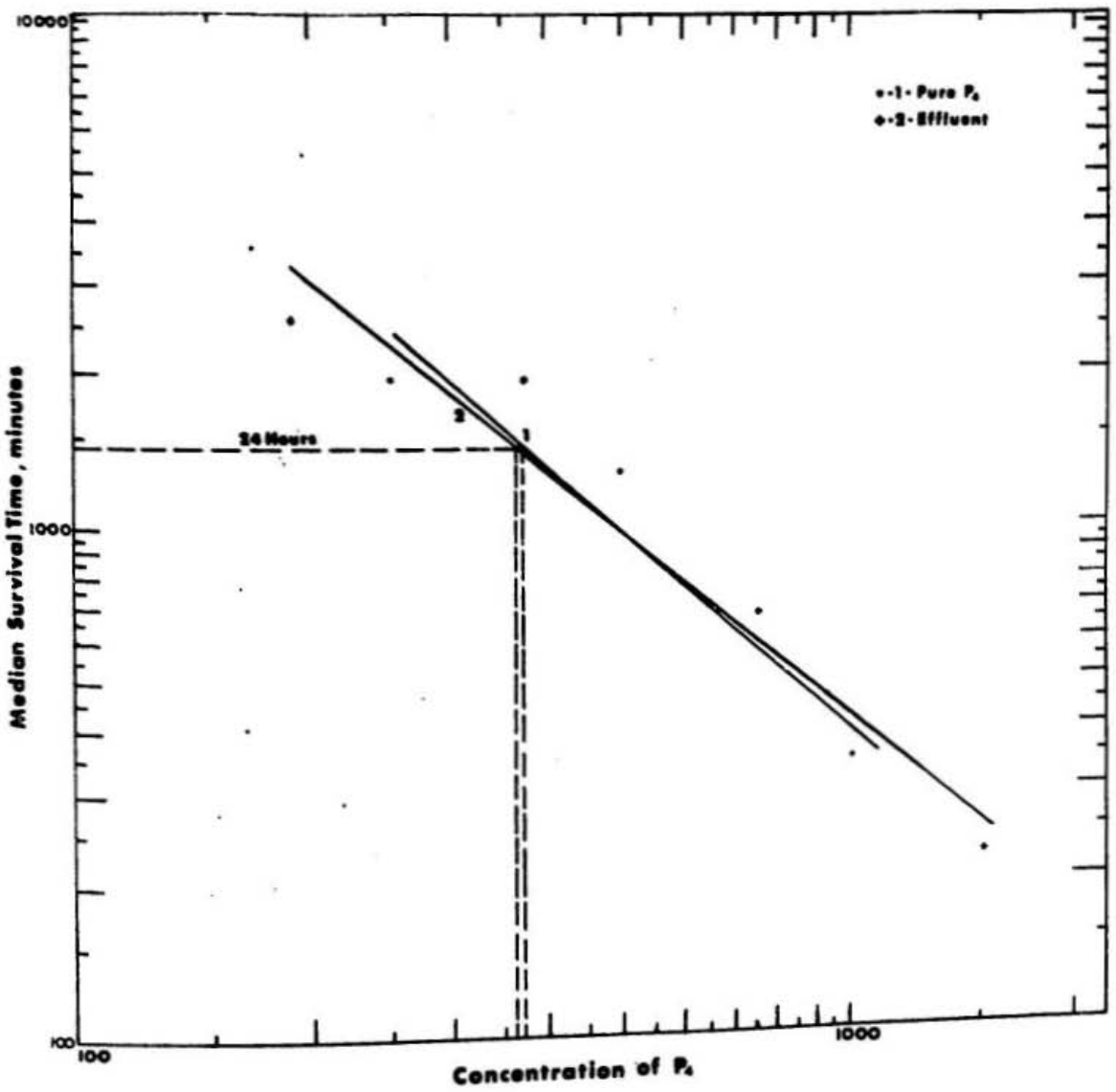

10000
1000
100
100
1000
Median Survival Time, minutes
Concentration of P4
24 Hours
1
2
•-1- Pure P4
•-2- Effluent

Figure 15.

attributed to components of the phossy water from the ERCO plant at Long Harbour. These two experiments were run at a temperature of 8.0°C. It should be stated that most of the experiments in this thesis were conducted at 8.0°C because it is an intermediate temperature in the range to which marine animals are subjected in Eastern Newfoundland waters. That temperature is reached twice during the year, that is, in early summer and again in the fall as the sea cools.

Figure 15 graphically demonstrates the results of this work. The variation between the lines obtained by plotting median survival time against concentration, is no greater than that obtained from the two experiments conducted to determine the reproducibility of bio-assay results (see fig.13). Times of death of the individual fish can be found in Appendices II and IV.

Toxicity of P_4 to the Three-Spined Stickleback

Six experiments were run on the three-spined stickleback. The toxicant used for the first three was ERCO phossy water and these tests were designed to relate temperature and toxicity of yellow phosphorus. The last three bio-assays with Gasterosteus aculeatus utilized a pure colloidal dispersion of P_4. Two of the latter were replicates of each other to show that the data obtained are reliable;the last experiment was run to demonstrate

Figure 16.

A toxicity curve for a 100 hour bio-assay on the three-spined stickleback.

In this graph, 95% confidence limits for the median survival times are included as well as the 24-hour and 48 hour TLm values.

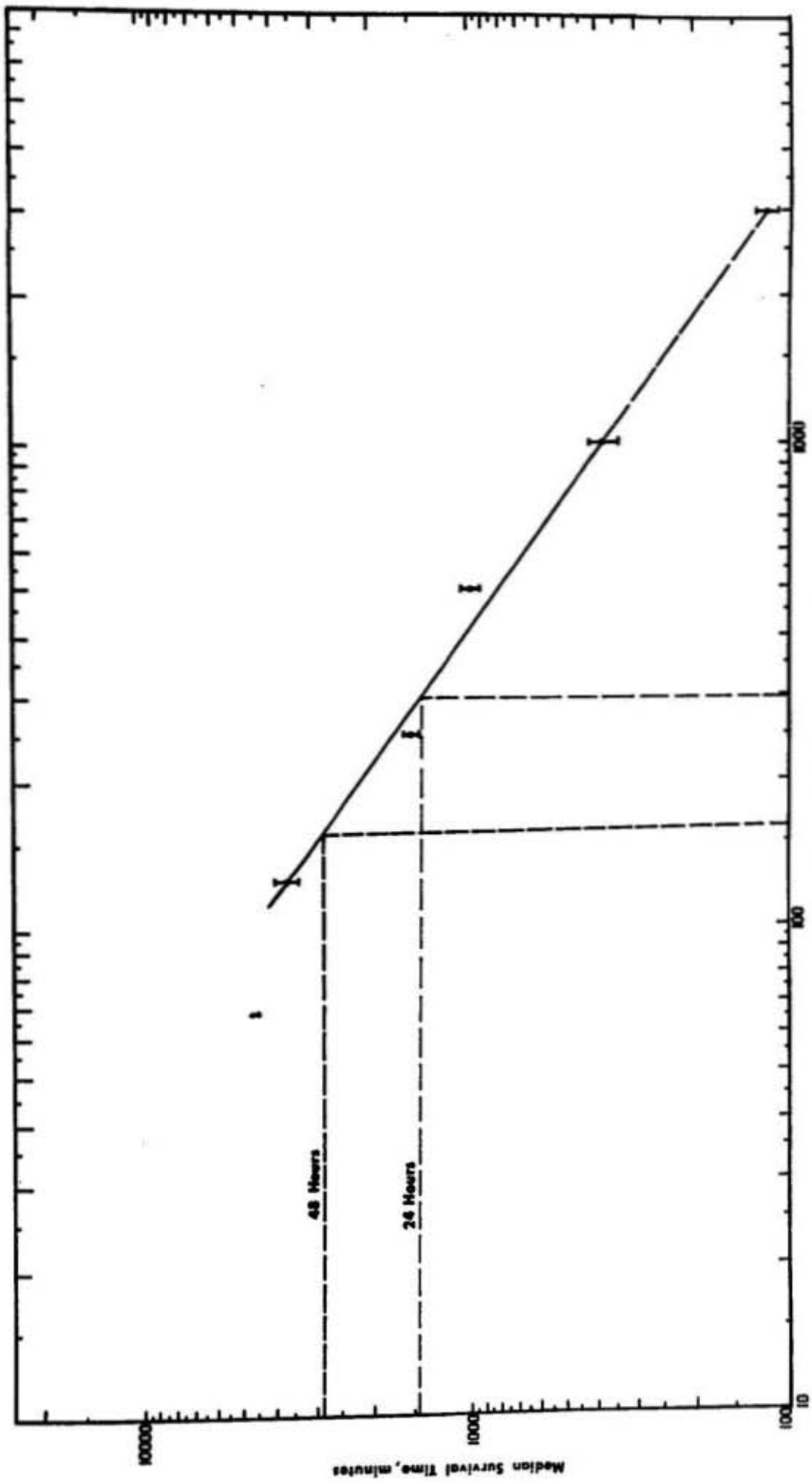

Median Survival Time, minutes
10000
1000
100
10
1
48 Hours
24 Hours
10
100
1000
10000
Concentration of Phosphorus, mg/l.

Figure 16.

an incipient lethal level - defined as that level of the environmental entity beyond which 50% of the population can no longer live for an indefinite period of time (Fry,1947).

The aquaria were continually monitored during the experimental period and temperature, pH and flow observations were made hourly. Time to death was recorded as closely as possible as well as any behavioral or morphological aberrations which were recognized. The median tolerance limit (TLm, the concentration at which there is 50% survival) was determined for 24 hours and 48 hours in each case. Appendices I through VI provided the raw data for these calculations.

Table 8. The 24 and 48 hour median tolerance limits for six bio-assays on Gasterosteus aculeatus.

Experiment	24 hour TLm	48 hour TLm
1	200 ugm/L.	110 ugm/L.
2	350 ugm/L.	180 ugm/L.
3	400 ugm/L.	190 ugm/L.
4	340 ugm/L.	190 ugm/L.
5	375 ugm/L.	200 ugm/L.
6	300 ugm/L.	160 ugm/L.

Toxicity curves have been drawn on log-log paper while graphs of mortality in individual tanks have been plotted on log-probit paper as suggested by Sprague (1969). In this latter case, survival or resistance time in minutes appears on one axis, while in estimating the TLm, concentration appears on that axis.

Figure 17.

Mortalities in individual aquaria during the 100 hour bio-assay on the three-spined Stickleback.

Survival times of the sticklebacks plotted against percentage effect on log-probit paper.

