1973

The Effect of the Herbicide Glyphosate on the Growth of Selenastrum Capricornutum

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THE EFFECT OF THE HERBICIDE GLYPHOSATE ON THE GROWTH OF *SELENASTRUM CAPRICORNUTUM*

by

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An honors thesis submitted in partial fulfillment
of the requirements for the degree

of

BACHELOR OF SCIENCE

in

BOTANY

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1973

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ABSTRACT

The Effect of the Herbicide Glyphosate on the Growth of

Selenastrum capricornutum

by

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Utah State University, 1973

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Department: Botany

The herbicide glyphosate (MON-0139), an isopropylamine salt of N-(phosphonemethyl)glycine, was tested to determine its effects on the growth of the fresh water green alga Selenastrum capricornutum. Experiments were carried out to determine algicidal, algistatic, and inhibitory concentrations of the compound.

Concentrations from 5 ppm to 1000 ppm were inhibitory to the growth of the alga, but the effects were algistatic, rather than algicidal. At concentrations from 0.1 ppm to 1.0 ppm the growth of the alga was stimulated by the presence of the herbicide, while at concentrations from 0.001 ppm to 0.05 ppm the effects of the herbicide were negligible.

Two chemical tests were used, and it was determined that in low pH systems (pH of about 4.5) glyphosate hydrolyzes with inorganic phosphate as one of the hydrolysis products. The concentrations of phosphate released is so low as to have no effect on the growth of S. capricornutum.

(24 pages)

INTRODUCTION

Herbicide use has, of late, become a very controversial issue. It has become increasingly important to develop herbicides which will be selectively effective, with little or no residual effect on non-target organisms.

Few investigations have been concerned with the action of various herbicides on the algae, yet this is becoming an area of increasing importance. Eutrophication of freshwater systems can lead to clogged filter pipes and drainage ditches, unusable boating and swimming facilities, loss of fish due to oxygen removal from the water, and taste and odor problems due to algal decomposition (Virmani, 1972).

On the other hand, herbicides not intended for aquatic use can have damaging effects on these systems if introduced into them.

The focus of this investigation is the effect on Selenastrum capricornutum, a freshwater alga, of a new, post emergence herbicide, glyphosate. This herbicide has poor plant selectively, but no residual effects in the soil (Technical Bulletin MON - 057 - 1 - 71). Glyphosate is examined both for its potential as an algicide, and for its residual effects in aquatic systems.

MATERIALS AND METHODS

Test organism

Selenastrum capricornutum, the organism selected for this study, is a freshwater member of the chlorophyta (green algae), widely distributed in both oligotrophic and eutrophic waters, notably in Europe. The alga is nonmotile, and solitary. Cells are up to 2 μ wide and to 20 μ long, and are characteristically lunate with acutely pointed apices. As far as is known the alga is an obligate autotroph, and is easily maintained in the laboratory (Skulberg, 1966). S. capricornutum is widely used as a bioassay organism (Joint Industry-Government Task Force on Eutrophication, 1969).

A stock culture of S. capricornutum was obtained from the Utah State University Water Research Laboratory, and maintained in Algal Nutrient Medium (Joint Industry-Government Task Force on Eutrophication, 1969). The trace metals used were provided as the P IV Trace Metal Mix of Provasoli and Pinter (1959). The complete medium, Modified Algal Nutrient Medium (see appendix), will hereafter be referred to as NAM. All media was sterilized by autoclaving at 15 psi and 121 C for 20 minutes. Medial formulations appear in the appendix.

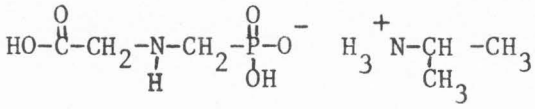
To obtain bacteria free cultures, 10 ml. of stock culture was placed in a 15 ml. centrifuge tube and spun at full speed in an International Clinical Centrifuge for 5 minutes. The supernatant was discarded, the cells resuspended in 10 ml. NAM, and recentrifuged. This process was repeated 10 times. Cells were then streaked out on the surface of sterile NAM containing 1.5 % agar. After 3 to 5 days bacteria free clones

were removed by means of a micropipette and transferred to the surface of sterile NAM solidified with 1.5 % agar.

Bacteria free cultures were maintained on NAM slants solidified with 1.5 % agar, at 15$\pm$ 1 C with constant illumination of 250-300 foot candles provided by "cool white" flourescent tubes.

