

**The Relative Significance of Phosphorus
and Nitrogen as Algal Nutrients**

by

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ABSTRACT
THE RELATIVE SIGNIFICANCE OF PHOSPHORUS AND NITROGEN AS ALGAL NUTRIENTS

By the examination of the interaction between nitrogen and phosphorus species relative to algal growth in several fresh water environments of differing trophic state, it has been possible to establish the relative significance of these elements as algal nutrients. The algal assay was carried out using membrane filtered samples derived from a series of oxidation ponds receiving secondary effluent from a trickling filter plant and from samples derived from sampling points on the New Hope and Haw Rivers. The latter represented a series of changing river qualities with particular respect to the oxidation states of nitrogen and phosphorus. The algal assay used pure cultures of five species; Euglena rostifera, Chlamydomonas reinhardtii, Pandorina morum, Scenedusmus quadricauda, and Chlorella ellipsoidea. Each of these species has been described as normally associated with polluted waters.

The results of both chemical and biological assay examined through multiple regression analysis of the independent variable involved in the algal assay as well as a quadratic analysis of covariance of $\text{NH}_3\text{-N}$, $\text{NO}_3\text{-N}$ and $\text{PO}_4\text{-P}$ established the relative significance of the nitrogen and phosphorus species in water containing high concentrations of these elements, concentrations normally found in discharges from biological waste treatment plants and following dilution in receiving streams. Under these circumstances it has been shown that the quantity of nitrogen rather than that of phosphorus determines the biomass of algae that might be expected to grow. This response holds true for the several species of algal that were used. It would thus appear that the question of algal nutrients takes on a somewhat more formidable dimension due to the considerably greater difficulty in removing in significant amounts the nitrogen that is normally found in municipal wastewaters.

SUMMARY AND CONCLUSIONS

Through the use of algal bioassay on wastewater samples and on samples derived from streams receiving wastewater discharges, the algal response as measured by biomass development indicates that the element nitrogen appears to be of more significance as a growth controlling element rather than phosphorus. The information developed by this investigation and substantiated by other reports in the literature indicate that these two elements hold this particular relationship when present in the relatively large amounts found in nutrient rich waters. If this demonstrable relationship is correct it is then evident that efforts to control eutrophication by phosphorus removal at wastewater treatment facilities will not resolve the problem until similar efforts are made to reduce the larger quantities of nitrogen that are also present.

RECOMMENDATIONS

It is recommended that the control of algal nutrients in wastewater discharges should not be focused solely on the amount of phosphorus present or capable of removal but should be expanded to include also the removal of nitrogen in order that conditions leading to eutrophication be controlled and minimized.

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INTRODUCTION

Phosphorus has been described as the most important ecological element since it is most likely to be deficient and therefore, more limiting to biological productivity than any other major nutritional element. However, in many areas its presence in surface waters is generally not dependent on local geo-chemical considerations. The quantities present are in most instances derived primarily from wastewater and land runoff from applications of agricultural fertilizer.

Contributions of nitrogen to aquatic environments are made in a more complex manner, being fixed directly from atmospheric nitrogen or enters the nitrogen cycle by the action of many species of microorganisms degrading nitrogen containing organic compounds. Other microorganisms may either oxidize reduced forms of nitrogen or reduce oxidized states. Agricultural fertilizers are also a major source of nitrogen in surface waters being carried in runoff or in return flow of irrigation water.

The use of phosphorus and nitrogen compounds by aquatic plants and particularly phytoplankton will depend on the specific species requirement for the element as well as environmental considerations. When the nutritional elements are present in quantities greater than is required for normal cell multiplication and seasonal factors of temperature and illumination are optimum, rapid cell multiplication leads to excessive numbers and generally undesirable water quality conditions. The water is then described as having reached a state of eutrophy reflecting the presence of high nutrient levels.

The behavior of phosphorus in water is further complicated by the fact that the number of chemical states that are found have different degrees of availability to algae. In addition to two principal oxidized states in water solution as well as phosphorus in particulate form, there have been demonstrated various soluble organic phosphorus compounds that are both secreted during growth or decay of living organisms and may even be reused directly if inorganic phosphate should become limiting. In a recent review, it was noted that the complex interactions of several levels of nutrients need to be fully described at any trophic level before a single primary cause-effect relationship can be defined (1).

In the investigation described in this report, it was proposed to investigate the cycling of various phosphorus species in several freshwater environments of different trophic states. These were to be a water supply impoundment (University Lake, Chapel Hill), a small stream (Morgan Creek) into which the treated wastewater effluent of Chapel Hill is discharged and a third environment was to be a series of small oxidation ponds that had been used for several years to study their effectiveness as a tertiary treatment facility at the Third Fork Creek Treatment Plant of the City of Durham. (Figure 1) In each of these environments it was intended to establish the relationship between the several chemical forms of phosphorus to determine the distribution of the total quantity of this element and to determine in what manner the various states showed any significant shift either in passage through the ponds, in movement down the stream, or in the natural cycling of the lake. In addition, the similar pattern of change of the states of nitrogen were to be followed and the relative growth properties of

the combined materials in their various states were to be assayed by the specific growth of five species of algae under controlled conditions.

In the preliminary phases of the investigation it was realized that the extremely low levels of phosphorus currently found in University Lake precluded any routine and systematic analytical methodology as well as the ability to measure with any reasonable time the growth response of assayed organisms under the particular conditions that were used to compare response in the more highly enriched environments of the streams receiving wastewater and the tertiary oxidation ponds. Thus, the emphasis was shifted to a comparison of the ponds and the stream and river locations which provided gradients of concentration and to include stations on the Haw River, a nearby drainage but which provided another gradient of nutrient levels for parallel comparison.

METHODS

Chemical analyses for nitrogen and phosphorus in their various states of oxidation were carried out on Technicon Autoanalyzers. Description of these procedures and the algal assay are described in the appendix. Samples, both for nitrogen and phosphorus, that required storage before analysis were collected and stored in polyethylene bottles and either frozen or 40 mg/l Hg^{++} ions were added for preservation plus storage at 1° C. These storage procedures minimized chemical change due to biological activity (2).

In addition to the analytical steps needed to describe the nature of the nutrient elements either in the oxidation ponds or in the

streams, water quality conditions in the ponds and streams were also collected and described according to procedures in Standard Methods (12th edition). These procedures and modifications will be presented in detail in the report in preparation on the water quality of the New Hope and lower Haw Rivers (3).

The water quality information of the New Hope and Haw River drainages will be of considerable interest to regulatory agencies concerned with the water quality of this area since many of the stations sampled for the current investigation are within that portion of the drainage that will be flooded on completion of the New Hope Dam. These same stations are continuing to be sampled in a subsequent investigation concerning itself with the potential effect of the quality of these waters on the new New Hope impoundment. The stream and river data will be presented in a subsequent report (3). Figure 2, a map of this area, shows all the sampling stations as well as other locations related to this investigation.

RESULTS

Algal Bioassay of Treated Wastewater on Passage through Oxidation Ponds

The basic premise employed in these investigations was that the comparative growth of 4 or 5 species of algae, species that are normally associated with high levels of nutrients, as might be found in streams receiving wastewater treatment plant effluents, would describe the potential effect of such nutrient levels. Since it was not feasible to directly isolate the relative nutrient significance of phosphorus or nitrogen in the aquatic environments,

the quantity of these elements and their various chemical states were determined and the growth of the various species of algae in a fixed period of time was used as the measure of the significance of these chemical species.

In the sampling and assay program for the oxidation ponds receiving treated sewage from the Third Fork Creek Treatment Plant of the City of Durham, four stages of effluent conditioning or additional stabilization were assayed. The first was the direct discharge from the sewage treatment plant, plant effluent (PE), the second following two days additional detention, the third after four days detention, and the fourth after a total of five days detention. As shown in Figures 3, 4, 5 and 6, the cell mass grown after ten days incubation under standard conditions of temperature and illumination for the five species of algae is described for each sample assayed. These figures are a comparison of species response to the same quality nutrient in the particular pond sample. All other species had been removed by filtration through a membrane filter before being re-seeded with the specific algae. Although general trends are not too evident, the set of diagrams for each sample date show for the plant effluent and two days detention a relative uniform growth response for all species, through the June 9 sample. After four and five days detention, the individual species response appears to be somewhat more erratic as if the quality of the substrate was more favorable to several of the species than others. The sample taken on April 28 is a good illustration of this particular

point. While all of the five species grew to varying degrees, it would appear that the growth response of Chlamydomonas reinhardtii and Scenedesmus quadricauda was consistently better than the other species.

Another procedure for examining the response of the algae to the particular environmental quality of the ponds and, in this case, the amounts of available nitrogen and phosphorus compounds is to examine the growth response on any one sampling date at the points of sampling and reflecting the changing flow-through conditions. This presentation is shown in Figures 7, 8, and 9. The mass of cells grown of each of the five species of algae is linked to describe the changing quantity of the growth response. The quantities of nitrogen and phosphorus compounds taken at the sampling point and characterizing the filtered substrate into which the algae were seeded is described in each instance by a histogram.

In all cases the nutrient levels were exceedingly high as compared to natural waters and would appear on casual observation to have no particular direct correlation with the nature of the algal growth response. In some instances, the change in growth response of the algal assay was dramatic as the substrate moved through the oxidation ponds. This is well illustrated in the sample of April 28. In other instances, little change was seen, such as in the samples of March 31 and May 12. In still other cases, such as April 14, there was an initial rise with four of the five species, a plateau and then a drop in growth response of three of the species. In nearly all examples, the algal growth response was more comparable in the plant effluent than in the

subsequent substrates whose growth supporting quality changed due to passage through the ponds. One exception was the sample of July 18. The earlier observation on the comparatively greater growth response of Chlamydomonas and Scenedesmus was quite evident in many examples of the assay, such as that of July 18, August 11, October 6, November 14, and December 1.

It would appear from these comments on the response of the algal assay that whereas the nutrient levels, nitrogen and phosphorus were in excess and never limiting, there were distinct species differences in response to the particular overall quality of the substrates.

Algal Bioassay of Water Sampled at New Hope and Haw River Stations

Quality characterization of a flowing stream, particularly those receiving inputs of wastewater from either treated or untreated sources, can be described by many parameters. As noted in the methodology for this investigation, river stations were systematically sampled over a period of 2½ years on the New Hope and 13 months on the Haw River for the material to be reported in this investigation. Investigations and collection of data at these river stations are continuing and a detailed analysis of the river quality parameters will be discussed in a separate report. However, for this report the nature of the algal nutrient characteristics of the river stations, with particular reference to the response of the algal assay, will be discussed.

