THE UNIVERSITY OF MICHIGAN

School of Natural Resources
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Technical Progress Report

NUTRIENT CYCLING AND PRODUCTIVITY OF DYSTROPHIC LAKE-BOG SYSTEMS (PART A)

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SCOPE OF WORK

December 1, 1969 - November 30, 1970

In the spring of 1970, I continued work on data from the in situ flow-through experiments conducted during the summer of 1969, and I completed a manuscript describing this work (Appendix A). Mr. Imes continued experiments dealing with phosphorus uptake by the phantom midge <u>Chaoborus</u>. These studies substantiated previous evidence that bacteria were a major source of phosphorus uptake by <u>Chaoborus</u> and were probably important in the biological transport of this element in the lake. A manuscript summarizing these data is attached (Appendix B).

A second ^{32}P labeling experiment designed to provide additional data on phosphorus transport and the phosphorus cycle of the upper water was conducted in early May. A mixture of ^{32}P and rhodamine WT was injected into the 7M zone from a boat after the spring overturn. This experiment was less successful than the 1970 treatment in isolating the tracer in the bottom water. In 1969 some vertical mixing took place which moved small amounts of our tracer into the upper water. Thus there were two possible sources of ^{32}P—(1) from <u>Chaoborus</u> transport into the upper water, and (2) from vertical mixing. The extent of the vertical mixing can be estimated from our rhodamine data. Hence it may be possible to quantitatively assign a fraction of activity to each of these two sources.

An extensive set of data on the phosphorus were collected in spring and summer of 1970 after labeling. These included both stable and ^{32}P activity (1) in the plankton, (2) in <u>Chaoborus</u>, (3) in <u>Umbra</u> (the only fish present), and (4) in the bog mat. Very few of these data have been compiled at the time of writing this report but are now being processed for computer analysis; hence most of the past summer's work has not been included. We have provided certain chemical data for the lake required by Professor Griffing in his analysis of the calcium cycle (Appendix D).

To further evaluate the role of the bog mat in the regulation of the chemical economy of the system, we collected several sets of samples from the following locations: (1) The mat-water interface, and (2) within the mat at distances of 3, 5, 8, and 15 meters from the open water. To collect a vertical series of uncontaminated water samples at each of these locations, we devised a simple but effective device for sampling. It consisted of a section of heavy plastic tubing sealed at one end (Fig. 1). The sealed end of the tube was easily forced into the bog mat. At the site of the tube a few inches behind the sealed end, we provided a small window covered with plastic screening. During penetration of the mat, this window was sealed by

an inflated rubber balloon. The balloon was deflated when the sampler was in place at the proper depth. This allowed the sample tube to fill with water. We collected samples within the bog mat down to a depth of 4M with this device. Filtered and unfiltered samples were analyzed for stable phosphorus, ^{32}P activity, and for soluble and total iron using the bathophenanthroline procedure. Representative data from this work are included (Appendix D).

Work continued on the identification of bog organic phosphorus compounds. During the spring I completed a literature survey dealing with the identification, origin, and regeneration of organic phosphorus within freshwater systems. Mrs. Kay Terry perfected a new procedure for isolating and analyzing the phosphorus compounds, and in August we used this procedure in the analysis of samples from three locations within the basin. This literature survey and the results of the past summer's sampling are reported in a third report (Appendix C).

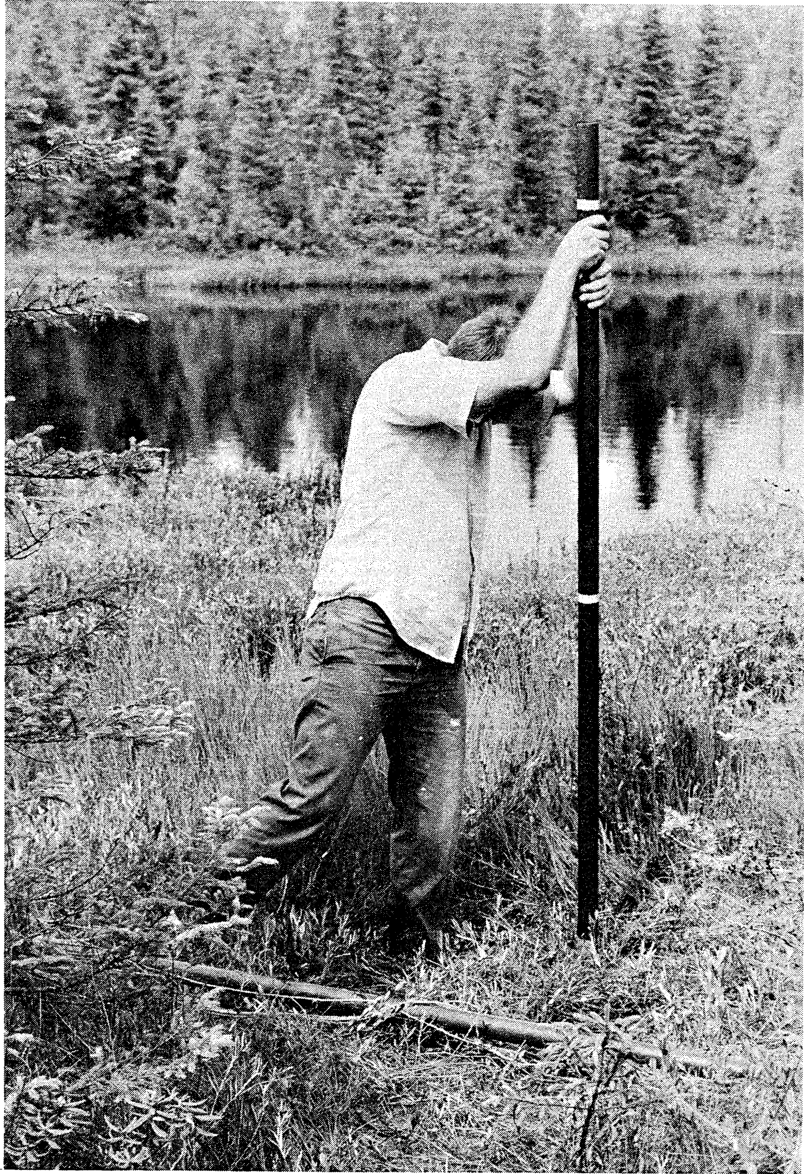

Fig. 1. Mat sampling apparatus.

APPENDIX A

TRANSFORMATION OF HYPOLIMNETIC PHOSPHORUS BY A PERIPHYTON COMMUNITY

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The redistribution of chemicals from the hypolimnetic zone during the vernal overturn is vital to the biological productivity of lakes. Gross features of this process have been adequately described (Welch, 1952; Hutchinson, 1957); however, certain aspects have been poorly investigated. These are (1) the nature of the chemical transformations that take place when organic phosphorus compounds originating in the hypolimnion are cycled in the surface water, and (2) the rate at which orthophosphate and organic residues arising in the hypolimnion are incorporated into the plant biomass of the surface water. To determine the fate of hypolimnetic phosphorus carried to the photosynthetic zone, we conducted a series of experiments in July, 1969, in which bottom water of a bog lake, labeled with ^{32}P, was pumped to the surface, where it was passed through a plastic tank containing a culture of periphyton and bacteria. This paper describes the transformations of the hypolimnetic phosphorus carried out by this system.

In our analysis of the phosphorus cycle of North Gate Lake, a small bog lake in northern Michigan, the dissolved organic phosphorus (DOP) was of particular interest because of its sporadic occurrence in the lake both in time and in space. During the summer of 1968 there was an abundance of DOP at all depths below the thermocline. That year, thermal stratification was normal and there appeared to have been some mixing throughout the entire water column throughout the entire summer. In the spring of 1969 there was an incomplete vernal overturn and a strong thermocline developed which largely prevented excursions of surface water into the deeper layers. This year, DOP was absent or very low in both the 6M and 7M strata during most of the summer. However, it increased strongly at all depths in late July when there apparently had been vertical mixing. This pulse of DOP was short-lived, however, and soon decreased or disappeared at all depths. To test the hypothesis that within the anaerobic zone of the lake DOP is produced by the aeration arising from mixing, was one purpose served by the present experiment.

The vernal overturn may take place in a few days. It is often difficult to analyze the processes taking place because the critical biological conversions may occur not only in a short span of time, but also, within restricted zones of the lake basin. By artificially pumping hypolimnetic water to the surface, we have previously demonstrated its effect upon primary production (Hooper, Ball, and Tanner, 1953). Of somewhat more fundamental interest, however, is the fate of specific phosphorus residues and their conversion into the organic phosphorus of the biota.

Recently there has been wide-spread interest in de-stratification of lakes and reservoirs for the purpose of (1) improving water quality, and (2) providing benefits to the fisheries (Fast, 1968; Mayhew, 1963; Koberg, 1964; Johnson, 1967). During aeration large quantities of nutrients are released and de-stratification represents a form of auto-fertilization. In view of the practical interest in redistribution of hypolimnetic water, it is of value to explore more deeply the transformations of phosphorus that take place.

Methods

The tank used in these experiments contained four compartments separated by plastic partitions. During passage, the water made contact with periphyton substrates suspended in the two center compartments as well as with the plastic walls of the container. Water was pumped through the system by means of an aquarium air-lift located in the downstream compartment (Figure 1). Flow from the air-lift entered a plastic tube which returned the water to the 5M depth. As water was removed from the downstream compartment, it was replaced by a flow of 7M water entering the upstream compartment. The flow could be varied within wide limits; however, during experiments described in this paper and during the equilibration process a flow of 60 ml/min was maintained. At this rate the residence time of water in the system was 9 hr. Each compartment of the tank was supplied with a flow of compressed air. This aerated the water and circulated the flow during its passage through each unit. Three weeks before our first experiment the system was filled with 7M water and innoculated with a net plankton sample collected from the surface water. The innoculum consisted of the catch made by a 20 μ net when 50 l of surface water were poured through at a rate of 5 l/min. The plankton of the lake at this time was sparse and the innoculum was estimated to contain less than 5 μg (dry weight) of plankton. Qualitatively it consisted of a variety of algae, including diatoms, dinoflagellates, chryosomonads, blue-green and green algae, and a few copepods. The intake of the tank system was screened with 20 μ nytex to prevent loss of zooplankters.

Within a week after the flow of 7M water was started, a dense growth of periphyton developed on the substrates and the walls of the tank. During the second and third week the growth increased. By the start of the experiment, the dry weight of the periphyton crop was estimated to 2789 μg or 558 times the initial innoculum.

Measurements of phosphorus transformation by the system were first made on July 3, three weeks after the unit was set in operation. A second set of measurements was made on July 10. On each of these dates two sets of measurements were made. The first was made at 6 a.m., after the system had been operating in the dark for approximately 9 hr. The second set was made at 3 p.m. after approximately 9 hr. of sunlight. Measurements of the affluent and

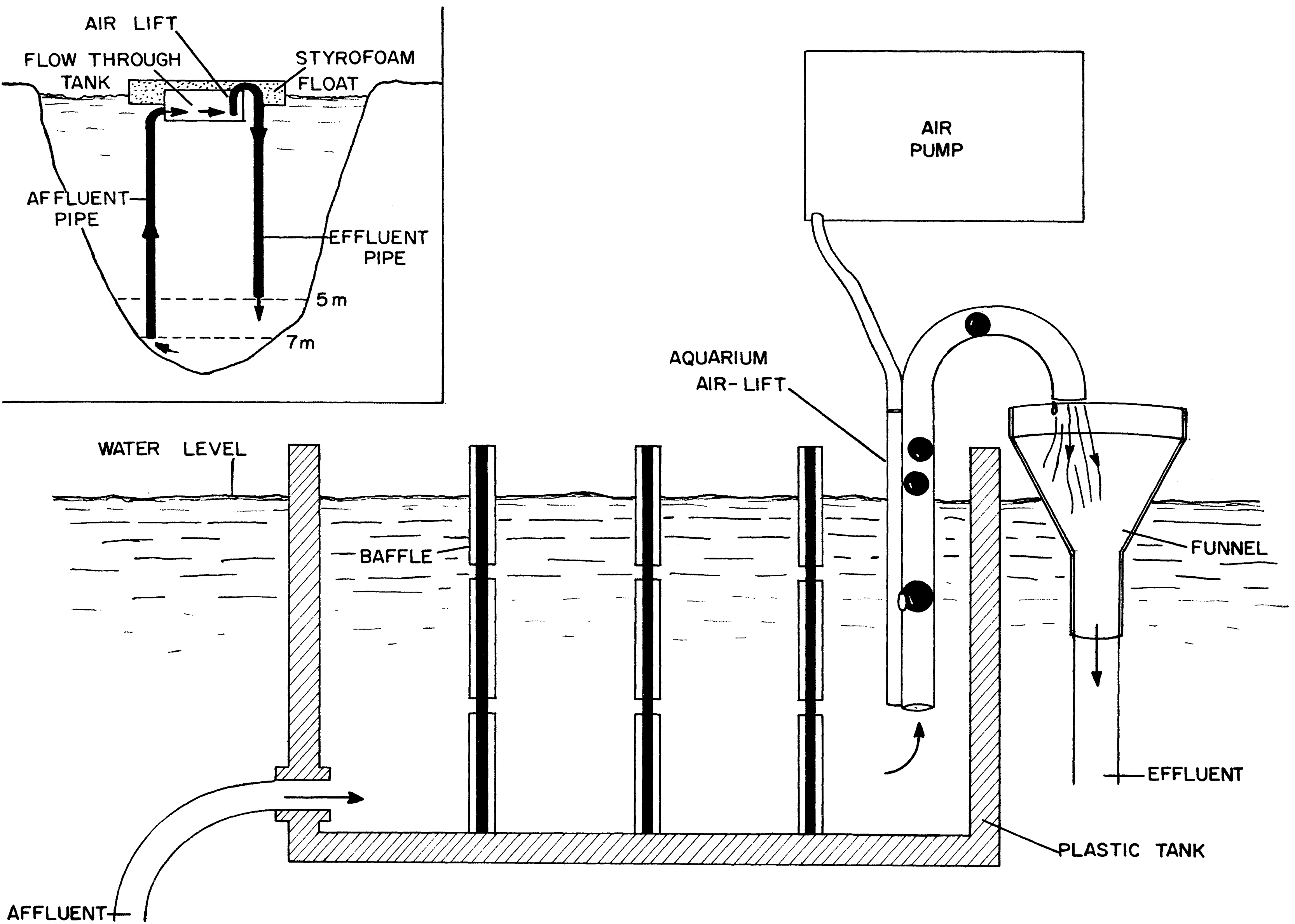

Fig. 1. Diagram of flow-through apparatus.

and effluent flows were made on aliquots from 3-l samples siphoned from the intake and discharge. Measurement of orthophosphate, dissolved organic phosphate (DOP), and particulate unreactive phosphorus (PUP) were made on three 100-ml aliquots. Radioactivity was measured by evaporating to dryness 50 ml of the solution used for stable phosphorus measurement and counting with a low background beta system.

Small but significant differences in the stable phosphorus of filtered samples from 7M before and after digestion indicated that DOP was present. To determine whether or not the DOP of the affluent and effluent were labeled, we extracted the heteropoly blue complex with 50 ml of hexanol after stable phosphorus had been measured, using the procedure given by Strickland and Parsons (1965). We then evaporated the aqueous phase to dryness and counted the residue. Since we did not detect a significant amount of activity in the water phase of the affluent samples we concluded that DOP of the 7M water was unlabeled. A small but detectable amount of activity present in the effluent indicated that labeled DOP was produced by the system.

Measurements of stable and radioactive phosphorus of samples from the 7M stratum were made at frequent intervals for a period of three months before transformation experiments were undertaken. During this period orthophosphate varied from 66 to 84 μg/l. The specific activity of filtered samples (corrected for decay) increased from 180 pci/μg under the ice on March 21 to a maximum of 234 pci/μg at the time of the spring overturn. After the overturn, specific activity fell slightly and remained close to the pre-overturn level. Mean specific activity values during May and June were not significantly different from the March 21 values. The filterable phosphorus fraction of the 7M stratum therefore appeared to be in isotopic equilibrium.

Direct calculation of the specific activity of the particulate phosphorus was not possible since in many cases measurements of activity and stable phosphorus were made on sets of samples collected on different dates. The 14 sets of measurements of particulate phosphorus between March 21 and June 29 can be compared with 11 measurements of particulate activity. In these measurements the percentage of the total stable phosphorus removed by filtration (24.2 ± 9.1%) agreed well with the percentage of activity removed by filtration (25.6 ± 8.1%). Although there was considerable sample-to-sample variation in the percentage of both particulate activity and particulate phosphorus, these variations were random and there were no detectable trends during this period. This agreement in the percentage of particulate activity and the percentage of stable particulate phosphorus indicates that the specific activity of particulates was relatively stable.

At the time of the transformation experiments, the flora of the system consisted chiefly of bacteria and the green alga *Oocystis*. Two species of epiphytic diatoms and the chrysophycian *Dionbryon* were also present. Counts of periphyton made at frequent intervals indicated that forms other than

Oocystis and large numbers of unidentified bacteria were qualitatively unimportant.

Uptake of Phosphorus Fractions

A major fraction of the orthophosphate was removed by the tank system. This fraction varied from 60 to 94% (Table I). A larger fraction of ortho-

Table I. Uptake (-) and export (+) of the fractions of labeled fractions of 7M water during passage through aerated tank at lake surface containing bacteria and periphyton. (Averages of 3 samples collected from affluent and effluent flow.)

Fraction	July 3				July 10			
	a.m.		p.m.		a.m.		p.m.	
	μg/l or pci/l	Net change ÷ affluent conc.	μg/l or pci/l	Net change ÷ affluent conc.	μg/l or pci/l	Net change ÷ affluent conc.	μg/l or pci/l	Net change ÷ affluent conc.
	Stable Phosphorus (μg/l)							
Orthophosphate	-52	0.60	-82	0.82	-77	0.83	-94	0.86
Dissolved Organic	+18	6.00	+12	4.0	+23	3.3	+26	6.5
Particulate	+23	0.96	+11	1.1	+27	0.64	+24	2.61
Storage	-11	0.096	-59	0.52	-27	0.19	-21	0.16
Estimated labeled dissolved phosphorus in effluent*	24		17		9		6	
	^{32}P Activity (pci/l)							
Dissolved	-79	0.89	-75	0.99	-35	0.92	-36	0.92
Particulate	+ 7	2.9	+ 7	7.2	+32	1.9	+14	1.8

*For calculation, see text.

phosphate was removed from the afternoon samples than from the corresponding morning samples on both July 3 and July 10. The difference between the means of morning and afternoon samples was significant at the 95% level on both dates.