The herbicide

The herbicide used in this investigation is an isopropylamine salt of N-(phosphonomethyl)glycine hereafter referred to as glyphosate. The structure is most probably:



This herbicide is very new, and is to date available only for experimental research, through the Monsanto Company of St. Louis, Missouri. The mode of action in higher plants is presumed to be translocation from the vegetative parts of the plant to the roots (Technical Bulletin MON - 057 - 1 - 71). As far as the author knows, no work has been done to determine the mode of action of glyphosate on algae.

Two methods were used to determine probable breakdown products of glyphosate, with special attention paid to inorganic phosphate. If inorganic phosphate was found to be a breakdown product, and present in sufficient quantities, it could have a significant influence on both the growth rate, and the total growth attained, of S. capricornutum.

Inorganic phosphate analysis. A series of standards were prepared using dilutions of 0.125 mM sodium phosphate. An appropriate volume of 0.125 mM sodium phosphate was placed in each of 9 test tubes, and water added to bring the total volume to 7.8 ml. (table 1). One test tube containing sterile distilled water alone served as a control. 1 ml. 2 N

sulfuric acid and 0.8 ml. molybdate reagent (formulation in appendix) were added to each test tube, and the test tubes thoroughly mixed on a Vortex-Jr. mixer. 0.4 ml. amino naphthol sulfonic acid reagent (formulation in appendix) was then added to each test tube, the tubes again mixed, and allowed to incubate in the dark. After 15 minutes the optical density was recorded on a Bausch and Lomb Spectronic 20 Spectrophotometer at 660 nm. and a standard curve constructed.

A dilution series of glyphosate was prepared in water and treated in the same manner as the standards. The concentration of inorganic phosphate in each of the glyphosate dilutions was determined by consulting the standard curve (Fiske and Subbarow, 1925 and 1929).

Electrophoresis. Electrophoresis was carried out on Whatman No. 3MM chromatagraphy paper in pyridine acetate buffer at pH 4.5. 30-40 milliamps were applied for 45 minutes. Potential difference across the paper was 1.5 kilivolts over 54 cm. (28.8 volts/cm).

The paper was spotted with 0.125 mM potassium phosphate, 1000 ppm glyphosate (2 spots), and glycine.

Ninhydrin in acetone was used to detect the amino acids and amino acid derivatives, the primary amine, and the undissociated glyphosate. Phenol red (adjusted to pink with ammonium hydroxide) in ethyl alcohol was used to detect the phosphate, under ultraviolet light.

Experimental design

A dilution series of glyphosate was prepared in NAM and each resulting solution filter-sterilized by passing through an autoclaved HA type Millipore filter of pore size 0.45μ. Experiments were carried out in 18 x 150 mm. optically matched culture tubes, with stainless steel caps, containing 10 ml. of NAM and appropriate concentrations of herbicide. Four

Table 1. Preparation of phosphate standards from 0.125 mM sodium phosphate.

	ml. 0.125 mM Na_2HPO_4	H_2O	µmoles PO_4
	0.1	7.7	0.0125
	0.2	7.6	0.0250
	0.4	7.4	0.0500
	0.6	7.2	0.0750
	0.8	7.0	0.1000
	1.0	6.8	0.1250
	2.0	5.8	0.2500
	4.0	3.8	0.5000
	7.0	0.8	0.8750
control	0.0	7.8	0.0000

replicates were prepared for each herbicide concentration examined; three were inoculated and one left uninoculated to serve as a control. An additional control was prepared using algae and medium, less herbicide. Culture tubes containing only the alga and medium served to establish normal growth curves for the test organism, while uninoculated control tubes containing herbicide and medium were used to detect any changes not related to algal activity.

Growth was measured by recording optical density at 700 nm. on a Bausch and Lomb Spectronic 20 Spectrophotometer. Before reading, cultures

were thoroughly stirred using a Vortex-Jr. mixer. Optical density was recorded over a period of 32 days at 48 hour intervals, beginning on the second day post-inoculation.

Algicidal and algistatic herbicide concentrations

A test was devised to determine whether algal cultures died, in which case the effects of the herbicide were considered to be algicidal, or merely ceased to grow while exposed to the herbicide, in which case the effects of the herbicide were considered to be algistatic. Forty-eight hours after the final optical density reading a small subsample of the alga was taken from one of each set of replicate cultures, and from one control. These subcultures were transferred to fresh, sterile NAM. Subcultures were grown under the same conditions as previously described.

The cultures were examined for regrowth over a period of two weeks. Herbicide concentrations were considered to be algicidal if cultures showed no regrowth, and algistatic if regrowth occured.