In the following series of figures representing eight separate sampling dates on the New Hope and Haw Rivers each (the NH or HAW

number designation being the sampling number of the overall river sampling series) are described in similar fashion to those figures used to illustrate the response of the algal assay to the oxidation pond qualities. However, on the river samples, only four species of algae were used. The Euglena was not available but the same letter identification code was used, eg. B - Chlamydomonas reinhardtii, C - Pandorina morum, D - Scenedesmus quadricauda, and E - Chlorella ellipsoidea. At each of the stations in downstream sequence, with the distances between adjacent stations noted, a histogram is used to describe the growth response of the algae when grown for 10 days at 22° on a diurnal day-night cycle. The nitrogen and phosphorus characterization of the filtered sample prior to re-seeding is similarly presented.

Since the New Hope River stations were not in direct downstream sequence, due to their being located on several of the feeder streams which converged to form the main river, the sequence from left to right may, in some instances, give the impression of a peak response mid-way in the series. As noted in the legend, the downstream sequence was stations 1, 6, 7, 10 and 5, 7, 10, all upstream feeder creeks having converged into the main river at station 7. However, as shown in Figure 10, describing sample NH-38, the total biomass formed appeared to have a direct relationship to the magnitude of the nutrient levels described in the river for both nitrogen and phosphorus. As described for the pond assays, not all of the species grew to the same degree but in samples taken from the New Hope River, Pandorina and Scenedesmus, appeared to

outgrow Chlamydomonas and Chlorella. It is of interest to note that although nutrient levels in the river remained relatively high and were highest at stations 6 and 5 throughout the sampling, the systematic response did not always appear to follow a direct relationship. That is, on some occasions growth, such as on sample NH-46, Figure 14, massive growth occurred throughout the assay regardless of the relative nutrient quantities and as compared to other samples. However, the growth in the samples from stations 6 and 5 was greatest and appeared to relate to the degree of nutrient levels in this particular sample set.

The relatively smaller growth response as well as smaller quantities of nutrients of the Haw River series is quite clearly related. Growth of Pandorina and Scenedesmus once more illustrated a response somewhat greater than the other two species. On the Haw River the downstream sequence is in straight line with no additive changes in nutrient quality except for small inflowing streams, and the total concentration of nutrients is small as compared to the concentrations found in the New Hope River. In the length of river sampled, over a distance of 21 miles, in some cases a slight decrease in assay response was evident, in others relatively no change, and in still others perhaps a slight increase, such as on samples of August 7, 1968, one of the samples with a larger growth response, and that of November 13, 1968. In part this apparent downstream change may only be a behavior of a particular species in that particular sample, although the general impression of the assay histogram would appear to be an overall changing dimension in the downstream direction.

Multiple Regression Analysis of the Independent Variables in the Algal Assay

From the preceding discussion of the algal assay of pond and river filtrates, the growth of the several algae appears to be related in some manner to the quantity of nitrogen and phosphorus species that are present in the sample. To establish the relative significance of the variables with reference to the growth of algae, a multiple regression analysis was carried out on the algal assays of the oxidation ponds, the New Hope and the Haw River samples. In each instance, the growth response of each species was tested against each of 16 independent variables to determine the most direct relationship indicated, the curve of best fit, and then by the nature of the statistical program insert additional variables to improve the fit until further addition produced no significant change.

The variables included the chemical information on the quality of the filtered substrate, which was then reseeded with algae, as well as certain statistical manipulations of these values (Table 1).

As summarized in Table 2 it would appear that phosphorus species in the pond filtrates had a more direct role in determining growth than the nitrogen compounds, whereas, in the river filtrates it would appear that the nitrogen compounds in general had a more significant relationship in supporting algal growth in the assay.

As previously noted in the pond assays, when growth differences were clearly evident, Chlamydomonas and Scenedesmus grew to greater mass than the other three species, Euglena, Pandorina, Chlorella, which together formed a group which grew in a similar fashion. In the river filtrates (Euglena was not used in the river assays) the growth response

Table 1

Independent Variables Tested in Multiple
Regression Analysis for Best Fit to Growth of Algae

- 1 - Total N
- 2 - Total N²
- 3 - NH₃-N
- 4 - NH₃-N²
- 5 - Organic -N
- 6 - Organic -N²
- 7 - NO₃-N
- 8 - NO₃-N²
- 9 - Orthophosphate -A
- 10 - Orthophosphate -A²
- 11 - Orthophosphate -U
- 12 - Orthophosphate -U²
- 13 - Phosphorus · NH₃
- 14 - Phosphorus · NO₃
- 15 - Total N / Total Orthophosphate
- 16 - Total N / Total Orthophosphate²

A - available at start of assay, U - amount used in assay by algae
A squared value defines the shape of the regression curve as being non-linear.
Items 13 and 14, cross product functions and 15 and 16, ratios, allow added flexibility to the fitted curve.

Table 2

Summary of Multiple Regression Analysis of Sixteen Independent Variables and Growth Response of Algal Assay

<u>Assay Organism</u>	<u>Best Fit to the 5 Percent Level*</u>		
	<u>Ponds</u>	<u>Substrate Source</u>	
		<u>New Hope River</u>	<u>Haw River</u>
A - <u>Euglena rotifera</u>	P·NH ₃	-	-
	OP-A		
B - <u>Chlamydomonas reinhardtii</u>	OP-U ²	Tot N	NH ₃ ²
	Ratio ²	Org N	NO ₃
	OP-U		
	NO ₃ ²		
	P·NO ₃		
C - <u>Pandorina morum</u>	OP-U	OP-U	P·NO ₃
	OP-A ²	OP-A	NH ₃ ²
			NO ₃ ²
			OP-A
D - <u>Scenedesmus quadricauda</u>	OP-A	NH ₃	OP-U
		NO ₃ -A	NO ₃ -A
E - <u>Chlorella ellipsoidea</u>	OP-U	NH ₃	Tot N
	OP-A ²	P·NO ₃	P·NO ₃
		OP-A	NH ₃ ²
			NH ₃

*By use of a "stepwise regression procedure" variables are added to improve the fit of the regression line to the data. An improvement in fit was considered significant when there was an increase of 5 percent or more in R², the proportion of the total sum of squares attributable to regression.

of the four species was again generally similar but if a difference was apparent, Pandorina and Scenedesmus, and the latter somewhat more consistently, grew to a greater degree than the other two. On the Haw River filtrates Pandorina perhaps grew a little more in the assays than Scenedesmus. But the growth responses of these two were better than either the Chlamydomonas or Chlorella.

The relative growth response of the algae used in these assays, three flagellates, Euglena, Chlamydomonas, Pandorina and two green algae, Scenedesmus and Chlorella appeared to follow a pattern. To ascertain whether such a pattern did exist, a summary of their growth responses, measured as total cell mass formed in 10 days at 22° C, has been assembled in Table 3. For each of the characteristic water sampling points whether it was an oxidation pond or a river station, the growth response at these points has been averaged, that is, all samples for the particular species for each of the bioassays. Growth differences between the algal species in similar filtrates as well as differences between growth in the oxidation pond filtrates and the river station samples are evident.

Analysis of Variance

To establish the significance of these apparent differences, an analysis of variance was carried out. The species differences and species response at each sampling point (streams and ponds) were highly significant at .0005. Less than five chances in 10,000 that these differences would have occurred by random chance had the differences not been real. However, all species responded similarly to similar changes in qualitative factors.

Table 3

Average Growth of Algal Species in Filtrates
Derived from Oxidation Pond and River Waters

<u>Source of Filtrates</u>	<u>Biomass; mg, 10 days, 22° C</u>				
<u>Oxidation Ponds</u>	<u>Euglena</u>	<u>Chlamydomonas</u>	<u>Pandorina</u>	<u>Scenedesmus</u>	<u>Chlorella</u>
Plant effluent	4.2 (.75)	5.8 (1.15)	4.3 (1.09)	7.0 (1.13)	4.9 (.67)
2-days detention	5.1 (.58)	6.4 (1.20)	4.2 (.85)	7.3 (1.01)	5.2 (.48)
4-days detention	4.5 (.60)	7.5 (.74)	4.0 (1.08)	7.3 (1.20)	5.2 (1.06)
5-days detention	4.8 (.84)	6.5 (1.48)	4.1 (1.20)	6.7 (1.34)	4.3 (.75)
New Hope Stations					
1	-	6.1 (3.29)	8.9 (5.69)	10.6 (5.30)	3.2 (1.90)
5	-	12.3 (4.21)	18.7 (9.39)	20.1 (7.40)	10.0 (5.11)
6	-	13.8 (5.27)	12.3 (9.48)	22.0 (9.25)	9.8 (5.03)
7	-	7.0 (5.13)	10.6 (6.25)	15.1 (4.70)	5.9 (3.73)
10	-	4.5 (2.50)	5.4 (3.24)	8.2 (3.31)	3.0 (1.51)
Haw Stations					
1	-	5.1 (3.08)	8.6 (5.39)	9.3 (4.36)	3.3 (2.30)
2	-	4.3 (1.85)	9.7 (5.34)	8.8 (4.15)	3.7 (2.20)
3	-	5.6 (3.21)	8.1 (5.36)	7.1 (4.41)	3.4 (1.17)
4	-	4.1 (2.26)	8.7 (5.41)	7.0 (4.34)	2.8 (1.58)
5	-	3.2 (1.82)	7.7 (6.3)	6.5 (4.77)	2.8 (1.42)

Number in parenthesis is a \pm value representing the 90% confidence limit.

In the oxidation pond series Scenedesmus clearly outgrew the other four species under the assay conditions. The growth of Chlamydomonas, in turn, was significantly greater than the remaining three species which statistically had about the same growth response. The samples from the New Hope Stations produced a difference in growth response for each species which in nearly every instance was statistically significant and confirmed for each station. The Haw River samples, in contrast, emphasized primarily the greater growth response of Scenedesmus and Pandorina, also seen in the New Hope samples, as compared to Chlamydomonas and Chlorella. The growth of Chlamydomonas was always slightly greater than Chlorella in every instance but in some cases was only marginally significant. Although Pandorina and Scenedesmus produced significantly greater cell mass in all river samples as compared to pond samples, Chlamydomonas and Chlorella again were only marginally significant in the growth response in the filtrates from these two sources.

If the pond and river stations can be so clearly characterized by the algal growth response, then this response must relate to the available nutrients. As in the case of the algal cell mass formed, the concentration of $\text{NH}_3\text{-N}$, $\text{NO}_3\text{-N}$ and $\text{PO}_4\text{-P}$ has been averaged for each of the pond and river station filtrates which were assayed and assembled in Table 4. Levels of significance show that the pond series were characteristically very rich in $\text{NH}_3\text{-N}$ and $\text{PO}_4\text{-P}$ and significantly greater in $\text{NO}_3\text{-N}$ than the Haw samples. Although the New Hope filtrates had very much less $\text{NH}_3\text{-N}$ than the ponds, at stations 5, 6, and 7 they were still significantly richer in this material than the Haw samples. The concentrations of $\text{PO}_4\text{-P}$ showed a similar relationship.