The DOP concentration was from 3 to 6 times greater in the effluent than in the affluent, but the difference in production of DOP between morning and afternoon samples was not significantly different. The specific activity of the particulates entering the system was not significantly different from the specific activity of the filterable fraction of the affluent (Table II). This confirmed the earlier indications that the water and the solids of the 7M stratum had arrived at isotopic equilibrium.

Table II. Specific activity of affluent and effluent fractions of flow-through system during experiment of July 3 and July 10 (pci/μg).

Date	Affluent		Effluent	
	Dissolved Mean and S.E.	Particulate Mean and S.E.	Dissolved Mean and S.E.	Particulate Mean and S.E.
July 3				
a.m.	0.97 ± 0.14	0.94 ± 0.065	0.27 ± 0.092	0.75 ± 0.19
p.m.	0.75 ± 0.065	-- --	0.11 ± 0.11	0.96 ± 0.014
July 10				
a.m.	0.52 ± 0.076	0.59 ± 0.195	0.04 ± 0.02	1.59 ± 1.2
p.m.	0.50 ± 0.061	0.55 ± 0.033	0.06 ± 0.06	1.98 ± 1.2

In two experiments on July 3 the specific activity of particulates in the effluent was not significantly different from the activity of entering filterable or particulate fractions. However, on July 10, the mean specific activity of effluent particulates was threefold greater than that of the entering particulates. Considerable variation, however, was evident in the particulate activity of the effluent. On this date, despite this large difference in means, the mean specific activity of the afternoon effluent samples was not significantly different from the mean of the affluent particulates in either the morning or afternoon (Table II). The mean of the morning samples of effluent particulates was significantly different from the morning and afternoon affluent. Thus the specific activity of the soluble fraction decreased during passage through the tank. All effluent means were significantly lower than affluent means at the 95% level of confidence or above.

The decrease in specific activity of the orthophosphate passing through the system can be either (1) the result of contamination from an undetermined source of unlabeled phosphorus, (2) production of unlabeled orthophosphate by discriminatory breakdown and release of an unlabeled fraction of particulate phosphorus entering the tank, or (3) an unlabeled polyphosphate or other complex inorganic phosphorus compound present in the 7M water that was hydrolyzed to orthophosphate during analysis. All of the orthophosphate should

have been labeled, however a complex inorganic phosphorus component would not be labeled. Such a fraction can be hydrolyzed by the acid molybdate reagent during analysis (Olson, 1966) and would give high values of orthophosphate in the affluent and effluent. This fraction would have been less susceptible to uptake than the orthophosphate fraction and if it appeared as unlabeled phosphorus in the effluent would give an apparent decrease in specific activity. Since a major fraction of soluble activity was removed, a small change in unlabeled phosphorus would give a large decrease in specific activity. This fraction would be less susceptible to biological uptake than orthophosphate, and would give an increase in the percentage of unlabeled soluble phosphorus in the effluent and an apparent decrease in specific activity.

Monitoring the 7M water before the experiment did not indicate any additions of unlabeled phosphorus as required by alternative (1) above.

To assess alternative (2) above, I calculated the expected amount of unlabeled compound in the effluents as follows:

$$OP_U = OP_e - \frac{^{32}P_e}{^{32}P_a} \times OP_a$$

where: OP_U = the unlabeled orthophosphate fraction, OP_e = the effluent orthophosphate, OP_a = the affluent orthophosphate, $^{32}P_e$ = the soluble activity of the effluent, and $^{32}P_a$ = the soluble activity of the affluent. These estimates indicated that on July 3 the expected unlabeled fraction in the effluent was greater than the quantity of particulate phosphorus entering the system (Table I). Thus alternative (2) does not appear likely since at least a part of the affluent particulate phosphorus was labeled and had a specific activity that was not significantly different from the soluble fraction (Table I).

Alternative (3) is the most attractive explanation. Labile phosphorus fractions were separated from the 7M stratum in 1969 and in 1970. In 1970 this fraction was separated by column chromatography and recovered by gradient elution with formic acid. It appeared as a UV absorption peak distinct from orthophosphate that corresponded to polymers of orthophosphate. It was hydrolized by 1 N HCl and it comprised 36% of the inorganic phosphorus of the 7M stratum (Hooper and Terry, 1970).

Transformation Rates

The system removed virtually all of the orthophosphate and utilized this phosphorus in the production of DOP and PUP. Production of DOP was in all cases about equal to production of PUP; the mean increase in DOP was not significantly different from the mean increase in PUP in any of the four

Table III. Rates of phosphorus uptake, storage and export by periphyton and bacteria in flow-through tank.

Transformation	July 3				July 10			
	a.m.		p.m.		a.m.		p.m.	
	μg P/hr/ mg dry biomass	Percentage of periphyton P/hr	μg P/hr/ mg dry biomass	Percentage of periphyton P/hr	μg P/hr/ mg dry biomass	Percentage of periphyton P/hr	μg P/hr/ mg dry biomass	Percentage of periphyton P/hr
Uptake of Orthophosphate	.100	1.38	.127	1.76	.111	1.53	.121	1.68
Storage as PUP	.021	.29	.091	1.25	.039	.54	.027	.37
Export of PUP and DOP	.079	1.09	.036	.51	.072	.99	.094	1.31

experiments. This close agreement is perhaps purely fortuitous.

Rates of transformation of DOP into organic compounds were calculated from uptake measurements (Table III). Rates were expressed in terms of the biomass of periphyton in the tank system. Biomass estimates used were those made after the experiment of July 3. Further biomass measurements were not made on July 11 because substrates removed one week later (July 20) had approximately the same biomass as the July 3 substrates.

The uptake rates of orthophosphate varied from 0.1 to 0.127 μg P/hr/mg of periphyton biomass. This uptake equaled 1.38 to 1.76% of the phosphorus present in the system per hour (Table III). From 21 to 71% of the phosphorus uptake was stored in the tank and apparently went into new growth of cells. The remaining fraction (0.51 to 1.31% of phosphorus present) was incorporated into organic compounds of the export. In addition to DOP, the export contained some living cells, but consisted chiefly of dead cells and debris with its covering of epiphytic bacteria. Thus the export consists of phosphorus being turned over within the system through the autolysis and replacement of old cells as well as phosphorus released by metabolic turnover.

Discussion

Most of the experimental systems used for the analysis of phosphorus cycling in the past have either involved the addition of a tracer (Hutchinson and Bowen, 1950; Hayes, et al., 1952) or have enriched the system by the addition of the stable element creating a perturbation which provides the basis for a dynamic analysis. Transformations of a chemical cannot be studied effectively in a standing water system when the system is under steady state conditions, even if a tracer is used. In a flow-through type system, the recycling is minimized and the net change between import and export provides a measure of total system response.

Tracer experiments using small microcosms such as aquarium systems have identified the direction of movements of tracers between pools of the stable elements within the biological system. Experiments with ^{32}P such as those conducted by Hayes and Phillips (1958) and Phillips (1964) have indicated the sites of active exchange, but are of doubtful validity when used as a measure of uptake and flow rates because the movement of a tracer is not necessarily a quantitative measure of the movement of the stable element when there is the possibility of absorption and isotopic exchange. Rates of uptake measured under these circumstances invariably show a very rapid flow of ^{32}P into bacteria of the water phase or into the bottom substrate. Flow rates calculated under these conditions assume uni-directional movement of the tracer and stable elements. Such a model does not allow for isotopic exchange arising from concentration gradients of the tracer.

Turnover rates of the order of 5 min suggested by Hayes and Phillips (1958) for aquaria containing algae and bacteria are an order of magnitude greater than the rates indicated by the present study (approximately 1-2% of the total phosphorus per hour). This turnover rate is greater than that reported for the marine copepod *Calanus* (10%/day; Conover, 1961), but less than excretion rate for mixed marine plankton (about 100%/day; Pomeroy, *et al*., 1963) and freshwater plankton (30 to 120% of body phosphorus per day; Barlow and Bishop, 1965). Figures from the tank study must include the phosphorus released by cell replacement in addition to that arising from metabolic turnover. In this study, recycling cannot be excluded but any recycling taking place must have been principally reutilization of orthophosphate. It is doubtful if the flow-through time (9 hr) would have permitted significant recycling of particulates and DOP.

The production of DOP by the aerated tank tends to substantiate the hypothesis that DOP is produced from orthophosphate whenever bottom water is aerated. Phillips (1964) and Hayes and Phillips (1958) found that bacteria produced DOP during a decline in cell numbers but not during the growth phase. Rates of production in the present study presumably represent an equilibrium condition when both senescent and growing cells are present.

Summary

During recycling of labeled hypolimnetic water through a tank containing periphyton suspended at the surface, there was nearly complete uptake of the labeled orthophosphate. The smaller uptake of stable inorganic phosphorus compared to the corresponding uptake of ^{32}P indicated that an unlabeled and nonexchangeable complex inorganic phosphate fraction was present in the hypolimnetic water. When the tank system operated under approximately steady state conditions, it removed between 89 and 99% of the hypolimnetic orthophosphate and exported approximately equal quantities of DOP and PUP. Uptake rates ranged between 1.38 and 1.76% of the system's phosphorus per hour. Phosphorus was exported from the system as DOP and as living and dead periphyton and bacteria at rates of from 0.51 to 1.31% of the system's phosphorus per hour.

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APPENDIX B

MECHANISMS OF ^{32}P UPTAKE BY INVERTEBRATES

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MECHANISMS OF ^{32}P UPTAKE BY INVERTEBRATES

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Laboratory experiments were performed with aquatic larvae of <u>Chaoborus americanus</u>, a midge, in an attempt to determine the mode of radiophosphorus uptake. The results of these experiments to date will be presented in this paper. Also, I will review the literature dealing with biological transport of phosphorus in aquatic systems; discuss the problem of radiophosphorus uptake—ingestion, absorption, and adsorption by freshwater invertebrates involved; and discuss the techniques that I used in laboratory experiments in relation to the techniques used by other workers for similar problems.

Biological Transport of an Element in an Aquatic Ecosystem

Hasler and Likens (1963) have summarized earlier work on biological transport, and from their research on iodine state:

> It seems reasonable, therefore, that if aquatic organisms can take up and transport trace elements such as radioiodine, they certainly would be able to concentrate and transport appreciable quantities of the radionuclides of the more abundant protoplasmic constituents. Thus, biologically significant quantities of radionuclides could be transported from the deep and relatively inaccessible part of a lake to the surface and thence to the terrestrial environment.

The biological transport of an element such as phosphorus could be important because it might make a lake more productive. Phosphorus from the bottom of the hypolimnion could be taken to the epilimnion where it could be used by phytoplankton and bacteria. Conversely, phosphorus can be transported from the epilimnion to the hypolimnion or to the terrestrial environment, thereby decreasing the productive potential of the lake. Movement of phosphorus from the hypolimnion to the epilimnion is evidently more exceptional. Aquatic insects appear to be of the greatest importance in the transport of nutrients from the hypolimnion to the epilimnion and to the terrestrial environment. Since the mass of adult dipteran larvae emerging from a lake is almost entirely derived from the lake bottom, Vallentyne (1952) believed that a depletion of the chemical energy of the lake basin must result. He therefore attempted to determine the quantity of chemicals removed by insects and found that from July 1 to July 15 the loss of phosphorus from an Indiana lake basin was

0.15 mg P/m^2/day. This figure cannot be used to compute an annual loss. Chaoborus was one of the insects responsible for phosphorus removal. Although Vallentyne (1952) found a greater rate of phosphorus sedimentation, the removal of phosphorus by insects could be important in small lake systems containing a high density of insects.

Whitten and Goodnight (1969) studied accumulation of radiophosphorus by tubificid worms from water, bacteria and sediment. Because of their abundance in some ecosystems they believed that tubificid worms may have an important part in the transfer of radioactive phosphorus from water and detritus to other components of the ecosystem such as fish. These worms acting in conjunction with bacteria recycle radiophosphorus from detritus and sediments. In stratified lakes with an oxygenated hypolimnion, fish might transport phosphorus from the hypolimnion to the epilimnion by feeding on tubificids and other benthic organisms in the sediments.

If aquatic organisms are important in nutrient cycling, further studies should be undertaken to ascertain the magnitude and mechanisms of their nutrient transport. Without labeled elements, it is difficult to determine the source of a chemical within the basin. A labeled element in part of a lake system, such as the hypolimnion or epilimnion, might easily indicate whether certain organisms are obtaining a certain element in these areas.

The Mode of Radiophosphorus Uptake

The problem of determining how an organism becomes radioactive often is difficult even though only a few possible mechanisms can be involved. Radiophosphorus may be taken up by an aquatic organism via absorption through membranes, via ingestion of food or inert particles, or via adsorption to exposed surfaces. External, as well as internal, bacteria of the larger aquatic organism may become radioactive. Determination of which of the possible mechanisms involved in radiophosphorus uptake occurs will probably not be made with certainty until the mechanisms themselves are better understood.

Determination of the movement of a stable element from data of a radioisotope is often difficult. Uptake of radiophosphorus by an organism only means that some radioactive atoms have been taken into the organism's phosphorus "pool"; it does not necessarily indicate that a certain quantity of stable phosphorus has also entered the organism's phosphorus "pool." Some scientists have probably incorrectly calculated the movement of stable phosphorus from one "pool" to another from their radioisotope data. The reason for the error is that a steady state distribution of an element usually exists between aquatic organisms and their environment; with an addition of a radioisotope in one part of the system, the radioisotope will tend to reach isotopic equilibrium with the other part(s) of the system. However, isotopic equilibrium can

be reached via isotopic exchange, in which the total content of the element in the organism is not affected, but there is replacement of the stable isotope by its radioisotope or vice versa (Polikarpov, 1966).

At various times certain phosphorus pools, or individual parts of the pool may have a net uptake of phosphorus. Phosphorus may be incorporated into insoluble tissue components, but there is no way of knowing if radiophosphorus atoms have been used as raw material for synthesis, or if the atoms have merely been partners in exchange reactions. Waddington (1959) states that "atoms may be utilized for the de novo synthesis of some fixable compound, such as a protein. But secondly, there is the possibility that the atom may enter such a compound as a consequence of an 'exchange reaction' in which parts of an already-formed macro-molecule become liberated from it and their place taken by a similar atomic group, containing the tracer, without any increase in the macro-molecular species."

Organisms become radioactive by adsorption when the radioactive substance physically adheres to the outer surface of the organism (Seymour, 1959). The outer surface also includes the surface of internal organs. During absorption the material passes through a living membrane; absorption of a substance may occur externally or internally. Absorbed materials do not have to be assimilated in tissues and may remain within an organism only temporarily. Absorption of a material can occur against an electrochemical gradient via active transport, or it can occur passively via diffusion. The material may have been brought into the digestive system selectively or by chance; the material may be in live or dead organisms, soluble organic matter, or inorganic matter. It may pass through the animal without being changed and/or used. An animal may appear to be radioactive, when in actuality the radioactivity may be in some foreign matter in its gut. The ingested material could also be adsorbed to internal organs. The presence of ingested material in itself does not indicate that the organism uses the material.

Whittaker (1961) gave an interesting appraisal of the problem of phosphorus uptake in organisms:

> "When, as in this experiment and presumably in most natural water bodies, surfaces are coated with films of microorganisms, then adsorption onto and uptake by these organisms are difficult to separate. Of surface effects in general it may be said that adsorption, absorption, and other processes are so interrelated that they cannot be sharply distinguished in practice, and perhaps should not be regarded as distinct and separate in their significance in the functioning ecosystem."

Rice (1953) presents two ways in which phosphorus may enter a cell. First, by diffusion of inorganic orthophosphate through the cell membrane. In this case, the inorganic phosphate then combines with the intracellular orthophosphate. Inorganic orthophosphate is considered the source of phosphorus for the

various organic phosphates in the cell. The second possible method is the entry of phosphorus into the cell through esterification at the cellular interface. The breakdown of organic phosphate would then produce intracellular inorganic orthophosphate. Working with yeast, Kamen and Speigelman (1948) concluded that the primary mechanism of the entrance of phosphate is by esterification.

Rice (1953) thought that the mechanism of entry was important in phosphorus exchange studies because it might require that phosphorus remain on the surface of the cell for some time. Phosphorus would probably not adsorb on cells if it could diffuse into the cell. Esterification, however, might require that phosphorus remain on the surface of the cell. Rice (1953) states that "there has not been sufficient consideration of the part adsorption plays in phosphorus exchange in microorganisms." He could not distinguish the phosphorus exchanged from the cell surface from that exchanged from inside the cells.

Ussing (1947) proposed that one-to-one exchange of ions across a membrane could occur by exchange-diffusion, in which the ions are supposed to saturate a carrier, in combination with which they pass across the membrane which they cannot traverse in the free state. Mitchell (1954) states that an enzyme may be that carrier. Roberts and Roberts (1950) found one-to-one exchange of inorganic radiophosphorus occurred across the cell membrane of _Bacterium coli_. They found the cell membrane freely permeable to phosphate ions, but that the internal inorganic phosphate was not free; it was adsorbed on a number of acid-labile sites within the cells.

All of the mechanisms involved in moving phosphorus in and out of cells may be considered part of the transport process. The transport mechanism for phosphorus going into a cell is not necessarily the same for phosphorus coming out of the cell. Christensen (1962) defines transport as "the mode by which a solute passes from one phase to another, appearing in the same state in both phases." Conway (1954) states that "active transport across a membrane connotes movement of the solute or ion across the membrane dependent on the activity or energy change of another system. Passive transport is equivalent to free diffusion or 'exchange-diffusion,' where the energy of the net movement comes from the same system." However, active transport of some material may set up a gradient for passive transport of some other material. Active transport is generally defined as transport that takes place against an electrochemical gradient. In passive transport an electrical or chemical gradient may exist so that material moves "downhill." The diffusion rate is determined by the nature of the membrane and by the concentration gradient.