RESULTS AND DISCUSSION

Effects of various concentrations of glyphosate on the growth of Selenastrum capricornutum

Concentrations of glyphosate from 0.001 ppm to 1000 ppm in NAM were examined to determine inhibitory effects on the growth of Selenastrum capricornutum. Maximum growth at each concentration was plotted as a percent of maximum growth of the control (figure 1).

No inhibitory effects on algal growth were observed at concentrations from 0.001 ppm to 0.05 ppm.

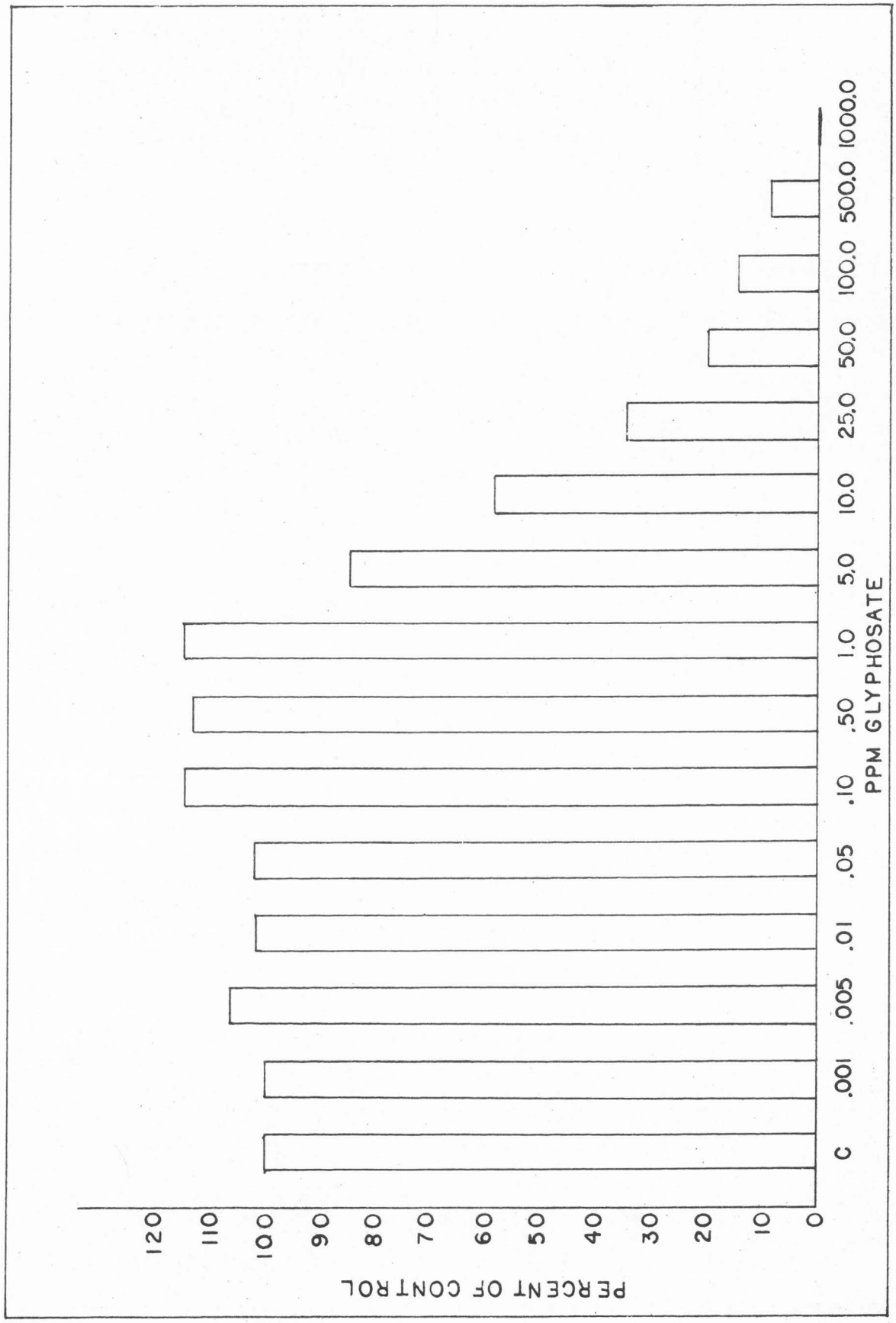
At concentrations from 0.1 ppm to 1.0 ppm algal growth was stimulated by the presence of the herbicide. (Maximum growth attained was 114 % of the maximum growth of the control.) Cells in these cultures appeared normal under microscopic examination.