Table 4

Average Concentration of $\text{NH}_3\text{-N}$, $\text{NO}_3\text{-N}$ and $\text{PO}_4\text{-P}$
in Filtrates Used for Algal Assays Summarized in Table 3

<u>Source</u>	<u>$\text{NH}_3\text{-N}$</u>	<u>$\text{NO}_3\text{-N}$</u>	<u>$\text{PO}_4\text{-P}$</u>
Oxidation Ponds			
Plant Effluent	15.94 (1.56)	3.67 (1.03)	9.39 (1.84)
2-days detention	17.19 (1.30)	1.91 (.74)	9.69 (1.50)
4-days detention	16.78 (1.26)	1.84 (.76)	10.73 (1.59)
5-days detention	17.05 (1.42)	1.77 (.78)	10.93 (1.65)
New Hope Stations			
1	.76 (.73)	1.26 (.37)	.81 (.40)
5	4.87 (1.84)	1.45 (.53)	2.97 (1.17)
6	8.39 (2.59)	.34 (.17)	3.89 (1.02)
7	2.02 (1.43)	1.68 (.36)	2.37 (1.09)
10	.32 (.09)	1.09 (.36)	1.30 (.78)
Haw Stations			
1	1.04 (.74)	.79 (.43)	1.35 (1.03)
2	.58 (.47)	.90 (.39)	1.38 (1.00)
3	.57 (.39)	.80 (.35)	1.33 (.97)
4	.34 (.37)	.55 (.36)	1.06 (.79)
5	.30 (.26)	.78 (.44)	1.09 (.70)

Number in parenthesis is a \pm value representing the 90% confidence limit

From the nature of the growth response on the river stations as compared to the oxidation ponds and from the information developed by the multiple regression analysis of the relationship of nutrients to growth, it would appear in this particular instance that the nitrogen compounds at the river stations were more significant in developing a growth response of the algae than the available phosphorus. This relationship would not be unjustified in light of the relatively small amount of phosphorus as compared to nitrogen that is needed for algal growth and the very minimal quantity of phosphorus that must be present if it is to be limiting. Thus, a growth limiting nutrient, the material which is required in least amount, may be of less significance in establishing a growth response than substances whose presence enhances growth in some proportional manner. In the quantities found in the filtrates prepared from the oxidation pond samples the phosphorus concentrations may actually be inhibitory.

Quadratic Analysis of Covariance of $\text{NH}_3\text{-N}$, $\text{NO}_3\text{-N}$ and $\text{PO}_4\text{-P}$

If the average growth for each of the four species, that were used in the pond and river assays, is related to the average concentration of $\text{NH}_3\text{-N}$, $\text{NO}_3\text{-N}$ and $\text{PO}_4\text{-P}$ in the filtrates in which the algae were grown, a distribution of points is found which in each case appears to generate a curve parabolic in form. These relationships are shown in Figures 22-27. In each instance, there appears to be a concentration above which the curve describing the relationship of biomass formed and nutrient concentration flattens and then decreases. The point of change in slope is approximately at 5 mg/l for $\text{NH}_3\text{-N}$, 2 mg/l for $\text{NO}_3\text{-N}$ and 4 mg/l for $\text{PO}_4\text{-P}$ under the conditions of the assay.

Since the three chemical species are interacting, being simultaneously present in all filtrates, the relative significance of each cannot be precisely described from these figures.

Analysis of these three interacting algal nutrient factors and the growth of the four species tested was made by a quadratic analysis of covariance. These results are summarized in Table 5. The conclusions that may be drawn from this analysis are as follows:

1. $\text{NO}_3\text{-N}$ is nearly twice the growth factor than $\text{NH}_3\text{-N}$ for Chlamydomonas and Chlorella and about four times for Scenedesmus and Pandorina.
2. In the presence of $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$, $\text{PO}_4\text{-P}$ appears to have a negligible growth effect except for Scenedesmus.

DISCUSSION

The investigations reported in this study establish the relative significance of nitrogen and phosphorus species as algal nutrients, with particular reference to the growth when these chemicals are present in relatively high concentration. It is not the description of the response of algal growth to quantities of nitrogen and phosphorus which may be barely detectable or present only in trace quantities. It is the response when these materials occur in concentrations that may normally be found in the discharges from biological waste treatment plants and when partially diluted in receiving streams.

Table 5

Quadratic Analysis of Covariance of $\text{NH}_3\text{-N}$, $\text{NO}_3\text{-N}$
and $\text{PO}_4\text{-P}$ on Growth of Four Algal Species

<u>Variable</u>	<u>B Values</u>			
	<u>Chlamydomonas</u>	<u>Pandorina</u>	<u>Scenedesmus</u>	<u>Chlorella</u>
Constant	2.459	3.524	3.404	1.198
(NH_3)	1.396	1.030	1.354	0.946
(NO_3)	2.616	5.376	5.553	2.229
(PO_4)	-0.238	0.148	0.513	0.080
(NH_3) ²	-0.046	-0.033	-0.038	-0.027
(NO_3) ²	0.167	-0.072	0.126	0.048
(PO_4) ²	0.011	0.012	0.0009	0.004
(NH_3) (NO_3)	-0.304	-0.301	-0.460	-0.196
(NH_3) (PO_4)	-0.011	-0.048	-0.053	-0.024
(NO_3) (PO_4)	-0.474	-0.697	-0.769	-0.320
(NH_3) (NO_3) (PO_4)	0.035	0.039	0.050	0.022

In the quadratic analysis of covariance the predicted growth = $b_0 + b_1(\text{NH}_3) + b_2(\text{NO}_3) + b_3(\text{PO}_4) + b_5(\text{NH}_3)^2 + b_6(\text{NO}_3)^2 + b_7(\text{PO}_4)^2 + b_8(\text{NH}_3)(\text{NO}_3) + b_9(\text{NH}_3)(\text{PO}_4) + b_{10}(\text{NO}_3)(\text{PO}_4) + b_{11}(\text{NH}_3)(\text{NO}_3)(\text{PO}_4)$

A least squares surface of the equation as given above was fitted to the growth of each species. Positive b values and their relative magnitude indicate positive effect on growth. Negative values of squared terms indicate a reversing curve or decreasing growth. A zero or close to zero value of a squared term indicates the curve is straight. A zero or close to zero value of a first order variable indicates the curve is horizontal, e.g. the growth does not vary with concentration if the concentration of other variables are held constant.

The implication of the quadratic analysis of co-variance as to the relative significance of nitrogen and phosphorus supports previous observations such as those made by Gerloff and Skogg (5). They noted that of the three possibly growth limiting elements, nitrogen, phosphorus, and iron, nitrogen was much more critical than either phosphorus or iron with approximately 5 mg. of nitrogen and 0.08 mg. of phosphorus necessary for each 100 mg. of algae produced. This was for the specific growth requirement of Microcystis aeruginosa. In other instances, nuisance algal blooms have been observed when nitrate nitrogen levels were above 200 ppb and soluble phosphorus levels were greater than 10 ppb as reported by Sylvester for Seattle's Green Lake (6). In his famous report on the lakes of the Madison, Wisconsin area, Sawyer noted that ammonia nitrogen was the most important nitrogen stimulant to explosive algal growth (as compared with nitrate nitrogen) and may be a factor in determining the type of bloom produced (7). However, he also had reported that nuisance conditions could be expected when the concentration of inorganic phosphorus exceeded or equaled 0.01 ppm and the critical level for inorganic nitrogen was 0.30 ppm.

Vollenweider (8), has noted that the bioassay experiments of E. A. Thomas attempted to determine experimentally which of the two factors, nitrogen or phosphorus, is usually the limiting factor in waters receiving or containing these elements in such quantity that appropriate remedial measures are in order. Thomas felt that there was no reason to suppose that any substance other than nitrogen and phosphorus need to be taken into consideration and concluded that where nitrogen becomes a minimum substance in summers it was possible to achieve success in reducing excess algal growth by reducing the nitrogen

income. In similar experimental approaches, Bringmann and Kuhn (also reported by Vollenweider) carried out a "Biomass test" in which three groups of water samples, one enriched with phosphates, one with nitrates and a third as a control were inoculated with a monoculture of Scenedesmus. On comparing the growth after a period of 7 days, they noted that where the basic nutrient level was low and overall production low the limiting factor appeared to be phosphorus; however, where overall production is high (such as in the test waters examined in this investigation) the relationship was reversed and the sources of nitrogen acted as the limiting factor.

It would appear from the references cited, as well as the information developed by this investigation, that under heavy pollution conditions, all other factors being compatible for algal growth, the quantity of nitrogen rather than phosphorus determines the biomass of algae that might be expected to develop. This appears to hold true for several species of algae that are normally associated with pollution conditions and might be expected to respond with excess growth if associated with appropriate nutrient levels of nitrogen compounds.

References

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- (8) Vollenweider, R. A. "Scientific Fundamentals of the Eutrophication of Lakes and Flowing Waters, With Particular Reference to Nitrogen and Phosphorus as Factors in Eutrophication." Organization for Economic Co-operation and Development Directorate for Scientific Affairs, 159 pp. Paris, France, 1968.

APPENDIX

Automated Procedures for Phosphorus and Nitrogen Species

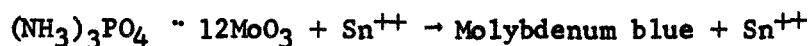
Phosphorus Species

1) Orthophosphate

The sample to be analyzed is introduced into a flow of excess acidic Ammonium Molybdate according to the following reaction



The molybdenum contained in the ammonium phosphmolybdate is reduced by stannous chloride according to the following equation:



The intensity of the blue color is measured colorimetrically at 660 m μ . The system obeys Beer's Law between .01 and 3.0 mg/l. Range expansion and the use of a 5 cm. flow cell allows for greater accuracy in the lower ranges.

2) Polyphosphate

The sample is treated with 2.5 N H₂SO₄ and heated for approximately 30 minutes at 95° C. This hydrolyzes the poly forms giving an end product which is mostly in the PO₄[≡] form. The sample is then treated similarly to the ortho form. The quantity of polyphosphate is determined by difference. Ortho and polyphosphate determinations are done on membrane filtered samples.

3) Organic Phosphorus and Total Phosphorus

The sample is manually digested by autoclaving in a sulfuric acid persulfate digestion mixture. The pH of the

molybdate solution of the ortho procedure is adjusted to correct for the acid added to the sample and analysis for orthophosphate is carried out. The values found for orthophosphate and polyphosphate are subtracted from this total value to obtain the Organic P. This total phosphorus determination is carried out on a nonfiltered sample to include both soluble and insoluble species.

4) All phosphorus forms are reported as P, mg/l

Nitrogen Species

Nitrogen determinations are made using two chemical principles: Oxidized forms can be reduced to the NO_2 specie and measured by diazotization, and reduced forms can be selectively oxidized to form Nitrosophenol compounds.

1) $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$

The automated procedure for doing NO_2 analysis is a modification of the Griess-Lowery diazotization method. Sample is introduced into a stream of acidic Sulfanilamide -N-(1 Naphthyl)-ethylenediamine dihydrochloride. The NO_3 sample is first reduced by hydrazine and CuSO_4 to NO_2 and is then measured as NO_2 . The earlier value (pre-existing NO_2) is subtracted from the reduced (total NO_2) value to determine NO_3 . The color solution is read at 520 m μ .