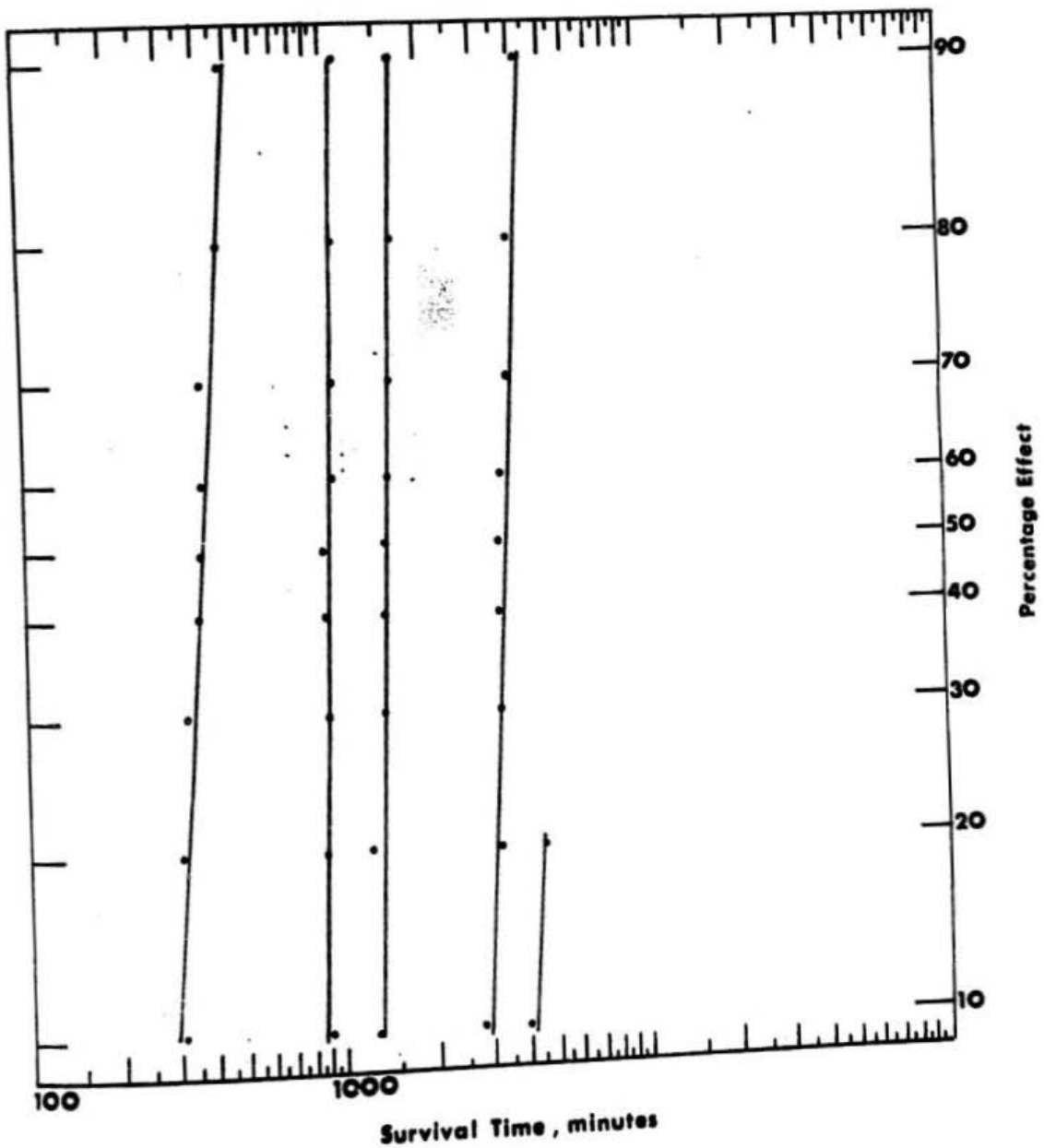

90
80
70
60
50
40
30
20
10
Percentage Effect
100
1000
Survival Time , minutes

Figure 17.

1. Observations on the Behavior of the Stickleback

Within 1 hour of the time that the test fish were introduced to the aquaria containing greater than 1 mgm/L., the movements of the stickleback became sluggish. In most cases, prodding with a glass rod did not accelerate movements. A short time before death, the fish would demonstrate some irregular activity: sinking to the bottom, then darting upwards, dropping to the bottom again then another effort to rise to the surface ... this was repeated as many as a dozen times before death.

Gasterosteus aculeatus in aquaria contianing 0.5 mgm/L. to 1.0 mgm/L. began moving more slowly within the first two hours but generally did not demonstrate the violent outbursts of activity of those fish in the stronger concentrations. Occasionally, a fish would swim to the surface, seemingly gulping for air, with its head completely out of water. This behavior conforms with observations made by fishermen on Long Harbour fish toxicated by ERCO effluent at the time of the pollution incident. As in the case of higher toxicant concentrations, the sticklebacks lying on the bottom were affected by body spasms.

Further observations of the sticklebacks during the pure phosphorus experiments have demonstrated very laboured breathing while the fish were resting on the floor of the test aquaria, two to three hours before death. Opercular movements were slow

and the opercula were widely distended during each movement. (20 opercular movements per minute as opposed to at least double that number in normal sticklebacks; the comparison was made with fish in a concentration of 0.5 mgm P_4 per liter) Occasionally, a fish would attempt to propel itself to the surface using only its pectoral fins.

The sticklebacks demonstrated a loss of equilibrium or coordination in concentrations greater than 0.5 mgm/L.. On prodding, they were not able to swim from point A to point B directly, with their bodies in a dorso-ventral orientation. In the same concentrations, at least 20% of the stickleback swam in circles for short periods of time. This fact, as well as fish often using their pectorals only, led one to speculate that the animals were losing or had lost control over their caudal areas.

As often occurred with the control fish previous to death, the toxicated animals spent a great part of their final hours near the surface, finally dropping to the bottom of the aquaria about 1 hour before death ensued. Similarly in control fish near death, the sticklebacks lost control over the melanophores on their dorsal surface so that the animals were much darker (very nearly black) at that time.

2. Temperature - Toxicity Relationship.

Three bio-assays were conducted,at temperatures of 13.5^{o}C, 8.0^{o}C and 3.5^{o}C respectively, with 100 fish in each case using

ERCO phossy water as the toxicant. Table 1 provided the total weights of fish per aquaria and suggested that differences in toxicity as demonstrated in this experiment could not be accounted for on the basis of weights.

Using the methods and calculations of Litchfield and Wilcoxon (1948), the following data were obtained. Expected and corrected values of percentage mortality (see original paper) were calculated for 48 hours only. A Chi-square test demonstrated that the line drawn in each case was a good fit (see fig.18).

Table 9. Some statistics developed from Litchfield and Wilcoxon (1948) to compare bio-assays run at temperatures of 13.5°, 8.0°, and 3.5°C

Type of statistic	Expt.1	Expt.2	Expt.3
48 hour TLm	230 ugm/L.	130 ugm/L.	225 ugm/L.
S- slope function*	2.32	1.42	1.27
95%confidence limits	327-161	162-104	249-202
f_s-factor for S	1.24	1.15	1.13
S.R.-slope function ratio**	1.62	1.12	1.82
f_{sr}- factor for S.R.	1.45	1.18	1.42
P.R.-potency ratio	1.70	1.70	1.00
f_{pr}-factor for P.R.	1.52	1.26	1.42

* slope function is the factor by which a concentration must be multiplied or divided to produce a standard deviation change in response.

**all ratios in column 1 are for 1/2; column 2 are for 2/3; column 3 for 1/3.

Figure 18.

Toxicity curves of bio-assays run on the three-spined stickleback at 13.5°, 8.0° and 3.5°C.

In the text, the bio-assay conducted at 13.5°C is referred to as experiment no.1, that run at 8.0°C as no.2 and 3.5°C as experiment no.3.

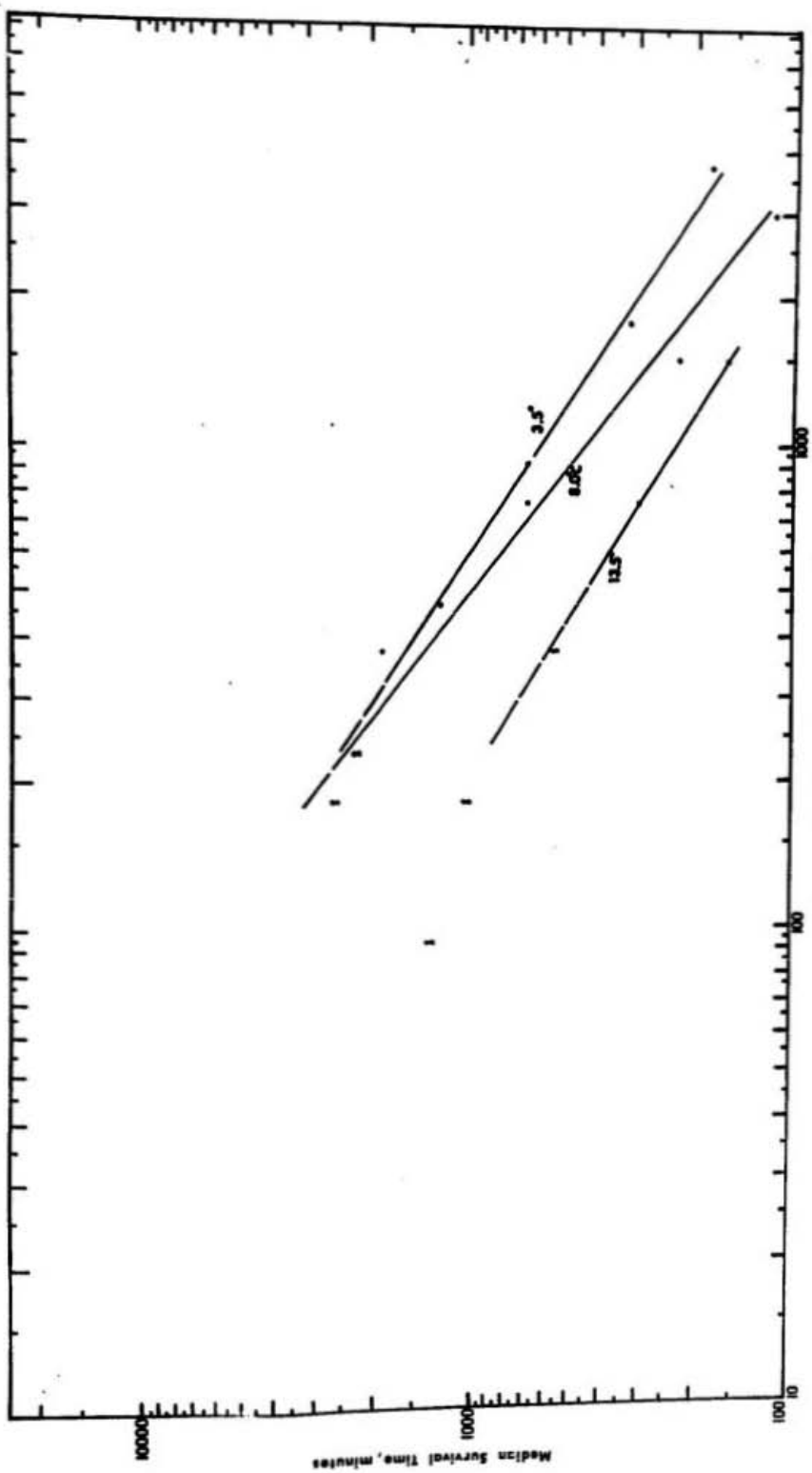

Median Survival Time, minutes
10000
1000
100
10
100
1000
Concentration of Phosphorus in Effluent, mg/l.
3.5°
8.0°
12.5°

Figure 18.

Toxicity of P_4 to the Atlantic cod

A preliminary bio-assay was conducted to give an indication of the gross toxicity of phossy water to the Atlantic cod, Gadus morhua. This bio-assay included eight dilutions and two controls though only two fish were introduced to each aquarium (see Appendix VII). A recording thermometer demonstrated that the temperature of the seawater was relatively uniform throughout the test period: 11.0 ± 0.5°C. A stock suspension of phossy water was obtained from ERCO, containing 150 mgm P_4 per liter ± 25%. (This factor applies to all ERCO phossy water in this thesis, as their laboratory methods of analysis were not accurate at that time.)

Within 110 minutes of the introduction of the young cod (size of aquaria restricted the cod to those of 2 years or less as verified by aging using the scales) into the aquarium containing 0.5 mgm/L., the fish began demonstrating behavior similar to that of the stickleback. The cod would swim with their heads out of water, apparently gulping air. Within 5 hours both cod in that tank were dead (defined as lack of response to prodding). Approximately 240 minutes later, both cod in the second tank (0.25 mgm/L.) had died. At 18 hours, those in aquarium 3 had died, all exhibiting the same behavior prior to death.

In this preliminary experiment, no cod demonstrated the redness prevalent in herring (Zitco,1970).

Figure 19.

A toxicity curve for the 48 hour bio-assay on cod using ERCO phossy water.

Points with arrows, on the figure, denote that all fish in the individual aquarium had not died and that the median survival time would likely be higher had the bio-assay been conducted for a longer period of time.