At concentrations from 5 ppm to 500 ppm growth of the alga was markedly reduced from growth of the control. At 5 ppm maximum growth was 84 % of the maximum growth of the control, at 10 ppm it was 59 % of the control, at 25 ppm it was 34 % of the control, at 50 ppm it was 20 % of the control, at 100 ppm it was 14 % of the control and at 500 ppm it was 8 % of the control (figure 1).

Upon microscopic examination of these cultures it was observed that the cells had lost their characteristic lunate shape, and had become isodiametric. Cell division was arrested and many cells in the process of division were observed to have increased to several times the size of normal cells.

The growth rate of S. capricornutum (figure 2) was uneffected by

Figure 1. Effects of glyphosate on growth of Selenastrum capricornutum. Maximum growth attained during 32 days of exposure to various concentrations of glyphosate compared with growth of control cultures with zero herbicide. Growth is indicated as optical density at 700 nm.


PERCENT OF CONTROL
0
10
20
30
40
50
60
70
80
90
100
110
120
C
.001
.005
.01
.05
.10
.50
1.0
5.0
10.0
25.0
50.0
100.0
500.0
1000.0
PPM GLYPHOSATE

glyphosate concentrations below 0.5 ppm. The growth rate of the alga in glyphosate concentrations between 0.1 ppm and 1.0 ppm. prior to the 24th day of the experiment, was very similar to the growth rate of the control. After the 24th day of the experiment the growth rate of S. capricornutum in glyphosate concentrations between 0.1 ppm and 1.0 ppm exceeded the growth rate of the control. The algal growth rate in glyphosate concentrations from 5 ppm to 500 ppm decreased with each higher concentration.

Phosphate analysis

Concentrations of glyphosate in water from 0.001 ppm to 1000 ppm were examined for the presence of inorganic phosphate. The technique used was not sensitive to the presence of inorganic phosphate in glyphosate concentrations of less than 50 ppm.

The concentration of inorganic phosphate in each dilutation was determined from the standard curve at 660 nm. (figures 3 and 4, table 2). In glyphosate concentrations of 1000 ppm the phosphate concentration was 0.365 μM, in 750 ppm it was 0.275 μM, in 500 ppm it was 0.175 μM, in 250 ppm it was 0.088 μM, and in 100 ppm it was 0.037 μM. Glyphosate was 80-84 % hydrolyzed at each dilution.

Electrophoresis

Under the conditions previously described, glyphosate was found to hydrolyze, giving 4 ninhydrin positive spots, and 2 phenol red positive spots (figure 5). The ninhydrin positive spots were identified as isopropylamine, glycine, glyphosate, and one other spot presumed to be N-methylglycine (sarcocine). The phenol red positive spots were identified as glyphosate (dark spot) and phosphate (light spot).

Algistatic and algicidal effects of glyphosate

Regrowth was observed in all algal cultures transferred from NAM

Figure 2. Growth of Selenastrum capricornutum in response to selected concentrations of glyphosate. Growth is indicated by optical density at 700 nm.