2) $\text{NH}_3\text{-N}$

The ammonia procedure is essentially an improved hypochlorite-phenol procedure. The sample is allowed to react with excess hypochlorite in a mixture of EDTA, citrate and acetone.

An alkaline phenol reagent is then introduced into the $\text{NH}_3\text{-OCl}$ -mixture forming a quinone compound. This compound in turn reacts with excess phenol in a basic solution to form the blue dye - indophenol. The final solution is measured colorimetrically at 630 m μ .

3) Organic Nitrogen

The raw sample is digested according to the Kjeldahl procedure in a concentrated sulfuric acid - perchloric acid mixture. This digestion utilizes the Technicon Helical Digester which retains the sample in the digestion fluid for approximately ten minutes at 360° C. The sample is neutralized and fed into the NH_3 manifold for color development. Pre-existing NH_3 , found in the earlier procedure, is subtracted from this total NH_3 value to give the Organic N results.

4) Total Nitrogen

Total nitrogen content of the sample is found by adding the total oxidized species to the Kjeldahl species.

Algal Bioassay

In the evaluation of the nutrient quality of surface waters a direct bioassay by growth response of specific species of algae often proves very informative. Although many of the criteria governing growth response of algae under different nutrient conditions still remains to be specifically defined, the quality of the effluent from the oxidation ponds receiving secondary wastewater as well as the streams in the New Hope and Haw River drainages were assayed in the following manner. Each river water

or pond sample was initially processed by filtering through membrane filters of 0.8 micron pore size. This filtrate, free of nearly all particulate matter, was then re-seeded with five different species of algae that have been described as organisms found in association with polluted waters (4). The test species were thus ecologically compatible with the waters that were to be assayed. The five species were as follows:

- A - Euglena rostifera
- B - Chlamydomonas reinhardtii
- C - Pandorina morum
- D - Scenedesmus quadricauda
- E - Chlorella ellipsoidea

Species A, the Euglena, was isolated from massive growths of the organism found in the oxidation ponds described in this report. Species B through E were purchased from the Indiana University algal culture collection and maintained as a single species culture by subculturing on a standard algal media until inoculated into the test filtrates. The test filtrates consisted of the filtered river or pond water, 50 ml volumes in 250 ml flasks. The flasks were either cotton plugged or lightly screw-capped to allow for gas exchange and grown in a day-night cycle of 14 hours light, 10 hours dark at a constant temperature of $22^{\circ} \text{C} \pm 1^{\circ}$. Light intensity was approximately 800 foot candles. Following ten days growth, the total cell growth was filtered onto pre-tared membrane filters dried and weighed to determine total cell production. Chemical determinations of the various nitrogen and phosphorus species

prior to seeding and after the ten day bioassay were made to determine the relative utilization of nutrient elements.

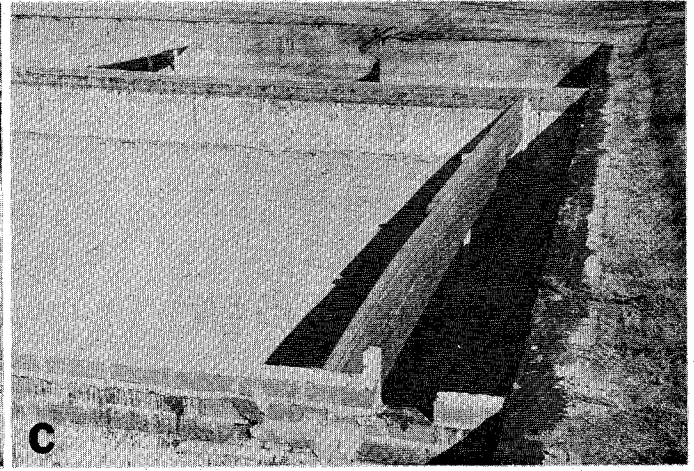
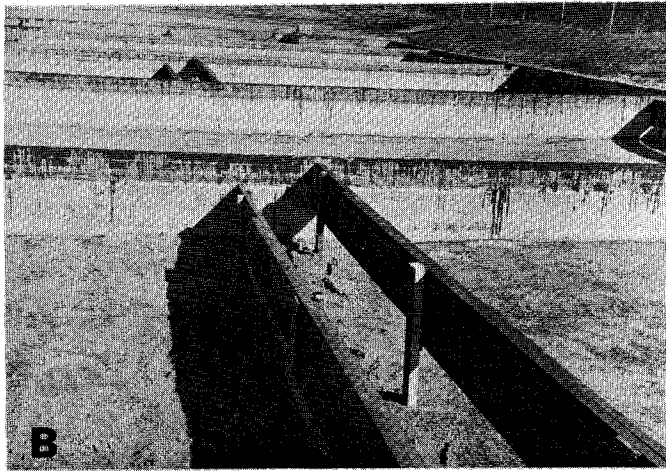
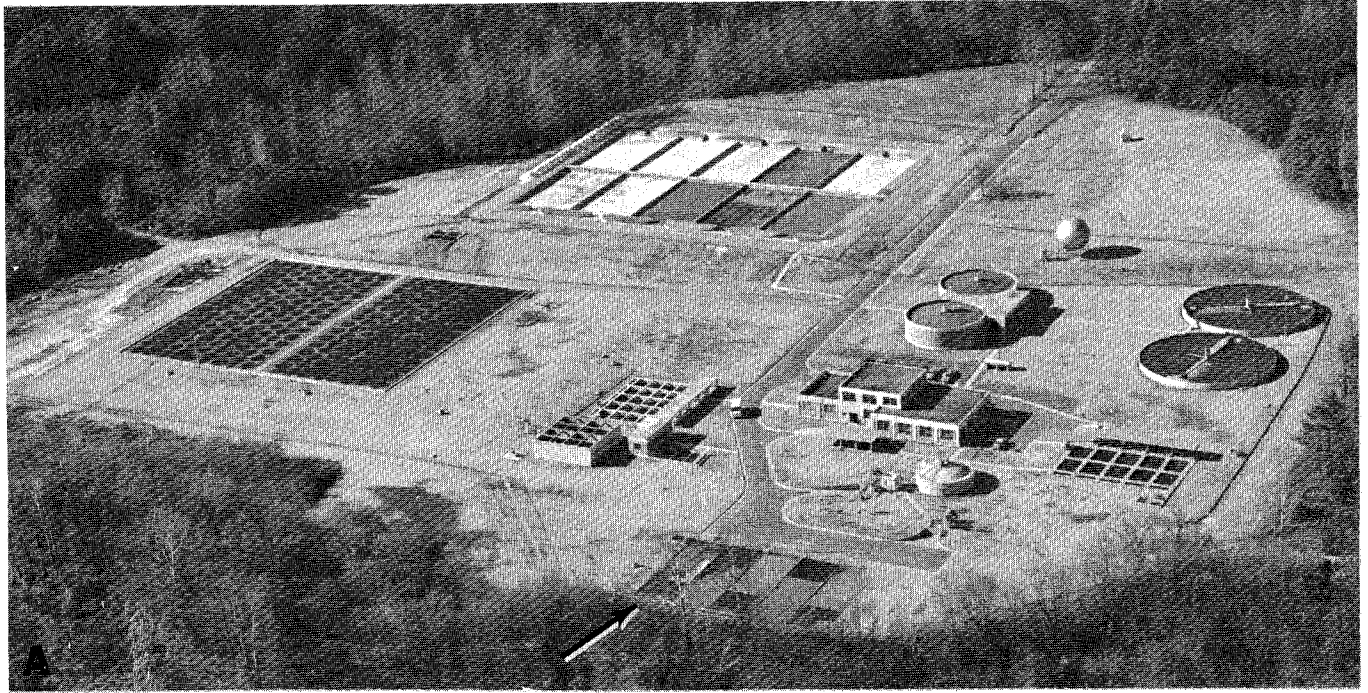


Fig.1. (a) Aerial view of Third Fork Waste Water Treatment Plant, November, 1966. Oxidation ponds in foreground, flow from left to right. (b) Detail of over-under weir at mid-point in pond. (c) Over flow from one pond to next and under-flow weir.