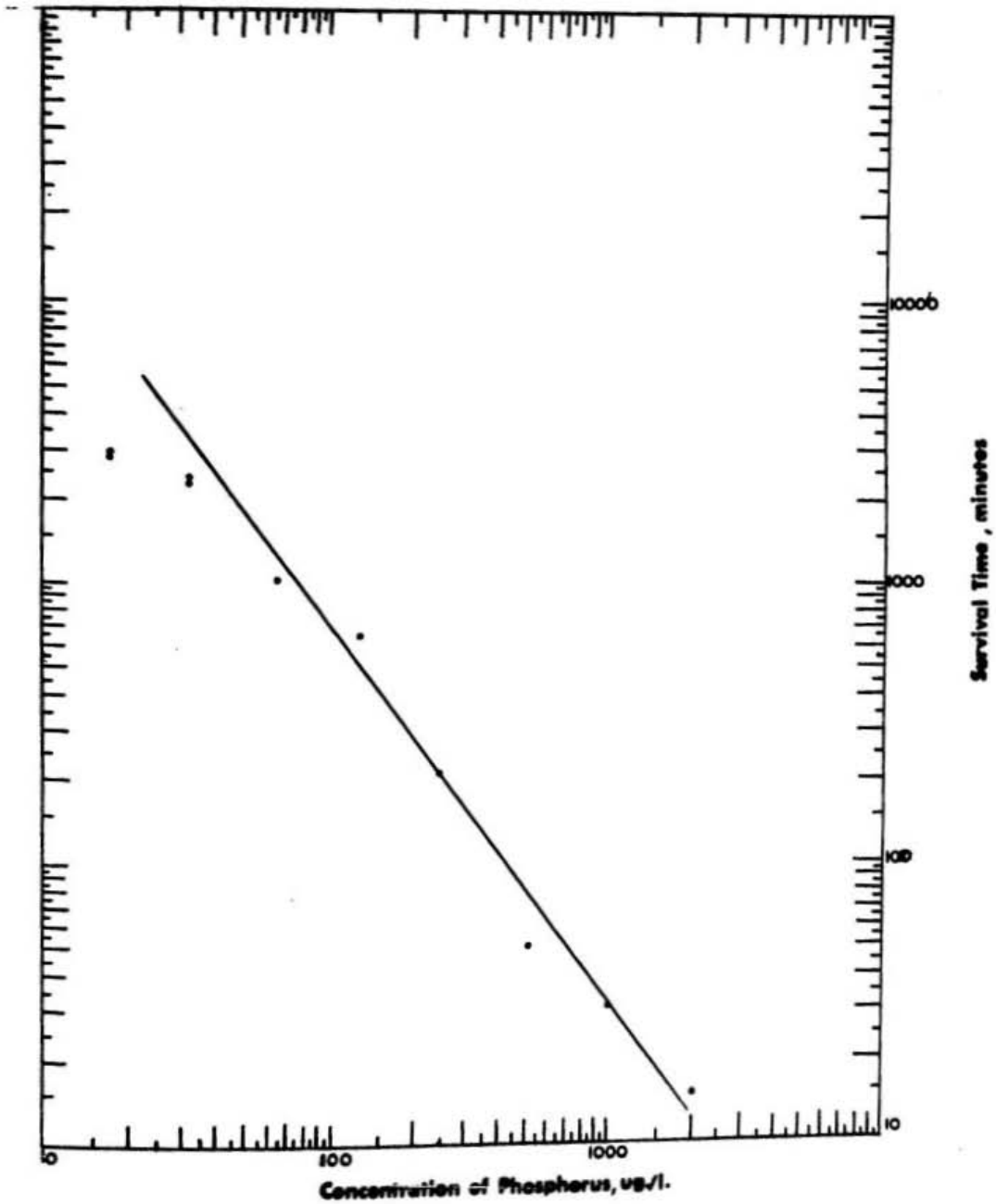

10000
1000
100
10
Survival Time, minutes
5
100
1000
Concentration of Phosphorus, ug-/l.

Figure 19.

Figure 20.

Graphical interpolation of the TLm values for 12, 24, and 48 hours of the 48 hour bio-assay on Atlantic cod using ERCO phossy water.

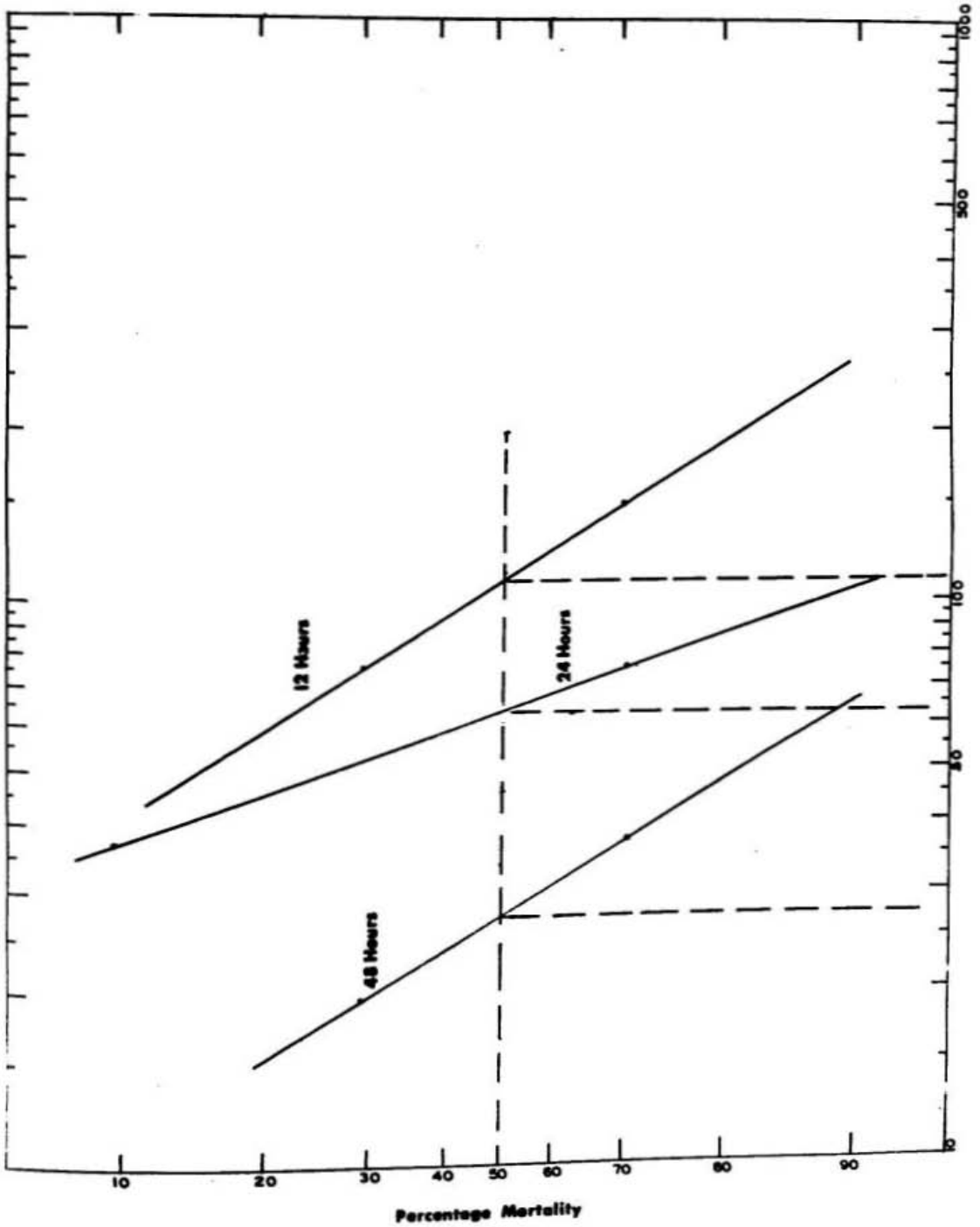

12 Hours
24 Hours
48 Hours
10
20
30
40
50
60
70
80
90
Percentage Mortality
1000
500
100
50
0
Concentration of Phosphorus, ug/l.

Figure 20.

Further observations on behavioral aberrations were gathered from other experiments conducted on Gadus morhua (see Appendices VIII and IX). Cod placed in dispersions containing 1 mgm P_4/L. and greater are affected within a few minutes of the time they are introduced to the aquaria. These fish alternately appeared sluggish and then excited; when the bio-assay unit is approached, the cod normally reacted by swimming excitedly around the tank (as do the control fish) but nearer the bottom; in the case of poisoned fish, neither approach, nor movement of hands elicited this reaction. Occasionally a cod would swim around the surface of the water with its head,at times, completely out of water.

Loss of equilibrium and swimming control was more marked in some cod, while not so easily recognized in others. The reason for this is not known. The fish have been seen swimming directly into the walls of the container, on their sides, as well as with their ventral surfaces uppermost. Tremors of the body were often noticed during erratic swimming and while the fish were lying on the bottom.

The above mentioned behavior is noticed but to a lesser extent as concentrations drop to 100 ugm P_4/L.. Below this concentration, the cod were overcome during a longer period but death followed a more normal pattern.

Figure 21.

Mortalities in individual test aquaria during the 10 day bio-assay on Atlantic cod using a pure P_4 dispersion.

Changes in slope of the lines may indicate a change in the mode of toxic action as well as the incipient lethal time.

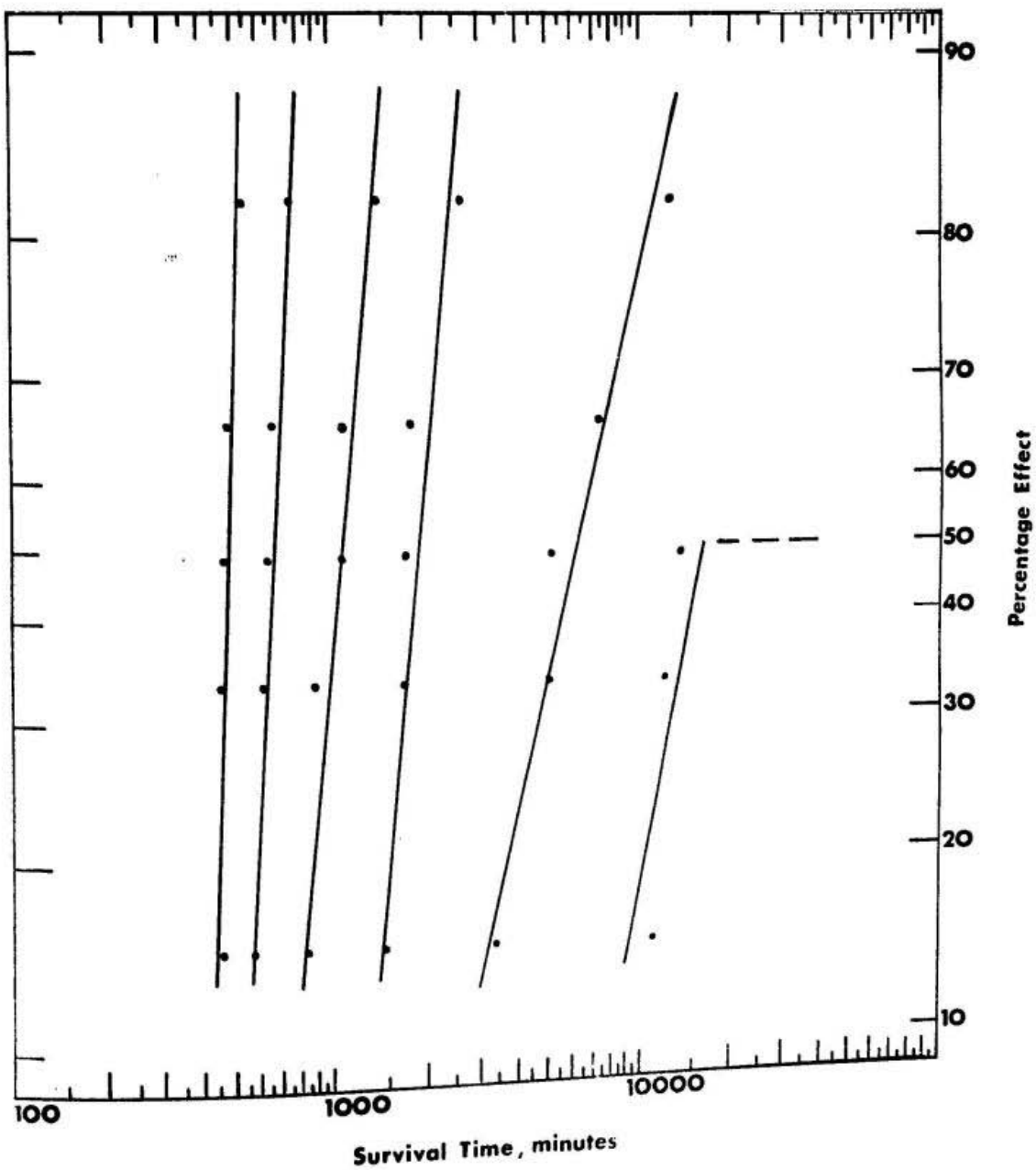

90
80
70
60
50
40
30
20
10
Percentage Effect
100
1000
10000
Survival Time, minutes

Figure 21.