Entry into cells of ions against a concentration gradient (i.e., active transport) requires energy from metabolic processes (Stumpf, 1952). Since phosphate molecules may be involved at several points in the process of energy-yielding reactions required to drive the active transport, studying phosphorus transport is difficult (Mitchell, 1954).

If an inorganic phosphate is incorporated into an organic compound at the plasma membrane of a tissue, and then a few hours later broken down into inorganic phosphate, true transport has not taken place. Christensen (1962) would consider this as "functional compartmentalization." If inorganic phosphate enters a cell by forming ATP and, successively, one or two other phosphoryl compounds before appearing seconds later as inorganic phosphate, then transport does occur. In this transport a phosphoryl translocation may prove to be the decisive step. The time lag is what differentiates transport from compartmentalization. Conover (1961) states that "radioactive phosphorus must be presumed to pass through several different physiological or chemical 'pools,' each with a different turnover time, before an equilibrium is approached." He found evidence for an additional phosphorus pool intermediate between external phosphorus and the large, stable pool of body phosphorus.

Undoubtedly phosphorus passes through the membranes of organisms in several different ways. There is a difference in the rate of radiophosphorus exchange among different animal tissues (Greenberg, 1952). It has been demonstrated that there is no chemical exchange between the inorganic radiophosphorus and atoms bound in organic compounds. Incorporation of radiophosphorus into these compounds must be through metabolic reactions (Chipman, 1959; Gourley, 1952). No methods are now known that clearly differentiate absorption by metabolic processes and adsorption by physical and ion-exchange phenomena (Cushing and Watson, 1968). Adsorption may also occur within a cell as well as on the outer surface of an organism.

When considering radiophosphorus uptake by invertebrates, the role of bacteria must be considered. In experiments with the beach flea Gammarus, Harris (1957) found that the absence of bacteria inhibited radiophosphorus absorption. Hayes (1963) states, "Evidently direct absorption of inorganic phosphorus does not occur appreciably either through the body wall, intestine, or gills...higher invertebrates obtain their food by digestion of particulate matter rather than by direct absorption of inorganic or organic solutions." Bond (1933) concluded that in general marine invertebrates were impermeable to water, salts, and organic solutes. The only way for such organisms to obtain radioactive phosphorus is by ingestion of water or particulate material. Harris (1957) believed that Gammarus obtained radiophosphorus from bacteria. Stuart, McPherson, and Cooper (1931) found that the cladoceran Morina macrocopa was unable to subsist on dissolved organic material, but could be cultured indefinitely on living bacteria. Fox (1952) reports that some crustaceans are known to swallow water and thus may absorb some phosphate. However, for most invertebrates phosphorus seems to be obtained from food.

Johannes (1964b) found uptake of ^{32}P by sterile amphipods (Lembos intermedius). Nonsterile animals, however, had greater uptake than sterile ones. The medium used by the nonsterile animals was initially sterile, so there appeared to be no ingestion of labeled bacteria. Radiophosphorus could have been taken up by exchange between the amphipod and the medium, however, Johannes' results suggest that a large fraction of the radiophosphorus taken up by the

nonsterile amphipods was by way of intestinal bacteria, thus making the phosphorus available to the animal. However, Rigler (1961), working with Daphnia magna, attributed the uptake of inorganic radiophosphorus to bacteria on the exoskeleton, and concluded that this phosphorus could not be used by the cladocerans. Both Rigler and Johannes found that almost 99% of the phosphorus taken up by the animals was obtained from their food. Hassett and Jenkins (1951) found that mosquito larvae obtained most of their radiophosphorus from food, but that in the fourth instar the anal papillae functioned in the absorption of phosphates (as well as chlorides). Although the anal papillae may only be an auxiliary path for phosphorus uptake, they apparently are capable of phosphorus absorption.

The reasons for the concentrations of radioactive substances by organisms has been summarized by Davis and Foster (1958) as follows:

(1) The mode of uptake which includes adsorption to exposed areas, absorption into tissues, and assimilation of ingested material.

(2) Retention, which is the function of the biochemistry of the particular elements and components involved, the site of deposition, the turnover rate, and the radioactive half-life.

(3) The mode of elimination, which may involve ion exchange, diffusion, excretion, and defecation.

Methods

In February several thousand Chaoborus larvae, mainly Chaoborus americanus, were collected from George Pond, on the Edwin S. George Reserve in Livingston County, Michigan. These larvae were kept in a jar and in two aquaria at 40°F in a cold room. The larvae were not exposed to light. Beginning on February 26, 1970, several experiments were undertaken to study radiophosphorus uptake in sterile media. Both direct counting and autoradiography techniques were used.

The first experiment was carried out with 60 larvae in 1 liter of nonsterile, George Pond water containing 2.5 μCi of ^{32}P to see if the larvae would become radioactive. After 18 hrs the larvae were removed from the medium. One aliquot was plated directly on planchets, one was washed in water before plating, and a third was washed in water and in dilute HCl (< 1% HCl) before plating. The larvae were dried and counted with a GM counter (efficiency of 19% with 1.17 Mev standard beta source). Untreated larvae from the storage jar were used to determine background counts.

Although rinsing is often used to measure adsorbed phosphorus, Whittaker (1961) states that "the problem is not simply solved by rinsing. On the one hand, some adsorbed phosphate enters chemical combinations on cell surfaces and resists removal without repeated rinses; on the other hand, repeated rinses may remove substantial, but variable, fractions of the more labile phosphate in the cells." Thus, washing might remove phosphorus inside the organism.

Whittaker (1961) measured the radioactivity of organisms held in radioactive media for various periods of time. He found that most of the initial uptake of radiophosphorus was adsorptive. This was followed by absorptive uptake. The absorption curve eventually reached a plateau as isotopic equilibrium was approached. Adsorption and absorption can occur simultaneously but the length of time the organism is exposed to the medium determines the relative importance of the two mechanisms.

In March, 1970, several experiments were initiated to study the importance of bacteria in radiophosphorus uptake. The procedure used was adapted from Rigler (1961). Chaoborus larvae were placed in a sterilized nutrient solution containing 100 units of penicillin/ml and 100 μg of streptomycin/ml. The Chaoborus were left in this antibiotic solution for 90 min, put into sterilized nutrient solution for 24 hr, returned to fresh antibiotic solution for 90 min, and cultured in radioactive sterilized nutrient medium for various time periods. All media were maintained at 40°F or 4.5°C. Pomeroy, et al. (1963), found that a combination of penicillin and streptomycin did not interfere with phosphorus analysis, and that they were effective against a broad spectrum of microorganisms. Plotz and Davis (1962) believed that penicillin and streptomycin had a synergistic action; penicillin lowered the threshold concentration of streptomycin and hastened the onset of killing by streptomycin. Penicillin interferes with synthesis of the cell wall, and streptomycin may cause membrane damage. However, streptomycin has a two-stage mechanism in which membrane damage precedes binding of the drug to intracellular sites.

Streptomycin and penicillin do not always sterilize. Pomeroy, et al. (1963), believed that sterility was not achieved because of continual seeding of the culture with viable bacteria from feces of the zooplankton, and antibiotics only maintained bacteria numbers at or below the number found in the sea water. Spencer (1952) could not obtain axenic cultures of diatoms with streptomycin and penicillin. Johannes (1964a) used a solution of penicillin, streptomycin, chloromycetin, terramycin, and polymixin B along with ultraviolet light in order to produce bacteria-free algae cultures. He found that the antibiotics did not kill, but only inhibited the growth of some species of bacteria. Therefore, he used antibiotics during, as well as preceding, his experiments. Antibiotics are simply antimetabolites whose influence on bacterial metabolism is somewhat greater than that on the metabolism of higher organisms. Pomeroy (1963) states that "metabolic rates of higher organisms also may be affected by antibiotics, and the difference observed in phosphate turnover may not be entirely the result of inhibition of bacterial metabolism."

Another source of bacterial contamination is the radiophosphorus solution added to cultures. Hayes (1963) noted that 60% of the carrier-free phosphate in Normal HCl will within a few days after it is diluted with distilled water, be converted to an organic form.

In my experiments, both the time of exposure of larvae and the strength of the antibiotic solution were greater than employed by Rigler (1961). In one experiment, antibiotics were introduced directly into the radioactive medium.

In a number of experiments I made autoradiographs of the larvae. After removal from the radioactive medium, larvae were frozen with dry ice. When all larvae were ready, they were taken to a dark room and allowed to thaw on a piece of absorbtive paper. They were then placed on Kodak no-screen medical X-ray film (NS54T and RPS7) in a manner which enabled them to be identified after film development.

Bruce-Chwatt and Hayward (1956) and Hassett and Jenkins (1951) used X-ray film to make autoradiographs of whole mosquito larvae that had been exposed to radiophosphorus, and Johannes (1964b) determined that ^{32}P was deposited in the nuclei of the muscle and hypodermis cells of amphipods by microautoradiography. Other workers have used ^{32}P for making autoradiographs but resolution is ordinarily poor in microautoradiography when X-ray film is used (Chapman-Andresen, 1959). These films have the advantage of speed, and may be useful to determine whether or not a cell has radioactive material.

The resolution of an autoradiograph is affected more by the nature of the isotope than by the type of film. Although various types of film will give different resolution, ^{32}P will never give the quality of tritium because of differences in beta energy. Lamerton and Harriss (1954) defined resolution as the distance, d, if the images of two uniformly active cylindrical sources of diameter d can just be resolved when the centers are separated by a distance 2d.

The most important factor in making good autoradiographs is to choose the right technique. Gude (1968), Boyd (1955), and Rogers (1967) thoroughly discuss the theory, problems and advantages of various autoradiographic techniques. Quimby and Feitelberg (1963), Chapman-Andresen (1959), Waddington (1959), Messier and Leblond (1957), and Leblond, *et al*. (1960), go into finer detail.

A variety of studies have been undertaken to isolate areas of absorption and diffusion. Koch (1938) and Wigglesworth (1933) found that the anal papillae of mosquito larvae served as osmoregulators and that many different ions could move in and out of the cells in the anal papillae. Wigglesworth could not determine whether phosphate could diffuse into these cells. Hasset and Jenkins (1951) found that the anal papillae of mosquito larvae functioned in the absorption of phosphorus. With ligatured larvae (human hair and/or fine silk thread) Schaller (1949) studied the osmotic regulation of *Chaoborus* and found that the anal papillae were the only salt and water permeable surfaces on the body. I attempted similar experiments with *Chaoborus*. Human hair was tied around

various segments of larvae. The larvae were then put in a sterile radiophosphorus nutrient medium. Later larvae were removed and autoradiographs were attempted.

Results

DIRECT COUNTING EXPERIMENTS

In the data presented, the activities of larvae have not been corrected for ^{32}P decay, but have been corrected for background. Experiments are not numbered in the order performed. Experiments numbered 1-4 were performed in nonsterile media; those numbered 5-12 were in supposedly sterile media.

Experiment 1

Sixty Chaoborus were put in 1 liter of George Pond water which contained 2.5 μc of radiophosphorus. They were stored in this solution for 18 hr, removed and put on planchets. One group of Chaoborus was first washed in distilled water, and a second was washed in both distilled water and dilute HCl (< 1% HCl). The difference between washed and unwashed samples indicates adsorption (Table I). There was only a small residual activity after washing, however some activity may have diffused out in the washing process.

Table I. Activity of Chaoborus larvae after 18 hours in George Pond water containing 2.5 μCi of ^{32}P. The larvae were prepared for counting by three different methods.

Plating Method	Number of Chaoborus	Counts/10 Min
Direct	10	160 ± 16
	10	132 ± 16
	10	148 ± 16
	10	130 ± 15
Water wash	10	31 ± 12
	10	36 ± 12
	10	33 ± 12
	10	18 ± 11
Water and HCl wash	10	2 ± 11
	10	7 ± 11
	10	6 ± 11
	10	0 ± 10

Experiment 2

Radiophosphorus was added to George Pond water to give 370 cpm/ml of medium. The same procedure as above was used. Most of the larvae were used in autoradiography, but some were saved for direct counting. Larvae were maintained in the medium for 10 days.

Results again show a large decrease in radioactivity with washing, but a significant amount of radioactivity remained (Table II).

Table II. Activity of Chaoborus larvae after 10 days in George Pond water with activity 370 cpm/ml.

Plating Method	Number of Chaoborus	Counts/10 Min
Direct	10	485 ± 24
Water wash	10	287 ± 20
Water and HCl wash	10	188 ± 17

Experiment 3

The medium used in the previous experiment was again used. Chaoborus were kept in the medium for 9 days. This experiment shows only a small loss of radiophosphorus with washing (Table III). Chaoborus were only rinsed once.

Table III. Activity of Chaoborus larvae after 9 days in George Pond water used in Experiment 2 (Table II).

Plating Method	Number of Chaoborus	Counts/10 Min
Direct	10	211 ± 18
	10	249 ± 19
Water Wash	10	244 ± 19
	10	181 ± 17

Experiment 4

The medium used in the previous two experiments was again used. A few Chaoborus removed and washed on March 30 were returned to the medium for eight more days (thus a total of 17 days in the medium). A significant amount of

radioactivity could not be washed from the larvae (Table IV), but a large difference in activities existed between the two samples.

Table IV. Activity of Chaoborus larvae after 17 days in George Pond water. Larvae were removéd after 9 days, washed and returned to the medium used in Experiment 3.

Plating Method	Number of Chaoborus	Counts/10 Min
Water and HCl wash	10	357 ± 22
	10	134 ± 16

Experiment 5

This experiment used normal Chaoborus and Chaoborus that had been treated with antibiotic according to Rigler's procedure (Rigler, 1961). Normal and treated Chaoborus were put in separate 1 liter flasks. The medium was the same in both flasks, except that the flask containing the larvae had twice as much radioactivity. Also, the density of normal larvae was greater than treated larvae. The larvae were in the media for 6 days. Sterilized nutrient media were used in this experiment and all the following experiments.

Results were similar for normal and treated larvae, except for a single high reading for water and acid-washed larvae (Table V). Since the treated Chaoborus were fewer in number and had more radiophosphorus available, they should have had higher activities than the normal larvae. Anwar and Middleton (1963) found the number of mosquito larvae per ml of solution limited the uptake of radiophosphorus. This experiment therefore indicates a decrease in uptake when larvae are treated for bacteria.

Table V. Activity of treated and normal Chaoborus larvae after 6 days in initially sterile nutrient media. Treated larvae were in a medium with 464 cpm/ml while normal larvae were in a medium with 192 cpm/ml.

Plating Method	Number of Chaoborus	Counts/10 Min	
		Normal	Treated
Direct	10	254 ± 19	118 ± 15
	10	143 ± 16	176 ± 17
	10		84 ± 14
Water wash	10	44 ± 12	64 ± 13
	10	58 ± 13	45 ± 12
	10		58 ± 11
Water and HCl wash	10	40 ± 12	200 ± 17
	10	60 ± 13	19 ± 11
			4 ± 11

Experiment 6

Treated Chaoborus were put in radioactive nutrient media which had various amounts of stable phosphorus. The radioactivity of each medium was approximately the same, with a range of 930-1200 cpm/ml. Normal Chaoborus were put in one medium as a control. The experiment lasted for 7 days. The exact number of individuals put in each medium was not noted, but more normal Chaoborus were used per ml of solution than treated Chaoborus. The amount of penicillin and the length of time in the antibiotics were increased from the previous experiment.

This experiment showed a large difference between normal and treated Chaoborus (Table VI). The normal Chaoborus had more activity for both washed and directly-plated larvae. The medium containing the normal Chaoborus not only had more larvae, it also had slightly less radioactivity before the start of the experiment. Thus a larger difference might have been expected with better control. These data would indicate an antibiotic effect either direct or indirect via epiphytic and/or endophytic bacteria. There was also a correlation between activity (for both washed and direct-plate larvae) and amount of stable phosphorus present. The exact number of treated larvae in each medium was not recorded although the numbers were very similar because I attempted to add the same volume of larvae (not numbers) in each trial.

Table VI. Activity of normal and treated Chaoborus larvae after 7 days in sterile nutrient media containing 930-1200 cpm/ml. Phosphorus carrier added to give differences in P content.

Plating Method	P in Medium ppbP	Number of Chaoborus	Counts/10 Min	
			Normal	Treated
Direct	0	10		313 ± 21
	0	10		240 ± 19
Water and HCl wash	0	10		164 ± 17
	0	10		163 ± 17
Direct	37	10		895 ± 32
	37	10		829 ± 31
Water and HCl wash	37	10		234 ± 19
	37	10		210 ± 18
Direct	186	10	7359 ± 86	2788 ± 54
	186	10	6588 ± 82	3756 ± 62
Water and HCl wash	186	10	2698 ± 53	288 ± 20
	186	10	2176 ± 48	298 ± 20

Experiment 7

The normal, washed Chaoborus used in Experiment 6 were returned to the medium for 8 days. The larvae had slightly more activity (Table VII) than in the previous study, but the difference would be larger if ^{32}P decay were calculated since the activity had decreased by 13% between the counting times.

Table VII. Activity of normal Chaoborus larvae after 8 days in the medium used in the previous Experiment 6.

Plating Method	P in Medium ppbP	Number of Chaoborus	Counts/10 Min (Normal)
Water and HCl wash	186	10	2948 ± 55

Experiment 8

Treated and normal Chaoborus larvae were put in media containing various amounts of stable phosphorus. More streptomycin was used than in earlier experiments. The same amount of radioactive solution (9 microcuries) was in each medium. The larvae were incubated for 26 hr.

For the normal, washed Chaoborus, it is apparent that there was no significant difference between the media, (Table VIII). For treated Chaoborus there was no distinct trend in either washed or directly-plated Chaoborus although there were differences between media. However, there was again a difference between normal and treated Chaoborus, especially for the washed larvae.

Table VIII. Activity of Chaoborus larvae after 26 hours in initially sterile media containing 9µCi ^{32}P. Phosphorus carrier added to give differences in P content.

Plating Method	P in Medium ppbP	Number of Chaoborus	Counts/10 Min Normal	Counts/10 Min Treated
Direct	186	10	645 ± 27	277 ± 20
	186	10	570 ± 26	
Water and HCl wash	186	10	182 ± 17	65 ± 13
	186	10	147 ± 16	
Direct	372	10	*	224 ± 18
	372	10	177 ± 17	
Water and HCl wash	372	10	190 ± 17	107 ± 15
	372	10	189 ± 17	
Direct	558	10	865 ± 31	429 ± 23
	558	10	480 ± 24	
Water and HCl wash	558	10	155 ± 16	33 ± 12
	558	10	164 ± 17	

*Sample lost.