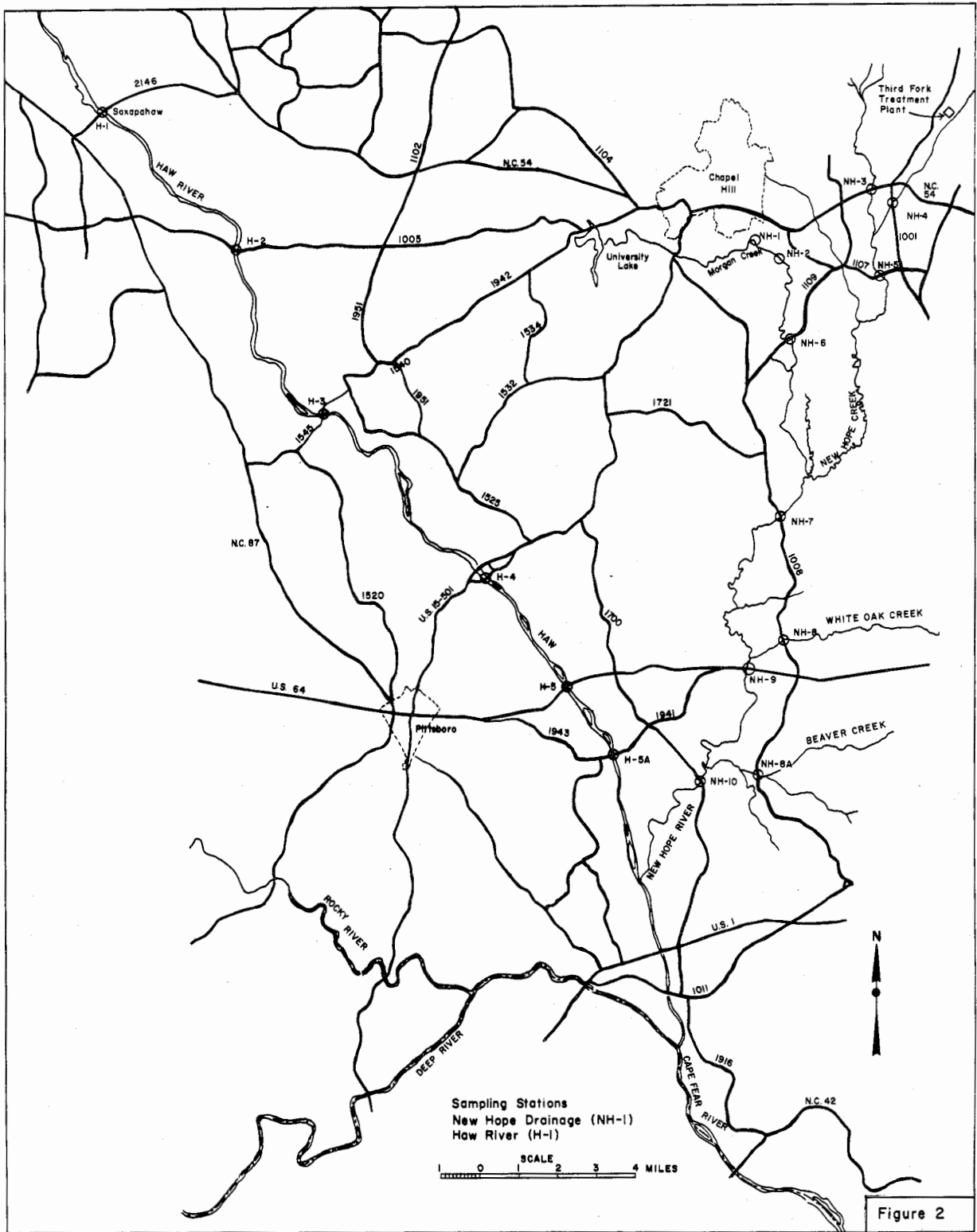


Figure 2

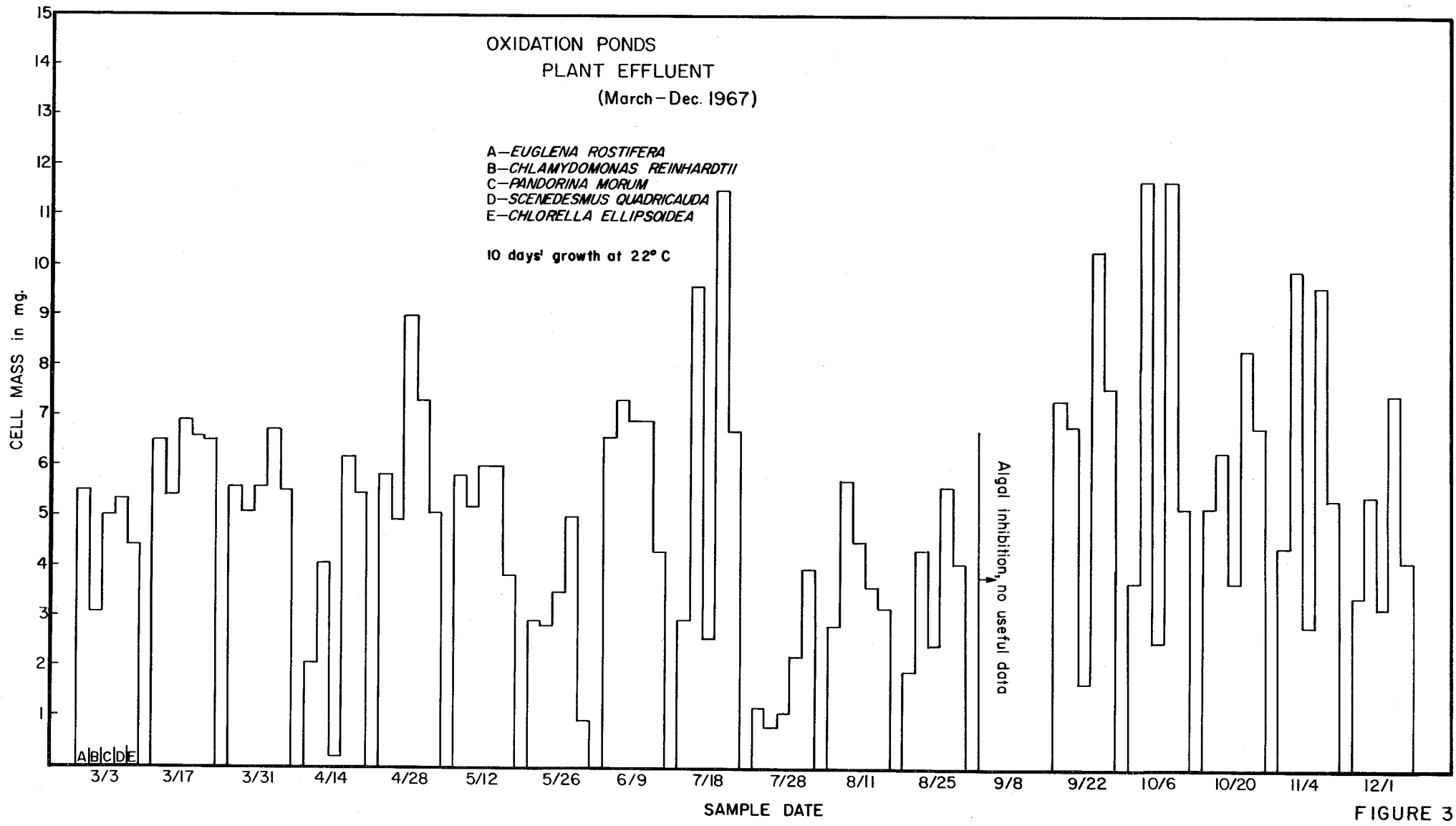


FIGURE 3

OXIDATION PONDS
(2 DAYS DETENTION)
(March-Dec. 1967)

- A-EUGLENA ROSTIFERA
- B-CHLAMYDOMONAS REINHARDTII
- C-PANDORINA MORUM
- D-SCENEDESMUS QUADRICAUDA
- E-CHLORELLA ELLIPSOIDEA