Figure 22.

Graphical interpolation of the TLm values for 12, 24, 48, and 96 hours during the 10 day cod bio-assay.

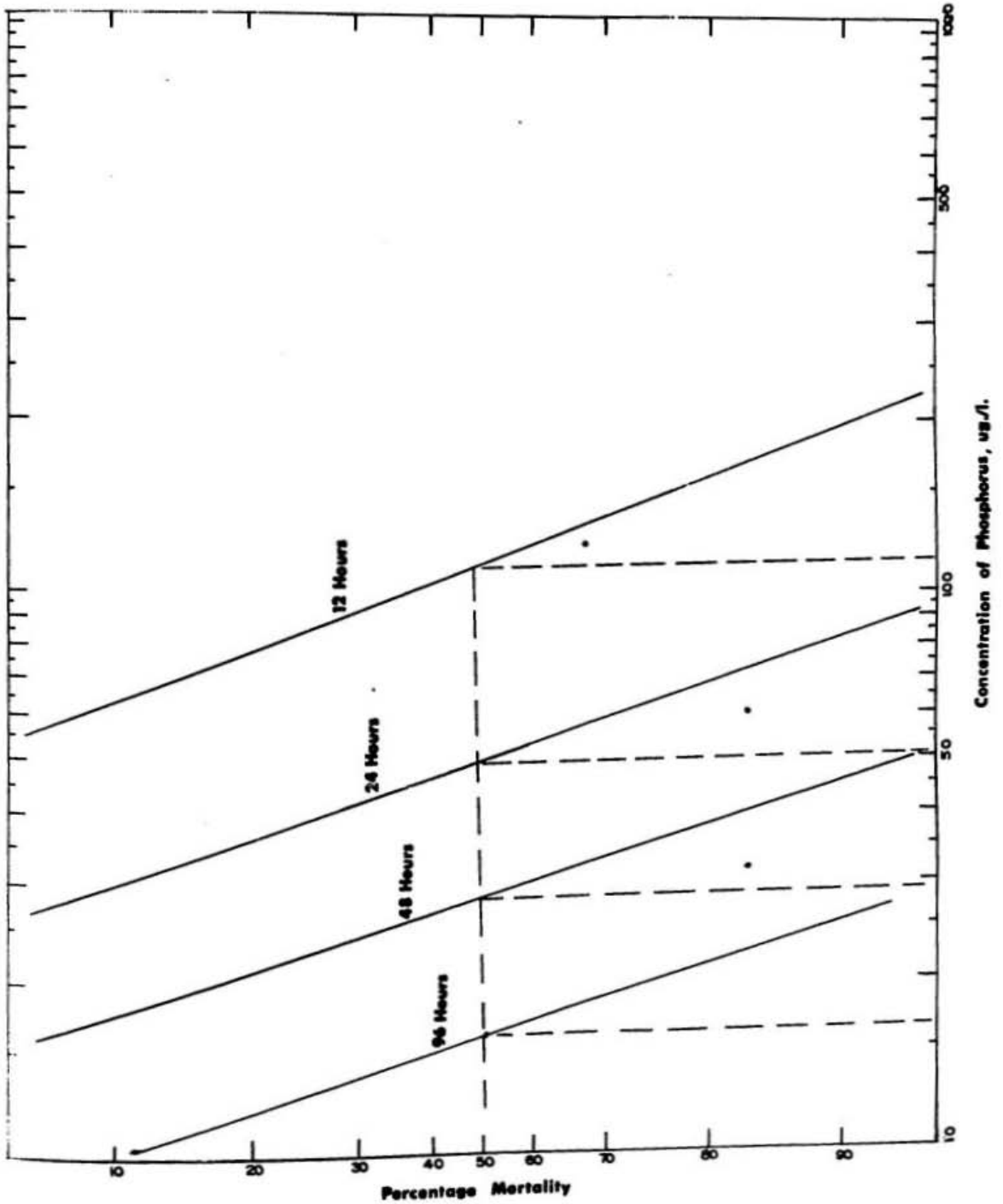

12 Hours
24 Hours
48 Hours
96 Hours
10
20
30
40
50
60
70
80
90
Percentage Mortality
1000
500
100
50
10
Concentration of Phosphorus, ug/l.

Figure 22.

Figure 23.

The toxicity curve for the 10 day cod bio-assay using a pure P_4 dispersion.

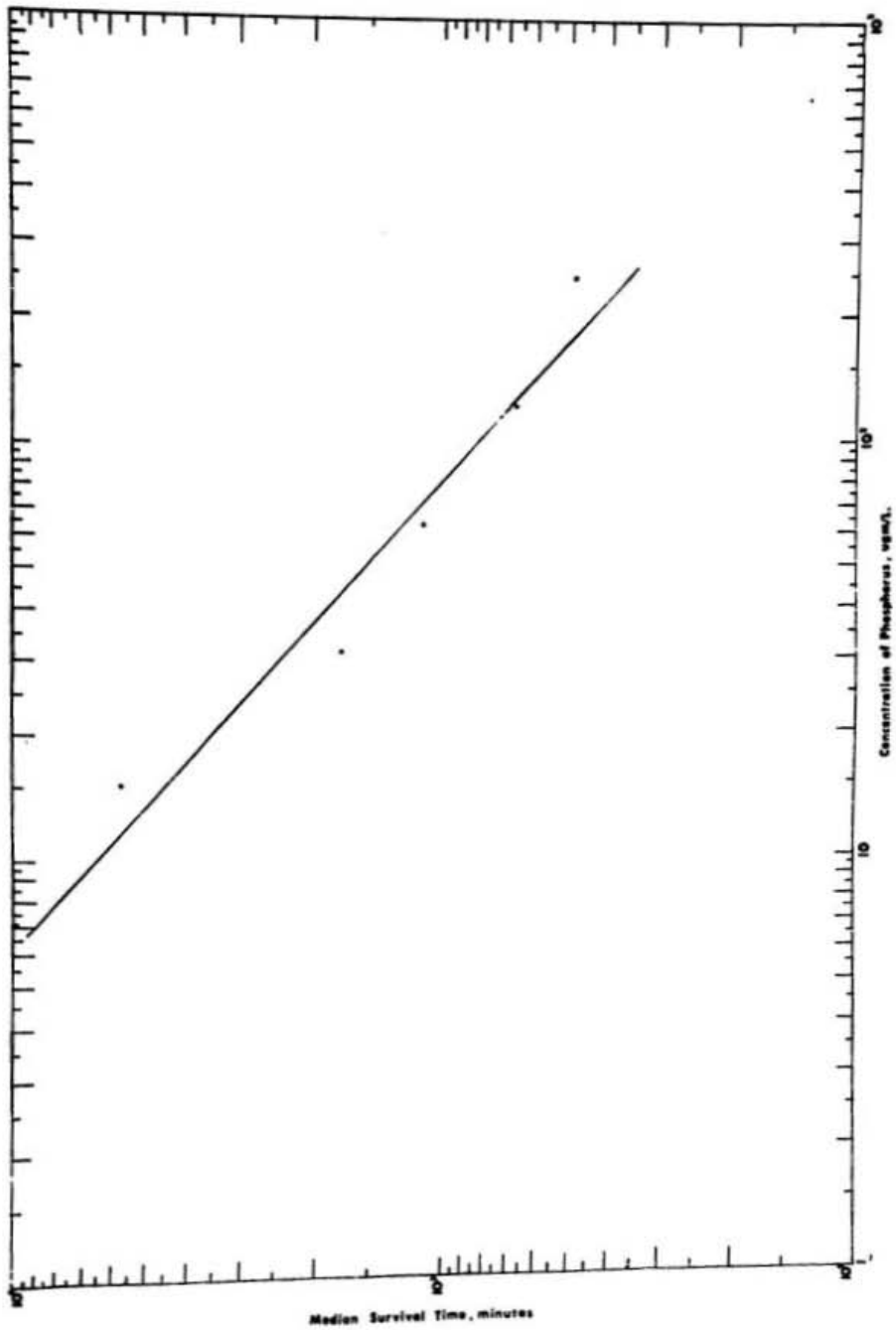

Concentration of Phosphorus, ug/ml.
Median Survival Time, minutes

Figure 23.

In the case of the second bio-assay on Gadus morhua,(see Appendix VIII) median tolerance limits were derived for 24 and 48 hours, these are 64 and 27 ugm.P_4/L. respectively.

TLm values for the 24,48 and 96 hour points in the 10 day bio-assay on cod (see Appendix IX) are 52,28, and 16 ugm.P_4/L. respectively. From this latter test and its toxicity curve it would appear that the incipient lethal level is below 16 ugm./L. The differences in TLm values are attributed to the fact that two different samples of cod were used, and because the curves are fitted by eye.

Toxicity of P_4 to the Winter Flounder

Behavior changes were not observed as carefully in the bio-assay on Pseudopleuronectes americanus. Thirty-six fish were employed in this case, and were distributed among six tanks, including 1 control aquarium.

These fish normally inhabit the muddy and sandy bottoms and become almost sessile animals, resting on the substrate for long periods of time. This probably bears some relationship to the fact that the young flounders in the phosphorus dispersion experiments did not move much during the 10 days. This was so, even in aquarium no.2 which received 250 ugm P_4/L.. The 48 hour TLm for the winter flounder is approximately 64 ugm/L. while the 96 hour TLm is roughly 23 ugm/L..

Appendix X can be referred to for the raw data on mortalities.

Figure 24

The toxicity curve for the 10 day bio-assay with pure P_4 dispersion on the winter flounder.

As can be seen from the points on the graph, the curve for Pseudopleuronectes americanus is a crude approximation.

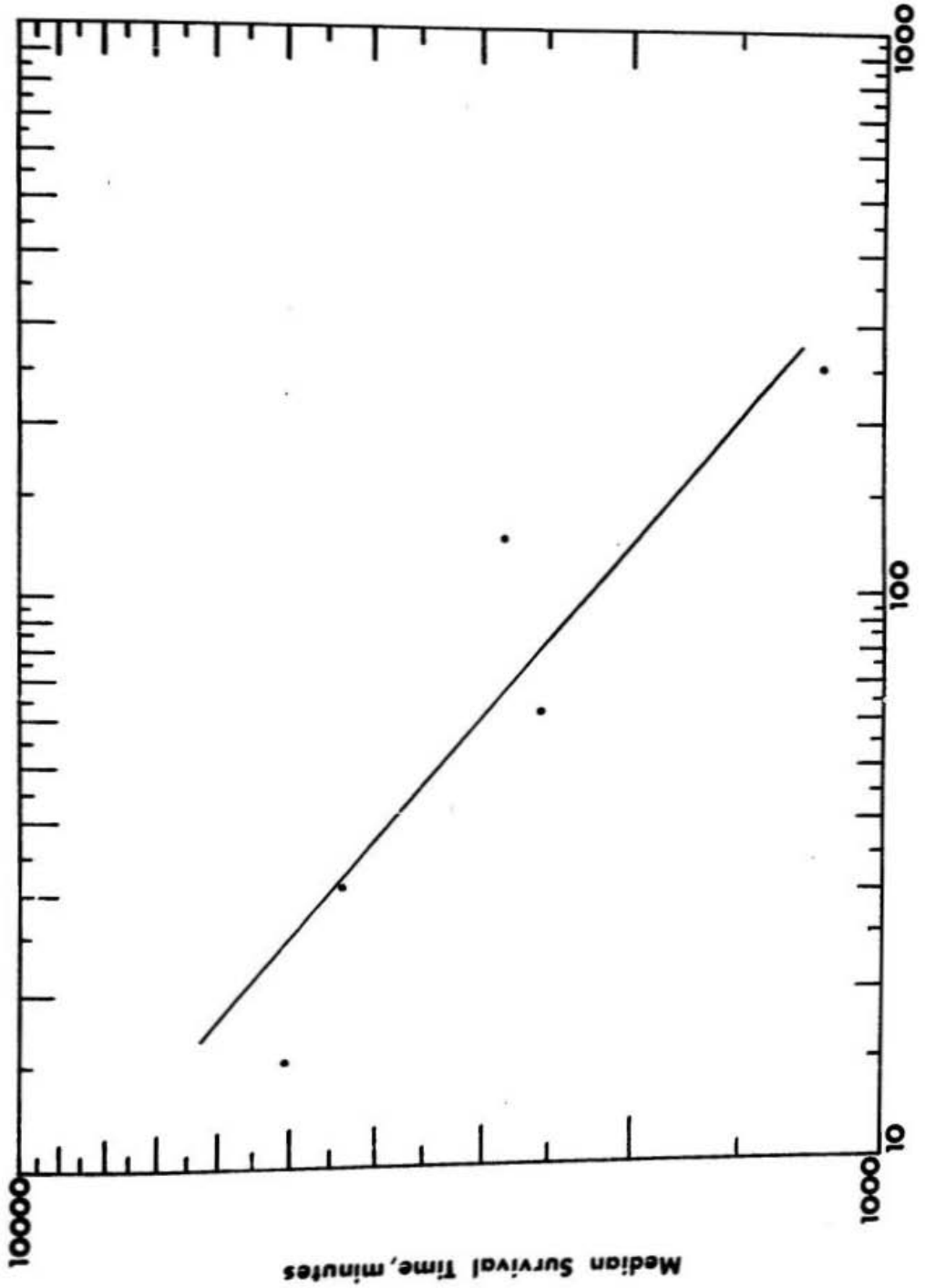

Median Survival Time, minutes
10000
1000
10
1000
100
Concentration of P_4, ug./l.