Experiment 9

This experiment used the media and organisms from Experiment 8. After the larvae were washed, they were returned to their respective media for 8 days.

Again there was a large difference between treated and normal Chaoborus in uptake (Table IX). There had been a large increase in uptake by normal Chaoborus after Experiment 8 and a significant increase by treated Chaoborus. The data indicate that isotopic equilibrium was not established in 26 hr (Experiment 8). The low radioactivity of the treated Chaoborus would indicate that most of the bacteria were killed instead of inhibited. However, the antibiotics might have affected uptake by the anal papillae or by some other part of the body.

Table IX. Activity of normal and treated washed *Chaoborus* larvae from Experiment 8 put back in media for 8 additional days.

Plating Method	P in Medium ppbP	Number of Chaoborus	Counts/10 Min
Normal *Chaoborus*			
Water and HCl wash	186	10	1412 ± 39
	186	10	1294 ± 38
Water and HCl wash	372	10	681 ± 28
	372	10	455 ± 24
Water and HCl wash	558	10	675 ± 28
Treated *Chaoborus*			
Water and HCl wash	558	10	195 ± 17

Experiment 10

Two 2-liter containers with the same nutrient medium and the same radioactivity (920 cpm/ml) were prepared; 170 normal *Chaoborus* were put in one container and 170 treated *Chaoborus* were put in the second container. Treated *Chaoborus* were prepared with weaker antibiotic solutions and exposed to the solution for a shorter period than in all earlier experiments except Experiment 5. Larvae were removed from the media after various exposures and were counted.

Since some radioactive medium was always present when the direct plating technique was used, these counts probably include some radiophosphorus from the medium and not adsorbed to the larvae. For the normal *Chaoborus* the direct plating counts start low, reach a maximum in 2 hr, and then decrease to a plateau level at 8 hr (Table X). The normal washed larvae show little activity until 8 hr, after which they show a steady increase (Fig. 1). The direct-plated, treated larvae increase in radioactivity more slowly and reach a maximum in 8 hr, after which they decrease to a plateau level at 16 hr (Table X). The washed, treated larvae contain little activity until the 4th hour (Fig. 1); then activity steadily increases, but they did not become as radioactive as the normal *Chaoborus* except early in the experiment. These results indicate radiophosphorus uptake via absorption.

Table X. Activity of normal and treated Chaoborus larvae after various times in initially sterile media with 920 cpm/ml.

Time in Medium and Plating Method	Number of Chaoborus	Counts/10 Min Normal	Counts/10 Min Treated
30 minutes			
Direct	10	89 ± 14	114 ± 15
H_2O and HCl wash	10	0 ± 10	0 ± 10
1 hour			
Direct	10	287 ± 20	105 ± 15
H_2O and HCl wash	10	19 ± 11	4 ± 11
2 hours			
Direct	10	1303 ± 38	472 ± 24
H_2O and HCl wash	10	15 ± 11	20 ± 11
4 hours			
Direct	10	653 ± 28	388 ± 22
H_2O and HCl wash	10	9 ± 11	55 ± 13
8 hours			
Direct	10	408 ± 23	918 ± 32
H_2O and HCl wash	10	114 ± 15	64 ± 13
16 hours			
Direct	10	486 ± 24	552 ± 26
H_2O and HCl wash	10	130 ± 15	80 ± 14
48 hours			
Direct	10	416 ± 23	473 ± 24
H_2O and HCl wash	10	170 ± 17	134 ± 16

Experiment 11

Seventy Chaoborus were put in media with identical nutrients and with 850 cpm/ml. One medium contained normal Chaoborus, the other medium contained treated Chaoborus. The treated larvae were prepared with large doses of antibiotics, and in this experiment the solution with the treated Chaoborus contained antibiotics. The larvae were incubated in their respective media for 7 days. No larvae died in any of the media.

This experiment was well controlled in that the conditions were identical in the beginning, except that one medium had normal Chaoborus and the other had treated Chaoborus and antibiotics. The differences between activities of normal and treated Chaoborus (Table XI) strongly suggest that bacteria account for most of the activity in Chaoborus larvae. If there were no bacteria in the treated larvae, the uptake of ^{32}P by the washed larvae would have occurred

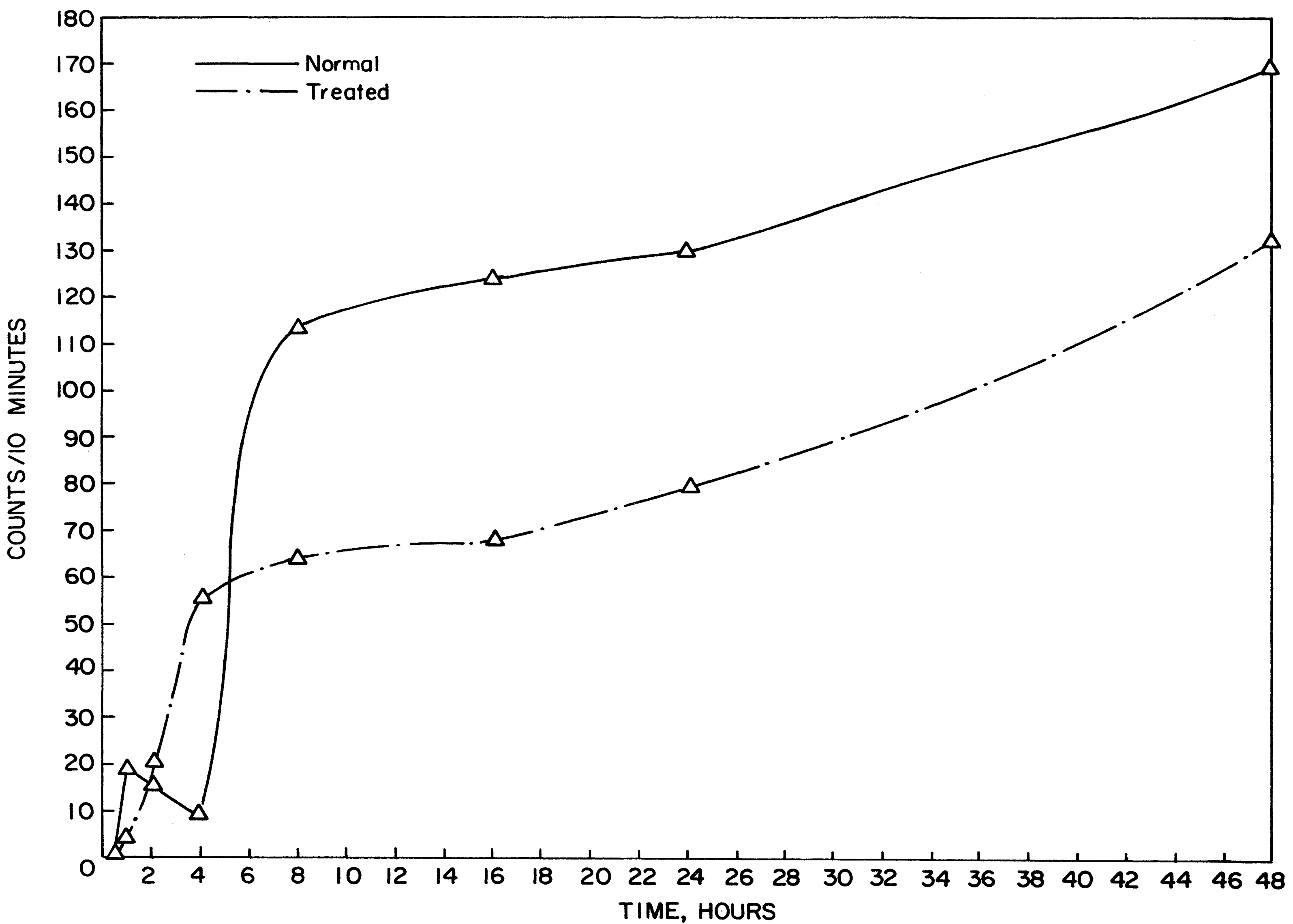

Fig. 1. Activity of washed _Chaoborus_ larvae in initially sterile nutrient media with 920 cpm/ml.

chiefly via adsorption. The adsorbed phosphorus of treated larvae is small compared to the adsorbed phosphorus of normal larvae. These data suggest that both endophytic and epiphytic bacteria are responsible for the principal activity of Chaoborus larvae.

Table XI. Activity of Chaoborus larvae after 7 days in initially sterile nutrient media with 850 cpm/ml.

Plating Method	Number of Chaoborus	Counts/10 Min Normal	Counts/10 Min Treated
Direct	10	1337 ± 38	187 ± 17
	10	1560 ± 41	223 ± 18
	10	1190 ± 36	170 ± 17
Water and HCl wash	10	647 ± 28	37 ± 12
	10	603 ± 27	82 ± 14
	10	487 ± 24	84 ± 14

Experiment 12

Three identical media with 750 cpm/ml of radiophosphorus were prepared for normal Chaoborus. One medium was kept at 45°C for 24 hr, one was kept at 21°F for 24 hr, and one medium kept at 45°C for 16 hr and at 21°C for 8 hr. Larvae were removed and plated at 8 hr intervals.

This experiment showed no increase, but perhaps a slight decrease, in radioactivity of washed larvae, (Table XII). The washed larvae in the warm medium for 24 hr had much more radioactivity than those in the cold medium (Fig. 2). This suggests a higher metabolic uptake at higher temperatures. No other patterns seem significant, except that the larvae plated directly decreased in radioactivity in the cold medium. The larvae that were changed from cold room to warm room showed a slight increase in radioactivity in the warm room.

Table XII. Activity of Chaoborus larvae in three initially sterile nutrient media with 750 cpm/ml at 8-hour intervals at 4.5°C and 21°C.

Time	Plating Method	Counts/10 Min 24 hr, 4.5°C	Counts/10 Min 16 hr, 4.5°C; 8 hr, 21°C	Counts/10 Min 24 hr, 21°C
8 hours	Direct	343 ± 21	156 ± 16	315 ± 21
	H_2O and HCl wash	65 ± 13	26 ± 12	180 ± 17
16 hours	Direct	202 ± 18	153 ± 16	288 ± 20
	H_2O and HCl wash	26 ± 12	26 ± 12	188 ± 17
24 hours	Direct	95 ± 14	268 ± 19	446 ± 24
	H_2O and HCl wash	25 ± 12	33 ± 12	172 ± 17

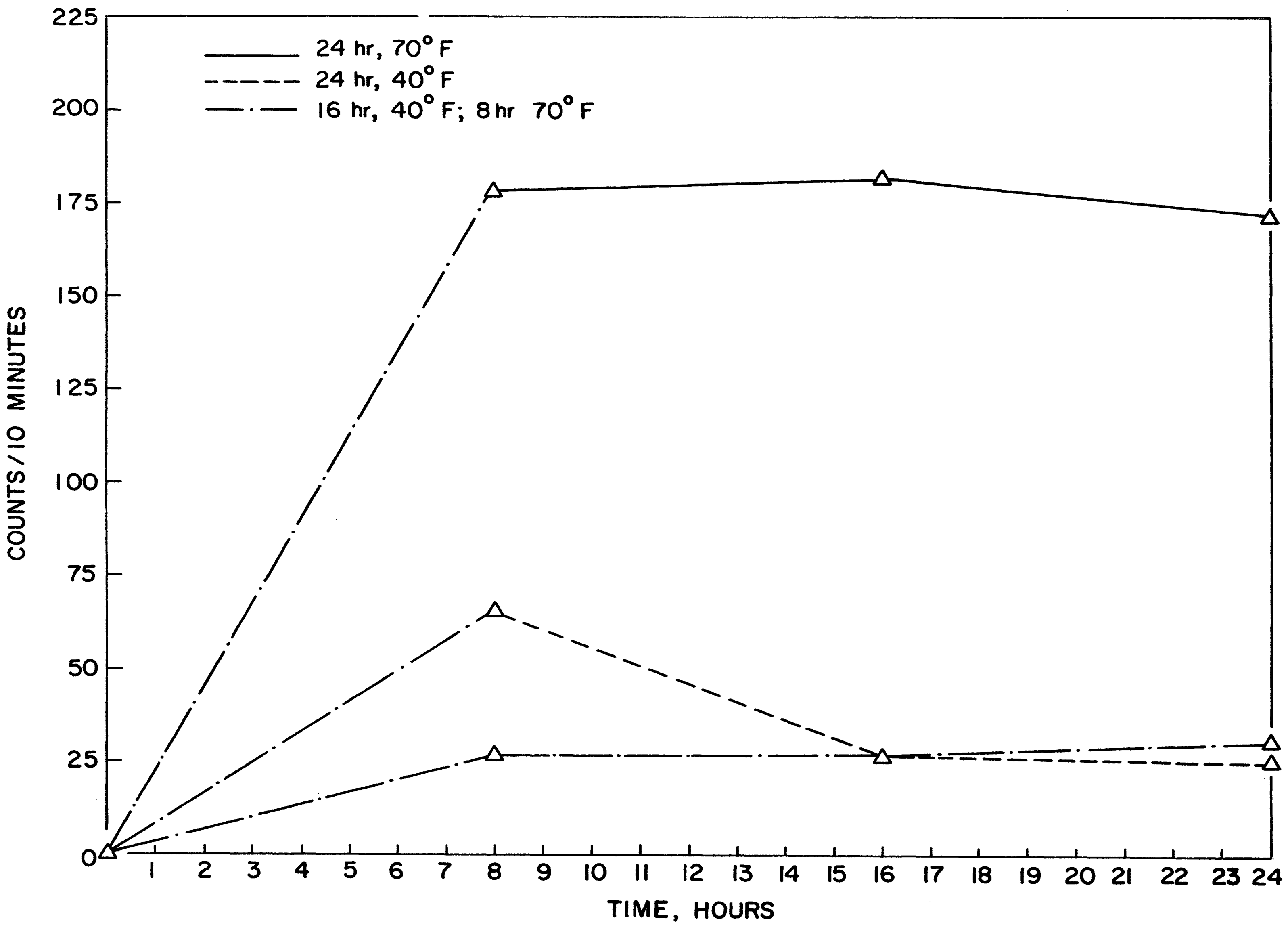

Fig. 2. Activity of washed *Chaoborus* larvae in three initially sterile nutrient media with 750 cpm/ml.

Autoradiography. Chaoborus larvae from Experiments 2, 3, 4, 6, 7, 8, 9, and a ligature experiment were autoradiographed. Several films did not produce good autoradiographs, but all the experiments except the ligature experiment are represented in Figs. 3-7.

Since the autoradiograph of the ligatured larvae was extremely poor, a heavier film i.e., one with a thicker emulsion, was used for all succeeding autoradiographs (Figs. 5-7). These results were not as good as the autoradiographs taken with lighter film (Figs. 3 and 4). The autoradiographs in Figs. 3 and 4 were made with a one-week exposure to the radioactive larvae while the autoradiographs in Figs. 5-7 were made with a six-month exposure to the radioactive larvae. Also, in Figs. 3 and 4 a picture of the larvae was taken by light as well as by radiophosphorus. Since the larvae did not change form, a change in position of the larvae, as in Fig. 4, is advantageous because the area of radioactivity is better known.

In Fig. 3, two washed larvae from Experiment 2 have a concentration of radiophosphorus in the head region. Other larvae have distinct black spots, but these were caused by the transparent body segments acting as a lens and bringing light rays to a focal point. Regardless of the experiment, the head region usually had the highest concentration of radiophosphorus. Although the pictures (Figs. 3-7) are not as clear as the X-ray film itself for determining areas of radiophosphorus concentrations, one can see from Fig. 4 that the head region is frequently the only noticeable radioactive part of the organism.

The entire body of normal, washed Chaoborus larvae from Experiment 6 is radioactive (row C, Fig. 4). These larvae were very radioactive (Table VI) compared with the other larvae used in this autoradiograph. Since the washed larvae should not have much adsorbed phosphorus, this autoradiograph shows that the absorbed phosphorus has been distributed throughout the body of one group. When there is not much radioactivity, the head region has the highest concentration of radiophosphorus.

Since the autoradiographs in Figs. 5-7 were exposed to radiation for 6 months, some radioactivity appears in all the samples. However, normal Chaoborus larvae from the storage jar in the cold room were put on these three films, and they are not at all visible. In Fig. 7 the nonradioactive larvae are above row A.

The activities of larvae within rows appear to be similar. The larvae from Experiment 4, however, show differences within a row (Fig. 6, row B). The large observed differences in counting (Table IV) are verified by variation in the activity of individual larvae. Other experiments produced equal or greater variation; however the similarity of activities within a row and the difference between rows is clear.