10 days' growth at 22° C

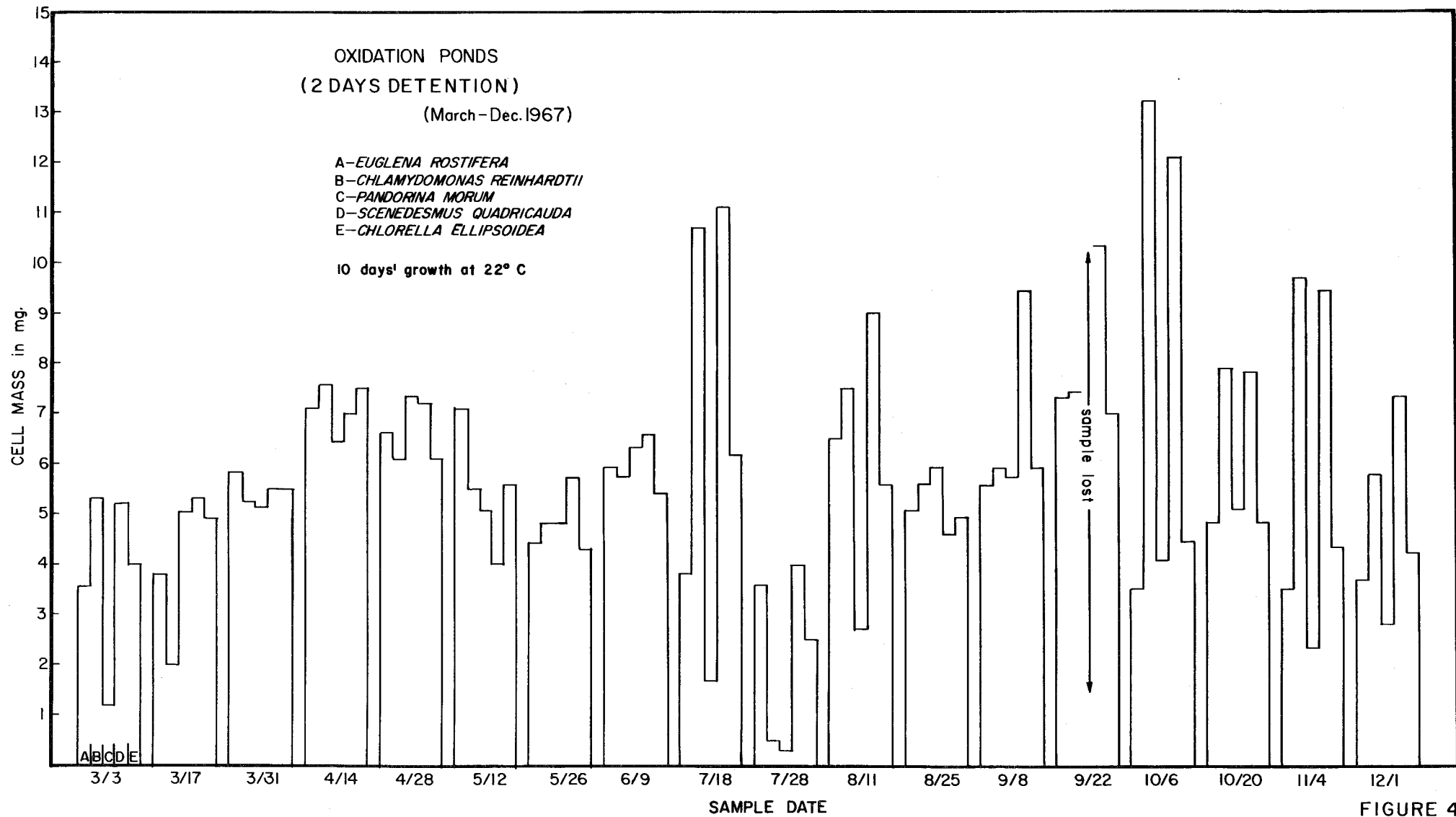


FIGURE 4

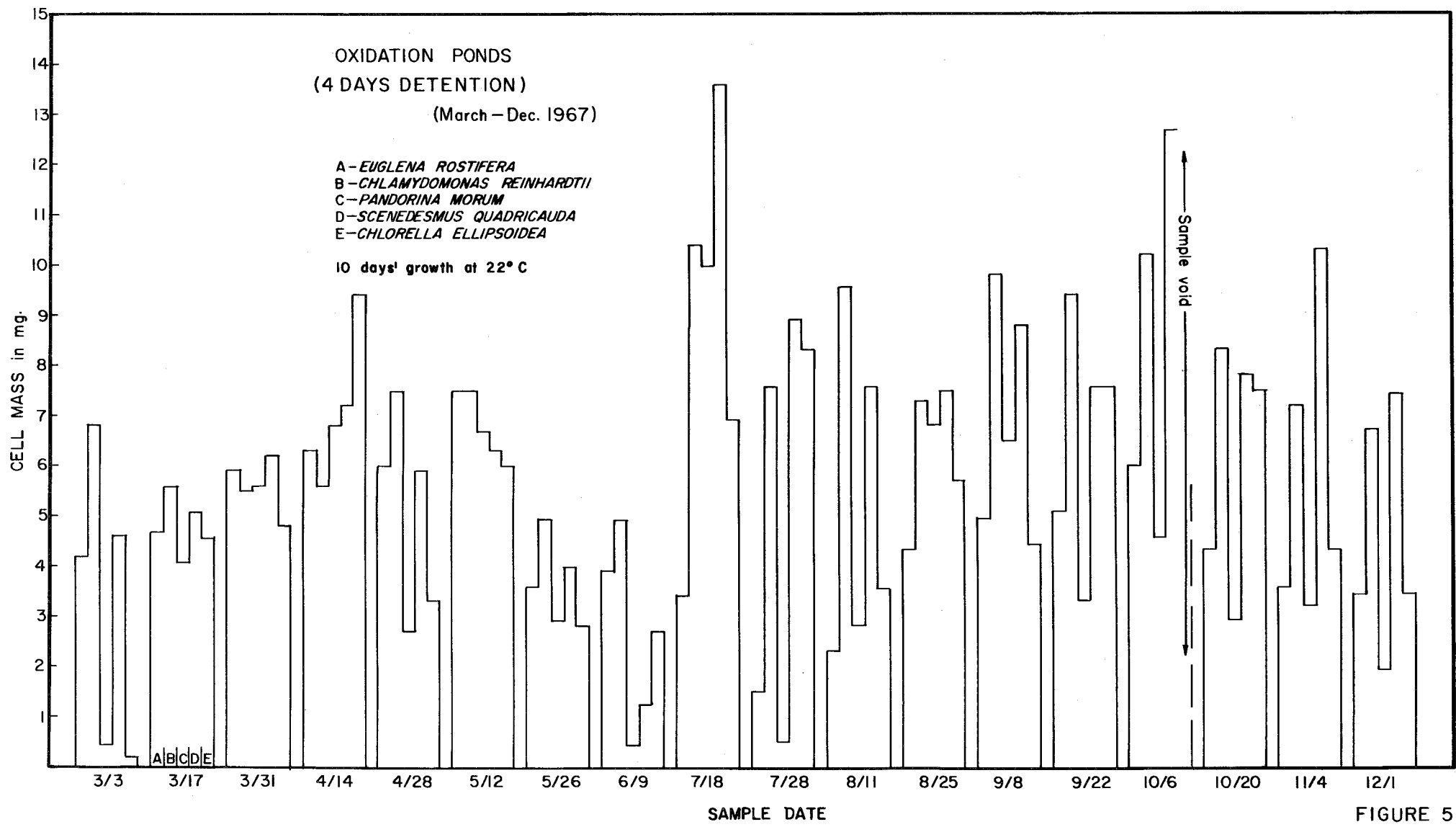
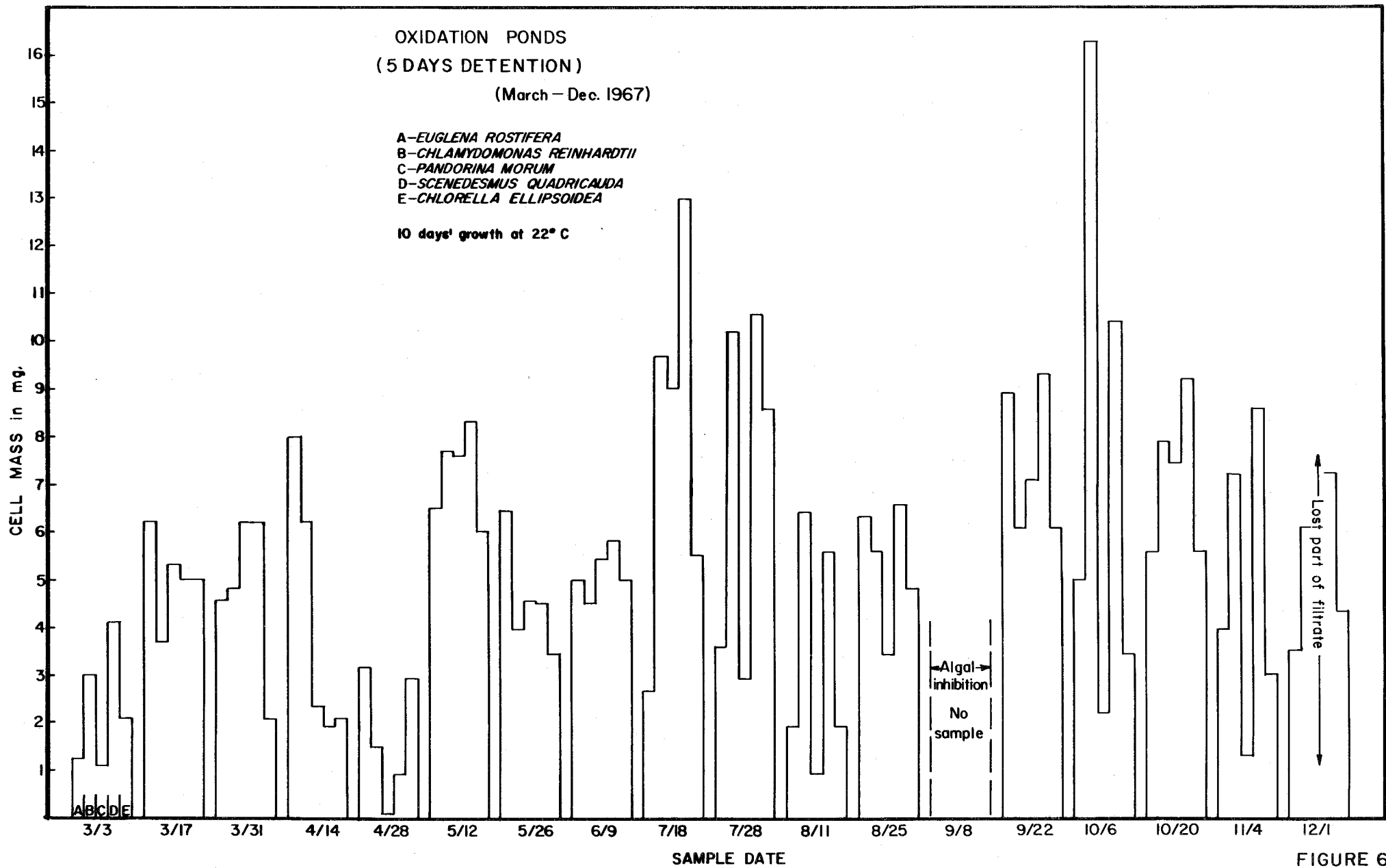