Figure 24.

Toxicity of P_4 to the Northern Starfish and Effect on its Righting Response

Since this test was primarily set up to measure a behavioral parameter, continuous monitoring was a necessity. Temperature, pH and flow rates were checked hourly, while the righting response was timed every three hours for each of the ten individual starfish in each aquarium. The righting response was defined as the movement by a starfish to overturn itself once it has been placed on its aboral surface,i.e.oral surface uppermost. For this bio-assay, I was concerned with the time taken by a starfish to execute that movement.(see fig.25)and Appendix XI).

When the northern starfish, Asterias vulgaris, is overturned under normal conditions, there is coordination in the manner in which it rights itself. Usually one or two arms begin to fold and adhere firmly to the substrate. Finally a third arm attaches itself and the remainder of the body turns over. In aquaria with concentrations greater than 1.5 mgm/L. the animal seemed to have difficulty coordinating its movements and on occasion tied itself in a knot before righting itself.

The 24 hour TLm is approximately 3 mgm P_4/L. while the 48 hour median tolerance limit is approximately 1 mgm P_4/L.

Figure 25

Relation between concentration of P_4 and the righting time of the northern starfish.

The curves were obtained by plotting the median righting time of starfish in individual aquaria in seconds against the concentration of P_4 in ERCO phossy water. The slope of the curve can be seen to increase from horizontal to vertical as time increases from 12 to 48 hours.

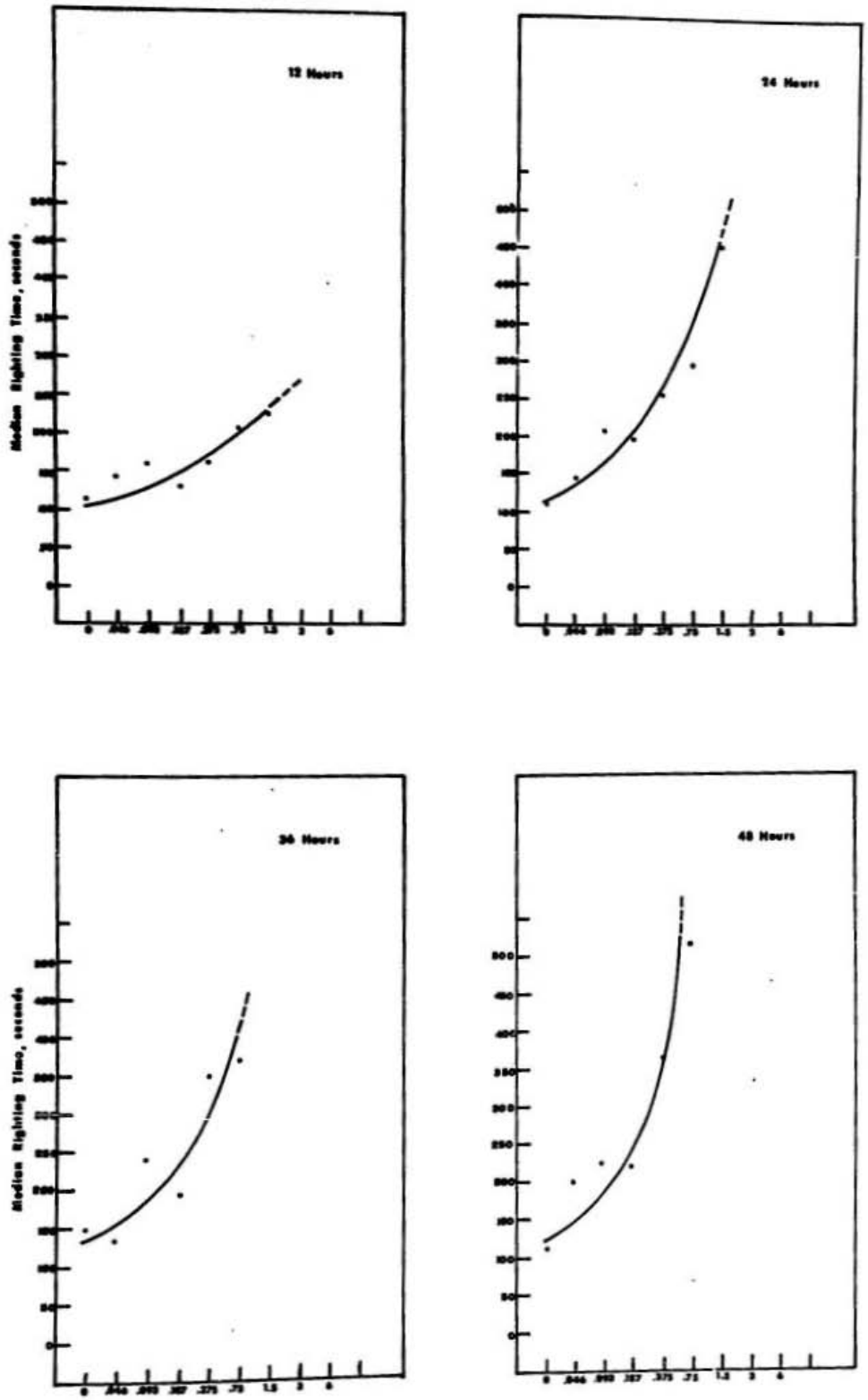

12 Hours
24 Hours
36 Hours
48 Hours
Median Righting Time, seconds

Figure 25.

Effect of P_4 on the Blood of the Atlantic cod and the Winter Flounder

1. Hematocrit.

Hematocrit values were obtained from the cod and the flounder during the 10 day experiments. The respective results for each species can be found in Tables 10 and 11.

Table 10. Hematocrit values for the Atlantic cod.

Aquarium No.	Concentration	Mean hematocrit	Range
2	250 ugm P_4/L.	15.1%	10-19%
3	125 " "	18%	15-22%
4	62.5 " "	18%	13-22%
5	31.2 " "	16%	12-22%
6	15.6 " "	12%	10-20%
7	7.5 " "	21%	17-27%
8	control	32%	27-38%
-	control	35%	34-37%

Table 11. Hematocrit values for the winter flounder.

Aquarium No.	Concentration	Mean hematocrit	Range
2	250 ugm P_4/L.	22%	15-30%
3	125 " "	13%	10-17%
4	62.5 " "	16%	10-23%
5	31.2 " "	9.3%	7-18%
6	15.6 " "	25%	18-36%
9	control	29%	20-33%

2.Differential counts.

Table 12 lists the counts for the three types of leucocytes most commonly encountered in the blood of the Atlantic cod. In this case, similar counts were not done for the winter flounder.

Table 12. Differential white cell counts for the Atlantic cod.

Concentration	Lymphocytes	Monocytes	Thrombocytes
250 ugm P_4/L.	87	8	5
	84	11	5
	82	12	6
	87	7	6
	86	8	6
Mean	87.2	9.2	4.4
125 ugm P_4/L.	89	7	4
	92	4	4
	88	4	8
	92	4	4
	90	4	6
	89	6	5
Mean	90	4.8	5.2
62.5 ugm P_4/L.	89	6	5
	89	4	7
	89	6	5
	88	6	6
	90	5	5
	92	5	3
Mean	90.1	5.3	4,6
31.2 ugm P_4/L.	91	7	2
	95	3	2
	94	4	2
	94	4	2
	93	5	2
Mean	93.4	4.6	2
Controls	96	2	2
	96	1	3
	90	1	9
	96	2	2
	94	4	2
	95	3	2
Mean	94.5	2.2	3.3

Effect of P_4 on the Cholinesterase Activity of the Brain of the Three-Spined Stickleback

Results of this experiment can be found in table 13 and figure 26. These comprise brain weight and the corresponding absorbance readings of the colour representing residual acetyl choline. These values were obtained for control brains, brains of fish removed from the toxicant water immediately prior to death and brains which were removed from stickleback which had spent 1/2 of the period required to kill the animals in 3 mgm P_4/L.

Table 13. Weight and absorbance readings of control and poisoned brains of stickleback for AChe activity.

Controls		Poisoned(3 hours)		Poisoned(6 hours)	
Wgt.	Absorbance	Wgt.	Absorbance	Wgt.	Absorbance
15mg.	0.37	43mg.	0.43	17mg.	0.22
21	0.43	10	0.26	34	0.21
27	0.35	42	0.25	47	0.28
30	0.46	58	0.36	36	0.27
39	0.44	39	0.29	38	0.24
32	0.42	25	0.22	30	0.19
34	0.38	52	0.26	23	0.19
27	0.38	49	0.28	19	0.22
25	0.35	35	0.22	38	0.26
16	0.41	27	0.23	36	0.24
22	0.55			20	0.17
33	0.50			22	0.17
23	0.50			15	0.21
19	0.53			13	0.13
24	0.49			17	0.19
15	0.49			15	0.15
15	0.49			22	0.18
29	0.44			13	0.12
19	0.54			11	0.16
26	0.46			9	0.14

Figure 26.

Relation between control stickleback brains and brains toxicated by P_4 re: AChe activity.

These points represent the distribution of brain weights plotted against absorbance readings in % transmittance as measured on a spectrometer, The crosses (+) represent the toxicated specimens while the points (·) represent the the control brains.