▲ control

△ 1 ppm

○ 5 ppm

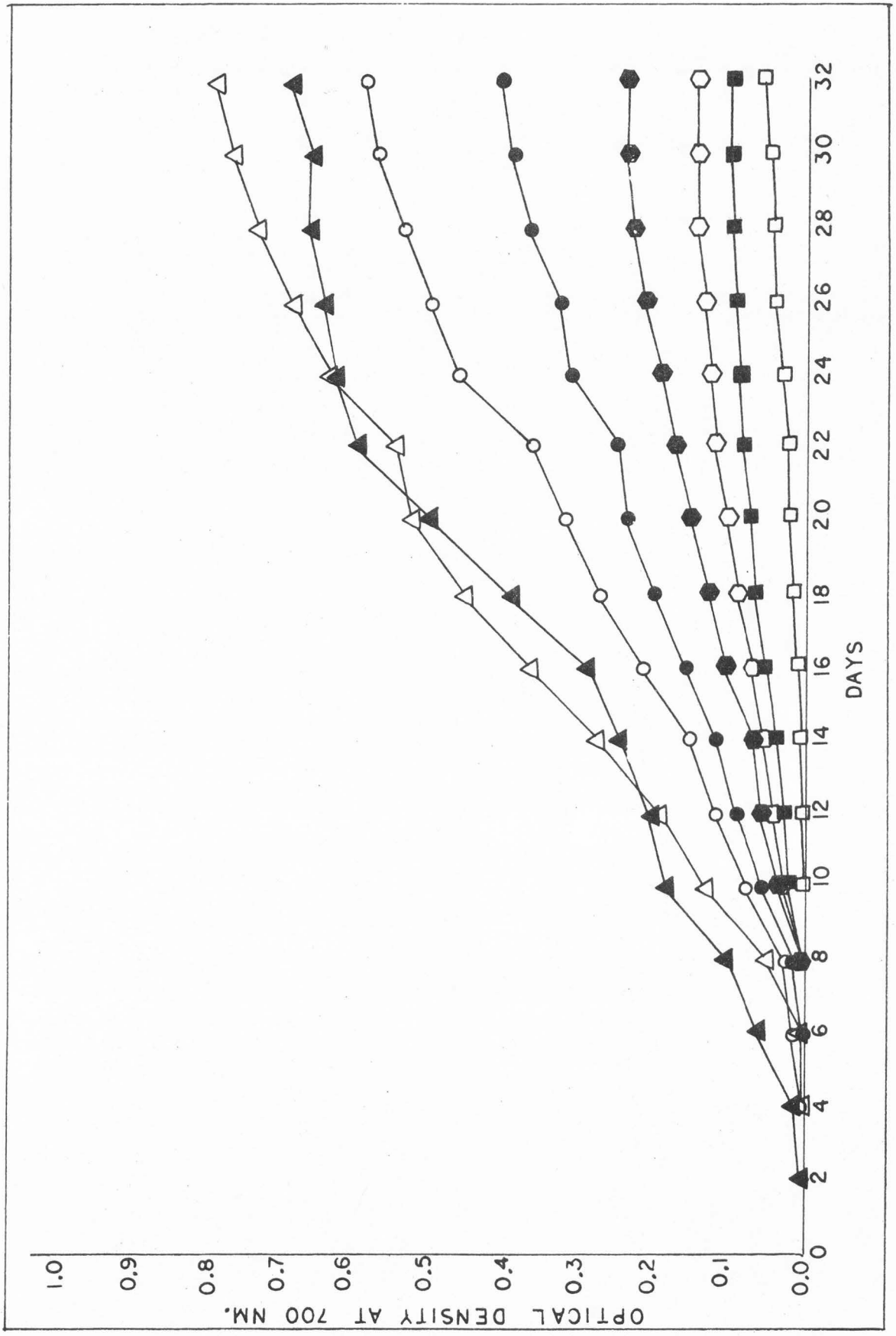
● 10 ppm

⬢ 25 ppm

⬡ 50 ppm

■ 100 ppm

□ 500 ppm


OPTICAL DENSITY AT 700 NM.
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
DAYS

containing glyphosate to fresh, sterile NAM. All concentrations of glyphosate between 0.001 ppm and 1000 ppm have an algistatic, rather than algicidal effect on the growth of S. capricornutum.

Discussion

In the course of this experiment glyphosate was found to be inhibitory to the growth of Selenastrum capricornutum only at relatively high concentrations (5 ppm and above). These concentrations were algistatic; if the alga was transferred to fresh media normal growth resumed.

Lower concentrations (0.1 ppm to 1.0 ppm) stimulated the growth of the alga. Exact reasons for this stimulation are unknown, although it is sometimes the case that toxins at very low levels will stimulate algal growth.

In low pH systems glyphosate hydrolyzes, releasing inorganic phosphate. The amount of inorganic phosphate released is not considered to be high enough to influence the growth of this alga.

More work is necessary to determine the exact modes of action of this herbicide on S. capricornutum.

Figure 3. Standard curve of inorganic phosphate concentration compared with optical density at 660 nm.