FIGURE 5



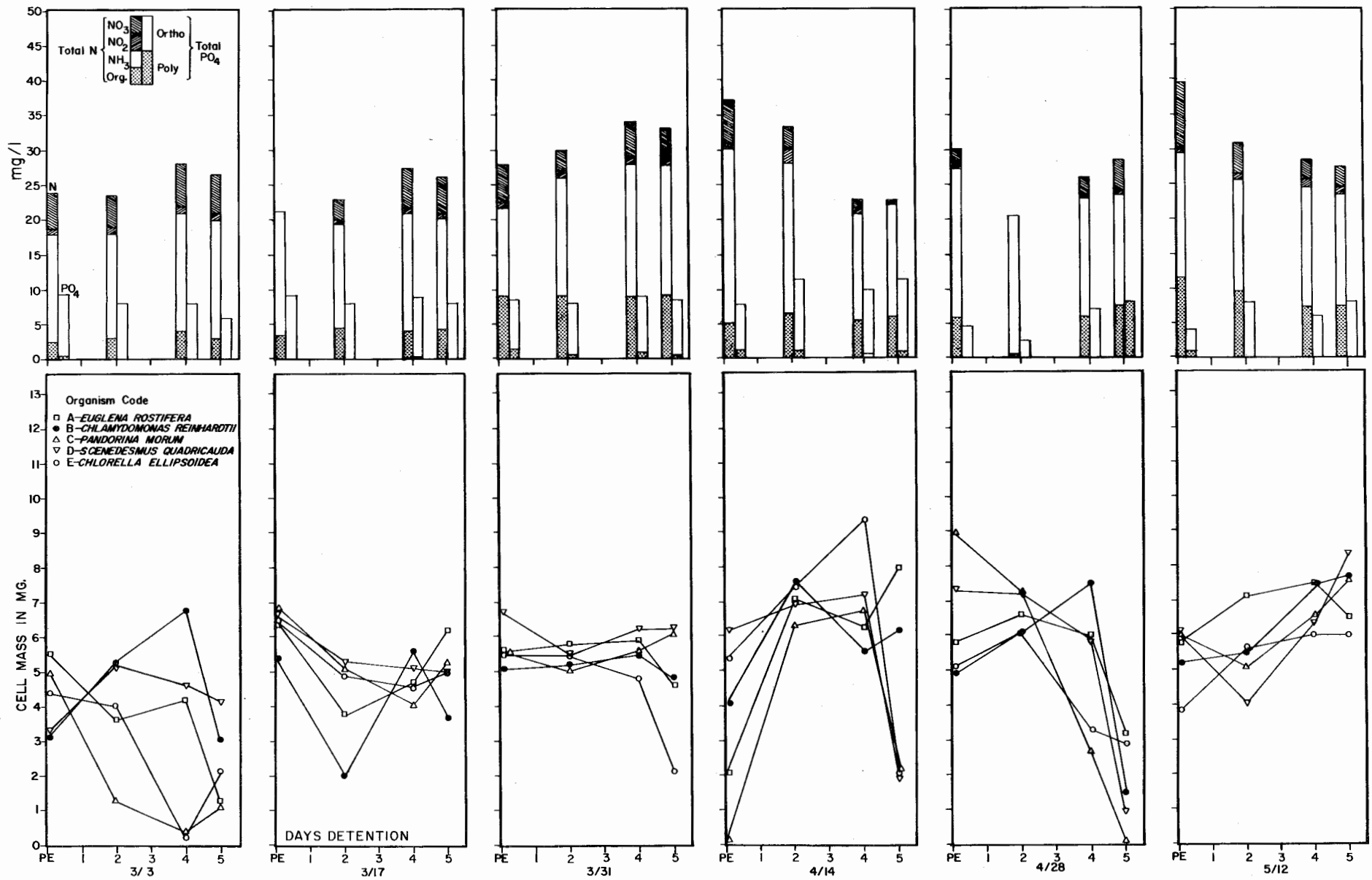


FIGURE 7

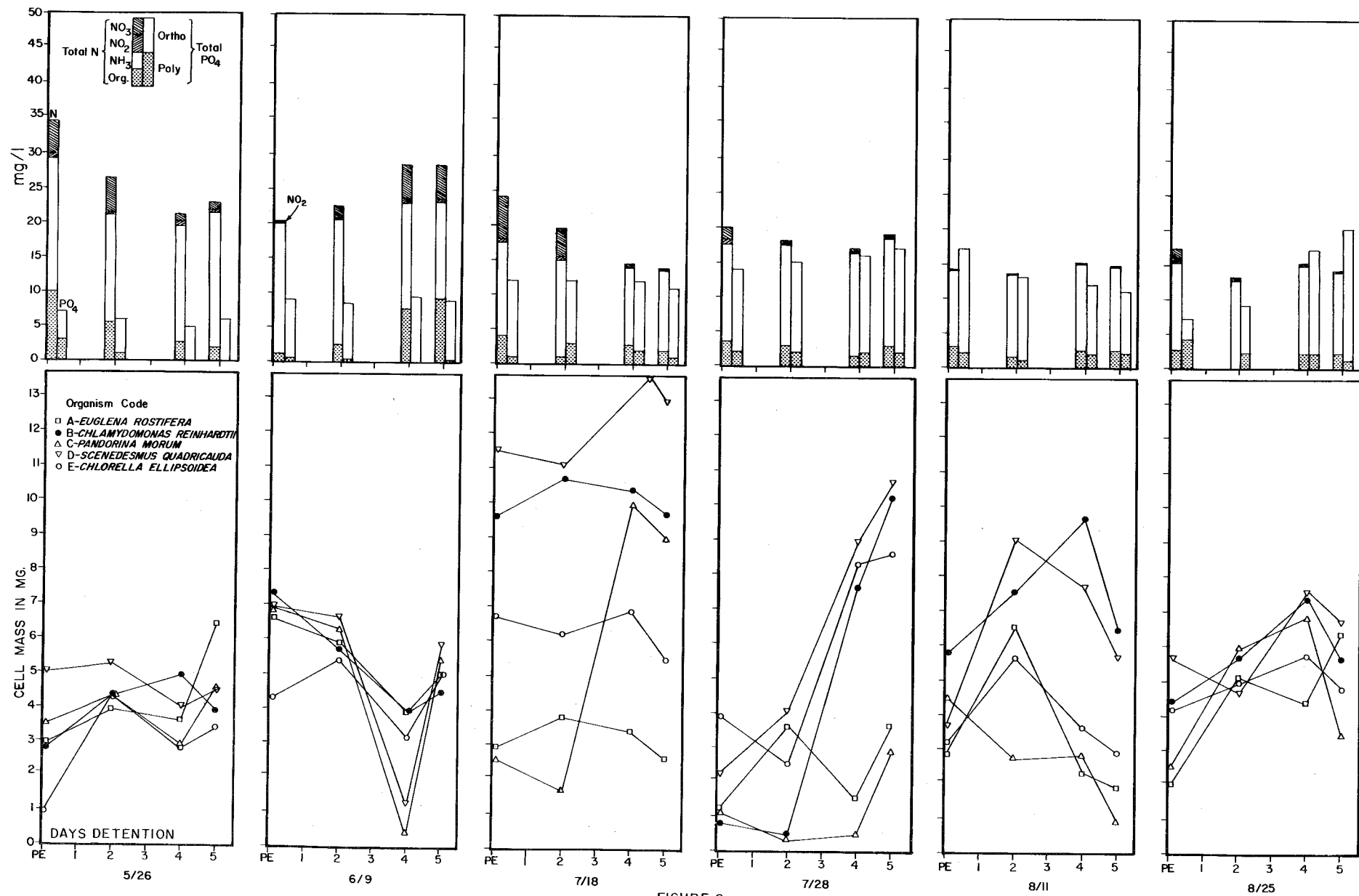


FIGURE 8

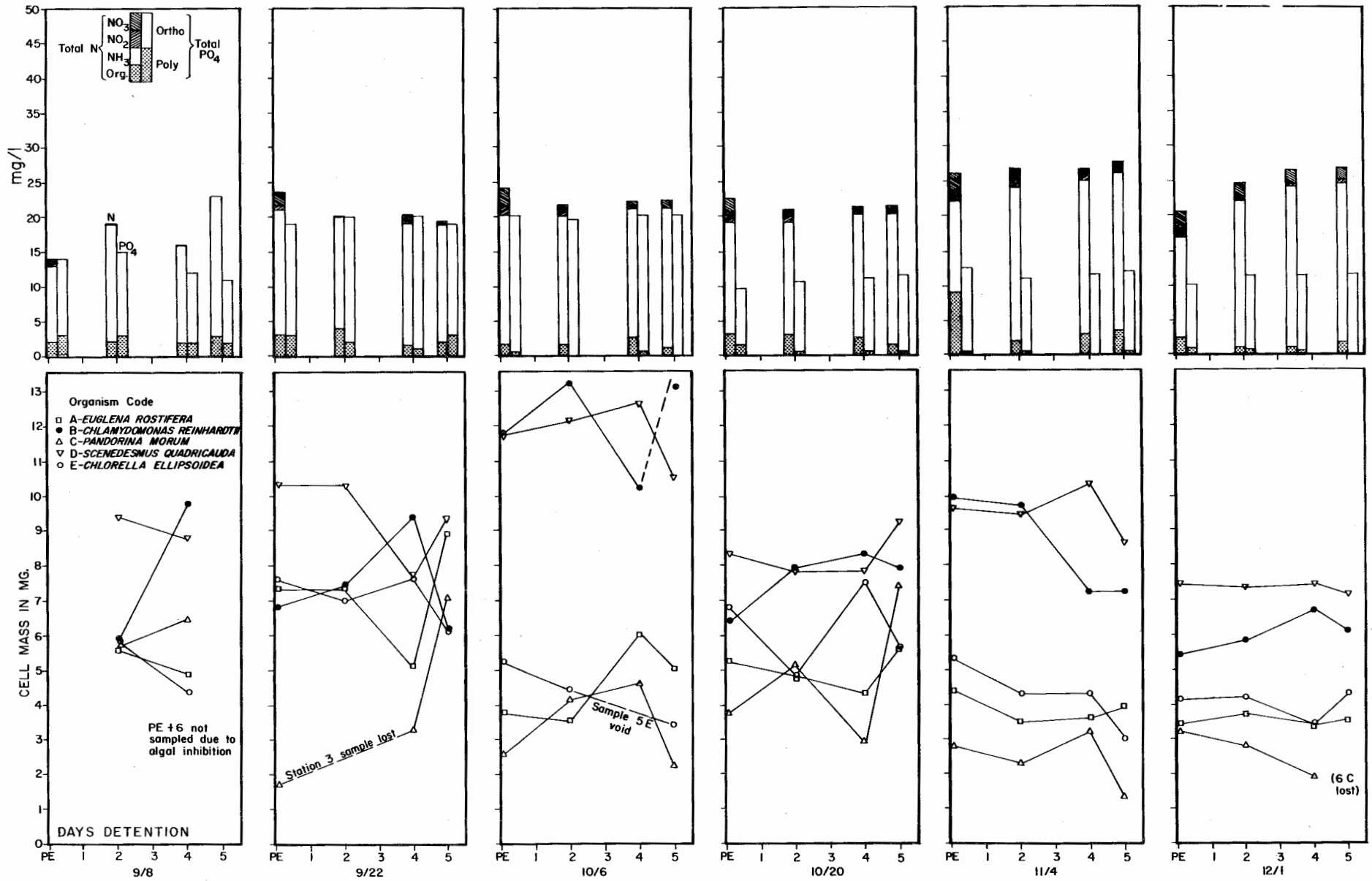


FIGURE 9

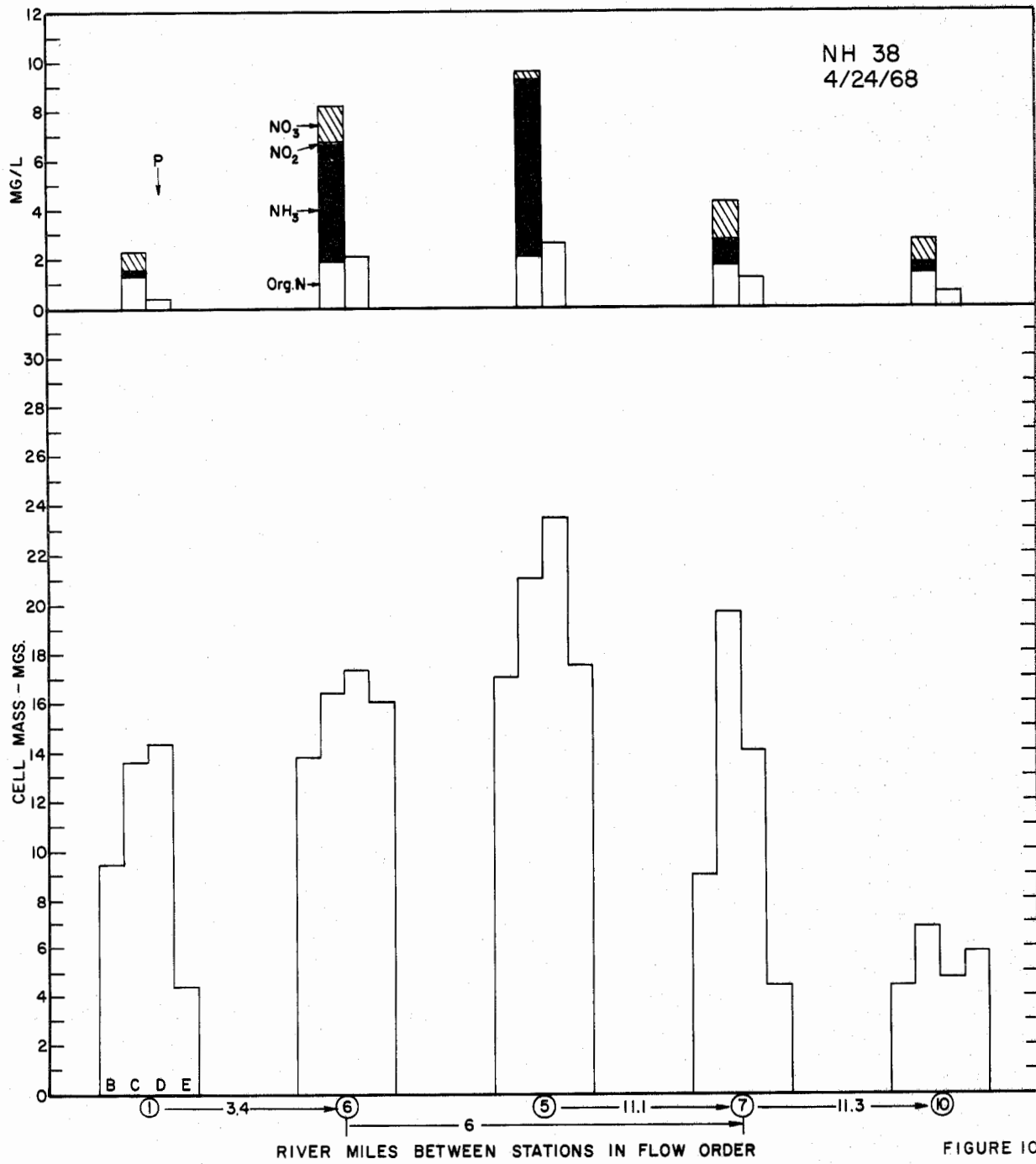