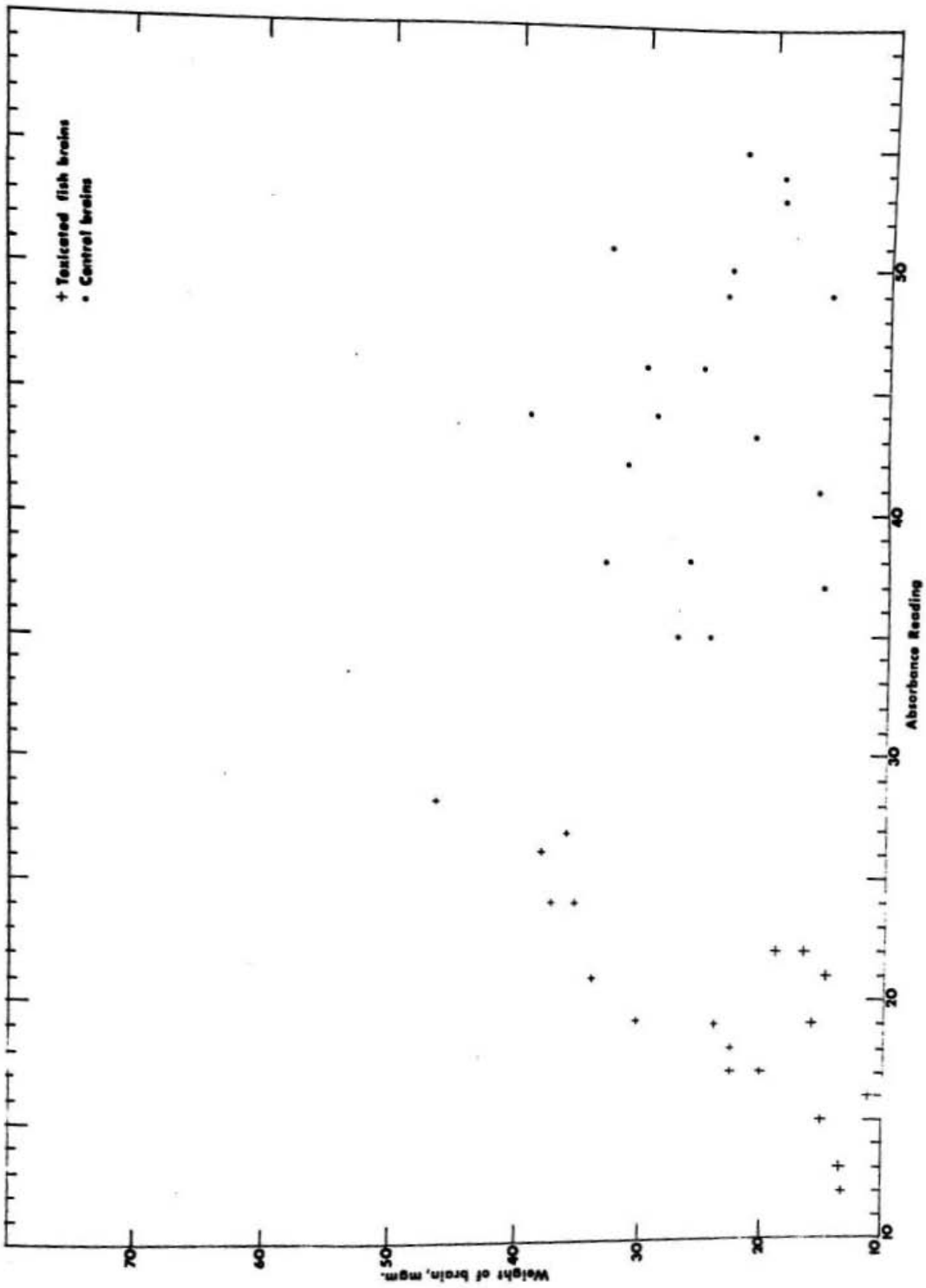

+ Toxicated fish brains
• Control brains
Weight of brain, mgm.
Absorbance Reading
10
20
30
40
50
60
70

Figure 26.

The mean values for absorbance of the color representing residual acetyl choline are 0.449, 0.280 and 0.197, with standard deviations of 0.060, 0.062, and 0.044 respectively for the controls, 3 hour toxicant group and the 6 hour toxicant group.

T-tests run on these absorbance values point out that at the 99% confidence level, the difference between the controls and the brains from the 3 hour toxicant group, and the difference between the controls and the brains of the 6 hour toxicant group, are significant. As can be seen from figure 26, the distribution of weights over the range of absorbance readings for the control brains and the brains of fish which had been in toxicant water for 6 hours, are quite separate.

DISCUSSION

Bio-assay Nomenclature and Interpretation

In determining the best method for reporting response times, two techniques were considered. The first was the use of the response time of the median fish, suggested as adequate by Doudoroff et al.(1951). Sprague (1969) points out that this is not the most informative parameter because response times of the other fish are not fully used. Brown (1967), on the other hand, used the geometric mean survival time , which is defined as the antilog of the mean of the logarithms of individual survival times.

In an effort to demonstrate the possible differences between lines plotted with the values obtained from each method, the times of death of the Atlantic cod in the 10 day bio-assay were used (see fig. 27 and Appendix IX).

The figure shows that the curves drawn in each case fall along the same axis. Any difference would be of very little consequence. For reasons of brevity, the median time was used throughout this thesis for toxicity curves.

The term Median Tolerance Limit (TLm) was used as a measure of toxicity in this manuscript. Sprague (1969) states that readers can substitute LC50 for TLm if they prefer that term.

When the times of death of the individual fish in the aquaria are used for derivation of the TLm, there is an experimental error

Figure 27

Relation of toxicity curves drawn with median and geometric mean survival times.

This figure was drawn with the results of the 10 day Atlantic cod bio-assay to show that the difference in plotting the median survival time and the geometric mean survival time is minimal.

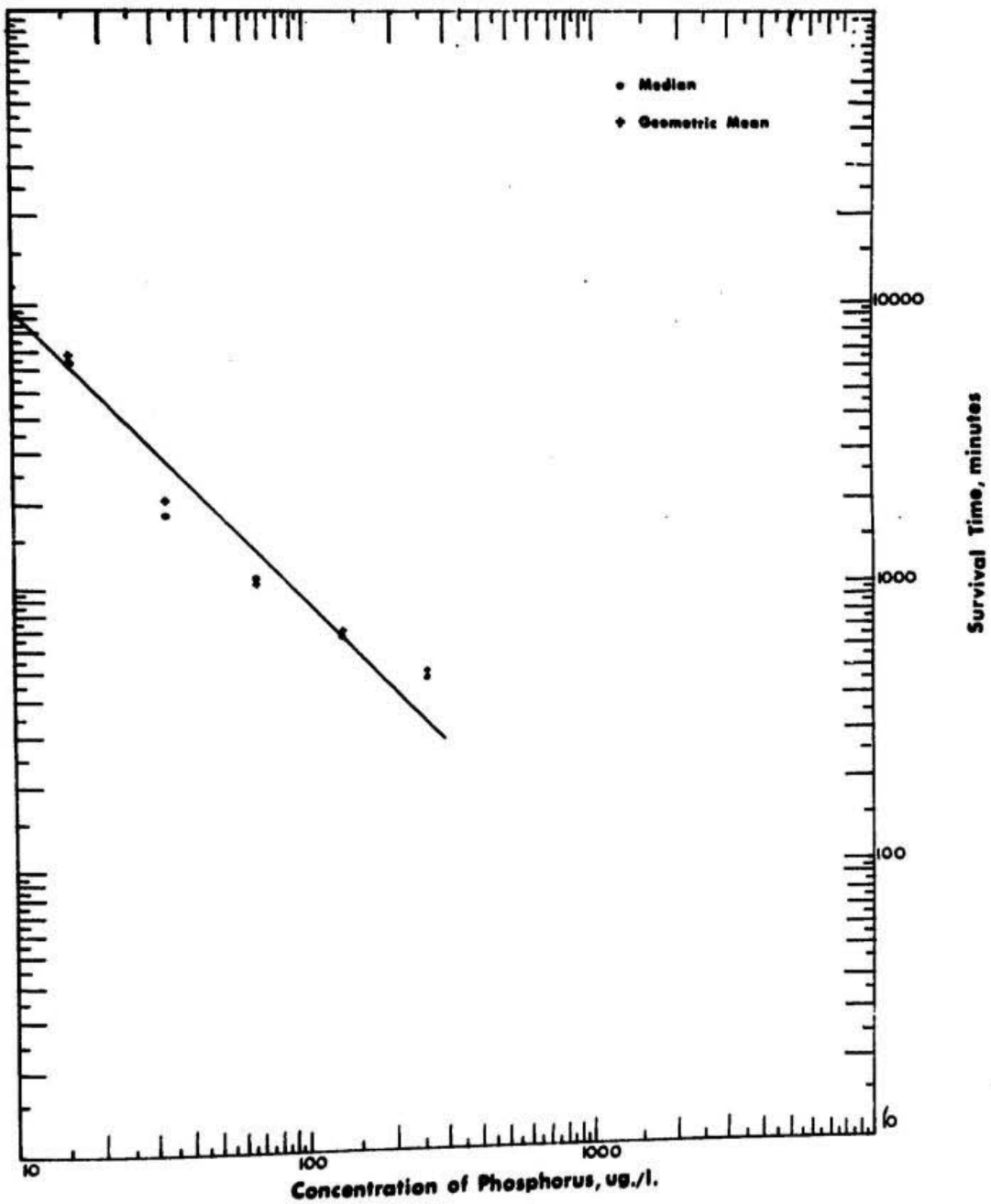

Median
Geometric Mean
10000
1000
100
10
Survival Time, minutes
10
100
1000
Concentration of Phosphorus, ug./l.

Figure 27.

in the bisection of the interval between deaths of fish if observations are only made periodically.

If there are ten animals per tank, the inclusion of an abnormal fish (physiologically weak or strong as compared to an average specimen of the particular species under investigation) will produce an error of 10% in the mortality. This error is theoretically increased as the concentration is decreased because there is a longer time interval between deaths and more noticeable individual variation.

Aberrant behavior is more readily noticed when the fish are under continuous observation. Though this is a more time-consuming method, the results justified it.

While log-log paper was used for the toxicity curves, log-probit paper was used to plot curves for TLm values and mortalities within individual aquaria. This paper is useful for three reasons: 1) differences in slope are more easily detected; 2) confidence limits may be placed on the TLm if required; 3) as with log-log paper, the logarithmic scale provides a means of plotting a wider range of time units or toxicant concentrations.

The changes in slope of lines plotted for individual test aquaria provide clues to different modes of toxic action. Two which come to mind are possible differences in the time taken to affect the cholinesterase activity of the brain and to affect

the blood of whatever animal is concerned. The change in slope also gives indications of the threshold between acute and chronic toxicity.

Reproducibility of Bio-assay Results

Brown et al. (1968) found that in the effect of phenol on rainbow trout, Salmo gairdneri, at different times of the year, the same stock of fish in the same laboratory gave LC50 values which differed by a factor of 2.5.

In figure 13, the difference amounts to 35 ugm/L. approximately, that is, a change from 325 to 360 ugm P_4/L. (10%) for a 24 hour TLm. This could easily be accounted for by a increase in dissolved oxygen which would decrease the elemental phosphorus available to the fish. The converse would also apply if there were a decrease in the TLm value.

It is partly for this reason that low application factors are set for allowable concentrations of a particular compound (if any is to be allowed into the water at all). Synergistic effects between compounds themselves as well as environmental factors which may modify the toxicity of a material, are also included in these considerations (Sprague, 1970).

pH Readings

The effect of a change in pH alone was not determined as it could not be dissociated from the toxicity of the compounds

themselves. Zitko (1969) using various acids, demonstrated that a drop in pH from 7.9 to 7.5 caused herring mortality. He does point out, however, that the toxicity is not a simple function of pH but depends very much on the anion present.

In consultation with Dr. M. Newlands of the Department of Chemistry, values have been calculated suggesting that at a concentration of 3 mgm P_4/L., even if all the phosphorus in a pure dispersion was converted to phosphoric acid (H_3PO_4), the maximum pH change would be 0.2 of a unit, that is, a change from 8 to 7.8.

Though the pH's in the aquaria with the stronger concentrations of ERCO phossy water are lower than those obtained by Zitko et al (1969), it would appear that the effect such a low pH would have on toxicity is neutralized by other factors.

Comparative toxicity of various particle sizes

The particle size hypothesis seemed to be the only remaining avenue when 500 mls. of 100 mgm. P_4/L. phossy water prepared by the ultrasonic generation method, were added to a static 12 liters of seawater into which a cod was introduced; the fish died but only after 6 hours exposure and more probably due to suffocation.

At first, I felt that the small difference in particle sizes, referred to in table 6, of the two methods of preparation would be unlikely to cause such a difference in toxicity of P_4.

Zitko et al. (1969) had found that phosphorus contaminated mud was toxic to fish even when the mud was separated from

the animals by a semi-permeable membrane. This suggests that dissolved phosphorus might be toxic to herring. Isom (1960) using bluegills in fresh water, demonstrated that elemental phosphorus showed no toxicity in a saturated solution at 26° C. In my work, however, dissolved phosphorus was toxic, though less toxic than dispersions of colloidal particles. The explanation of the difference in toxicity of dissolved P_4 in fresh water and in seawater may be related to chemistry of fresh and sea water.

The single piece of phosphorus (see page 59, aquarium 2, and table 7) introduced to the aquarium revealed no toxicity. It is likely that phosphorus did not dissolve into the water because the surface of the piece had oxidized, thus preventing further action.

Aquaria 3 to 6 in this experiment demonstrated that dispersions of colloidal particles of a range from approximately 0.6 u downward to dissolved phosphorus are toxic in seawater. It is concluded that there is a critical size of P_4 particles between 0.6u and 0.9u, above which phosphorus is no longer toxic, at least in the case of the three-spined stickleback. Research by Ackman (1970), Dyer (1970), and McIntosh (1951) substantiates this hypothesis to a certain extent.

Ackman (1970) reported that variations in the results for the herring, could indicate exposure to different levels of elemental phosphorus but it could also be caused by chance encounters with different sized particles in a given body of polluted water.