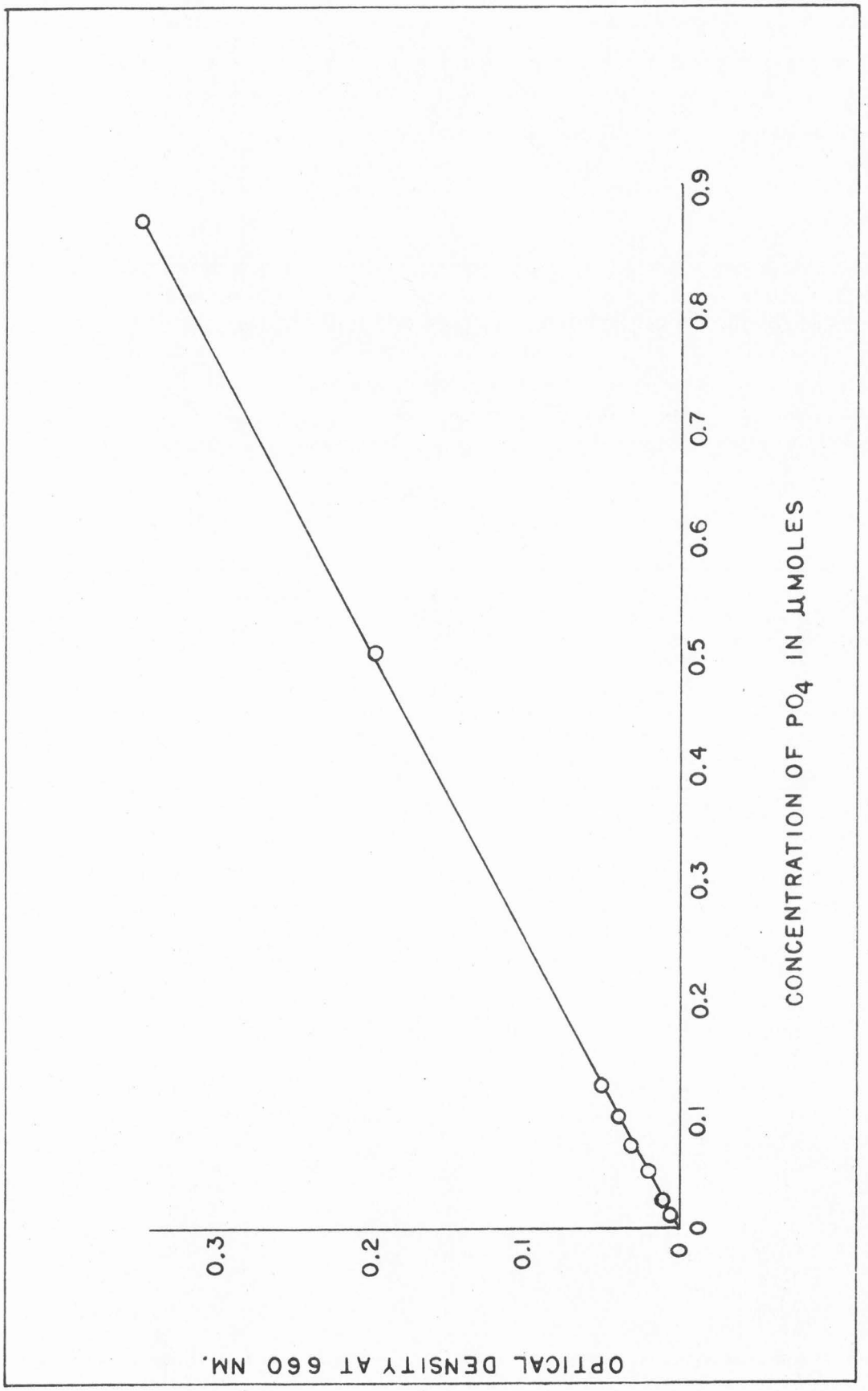

OPTICAL DENSITY AT 660 NM.
0.3
0.2
0.1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
CONCENTRATION OF PO_4 IN μMOLES

Figure 4. Concentration of inorganic phosphate in various dilutions of glyphosate. Concentration was determined from standard curve (figure 3).