The normal Chaoborus with the greatest activities produced the largest autoradiographs, i.e., largest black spots (Figs. 4-7). The treated Chaoborus

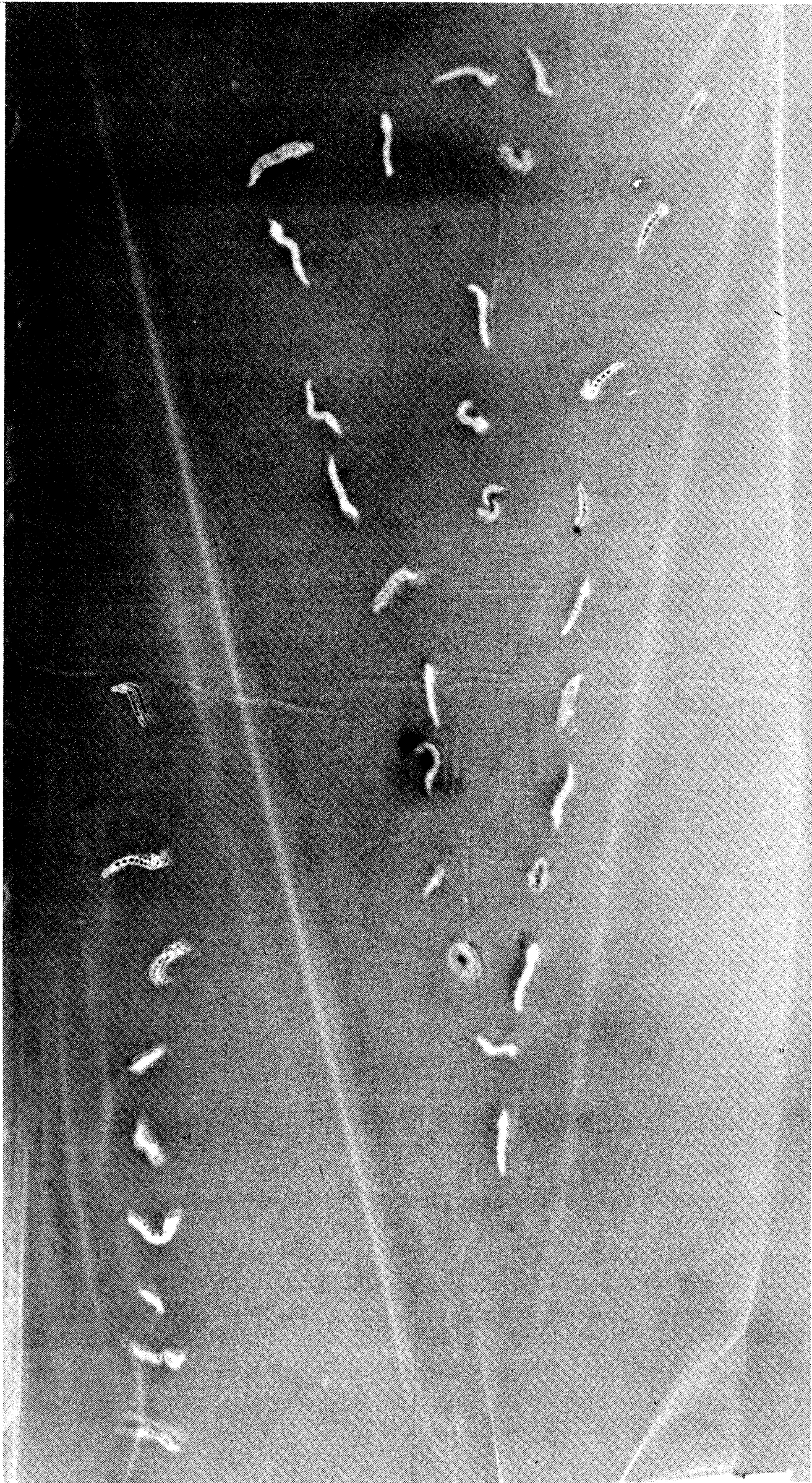

Fig. 3. Autoradiograph of washed Chaoborus larvae maintained in George Pond water for 10 days (Experiment 2).

Fig. 4. Autoradiograph of washed *Chaoborus* larvae from Experiments 3, 6, and 8.

A. Expt. 6 - Treated, washed, 37 ppb P.
B. Expt. 6 - Normal, washed, 8 ppb P.
C. Expt. 6 - Normal, washed, 186 ppb P.
D. Expt. 6 - Treated, washed, 186 ppb P.
E. Expt. 3 - Normal washed.
F. Expt. 8 - Treated, washed, 558 ppb P.
G. Expt. 8 - Normal, washed, 558 ppb P.
H. Expt. 8 - Normal, washed, 186 ppb P.
I. Expt. 8 - Treated, washed, 372 ppb P.
J. Expt. 8 - Treated, washed, 186 ppb P.
K. Expt. 8 - Normal, washed, 372 ppb P.

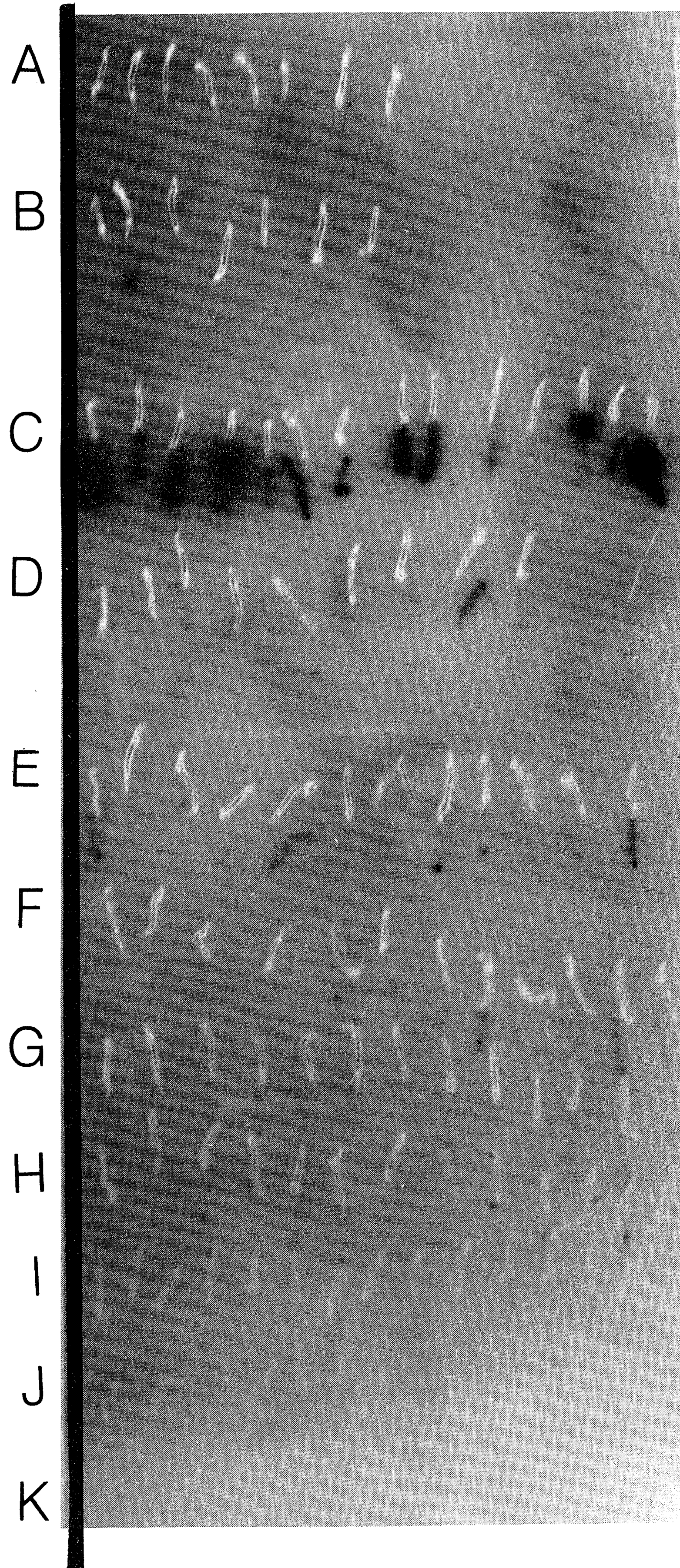

Fig. 4.

Fig. 5. Activity of normal and treated, directly plated and washed, *Chaoborus* larvae from Experiment 9.

A. Expt. 9 - Treated, washed, 372 ppb P.
B. Expt. 9 - Normal, washed, 372 ppb P.
C. Expt. 9 - Normal, plated, 372 ppb P.
D. Expt. 9 - Normal, plated, 186 ppb P.
E. Expt. 9 - Normal, washed, 186 ppb P.
F. Expt. 9 - Treated, plated, 558 ppb P.
G. Expt. 9 - Treated, washed, 558 ppb P.

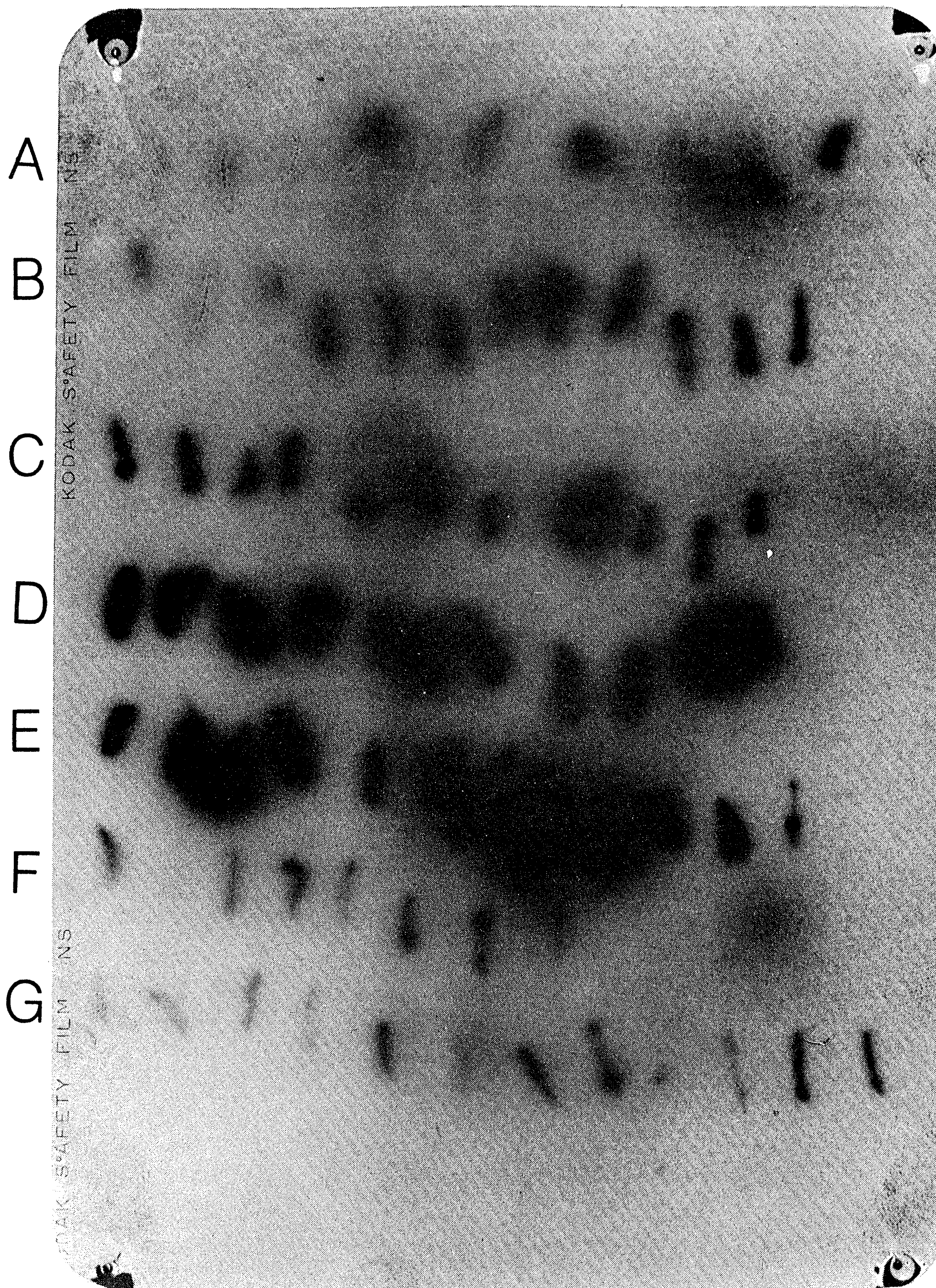

Fig. 5.

Fig. 6. Autoradiograph of normal and treated, directly plated and washed _Chaoborus_ larvae from Experiments 4, 7, and 9.

A. Expt. 9 - Normal, plated, 558 ppb P.
B. Expt. 4 - Normal, washed.
C. Expt. 9 - Treated, washed, 186 ppb P.
D. Expt. 9 - Treated, plated, 186 ppb P.
E. Expt. 7 - Normal, washed, 186 ppb P.
F. Expt. 4 - Normal, plated.
G. Expt. 7 - Normal, plated, 186 ppb P.

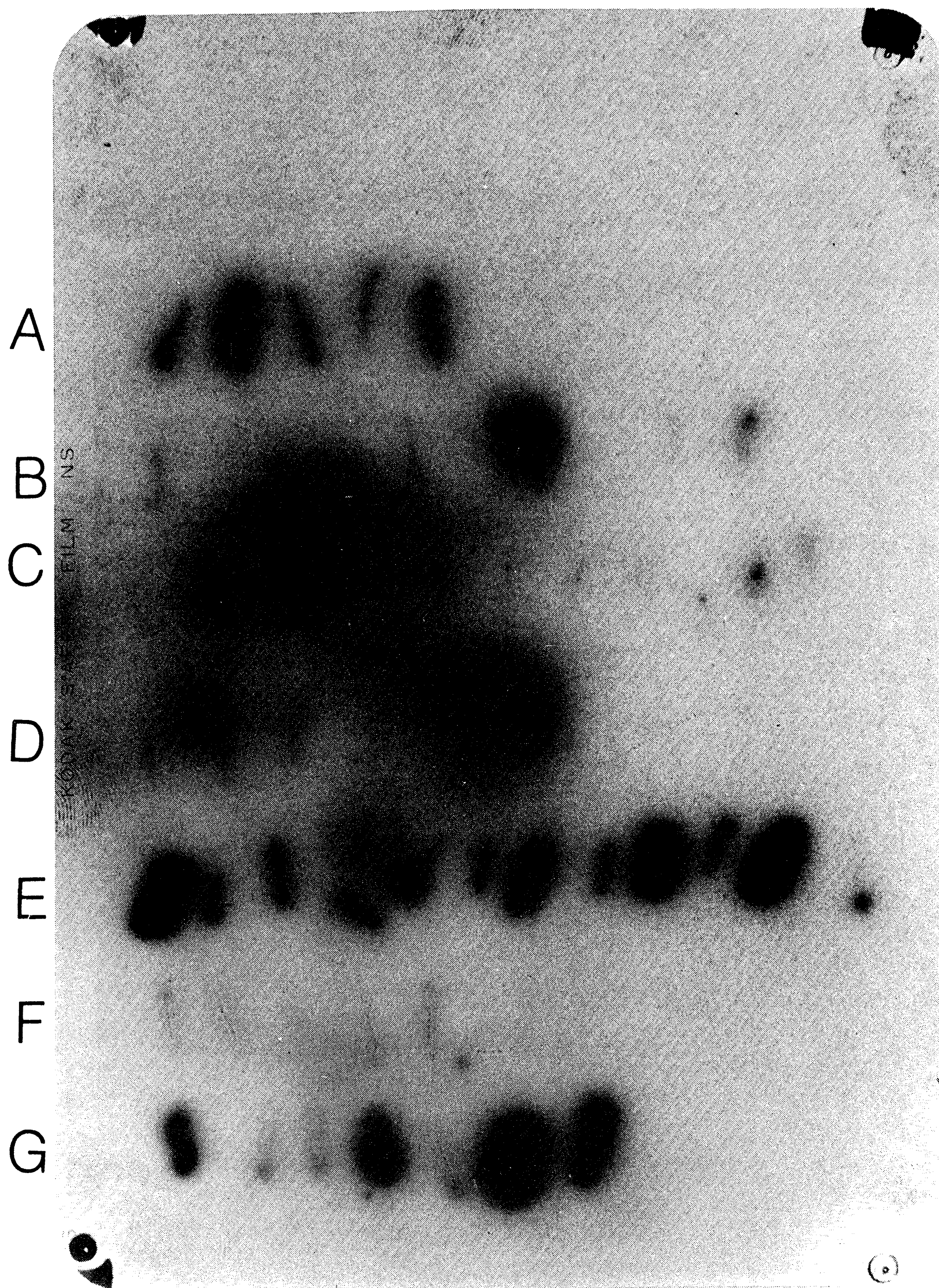

Fig. 6.

Fig. 7. Activity of normal, washed, and directly plated *Chaoborus* larvae from Experiments 4, 7, and 9.

A. Expt. 9 - Washed, normal, 558 ppb P.
B. Expt. 4 - Washed, normal.
C. Expt. 9 - Plated, normal, 558 ppb P.
D. Expt. 4 - Plated, normal.
E. Expt. 7 - Washed, normal, 186 ppb P.
F. Expt. 7 - Plated, normal, 186 ppb P.

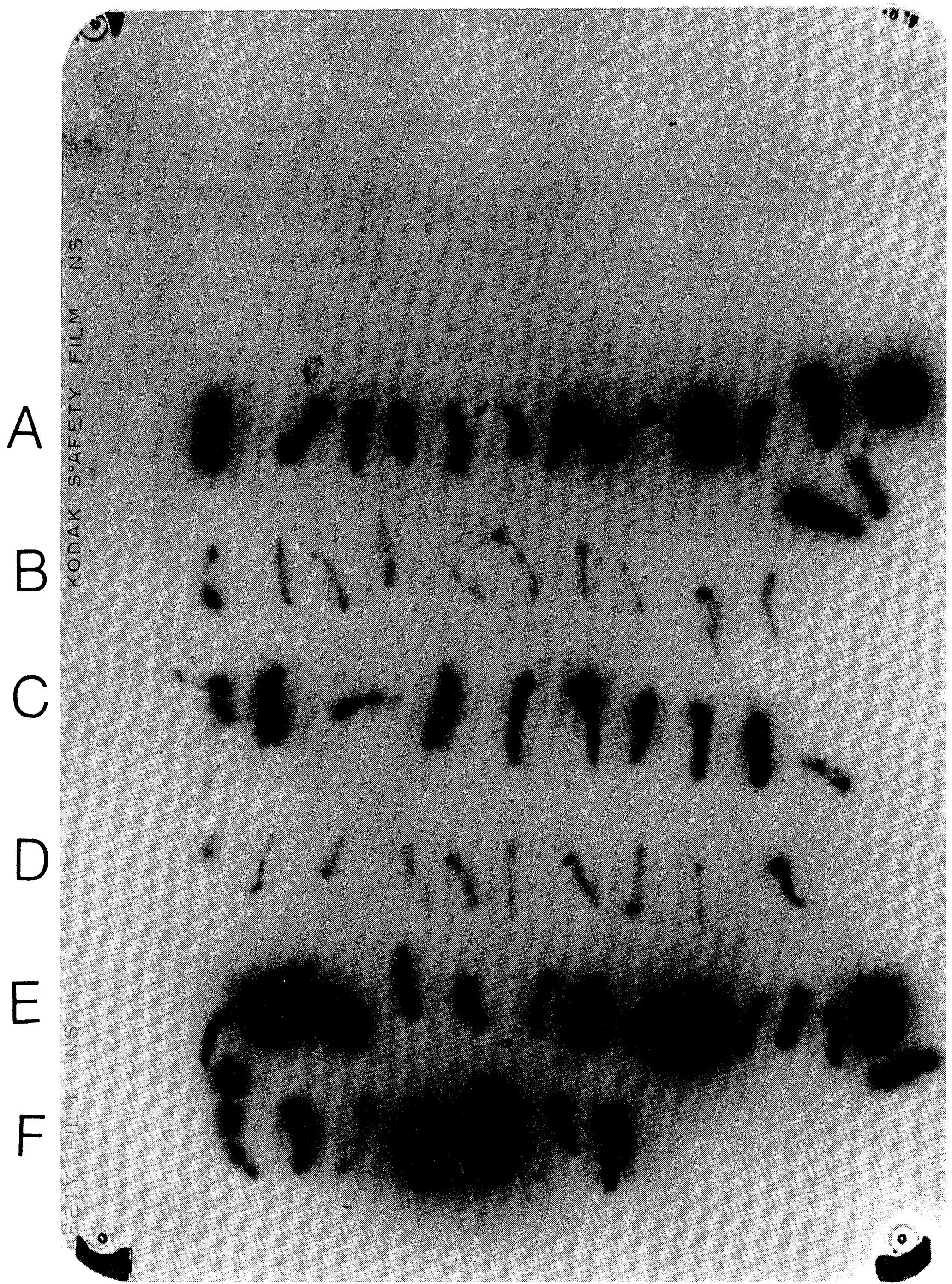

Fig. 7.

produced autoradiographs similar to the autoradiographs of normal *Chaoborus* with equivalent activities; thus the autoradiographs can be used to determine relative activities.