In work on particle size and the toxicity of insecticidal poisons on insects, McIntosh (1951) suggested that toxic action is caused by the poison crossing into the aqueous phase and carried to its site of action by diffusion and by the circulation of the blood. It is not clear how this occurs with pure poisons which are insoluble in water; insecticides may be more soluble in insect blood than in water itself. The research of Dyer et al. (1970) on the assimilation of P_4 by cod muscle and liver shows that phosphorus concentrates in tissues with a high lipid content..

McIntosh refers to Jones and Partington (1915) who found that the solubility of a solid in a solvent at any temperature depends on its particle size. The particle size below which the increased solubility becomes noticeable, depends on the solute-solvent pair, but for inorganic precipitates in water, is commonly in the region of 0.5u. This finding, though lower than the 0.8u which appeared to be the threshold for stickleback at 8.0°C, suggests that values found in my work relative to the toxicity of various P_4 particle sizes (see tables 6 and 7) though crude, are an important facet in the toxicology of elemental P_4 to marine fish.

McIntosh (1951) found great differences in potency and speed of action between various sized crystalline suspensions and colloidal rotenone. He also demonstrated that temperature has an effect on the toxicity of rotenone, probably because it

affects the solubility of the poison in fat or wax.

The point to be made is that there is more than the concentration itself which affects the toxicity of a compound; in the case of phosphorus, temperature and particle size may operate independently to modify the effect of concentration, but they may also work concurrently(Sprague,1970).

Toxicity of Pure P_4 Dispersion vs ERCO Phossy Water.

As can be seen from figure 15, the difference in the lines plotted for each experiment (see Appendices II and IV) at a temperature of 8.0°C, is not significant.

The toxicity of the materials other than phosphorus in the ERCO phossy water, as yet not analyzed, can only be gauged by comparing the toxicity of the ERCO phossy water to that of a pure dispersion of P_4. These materials can of course, be antagonistic or synergistic in their action but this is outside the scope of the present work.

Two possible conclusions can be drawn, however, from these bio-assays:

1. The ERCO phossy water is not more toxic than synthetic phossy water at 8.0°C;
2. Some compounds in the ERCO phossy water may have synergistic effects but these appear to be neutralized by others which work antagonistically.

Toxicity of P_4 to the Three-Spined Stickleback

The results listed in table 8 are consistent with the data obtained by the Fisheries Research Board of Canada for brook trout (Fletcher, 1970) and bluegill sunfish in fresh water (Isom, 1960).

It must be remembered, however, that the three-spined stickleback, Gasterosteus aculeatus, is among the hardier species of fish to be found in our coastal marine environment.

None of the sticklebacks used in the pure P_4 and ERCO phossy water bio-assays could be said to have demonstrated the"red herring" effect. This"red herring" is described as a red discoloration of the tissue, specifically in the head region and the fins. According to Department of Fisheries personnel who collected specimens in the field, the redness was primarily observed in the Atlantic herring (Ackman,1970). Though some reddish cod were taken, the feeling is that these hemorrhaged as a result of hitting the floorboards of the fishing boats they were collected in, rather than as a consequence of phosphorus poisoning. Redness in fish is not peculiar to this pollution incident when one considers the roughness with which they are handled (Ackman, 1970). The only hypothesis that has been advanced as to the reason some of the other species such as the stickleback did not exhibit the red

discoloration was suggested by Fletcher et al.(1970). He points out that the erythrocytes of the other species may not be as susceptible to the effects of phosphorus as are the erythrocytes of Atlantic herring.

It is interesting to note the similarities between the aberrant behavior displayed by fish poisoned by elemental phosphorus and those poisoned by organo-phosphate and organo-chlorine insecticides. A paper by Henderson and Pickering (1958,p.45) on the toxicity of organo-phosphate compounds to some species of fresh water fish, described the physiological and behavioral reactions as follows:

> Usually the first noticeable change was a darkening of the skin. This was followed by a period of high excitability with body tremors and complete loss of equilibrium. Sometimes death did not occur for many hours after equilibrium was lost. This response was similar to that observed when working with some of the chlorinated hydrocarbon insecticides.

Indications are that the sticklebacks which were not killed outright by phossy water, recuperated when the toxicant pump was turned off and normal seawater allowed to flow through the aquaria. In two experiments, after the 48 hour bio-assay had been completed, no deaths were reported up to 10 days later. No conclusion could thus be reached, as suggested by Zitco et al. (1970,p.23) of the apparent irreversibility of the toxic effects of phosphorus:

The toxic action of P_4 is irreversible. LT50 of herring exposed to a suspension of the phosphorus contaminated mud for 3 hours and then transferred into clean running water is almost the same as the LT50 of herring kept in the suspension.

The approach used in these experiments on Gasterosteus aculeatus did not provide the maximum amount of information because the only response measured was death. Though death is a definitive criterion in dealing with toxicity of materials, it may be, however that chronic sub-lethal effects are as devastating to the species but this is yet to be determined. Physiological changes, for example, may disrupt the biology of the animal without the typical all-or-none response being displayed.

1. Temperature - Toxicity Relationship.

In experiments 1 and 2 (see table 9 and figure 18) the difference is not so great as to rule out parallelism (if S.R. is larger than f_{sr}, then the lines deviate significantly, 95% probability, from parallelism); for this reason, the potency ratio was determined as a final test.

In bio-assays 1 and 3 (see table 9 and fig. 18), however there is a significant deviation from parallelism.

The lines for experiments 2 and 3 are considered parallel at the 95% confidence level and as such suggest that temperature has an effect on the toxicity of yellow phosphorus at temperatures of 8.0^{o} C and 3.5^{o} C.

Experiment 1 at 13°C may not have provided completely reliable information even though the line is greatly displaced from lines for bio-assays 2 and 3. The fact that there was a death in one of the control aquaria suggests that the fish may not have been in the best condition.

The line for experiment 3 upon analysis demonstrates a significant difference in potency from that for bio-assay 2.

Bio-assays at 13.5°C and 8.0°C indicate a difference in potency although there was no parallelism indicated in the lines. It should be pointed out that computations for these temperature toxicity analyses are done with the raw data from the Appendices while the lines in fig. 18 are only best fit by eye. No conclusion can be reached when comparing 1 and 3 because the deviation from parallelism was too great, as established by Litchfield and Wilcoxon's methods (1948).

The inference from the values obtained is that temperature has an effect on the toxicity of elemental phosphorus.

Toxicity of P_4 to the Atlantic cod

Many factors were involved in the decision to use this species of the family Gadidae as experimental subjects. The main consideration was that Gadus morhua is of economic importance, mainly as a food supply for man; its fishery also supports many Newfoundlanders. The species is also of ecological importance

in the sea as a prey and as a predator species. The Atlantic cod is common in the inshore areas and readily found in Placentia Bay. Though it appears to be a hardier fish than the Atlantic herring under laboratory conditions and less hardy than Gasterosteus aculeatus, relatively minor changes in water quality are presumably detrimental. Sprague (1970) discusses various reasons why fish are chosen for bio-assays.

Behavior before death, in the Atlantic cod, is similar to that exhibited by the three-spined stickleback, but differs from that of the winter flounder. The cod demonstrated the symptoms of the herring, as reported by fishermen of Long Harbour.

Its incipient lethal level would appear to be in the same range as that of the Atlantic herring, as deaths were still recorded with this species at 2.5 ugm P_4/L. (Zitco et al.1970) and mortalities for the cod were recorded at approximately 7 ugm P_4/L.

Toxicity of P_4 to the Northern Starfish and Effect on its Righting Response

Because death is difficult to determine in many of the macro-invertebrates, it was concluded to employ behavioral responses as sublethal indicators of the effects of phosphorus. For purposes of calculating the TLm's however, no response after 600 seconds when placed on its aboral surface and lack of movement by the tube feet was regarded as death. The righting time was

chosen as an esily measurable response. The ecological consequences suggested by the results are of importance; affected would be the speed in travelling and ability in obtaining food for example.

Field observations of invertebrates, by the Federal Department of Fisheries personnel, in Long Harbour at the time of the incident indicated that they may be less sensitive to the pollutant than marine fish. Sea urchins, for instance, were found crawling on the bottom muds near the ERCO wharf at Long Harbour. Therefore concentrations in the laboratory tests on starfish were increased. This was borne out by the results of the bio-assay where the 48 hour TLm was 1 mgm P_4/L. or almost 20 times higher than the tolerance limit for the three-spined stickleback, for example. Sprague (1970) refers to the comparison of resistances of invertebrates and fish.

Allowances must be made, if application factors are considered for differences between field and lab conditions and different animal species (Sprague,1970); little P_4 settled on the bottom of the assay tanks as compared to that deposited on the bottom muds of Long Harbour (Zitco,1969;MS report).

Effect of P_4 on the Blood of the Atlantic Cod and the Winter Flounder

The results obtained are believed reliable, as all fish demonstrated nothing other than the symptoms of phosphorus poisoning, throughout the 10 day test. The demonstrated decrease in hematocrit values, though not quite as marked, corroborates

data reported by Fletcher et al.(1970) for herring and brook trout. Mean hematocrits of 32% and 35% for two samples of control cod only rangeto 12% in poisoned fish and not as low as 1% as recorded by Fletcher et al.(1970). He also reported that:

> An examination of the hematocrits of the various groups of trout tested, indicated that fish that died in low concentrations of phosphorus had correspondingly low hematocrits. Fish that died in low concentrations of yellow phosphorus had been exposed to yellow phosphorus for longer periods of time; therefore it follows that reduced hematocrits were also related to the time taken for the fish to die.

Though the depressions of hematocrit occurred in a more or less corresponding order, the relationship between low concentrations of P_4 and low hematocrits was not fully corroborated by the work on the Atlantic cod and the winter flounder. In tank 7,in particular, at approximately 7 ugm.P_4./L., that relationship did not hold for either cod or flounder, though a depression was still evident.

Ackman (1970) reported that for herring which were gill-netted near Long Harbour, the hematocrit evidence from a few suspect red herring was inconclusive and did not correlate with the presence or absence of elemental phosphorus. He suggests this may be caused by sub-lethal concentrations, as there were no reports of kills in early 1970. Of special interest in these herring was the detection of phosphorus in the gills which Ackman suggests as particle adhesion, possibly from the stirred-up sediments.

The data for the flounder suggest a depression in hematocrits

but it is not as marked a change as cod or other species (see table 11).

The difficulties of studying the effects of phosphorus on fish blood are amply set out in the following points:

1. Redness, thought to be caused by hemolysis of the erythrocytes, is demonstrated in the Atlantic herring and the brook trout but it is not observed in the smelt, the Atlantic cod, the winter flounder and the three-spined stickleback.
2. There is a great depression in the hematocrit of the herring while that of the cod and trout can be referred to as marked and the depression of the hematocrit of the winter flounder and smelt as moderate.
3. Fletcher et al.(1970) suggests these differences could indicate that the erythrocytes of smelt and similarly the winter flounder, are not as susceptible to the effects of phosphorus as are those of the Atlantic cod and the brook trout; none of which are as susceptible to the effects of P_4 as are the erythrocytes of herring.

Explanations for these observations are not presented. Research into these problems is suggested to properly analyze the effects of phosphorus on marine life; a pollution problem of great complexity.

It is evident that there is a change in the ratios of lymphocytes to thrombocytes to monocytes. The meaning of this change however, is not understood.

The purpose of the counts (see table 12) was to discover whether there was a change. Further experimentation will be required for more accurate qualitative and quantitative measurements of the changes as well as studies of the mechanisms which cause cellular damage. Phosphorus has been shown to cause hemolytic anemia in mammals (Kracke, 1941) and though it is said to affect the blood cells and hemopoeitic tissue, blood chemistry changes in mammals are not fully understand. For example, since organophosphate insecticides have caused inhibition of the cholinesterase activity of plasma and red cells (Fitzhugh, 1960) and elemental phosphorus has been shown to cause similar inhibition in the brain, it is possible that elemental P_4 affects the cholinesterase in the plasma and red cells of fish blood.

Effect of P_4 on the Cholinesterase Activity of the Brain of the Three-Spined Stickleback

Acetyl cholinesterase is a compound present in the nervous system at neuronal surfaces, such as the endings of axons and dendrites. It is of vital importance because normal synaptic functioning depends on its presence; it hydrolyzes acetylcholine which is the intersynaptic transmitter substance. The absence or gross reduction (to 50-60% of normal) of acetylcholinesterase (AChe) in the central nervous system causes death (Weiss,1959).