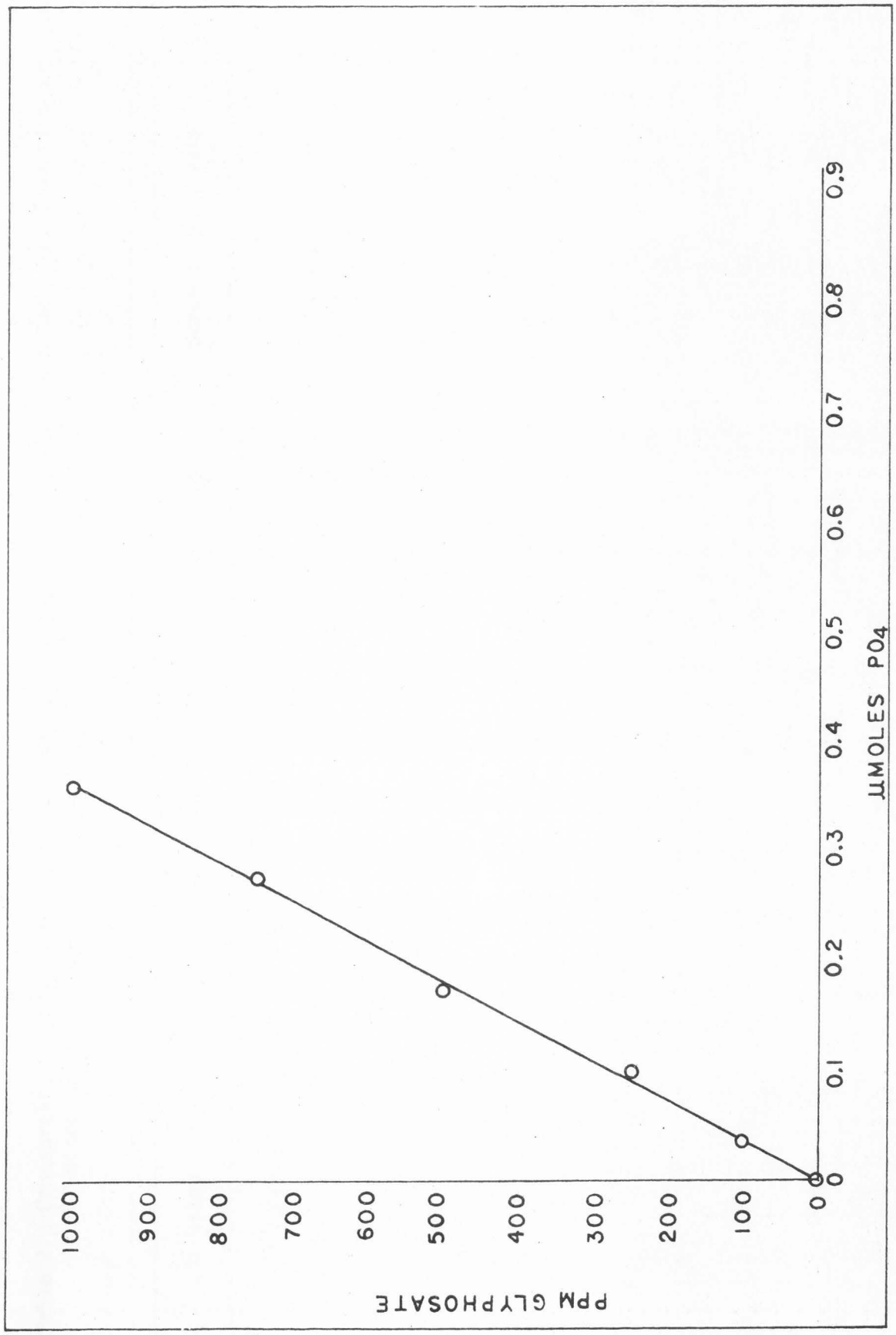
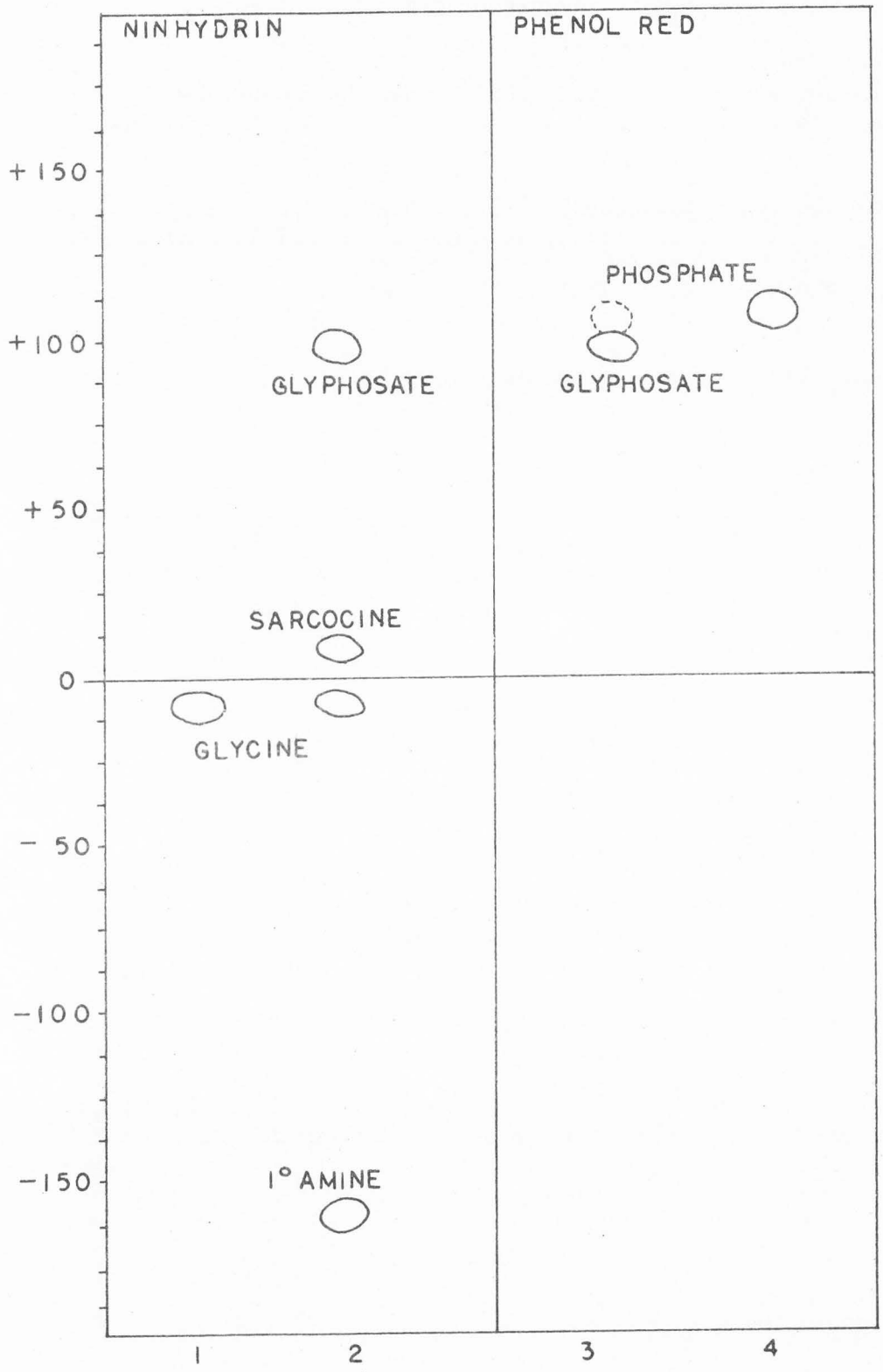