Summary

Direct counting and autoradiography experiments indicated that bacteria play a major role in radiophosphorus uptake by the midge larvae *Chaoborus americanus*. The initial radioactivity uptake of normal *Chaoborus* put in radioactive nutrient media was predominantly ^{32}P adsorption. Uptake by absorption became more important with time. Epiphytic bacteria did not play a major role in phosphorus absorption; however, endophytic bacteria are very important even when food, i.e., particulate matter, is not present.

In several experiments, the amount of adsorbed phosphorus reached a maximum, then decreased or remained constant. However there was no decrease in activity in any experiment with washed larvae (and thus absorbed ^{32}P).

Chaoborus treated with antibiotics picked up less radioactivity than untreated *Chaoborus*, except at the beginning of an experiment. The antibiotics apparently killed bacteria and/or inhibited bacterial metabolism, but they did not produce bacteria-free conditions because treated larvae slowly increased in activity. The rate of ^{32}P uptake was temperature-dependent, and thus probably depends upon metabolic rate.

Autoradiography indicated that the head region concentrated radiophosphorus more than any other part of the body. Many normal (untreated) larvae had ^{32}P distributed throughout their bodies, but the head appeared to be the most active region. These results suggest that *Chaoborus* have large numbers of bacteria in the mouth and foregut where the food is broken down. These bacteria apparently survive during extended periods when food is not supplied.

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APPENDIX C

DISSOLVED ORGANIC PHOSPHORUS COMPOUNDS OF A BOG LAKE ECOSYSTEM

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In an earlier report we presented preliminary analyses of dissolved organic phosphorus compounds (DOP) of North Gate Lake, a small Michigan bog lake (Hooper, 1969). These studies have indicated that the phosphorus of nucleic acids and polyphosphates were important fractions of the DOP in this ecosystem. In the present report, we wish (1) to review the literature dealing with the quantity and chemical nature of dissolved organic phosphorus compounds, their possible biochemical origin and function, and their relationship to the phosphorus cycle of aquatic systems, and (2) to report additional data on the dissolved organic compounds of North Gate Lake.

Literature Review—The Organic Phosphorus of Aquatic Systems

CELLULAR PHOSPHORUS COMPOUNDS

The particulate phosphorus found in natural waters consists chiefly of cellular phosphorus within a variety of microorganisms including planktonic algae and bacteria, and also nonliving detritus particles undergoing decomposition. During the degradation process within natural waters, many of these organic compounds may be released at least temporarily, into the water in a free state. In their free state, the cellular products constitute a part of the "dissolved" fraction. To adequately review the role of organic phosphorus in natural waters, it is of value to first discuss the intracellular compounds, consider their function in the cell, and review the present state of our knowledge regarding their metabolism.

Phosphorus is involved in the entire range of metabolic processes. It is connected in an essential way with virtually all energy transformation systems in the living cells. Phosphorus enters the cell as inorganic phosphate by means of an active transport process, and once inside the cell it becomes incorporated (1) into a variety of organic phosphorus compounds, and (2) into

condensed inorganic phosphate (polyphosphate). In algae, the disposition of phosphorus into these compounds arises from reactions involved in two major energy-yielding chains present in all living plant cells. These are (1) the process of photophosphorylation within chloroplasts whereby light energy is used for esterification of inorganic phosphate into ATP (adenosine triphosphate), and (2) phosphorylation coupled with respiration which is associated with mitochondria (oxidative phosphorylation).

In all organisms, most of the energy-consuming reactions are coupled with the hydrolysis of the energy-rich phosphate bond of ATP. In these reactions phosphate groups are actively transferred by the action of enzymes.

The ATP arising from photophosphorylation is used by the plant cells to support growth and all energy-requiring metabolism such as CO_2 assimilation, uptake of various organic and inorganic compounds, synthesis of amino acids, synthesis of proteins and nucleic acids, and synthesis of inorganic polyphosphates. This close coupling of phosphorus with cell energetics and photosynthesis makes photosynthetic production dependent upon available phosphates. It is clear that a phosphate deficiency in algae soon reduces photosynthesis and other metabolic reactions (Pirson, 1955), and may lead to unusual accumulations of fat, starch, and other cell wall substances. This, in turn, suggests a secondary interference with the nitrogen metabolism of the plant (Bergmann, 1955).

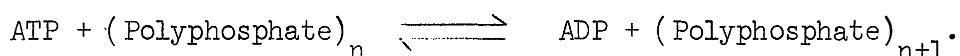
Of the numerous intracellular phosphorus compounds of bacteria and algae, polyphosphates have received a great deal of attention because for many years they were believed to be the compounds used for energy storage within the cell (Hoffman-Ostenhof, 1962). Polyphosphates are one of three types of condensed inorganic phosphate compounds containing pentavalent phosphorus, in which various numbers of tetrahedral PO_4 groups are linked together by oxygen bridges. The chain length of polyphosphates ranges from 2 units (pyrophosphates) to the insoluble Maddrell's salts of chain length up to 10^4. Only the molecules of lower chain length have been identified as individual compounds. Nearly all the polyphosphates from living cells are regarded as mixtures of various proportions of polyphosphates of different molecular size. Polyphosphates of varying chain lengths have been extracted from yeast cells using a variety of solvents and pH ranges. Fractions have varied in chain length from 4 to 260, and molecular weights range from 530 to 30,700 (Langen, Liss, and Lohmann, 1962). Column and paper chromatography have been used to separate polyphosphates and metaphosphates of shorter chain lengths from living cellular materials.

Polyphosphates appear to be characteristic of microorganisms and have been isolated from a wide variety of bacteria, fungi, protozoa, and all major groups of algae (Harold, 1966; Kuhl, 1962). In addition, they have been identified from some higher plants and aminals (Miyachi, 1961; Lynn and Brown, 1963).

Some controversy exists regarding the chemical state of polyphosphates within cells. Since some polyphosphates cannot be extracted with acid, for

many years they were believed to be bound with protein or ribonucleoprotein complexes (Katchman and von Wazer, 1954). Harold (1966) now favors the view that complexes do not exist *in vivo* but are formed during the extraction process and that polyphosphates exist free in living cells. In bacterial cells much of the polyphosphate may be localized in granules that stain metachromatically with basic dyes. These granules can be identified in cells with either the light or electron microscope and have been called volutin granules. In addition to polyphosphates, volutin granules may contain RNA, lipid, protein and Mg^{+2} (Widra, 1959). In yeast and in *Chlamydomonas* phosphates may be localized in vacuoles.

The biosynthesis of polyphosphate is accomplished in the living cell by the addition of the terminal phosphoryl group of ATP to the chain according to the following reaction:



$$\text{ATP} + (\text{Polyphosphate})_n \rightleftharpoons \text{ADP} + (\text{Polyphosphate})_{n+1}.$$

The discovery by Kornberg (1957) of the polyphosphate kinase catalyzing the above reaction led to the hypothesis that polyphosphate was in some way related to energy process within the cell. The above reaction appears to be reversible at low ATP-ADP ratios. Other enzymes have been isolated from cellular material which bring about the transfer of phosphate from polyphosphate to adenosine monophosphate (Winder and Denneny, 1955) and which bring about the phosphorylation of glucose and fructose (Szymona, *et al.*, 1962; 1966). A number of enzymes which have been isolated from living tissues will hydrolyze pyrophosphates, tripolyphosphates, tetrapolyphosphates, metapolyphosphates, and polyphosphates of high molecular weight to orthophosphate (Grossman and Lang, 1962; Muhammed, 1961). At this time the evidence is not clear whether hydrolysis is direct to orthophosphate or through intermediates such as ATP.

The polyphosphate content of living cells has been shown to fluctuate within a wide range. Polyphosphates usually cannot be detected in phosphorus-starved cells. They are low or undetectable during the exponential growth phase of cells. On the other hand, they accumulate to very high levels in cells with nutritional deficiencies (Wilkinson and Duguid, 1960), up to 20% of the dry weight of yeast cells (Liss and Langen, 1962). When sulfur, carbon, or nitrogen is limiting, *Nitrosomonas* converts nucleic acid into polyphosphate. When the limiting element is restored, polyphosphate is converted back into nucleic acid (Terry and Hooper, 1970).

The cycle of polyphosphate in *Aerobacter aerogenes* and its genetic control has been outlined by Harold (1964, 1965), and Harold and Harold (1965). Briefly, it appears that in growing cells, the synthesis of nucleic acid inhibits polyphosphate synthesis and stimulates the degradation of polyphosphate. Consequently little or no polyphosphate is deposited. If growth and nucleic acid synthesis stop because of the exhaustion of a nutrient, net polyphosphate synthesis is promoted. This is presumed to occur because competition for ATP is relieved

and polyphosphate accumulates at a rate determined by the level of polyphosphate kinase. It has also been shown that cells subject to prior phosphate starvation contain elevated levels of polyphosphate kinase and are thus capable of very rapid polyphosphate synthesis when orthophosphate is provided. Thus in Aerobacter, under favorable conditions for growth, net polyphosphate synthesis occurs at a very low rate and nucleic acid synthesis proceeds, whereas under unfavorable environmental circumstances, polyphosphate synthesis takes place and rapid accumulation occurs.

In algae, Kuhl (1960) found that there is rapid incorporation of orthophosphate into polyphosphate in the presence of light. In the dark, incorporation is depressed but not abolished. Thus in algae it appears that the formation of polyphosphate is stimulated by light but is not totally dependent on it. In algae as in other microorganisms the polyphosphate content is a function of growth phase and is lowest in the exponential phase and highest in old cultures. There also appears to be a reciprocal relationship between RNA and the polyphosphate content (Smillie and Krotov, 1960).

Harold (1966) reviews theories on the function of polyphosphate in living cells. His review raises doubts as to the validity of the earlier hypothesis that polyphosphates are stores of phosphate-bound energy from which phosphoryl groups may be transferred to ADP from ATP. He provides evidence that polyphosphates serve as a phosphate reserve and storage center for phosphorus within cells from which rapid biosynthesis of nucleic acid and phospholipids can take place. This leads to the general concept of polyphosphates acting as regulatory agents of cellular phosphorus. On the one hand, there is the cyclic regeneration of ATP and its participation in numerous reactions in which metabolic energy is produced and utilized. On the other hand, in growing cells ATP is continually drawn into nucleic acids and phospholipids. There appear to be valid biochemical reasons for believing cells must control the steady state concentration of orthophosphates, ADP, and ATP. Polyphosphate appears to be the compound regulating these levels by serving, in effect, as a metabolic buffer.

THE QUANTITY OF PHOSPHORUS FRACTIONS IN LIVING CELLS

The percentages of the various phosphorus fractions within bacterial and algal cells fluctuate considerably depending on light conditions, concentration of the phosphorus in the environment, pH, and temperature. Overbeck (1963) provides data on four of the above fractions in Scenedesmus quadricauda. The TCA soluble fraction made up 27% of the phosphorus of normal Scenedesmus cells. About half of this faction was orthophosphate and half was 7-min hydrolyzable phosphate (acid-soluble polyphosphate). The TCA insoluble phosphorus made up the remaining 73% of the cell phosphorus. This consisted of a fraction hydrolyzable by the 7-min hydrolysis (50.6% of cell phosphorus) and stable organic phosphorus (22.4%).

Correll (1965) fractionated particulate material of two size categories collected from Antarctic waters into five chemical fractions. The large size particles consisted chiefly of zooplankters while the smaller category was chiefly diatoms. The larger size particles contained higher quantities of RNA and polyphosphate (27-55% of total phosphorus) than the smaller size particles. On the other hand, the smaller category of particle was richer in phospholipids (13-29% of total phosphorus). Both classes of particle contained more orthophosphate, oligopolynucleotides, and oligophosphates than healthy laboratory plankton, which indicated that enzymatic degradation of cells had occurred. By incubating aliquots of these two samples with ^{32}P, rates of incorporation of the label into various fractions were observed. As expected, the smaller particles took up radioactivity about six times faster than the larger size fraction. The RNA-polyphosphate fraction was labeled more rapidly than any of the other four organic fractions separated. However, phospholipids were also labeled rapidly and reached a high specific activity.

REGENERATION OF PHOSPHORUS FROM ORGANIC COMPOUNDS

Demonstration that the stores of soluble phosphate measured in the open water of the photosynthetic zones of lakes and the ocean were inadequate to provide the necessary phosphorus for production of phytoplankton for any extended period (Hutchinson, 1941; Juday, et al, 1928; Redfield, Smith, and Ketchum, 1937) made it necessary to hypothesize that (1) phosphorus was being brought into open water from greater depths and/or bottom sediments, or (2) that major stores of phosphorus were regenerated from the phosphorus of the phytoplankton, zooplankton, or the dissolved organic phosphorus. It is now clear that recycling the phosphorus may be rapid and that regeneration can account for a major share of the needs of the system.

In the sea and in lakes it is difficult or impossible to determine the origin of soluble phosphorus compounds that appear in the open water since these compounds may arise by (1) in situ decomposition of the phytoplankton and zooplankton organisms themselves, (2) excretion by the plankton organisms, (3) regeneration from bottom sediments and transport to the photosynthetic areas, or (4) in situ release of dissolved organic compounds by algae and bacteria, and subsequent breakdown of the organic compounds into soluble phosphate. All of these processes may be operating simultaneously or one process may predominate to the exclusion of others.

Perhaps the most successful approach to the study of cycling of phosphorus in shallow systems where there are several regeneration processes operating simultaneously has been made by the use of radiophosphorus. Kinetic analysis of the movement of the label from the water into the various "pools" or "compartments" within the system has shown a logarithmic decline followed by a decrease at a much lower rate as the tracer is returned to the open water from pools. Pomeroy (1960) reviewed data on the residence time and turnover

rate from ^{32}P experiments in water systems and found the residence time range to be from 0.05 to 200 hr. He suggests that a short residence time indicates either depleted phosphorus resources, or high metabolic activity, or a combination of these two conditions. He found that turnover rates fell between 0.1 and 1.0 μg P/m^3/hr regardless of the phosphate concentration of the water except in biologically active systems where values between 1.0 and 20 μg P/m^3/hr are encountered. Turnover time was considered more important than phosphate concentration in maintaining high productivity.

In many unpolluted freshwater systems, rapid regeneration within the water is believed to take place because the concentration of orthophosphates is frequently 1 mg/m^3 or lower and variations in quantity of orthophosphate may be small despite large changes in plankton biomass (Juday, et al., 1928). The transient state of orthophosphate can be demonstrated in a striking way by simple aquarium experiments in which radiophosphorus is added as a tracer (Hayes and Phillips, 1958; Phillips, 1964). Aquarium experiments as well as in situ lake measurements by Rigler (1956) suggest that the rate of turnover in bacterial cells is a matter of a few minutes and that turnover of the dissolved organic phosphorus pool in the water may be a matter of a few hours.

There can be little doubt that the major share of the phosphorus of the hypolimnia of stratified lakes comes from regeneration of the phosphorus from plankton settling below the thermocline. Radiophosphorus added to the epilimnion promptly appears as a soluble phosphorus in bottom strata (Hutchinson and Bowen, 1950; McCarter, Hayes, Jodney, and Cameron, 1952). However even in the deepest layers of lakes regeneration is a complex problem since in this zone phosphorus may also come from sediments. The classical study by Mortimer (1941, 1942) in Esthwaite Water demonstrates that regeneration of phosphate from bottom mud proceeds when the surface layer of the mud becomes chemically reduced and an iron colloid layer is broken down. Mortimer's studies indicate that the dissolved phosphate which moves from the mud into water may be transported vertically in the hypolimnion by currents arising from internal waves.

In the open ocean a major share of the phytoplankton cells produced in the euphotic zone is consumed by herbivorous zooplankters living in this zone, and is not lost by sedimentation (Ketchum, 1962). After digestion and assimilation by zooplankton it is subsequently excreted into the water. Ketchum and Corwin (1965) recorded changes in the phosphorus fractions within the euphotic zone during a plankton bloom in the Gulf of Maine. The magnitude of the regeneration process can be deduced from their field measurements. During the ten-day period in which the bloom was studied, 14.2 μg- at P/m^2 was removed from the 0-50 M stratum. This phosphorus gave an observed increase of 7.58 μg-at /m^2 of new particulate phosphorus (plankton) and 1.05 μg-at/m^2 increase in dissolved organic phosphorus. All of the organic phosphorus was liberated in the upper 25 M. Below the euphotic zone (137 M) 2.7 μg-at/m^2 of inorganic phosphorus appeared in the water, presumably from regeneration of particulate matter sinking to this zone. The overall rate of regeneration of phosphorus

in this study was 4.0×10^{-3} μg-at/m^3/day. This rate under bloom conditions can be compared to rates in the Gulf of Maine under non-bloom situations of 2.0×10^{-3} μg/at/m^3/day, and the average, 0.01×10^{-3}, and the maximum 0.30×10^{-3} μg-at/m^3/day, for the North Atlantic ocean given by Riley (1951).