It has been reported in the literature that organic phosphorus compounds have an effect on the AChe activity of fish brain (Henderson and Pickering,1958).This occurs due to the phosphorylation of cholinesterase. Weiss (1959) reports that small fish exposed to these insecticides had a reduction in brain AChe, proportional to the concentration and extent of exposure. Henderson and Pickering (1958) suggest another interpretation: namely that at a specific concentration and time, an equilibrium is reached in which the inhibition of AChe is counterbalanced by its rate of resynthesis. It is also possible that mortality of fish from the organic phosphorus insecticides is the result of some physiological action in addition to or other than total brain AChe inhibition.

Gibson et al.(1969) state that differences within and among populations indicate that AChe activity of a species fluctuates with time. Their work showed that the degree of AChe inhibition is not always related to the concentration present or to the length of exposure. They also demonstrated that recovery occurs after organo-phosphorus poisoning in fish.

Cameron and Patrick in their work on mammals reported that (1966, p.212):

> From our extraction experiments, it seems possible that some inorganic yellow phosphorus can become incorporated into substances such as inorganic phosphate, phospholipids and other compounds extractable by aqueous and fat solvents. It is also apparent that a substantial amount

can become incorporated in some way into protein and lipo-protein complexes resistant to these solvents. The probable association between such binding and the toxic action of phosphorus on cells is not clear.

All these observations by other researchers are of interest when one considers the toxicology of elemental phosphorus poisoning to fish. Since the death of the fish occurred so rapidly in the high concentrations (15 minutes at 3 mgm P_4/L. for cod) it was felt that the effect of phosphorus on AChe might provide a reasonable answer.

The mean absorbance reading of the control fish (table 13) was more than twice that of the toxicated specimens after 6 hours (dead or moribund). This value is 60% of the normal and according to Weiss (1958) is sufficient to cause death of the fish.

The experiment was further reinforced by the analysis of residual acetylcholine in samples from the ten sticklebacks which had been placed in 1 mgm.P_4/L. dispersion but which were removed after they had been in the phossy water for one-half the time of the initial twenty (see table 13). The absorbance value, as expected was somewhat higher.

The conclusion drawn from this experiment is that elemental phosphorus, in some manner, affects the AChe activity of the brain of the three-spined stickleback.

CONCLUSIONS

1. The temperature-controlled, serial-dilution bio-assay unit performed as it was designed to, diluting a stock concentration sequentially in a 2:1 ratio. The unit also required very little care during the experimental period.
2. Particle size of the phosphorus was shown to be an important parameter in considering the toxicity of elemental phosphorus to the three-spined stickleback. Particles greater than 0.9u appear to have little or no effect on this species.
3. Phosphorus demonstrates a linear logarithmic relation such as that demonstrated by cyanide and flouride toxicity.
4. The toxicity of the ERCO phossy water is essentially the same as that of the pure P_4 dispersion in the case of Gasterosteus aculeatus and Gadus morhua.
5. Elemental phosphorus is extremely toxic to the three species of marine fish tested in my research. The 48 hour TLm values for the three-spined stickleback, the Atlantic cod and the winter flounder at 8.0°C are 185, 28 and 70 ugm.P_4/L. respectively.
6. The toxicity of yellow phosphorus to the three-spined stickleback is affected by water temperature; the toxicity increases with temperature as tested at 3.5°C, 8.0°C and 13.5°C.
7. Elemental phosphorus retards the righting response of the starfish at concentrations much lower than that required for lethality.

8. Elemental phosphorus affects the blood of the cod in at least two ways: it lowers the hematocrit (red cell volume) and decreases the relative number of lymphocytes while increasing the number of monocytes and thrombocytes.
9. The acetyl cholinesterase activity of the brain is depressed a sufficient amount to cause death, in water containing 3 mgm. P_4/L.
10. In view of the relative ease with which P_4 is oxidized, the actual effective concentrations may actually be somewhat lower than those reported in the thesis. The inference being that if it is the case, phosphorus is really more dangerous than demonstrated in this thesis.
11. The research reported in this thesis and that conducted by the Fisheries Research Board of Canada is only a beginning at understanding the effects of elemental phosphorus on marine life. One thing seems clear, however, elemental phosphorus is too lethal to be released into the marine environment, and its allowable level should be set at zero.

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LIST OF MAJOR INSTRUMENTS AND SUPPLIERS

1. The basic Serial Dilution Unit and the 10 foot Effluent Trough were built by Plastics Maritime Ltd.,Halifax. It was then modified by plexiglass which was obtained locally from Day-Nite Neon.
2. The magnetic stirrer was purchased from Cole-Parmer Instrument Co. Chicago,Ill. (Model No. 4815)
3. The peristaltic pump, the controller and the tubing were bought from Cole-Parmer Instrument Co. Chicago,Ill.(Model 7020VK).
4. The immersion heaters (1000 watt) Vycor Brand Glass, were purchased from Fisher Scientific Co.Ltd. Montreal.
5. The relay unit was built by Technical Services, Memorial University, to accept the 3000 watts of the immersion heaters.
6. The flowmeter kit (Gilmont Model No. 3200) was obtained from Cole-Parmer Instrument Co.Ltd. Chicago, Ill.
7. The filter unit was bought from Commercial Filters Canada Ltd. It wascomposed of a Hypur Water Conditioner H15-10 and Honeycomb filters W17R10-AV.
8. The hematocrit centrifuge was purchased from Clay-Adams Co.
9. The 10 gallon glass and stainless steel aquaria were purchased at a Woolco Department Store, St.John's.
10. Glass ware was obtained from Fisher Scientific Co. and Zeiss Instrument Co.

11. The phossy water was provided by ERCO at Long Harbour. The pure yellow phosphorus was provided by ERCO and purchased from Fisher Scientific Co.Ltd.

APPENDIX I

Mortality times of the three-spined stickleback, Gasterosteus aculeatus, in ERCO phossy water at 13°C during a 48 hour bio-assay. Times are listed in minutes.

TANK 0	TANK 1	TANK 2	TANK 3
60	240	420	660
120	240	480	900
180	240	540	1140
180	300	540	1260
180	300	600	
180	300	1200	
180	300		
180	300		
180	360		
240	360		
(1.5 mgm/L.)			

TANK 4	TANK 5	TANK 6	TANK 7
1200	660		
1320			

There was one death in control tank 8 during the experiment and none in control tank 9.

APPENDIX II

Mortality times of the three-spined stickleback in ERCO phossy water at 8°C during a 48-hour bio-assay. Times are listed in minutes.

TANK 0	TANK 1	TANK 2	TANK 3
90	199	530	1110
100	205	535	1545
105	220	577	1660
120	221	665	1895
120	221	669	1930
120	227	670	1933
125	235	672	1935
127	235	733	1960
130	237	735	1985
133	245	735	2052
(4mgm/L.)			

TANK 4	TANK 5	TANK 6	TANK 7
2445	2641		
2505	2645		
2507			
2565			
2575			
2630			
2632			
2640			

No deaths were reported in tanks 8 and 9 during the experimental period.

APPENDIX III

Mortality times of the three-spined stickleback in ERCO phossy water at 3.5°C during a 48-hour bio-assay. Times are listed in minutes.

TANK 0	TANK 1	TANK 2	TANK 3
170	285	483	1110
175	310	640	1118
179	315	646	1127
182	317	647	1174
186	330	651	1175
187	342	662	1221
192	345	680	1225
193	345	702	1230
194	350	720	1238
200 (2mgm/L.)	370	735	1261

TANK 4	TANK 5	TANK 6	TANK 7
1885	2810		
1890			
1980			
2532			
2640			
2825			

No deaths were reported in control tanks 8 and 9 during the 48 hour period-

APPENDIX IV

Mortality times of the three-spined stickleback, in pure phosphorus dispersion at 8.0°C during a 48-hour bio-assay. Times are listed in minutes.

TANK 0	TANK 1	TANK 2	TANK 3
240	1100	1810	
290	1150	1850	
320	1220	1895	
335	1295	1900	
336	1342	1925	
357	1349	1975	
372	1425	2002	
390	1465	2030	
400	1555	2065	
(1mgm/L.)			

No deaths were reported in any of the other aquaria, including the controls.

APPENDIX V

Mortality times of the three-spined stickleback in pure phosphorus dispersion at 8.0°C during a 48-hour bio-assay. Times are listed in minutes.

TANK 0	TANK 1	TANK 2	TANK 3
285	970	1362	
300	1005	1400	
307	1025	1635	
320	1040	1643	
355	1042	1710	
380	1118	1750	
395	1135	1762	
405	1160	1800	
460	1295	1895	
550	1360	1960	
(1mgm/L.)			

No deaths were recorded in any of the other aquaria.

APPENDIX VI

Mortality times of the three-spined stickleback in pure phosphorus dispersion at 8.0°C during a 100-hour bio-assay. Times are listed in minutes.

TANK 0	TANK 1	TANK 2	TANK 3
310	900	1280	2825
315	900	1282	3270
340	960	1450	3285
374	960	1490	3310
380	960	1545	3390
397	1020	1598	3635
398	1020	1605	3900
455	1080	1710	3960
475	1175	1732	4210
480 (1mgm/L.)	1200	1800	4228

TANK 4	TANK 5	TANK 6	TANK 7
3900			
4551			

No deaths were reported in any of the other aquaria during the 100 hour bio-assay.

APPENDIX VII

Mortality times of the Atlantic cod, Gadus morhua, at 11.0°C during a preliminary bio-assay over a 72-hour period. Times are listed in minutes.

TANK 0	TANK 1	TANK 2	TANK 3
180	450	1005	2700
300	470	1055	
(0.5mgm/L.)			

No deaths were reported in aquaria 4 through 9, though only two fish were used per tank.

APPENDIX VIII

Mortality times of the Atlantic cod in diluted ERCO phossy water at 8.0°C during a 48-hour experiment.

TANK 0	TANK 1	TANK 2	TANK 3
15 (2 mgm/L.)	32	45	135
		65	150
		75	155
		88	167
		91	220
		100	225
		108	255
		112	260
		115	290
		120	300

TANK 4	TANK 5	TANK 6	TANK 7
230	500	1440	2585
320	672	1480	2595
480	763	2095	2740
563	855	2386	
645	980	2390	
670	1380	2394	
692	1408	2725	
825	1510		
881	1810		
927	2345		

No deaths were reported in the control tanks during the 48-hour bio-assay.

APPENDIX IX

Mortality times of the Atlantic cod in pure phosphorus dispersion at 8.0°C during a 10 day bio-assay. Times are listed in minutes.

TANK 0	TANK 1	TANK 2	TANK 3
		460	560
		460	610
		465	640
		480	660
		545	760
		555	970
		(0.25mgm/L.)	

TANK 4	TANK 5	TANK 6	TANK 7
835	1470	3360	11920
900	1710	5045	12000
1125	1723	5100	13500
1132	1805	7510	
1410	2600	12720	
1440	3460	13200	

No deaths were recorded in tank 8 which served as the control tank. The control cod remained in good condition for a two week period. Due to the difficulty of obtaining specimens, only 6 cod were used in this bio-assay.

APPENDIX X

Mortality times of the winter flounder, Pseudopleuronectes americanus, in pure phosphorus dispersions at 8.0°C during a 10 day bio-assay.

TANK 0	TANK 1	TANK 2	TANK 3
		1210	1645
		1255	1660
		1262	2510
		1270	3110
		1390	4305
		1400	4500
		(0.25mgm/L.)	

TANK 4	TANK 5	TANK 6	TANK 7
1810	960	4740	
2520	3900	4800	
2590	4320	4980	
3930	4680	5150	
4020	13260	7590	
-	-	13500	

One death was recorded in the control tank after 4 days. Only 6 flounder were used in this bio-assay.

APPENDIX XI

Righting times of the starfish, Asterias vulgaris, in diluted ERCO phossy water at 12, 24, 36 and 48 during a 48-hour experiment. Times listed below are median times of 10 starfish in seconds for each experiment.

	12 HOURS	24 HOURS
tank 0	600+	-
1	300	600+
2	225	450
3	210	282
4	160	252
5	122	195
6	158	215
7	142	145
8	120	120
9	112	105

	36 HOURS	48 HOURS
tank 0	-	-
1	-	-
2	600+	-
3	370	520
4	350	367
5	197	220
6	245	225
7	135	200
8	150	130
9	150	112

Deaths in the aquaria are denoted by -.