PPM GLYPHOSATE
1000
900
800
700
600
500
400
300
200
100
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
μMOLES PO4

Table 2. Concentration of inorganic phosphate in various dilutions of glyphosate as determined from the standard curve (figure 3).

dilution	µmoles glyphosate	µmoles phosphate	percent hydrolysis
1000 ppm	0.435	0.365	84
750	0.327	0.275	84
500	0.218	0.175	81
250	0.109	0.088	80
100	0.044	0.037	84

Figure 5. Diagramatic representation of electrophoresis of glyphosate in pyridine acetate buffer (pH 4.5) at 27.8 volts/cm. Spots 1 and 4 are controls; spot 1 is glycine, spot 4 is 0.125 mM potassium phosphate. Spots 2 and 3 are glyphosate at 1000 ppm.


NINHYDRIN
PHENOL RED
+150
+100
+50
0
-50
-100
-150
PHOSPHATE
GLYPHOSATE
GLYPHOSATE
SARCOCINE
GLYCINE
1° AMINE
1
2
3
4

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APPENDIX

Algal Nutrient Medium (Joint Industry-Government Task Force on Eutrophication, 1969)

Macronutrients - Following salts, Biological or Reagent grade, in mg. per liter of glass distilled water.

$NaNo_3$	85.00
K_2HPO_4	3.48
$MgCl_2$	19.00
$MgSO_4 \cdot 7H_2O$	49.00
$CaCl_2 \cdot 2H_2O$	14.70
Na_2CO_3	50.00
$FeCl_3$	0.32

P IV Trace Metal Mix (Provasoli and Pinter, 1959)

To 500 ml. of glass distilled water add the following amounts of chelating agent and metal salts:

$FeCl_3 \cdot 6H_2O$	0.097 g
$MnCl_2 \cdot 4H_2O$	0.041 g
$ZnCl_2$	0.005 g
$CoCl_2 \cdot 6H_2O$	0.002 g
Na_2MoO_4	0.004 g
Na_2EDTA	0.750 g

Molybdate Reagent (Fiske and Subbarow, 1925)

2.5 % ammonium molybdate in 2.5 N H_2SO_4

Amino Naphthol Sulfonic Acid Reagent (Fishe and Subbarow, 1925)

15 % $Na_2S_2O_5$	195.0 ml
1-amino-2-naphthol-4-sulfonic acid	0.5 g
20 % Na_2SO_3	5.0 ml

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