In field studies such as the above, it is seldom possible to identify the source of the organic material being decomposed. Ketchum and Corwin (1965) point out that the dissolved and organic phosphorus appearing in their study may have come from excretion of phytoplankton as well as zooplankton. Cultures of phytoplankton cells may liberate up to 25% of the organic carbon fixed during the 24-hr period (Hellebust, 1965). Carbon excretion presumably would be accompanied by sizable loss in organic phosphorus. However autolysis and bacterial degradation of phytoplankton and zooplankton together with excretion of living zooplankton organisms are believed to account for a major share of regeneration in the sea. The relative importance of autolysis as compared to bacterial decomposition of phytoplankton and zooplankton cells has not been adequately evaluated. There are several laboratory studies in which the breakdown of plankton has been followed. Hoffman (1956) found that 20-25% of the inorganic phosphorus and 30-40% of the organic phosphorus was liberated shortly after death, and between 80 and 90% was liberated within 24 hr. Marshall and Orr (1961) found a complete breakdown of the phosphorus of dead marine copepods of the genus Calanus within 2 days. The rapidity of decomposition led the above authors to conclude that autolysis, rather than bacterial action or the action of free enzymes, was the most important regenerative process. Grill and Richards (1964) followed the regeneration of phosphorus from a culture of phytoplankton (chiefly a centric diatom) which had been incubated in sea water and then stored in the dark. During the first day they noted that a sizable fraction of the inorganic phosphorus passed into particulate form, presumably uptake by bacteria. The increase in particulate phosphorus continued for approximately 8 days, at which time there was a sudden increase in the dissolved organic phosphorus and a decrease in particulate phosphorus. This change, they believe, was the result of autolytic release similar to that described by Hoffman. The dissolved organic phosphorus liberated was quickly acted upon by the bacteria population which was rapidly increasing so that after 17 days it was almost completely reassimilated into particulate matter. After 4 weeks there was again decomposition of particulate phosphorus and an increase in inorganic phosphorus. This continued until the end of the experiment. By plotting the decay of various components, Grill and Richards developed a simple kinetic model of the mineralization process. The process was approximated by the resultant of the first order decay rates of two labile organic fractions plus a refractory fraction which did not change during the experiment. They believed the initial decay rate represented organic phosphorus released from diatoms. Decay of this fraction gave a second fraction which appeared to be the phosphorus contained within the bacterial cells. This fraction, in turn, gave rise to (1) inorganic phosphorus and (2) refractory organic products of bacterial matabolism and the undecomposed particulates of bacterial cells.

Cooper (1935) observed the release of phosphorus from the decomposition of a mixed culture of marine zooplankters and from a mixed cluture of marine diatoms. He observed a large release of inorganic phosphorus from the zoo-

plankton within the first 12 hr. An amount of phosphorus equivalent to that in the plankton was set free within a period of 6 days. Regeneration of phosphorus from the diatom culture was somewhat delayed. Very little phosphate appeared within the first 3 days but thereafter rapid liberation took place, reaching a maximum amount in approximately one month. After this maximum there was a slow decrease in phosphate for a 4-month period.

Although there are few or no data on excretion rates of the zooplankters under field conditions, there are data from laboratory studies and from in situ field measurements in closed systems. The excretion rates of herbivores depend upon feeding rates, density of herbivores, density and quality of the food, temperature, and other variables. A wide variation in regeneration rates is encountered. Although most attention has been paid both in marine and freshwater studies to the larger herbivorous zooplankters, the microzooplankton may be of great significance.

Johannes (1964) demonstrated with a series of marine animals of varying sizes that the rate of excretion of dissolved phosphorus per unit of body weight increased as the body weight decreased. This suggests that the smaller short-lived species may play a very important role in mineralization. The large numbers of protozoa, rotifers, and other small metazoa inhabiting the mud-water interface as well as the open water are capable of liberating large quantities of phosphorus. Excretion of phosphorus is not confined to forms grazing upon living cells. Many of the microzooplankters as well as benthic filter feeders may ingest both living and dead cells (Edmonson, 1957). Dead cells, together with their covering of epiphytic bacteria may constitute a large fraction of the organic material in many freshwater ecosystems. However, the microplankton may release inorganic phosphorus in the absence of bacteria. Hooper and Elliot (1953) demonstrated the release of inorganic phosphorus from bacteria-free cultures of the ciliate Tetrahymena pyriformes that contained autoclaved extracts of the organic matter from lake mud. Margalef (1951) observed release of inorganic phosphorus from cultures containing cladocera and the algae Scenedesmus. Release of phosphorus increased as the number of cells of Scenedesmus increased. Margalef believed that the algae stimulated phosphotase activity in the digestive tract of the cladocera. Rigler (1961) verified Margalef's finding that living crustacea secrete phosphateses into the medium and Overbeck (1961) found that a bacterial suspension releases phosphate enzymatically from sodium glycerophosphate and makes this phosphorus available to the alga Scenedesmus quadricauda.

The early experience of Gardiner (1937) using mixed cultures of the larger marine zooplankters demonstrated that large stores of inorganic phosphate are excreted by these animals within a 3-hr test period. These and later studies by Marshall and Orr (1955) provide estimates of excretion rates of cultured animals under different conditions of feeding. More refined measures of excretion rates come from studies in which diatoms labeled with ^{32}P were fed to the marine copepod Calanus and the rate of loss of metabolized label from the medium was measured (Marshall and Orr, 1961). In these studies the biological

half-life of phosphorus was estimated to be 14 days. A kinetic analysis of Marshall and Orr's data by Conover (1961) indicated that the phosphorus of Calanus existed in two pools, one of labile phosphorus with a short biological half-life (approximately 0.375 days), and a second pool of more stable phosphorus with a half-life of approximately 13 days. It was estimated that between 94 and 99% of the total phosphorus in Calanus was in the stable form and that the mean turnover rate from both pools was about 10% of the body phosphorus per day. Ketchum (1962) summarizes unpublished data of Conover on excretion rates of a variety of marine organisms. The rates vary from 0.03 to 6.9% of the total phosphorus content of the organisms per day.

Pomeroy, Mathews, and Min (1963) found much higher excretion rates for estuarine plankton than reported above. Net plankton excreted an amount of phosphorus nearly equal to its total phosphorus content in a day. Slightly more than half of the excreted phosphorus was phosphate; the remainder was insoluble organic compounds. Rigler (1961) found that Daphnia magna excreted inorganic phosphorus into the medium at the rate of 8.4×10^{-3} μg/animal/hour. This loss was independent of epizootic bacteria, and was independent of the production of feces. He was not able to detect loss of organic phosphorus. His excretion rates for Daphnia appear to be much higher than those reported by Gardiner (1937) and are slightly higher than those reported by Marshall and Orr for Calanus. Barlow and Bishop (1965) followed the regeneration of phosphorus from zooplankton by collecting net plankton from the epilimnion (5 M) and from the hypolimnion (45 M) of Cayuga Lake and re-suspending the plankton at 100 times its original concentration in bottles of water taken from the depth from which the plankton was collected. Excretion rates ranged from 30 to 120% of body phosphorus per day and were considerably higher than those reported by Ketchum (1962) but within the range reported by Pomeroy, et al. The epilimnetic zooplankton population of Cayuga Lake consisted chiefly of cladocera while the hypolimnetic plankton consisted chiefly of copepods. The latter were, on the average, of the larger size. Excretion rates were consistently lower for the epilimnetic zooplankton as compared to the hypolimnetic forms of the same body size.

The fecal pellets produced by herbivorous zooplankton have been suggested as a source of debris that may be converted into phosphorus compounds. Marshall and Orr (1961) found that Calanus produced pellets at the rate of one pellet every 5 to 6 min. However these authors believe the phosphorus content of pellets is low and their studies on digestion indicate that the phosphorus assimilation efficiency of Calanus is high. Thus the role of fecal pellets in the regeneration cycle of phosphorus is not clear.

The role of bacteria in the regeneration process appears to be complex. Both the tracer studies on aquatic microcosms (Hayes and Phillips, 1958; and Phillips, 1964) and the direct measurements of decomposition in sea water (Grill and Richards, 1964) indicate that living bacteria not only incorporate phosphorus rapidly, but following uptake much of the phosphorus is released as dissolved organic phosphorus. Although most of the direct evidence for

bacterial production of dissolved organic phosphorus comes from experiments with laboratory microcosms, much of the dissolved organic phosphate in nature may be of bacterial origin.

In following the uptake of ^{32}P by bacteria, Phillips (1964) noted that normally there is not a release of dissolved organic phosphorus during the growth phase of cells and that release is usually associated with a decline in cell numbers. He concluded that release was associated with death of microorganisms. Bacteria also appeared to be the agents responsible for the conversion of dissolved organic phosphorus to inorganic phosphate. Phillips (1964) added labeled dissolved organic phosphorus to sea water under sterile and nonsterile conditions. With bacteria present, labeled dissolved organic phosphorus declined rapidly and the label entered the particulate phase (bacteria). Later, inorganic phosphorus appeared. On the other hand, the label remained in organic form in sterile cultures. From these experiments, Phillips concluded that there was no conversion of dissolved organic phosphorus to inorganic phosphate without bacteria.

Johannes (1964b) found that marine bacteria utilized 80% of the dissolved organic phosphorus released by an amphipod. He also found that a large fraction of this organic phosphorus was hydrolyzed in a sterile medium. He noted a release of organic phosphorus by bacteria-free diatoms after their growth phase had ceased, but was unable to report regeneration of dissolved inorganic phosphorus from dissolved organic phosphorus in the presence of bacteria; contrary to the findings in freshwater systems, he believes that marine bacteria (living or dead) released little dissolved organic phosphorus.

Under anoxic conditions, cultures of bacteria and other organisms have been shown to release large quantities of orthophosphate very rapidly (Shapiro, 1967). When oxygen is restored to the medium, much of this phosphorus is reabsorbed. The phosphorus liberated appears to have been cellular, since release was inhibited by mercuric chloride and by 2, 4-dinitrophenol. The fractions of the phosphorus compounds in the systems at various times during the release and uptake process indicated that the phosphorus liberated during the early stage of release was phosphorus that could be extracted by acid. Later in the release process RNA and DNA fractions were produced. There did not appear to be a release from phospholipids or phosphoproteins.

IDENTIFICATION OF DISSOLVED ORGANIC COMPOUNDS

Despite the wealth of data on the quantity of dissolved organic phosphorus in marine and freshwater systems, only fragmentary and isolated efforts have been made toward the identification of these compounds. More important, little or no effort has gone toward linking these organic fractions with the metabolic activities of bacterial and plant cells from which many of these materials must arise. Other than suggestions that the dissolved organic phosphorus represents the more refractory compounds that decompose slowly (Ketchum, 1962; Grill and

Richards, 1964) and that they are frequently liberated by bacterial cells, there are few guides as to their role in the biochemical cycles of either marine or freshwater systems.

Phillips (1964) separated the dissolved organic phosphorus of sea water that had been equilibrated with ^{32}P into 6 fractions. Three were identified as nucleotides or polynucleotides on the basis of adsorption of Norit A charcoal. Three other chromatographic peaks were not absorbed and were believed to represent phosphorylated hydrocarbons. The fractionation of dissolved organic phosphorus of bog water samples (Hooper, 1969) showed that in the aphotic zone, between 0.3 and 4.2% of the total phosphorus was absorbed by charcoal and presumably was nucleic acid. This fraction, however, was not present in the euphotic zone. From 1.7 to 12.3% of the total phosphorus was labile inorganic phosphorus, presumably polyphosphate or pyrophosphate. This fraction was present at all depths. The identified fractions however represent a small portion of the dissolved organic phosphorus since 35 to 47% of the organic phosphorus remained unidentified.

Since algal and bacterial cells are known to accumulate large quantities of polyphosphates, the question arises whether or not these fractions exist free in the euphotic zone since they would be expected to be liberated by the lysis of cellular material. Solórzano and Strickland (1968) and Armstrong and Tibbitts (1968) found only small quantities of polyphosphates in unpolluted coastal waters. Solórzano and Strickland suggest that polyphosphate does not accumulate to any extent in unpolluted situations since it appears to be taken up rapidly by algal cells even in the presence of bacteria. However since it accumulates when there are deficiencies of other nutrients (Harold, 1966) and under adverse environmental conditions, it may occur in tropholytic waters and its presence in the euphotic zone may be an indication of a limiting micronutrient.

Because little of the organic phosphorus of sea water is easily hydrolyzed by enzyme phosphate esters Solózano and Strickland suggest that the organic phosphorus isolated from sea water is mainly nucleic acid. Particulate DNA has been isolated from sea water by Holm-Hansen, Sutcliffe, and Sharp, (1968). However, Armstrong, Williams, and Strickland (1966) found that nucleic acids were easily degradable by ultraviolet light, and thus might not be expected in the surface water but might appear in the aphotic layers, as reported for bog lakes (Hooper, 1969).

Methods

Analyses made during 1969 were on samples concentrated by flash evaporation. This concentration procedure produced substances that interfered with the reduction of the molybdate complex in orthophosphate measurement. Removal of the interfering materials proved difficult and time-consuming (Hooper, 1969).

In 1970, we perfected a procedure for concentrating the organic residues on an ion-exchange resin. This proved more efficient and more reliable.

For these analyses we collected 600-ml water samples in clean polyethylene bottles from three locations in the lake: (1) the center of the lake at a depth of 1 M, (2) the center of the lake at a depth of 7 M, (3) at a depth of 0.2 M in the bog mat at the southeast corner of the lake, 5.5 M from the edge of the open water.

The 600-ml samples were applied to a 2 x 15 cm column of Dowex 1 x 8 anion exchange resin, 100-200 mesh, formate form. The effluent and the subsequent water washes of the resin were colorless, contained no phosphate, and did not absorb light at 260 nm, as compared to a water blank. A gradient elution was set up between 0.01 and 0.5 formic acid and allowed to flow at a rate of 0.5 ml/min. Measured fractions were collected on a Buchler fraction collector. Fractions were checked for absorbance at 260 nm against appropriate formic blank. Phosphate was analyzed using the procedure given by Strickland and Parsons (1965). Selected fractions were assayed for ribose content, using the orcinol method (Ashwell, 1957) and UV absorption spectra were determined on a Beckmann DB-G scanning spectrophotometer. Some fractions were assayed for labile phosphorus by adjusting the pH to 1.0 with HCl, boiling for 10 min, and assaying for orthophosphate. Fractions were digested with perchloric acid to determine total phosphorus content.

Results

Phosphorus compounds such as phosphoric acid, polyphosphoric acid, nucleotides, and nucleic acid can be absorbed on an anion exchange resin. Elution with increasing normality of acid or salt can successively elute and separate various compounds (Cohn, 1957). When lakewater from a depth of 7 M was analyzed in this manner, it was shown to contain several different ribose and phosphate-containing compounds which absorbed in the 250-260 nm, wavelength region (Table I). Such properties are characteristic of ribose nucleic acids. It was not determined whether these compounds were ribose nucleotides or polymers. Free ribose nucleosides, which also absorb in the 250-260 nm range, do not absorb to the resin and would have been detected in the pre-elution wash. Nucleosides were not present in these samples. Mat water and lake water from a depth of 1 M contained smaller amounts of RNA than the 7 M water. All three samples had a clearly defined orthophosphate peak and a second inorganic peak that corresponded in order of elution to polymers of orthophosphate (Beukencamp, *et al*., 1954). Since earlier work has shown that this lake water contains a labile inorganic phosphate, similar in properties to polyphosphate, this peak is described in Table II as polyphosphate.

Table I. Characterization of Nucleic Acid Fractions

Depth	Sample Number	RNA Peak Absorption (nm)	RNA $m\mu$ mol/ml (using extinction coefficient of 14,000 cm^{-1} M^{-1})	Ribose, $m\mu$ mol/ml	Phosphorus, $m\mu$ mol/ml
7 M	9	258	12	20	--
	10	258	7	16	1.8
	15	Broad	4.7	5.4	1.5
	25	Broad	1.7	1.4	1.2
1 M	17	Broad	6.4	6.5	1.2
	18	260	10.2	6.5	1.2
Mat	14	Broad	1.1	1.6	0.1
	26	Broad	2.1	2.4	0.3

Table II

Sample	RNA*		Orthosphate,	Polyphosphate,
	μmol/l	μg P/l	μg/l	μg/l
Lake water, 7 M	1.56	48.3	22.7	13.0
Lake water, 1 M	0.52	16.1	6.6	4.3
Mat water	0.40	12.4	32.8	5.0

*Based on UV absorption, using an average extinction coefficient of 14,000 $cm^{-1}M^{-1}$. P content assumes molar equivalence between phosphate content and base content.

Discussion

There were sharp differences between samples from the three locations in the amount and character of the 260 nm absorbing materials. This may indicate differences of origin of the RNA fractions or differences in state of degradation. Peaks in the 250-260 nm range were broad and lacked prominent shoulders, which indicated that the material was not single nucleotides but some size of polymeric form. The lack of agreement in stoichiometry between UV absorption, ribose, and phosphorus leaves some uncertainties. These differences arose because we were below the desirable range of accuracy in certain of the procedures.

The larger quantity of RNA at 7 M than at 1 M may in part arise from the greater degradability of nucleic acids in light (Armstrong, Williams, and Strickland, 1966). However, larger quantities of RNA might be expected at 7 M from autolysis of cells settling into this layer. The lower RNA content of the mat water compared to 7 M and 1 M suggests a limnetic origin of this RNA since water exchange takes place between the mat and the lake. However, differences in character of the RNA fraction were also apparent; therefore, origin in the mat cannot be excluded.

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APPENDIX D

CHEMICAL DATA, NORTH GATE LAKE, 1970.

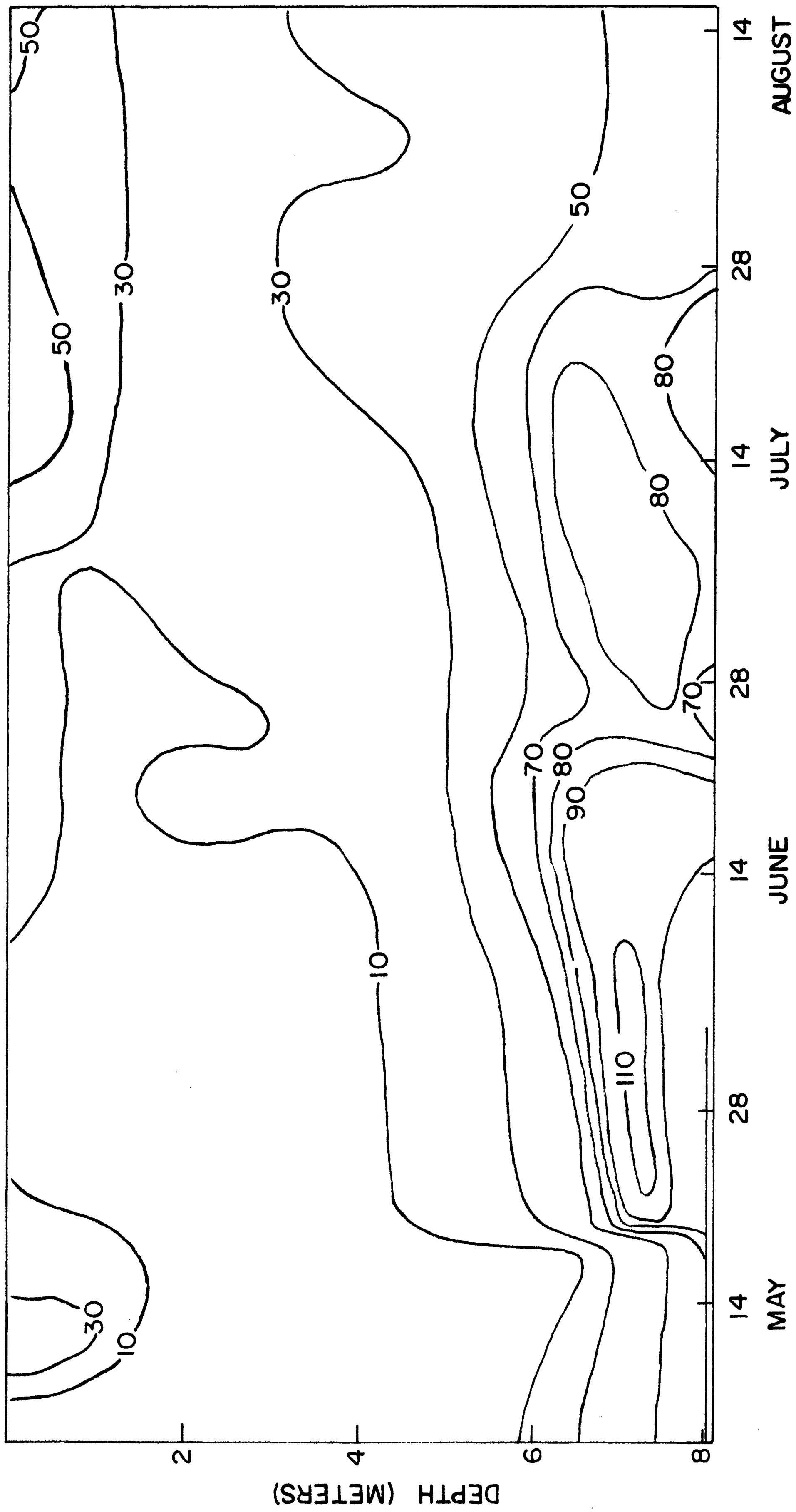

Fig. 1. Rhodamine WT (ppb).

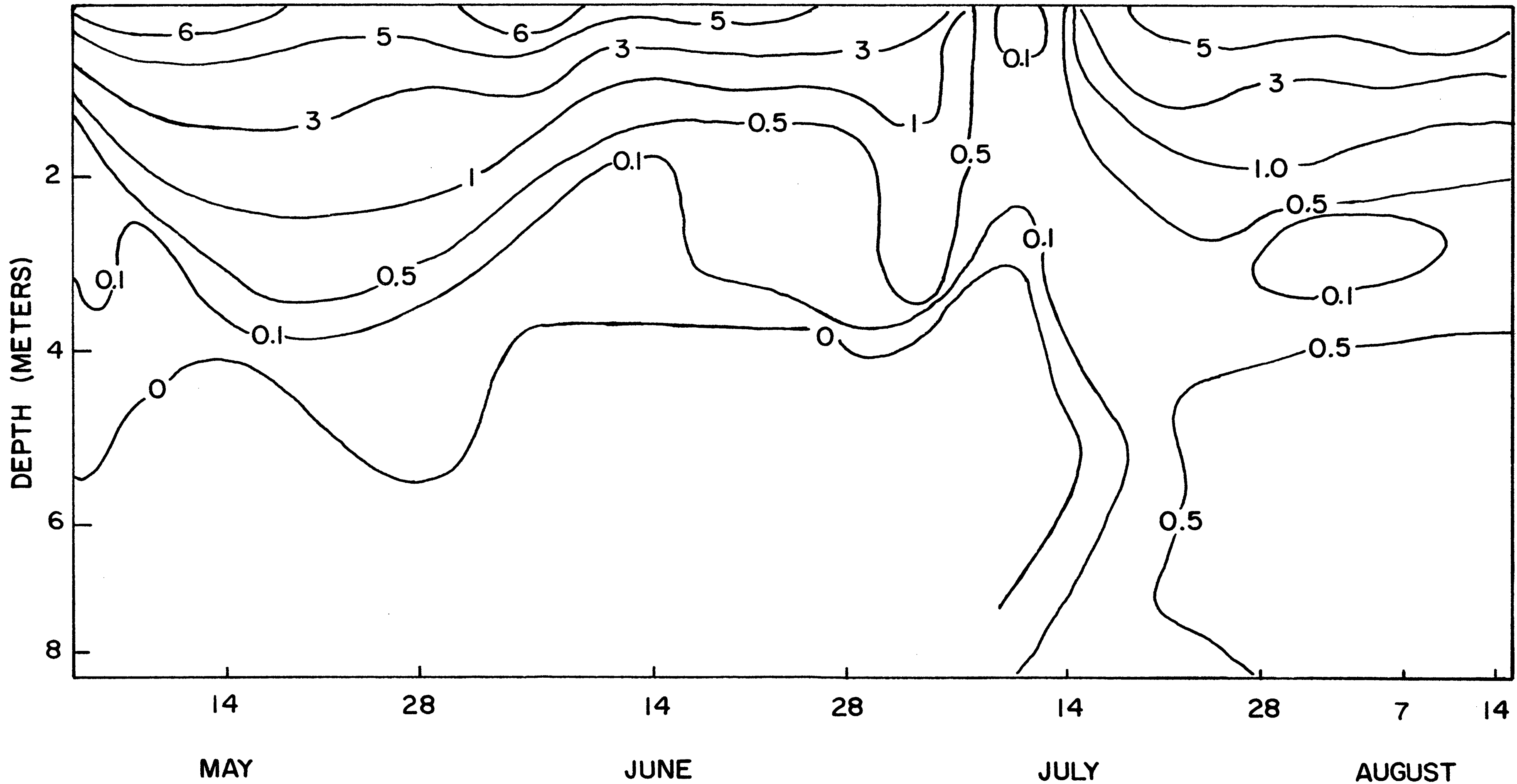

Fig. 2. Dissolved oxygen

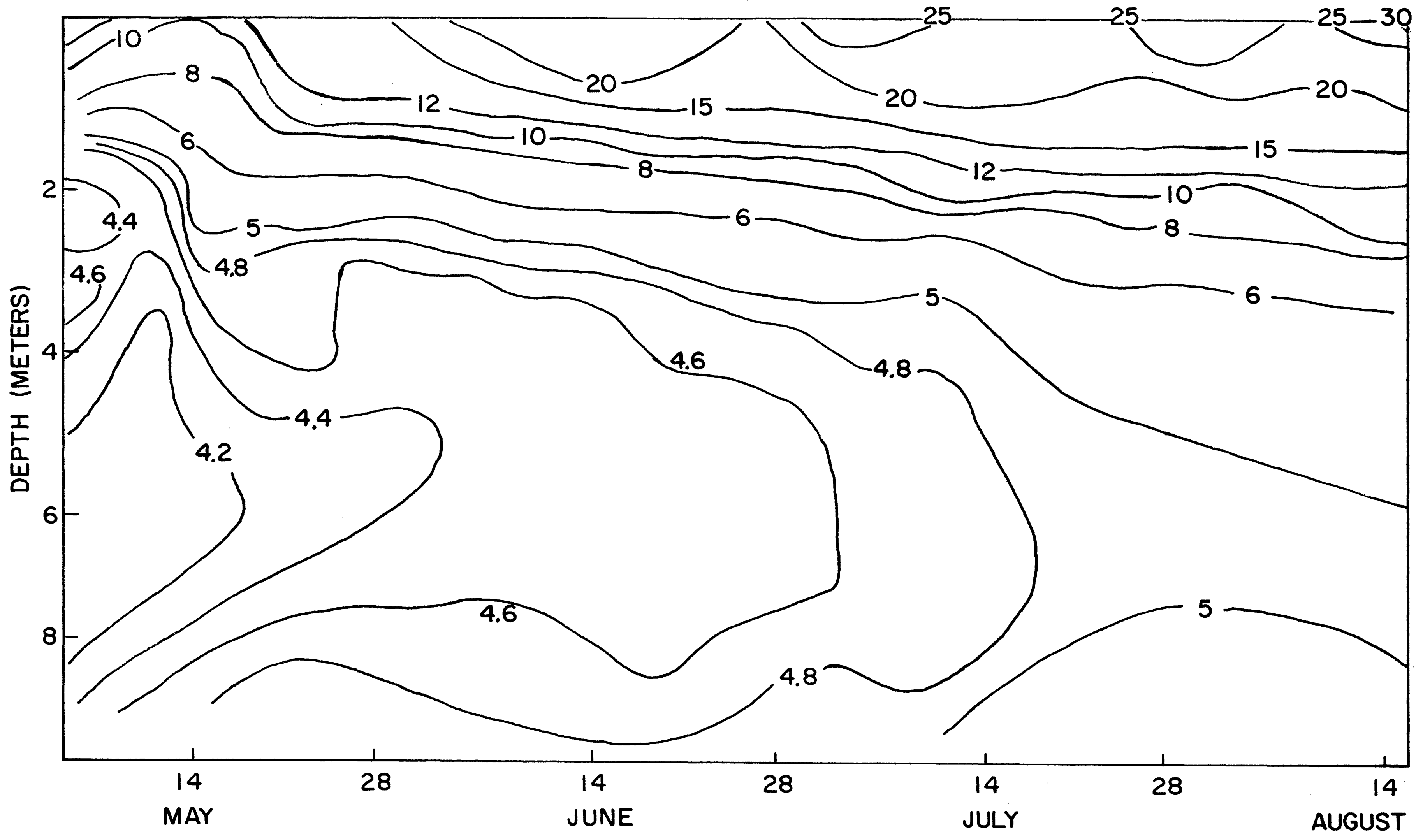

Fig. 3. Temperature (°C).

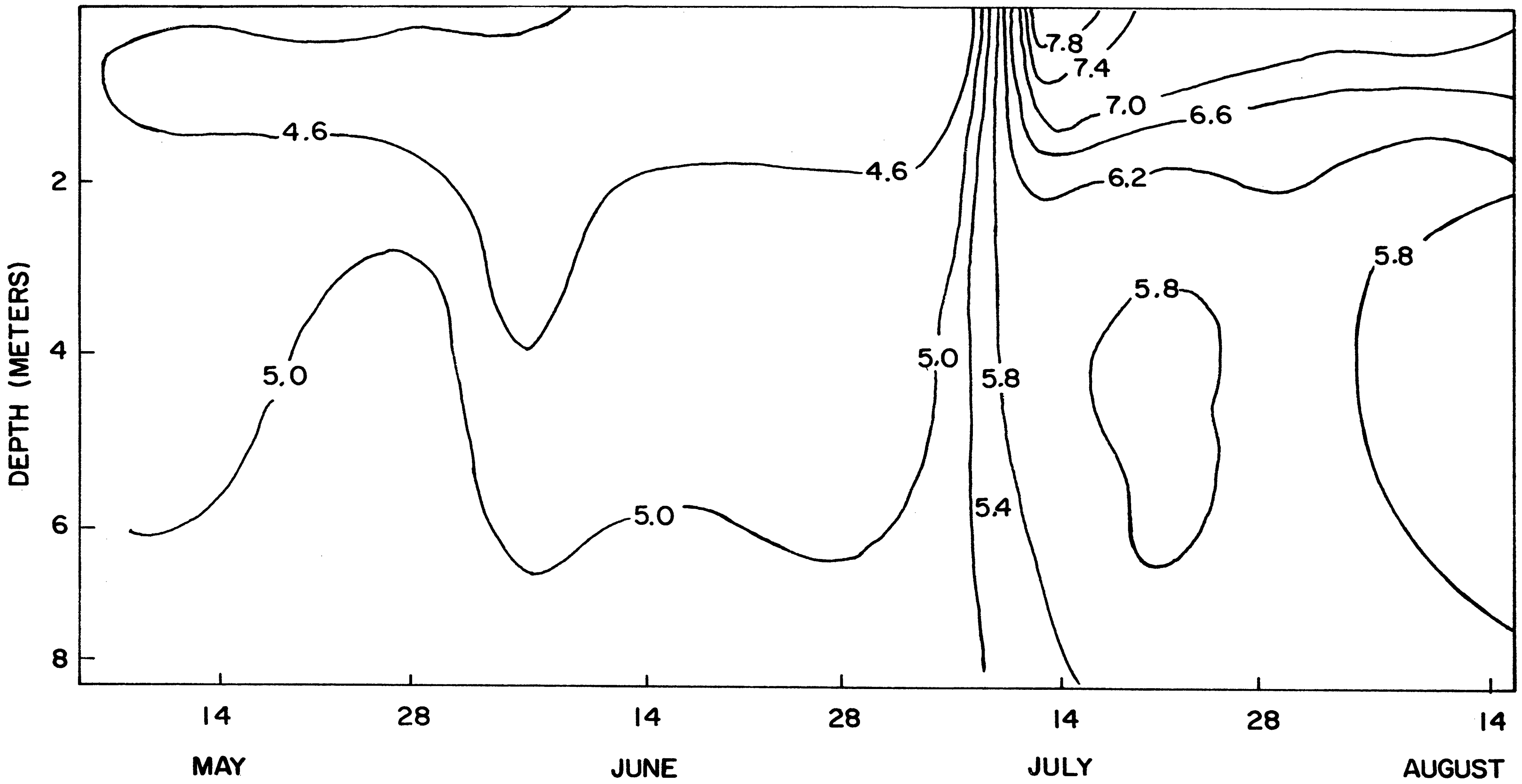

Fig. 4. pH

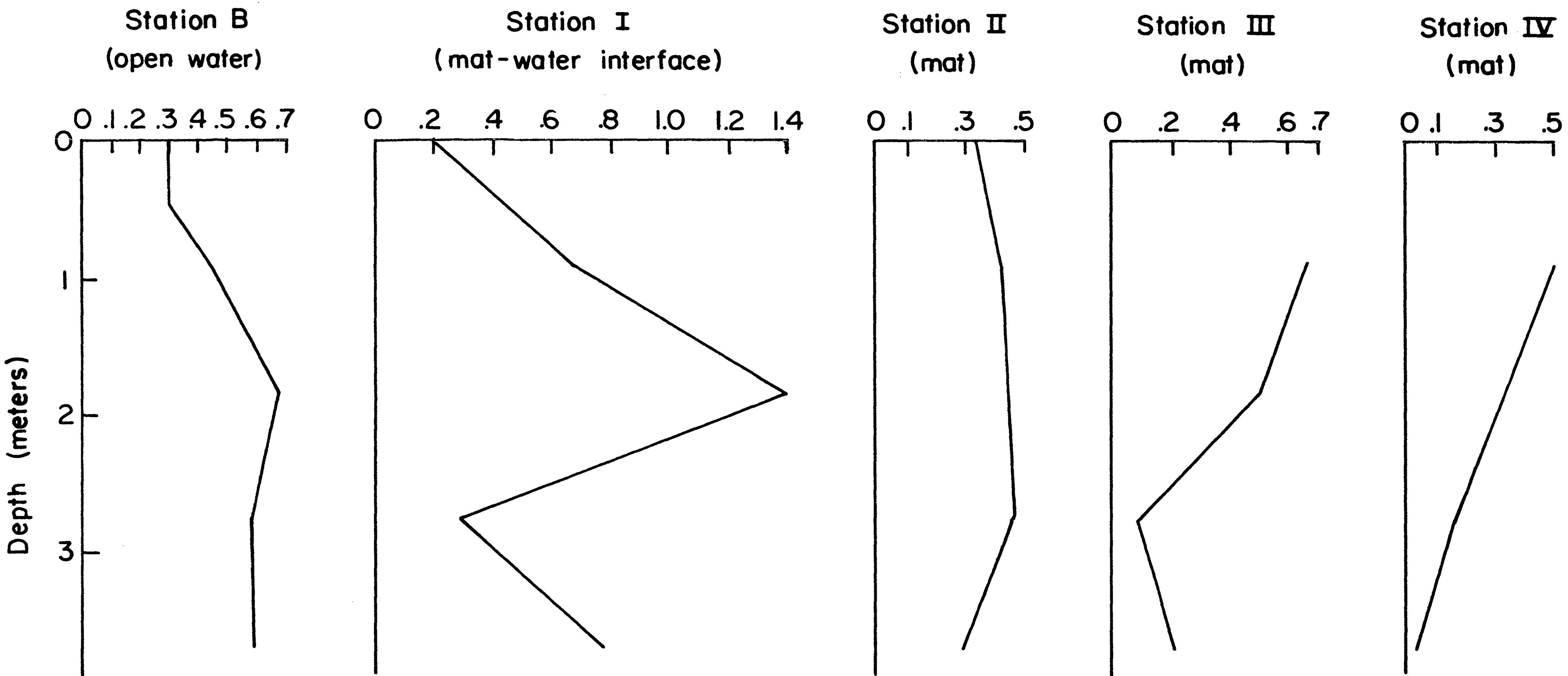

Fig. 5. Filterable iron (ppm), open water and bog mat. July-August, 1970.