FIGURE 10

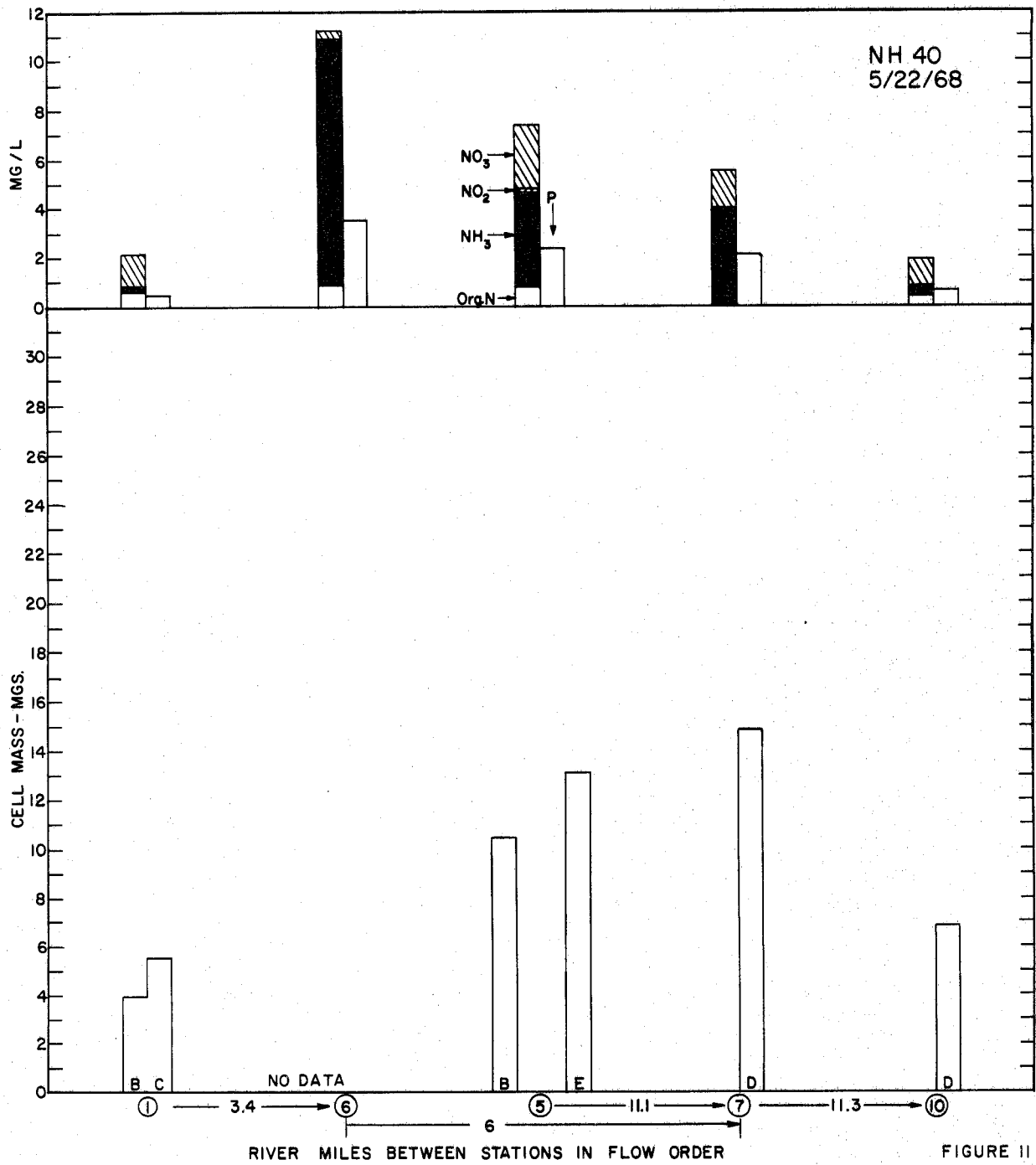


FIGURE II

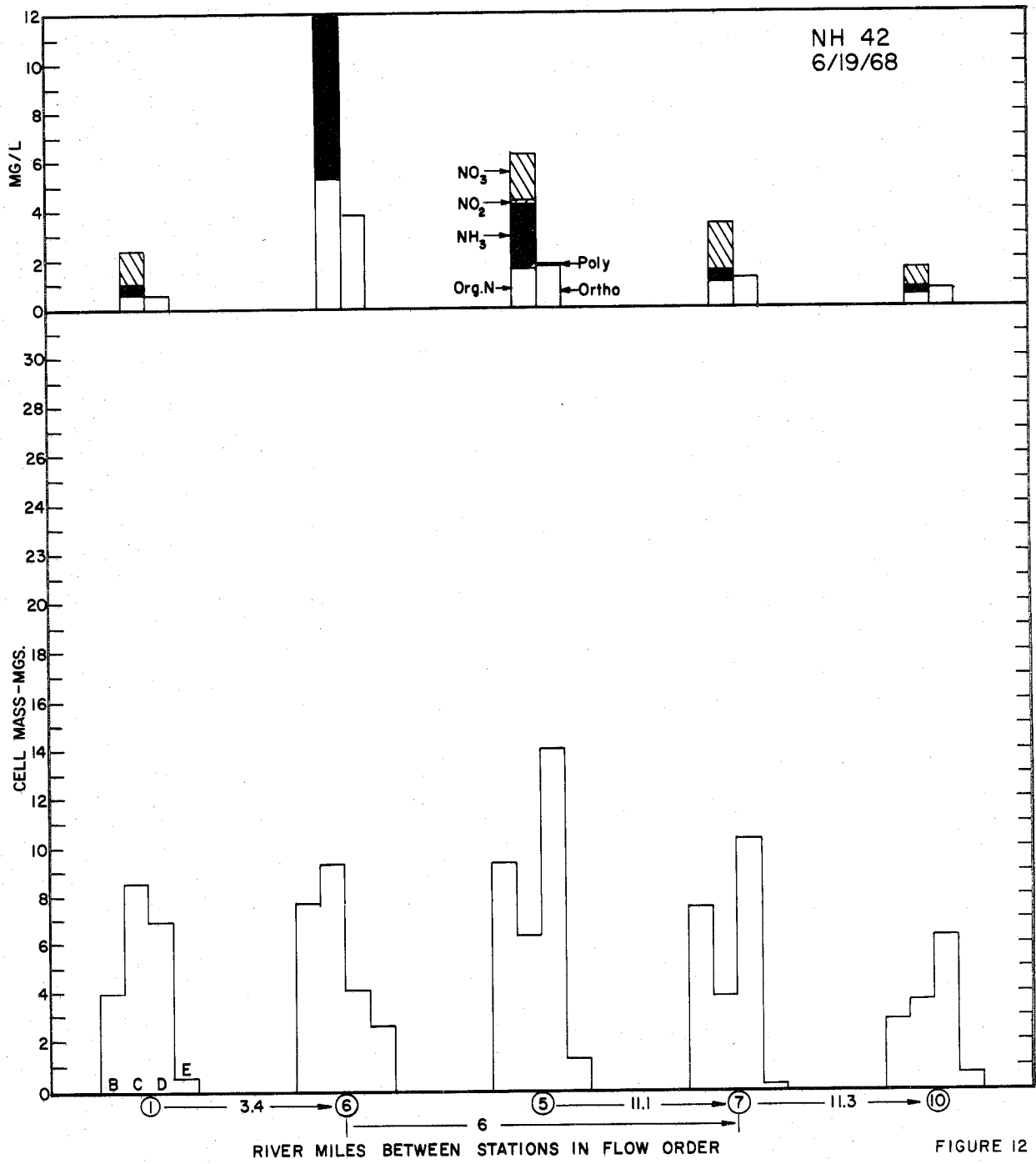


FIGURE 12

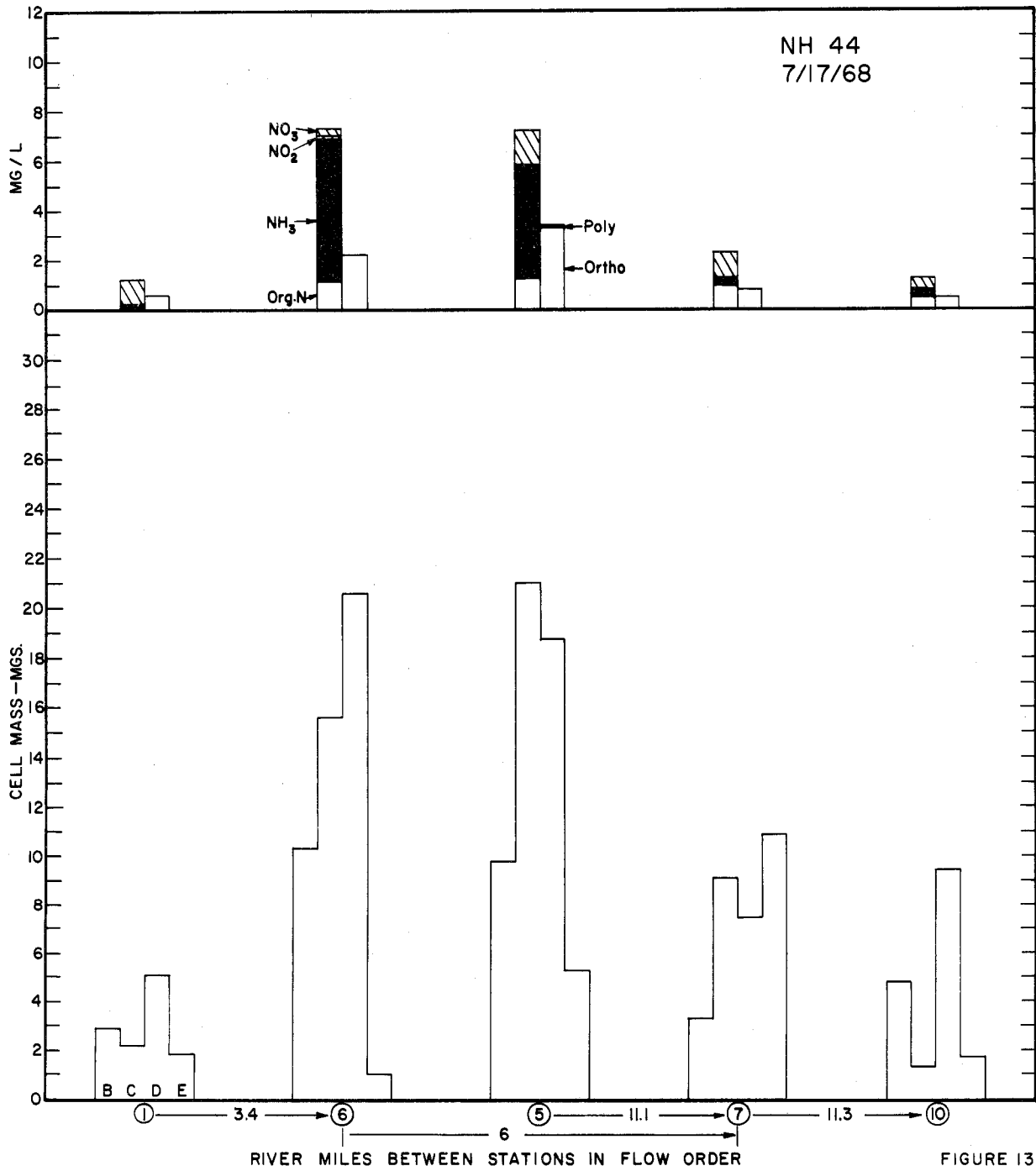


FIGURE 13

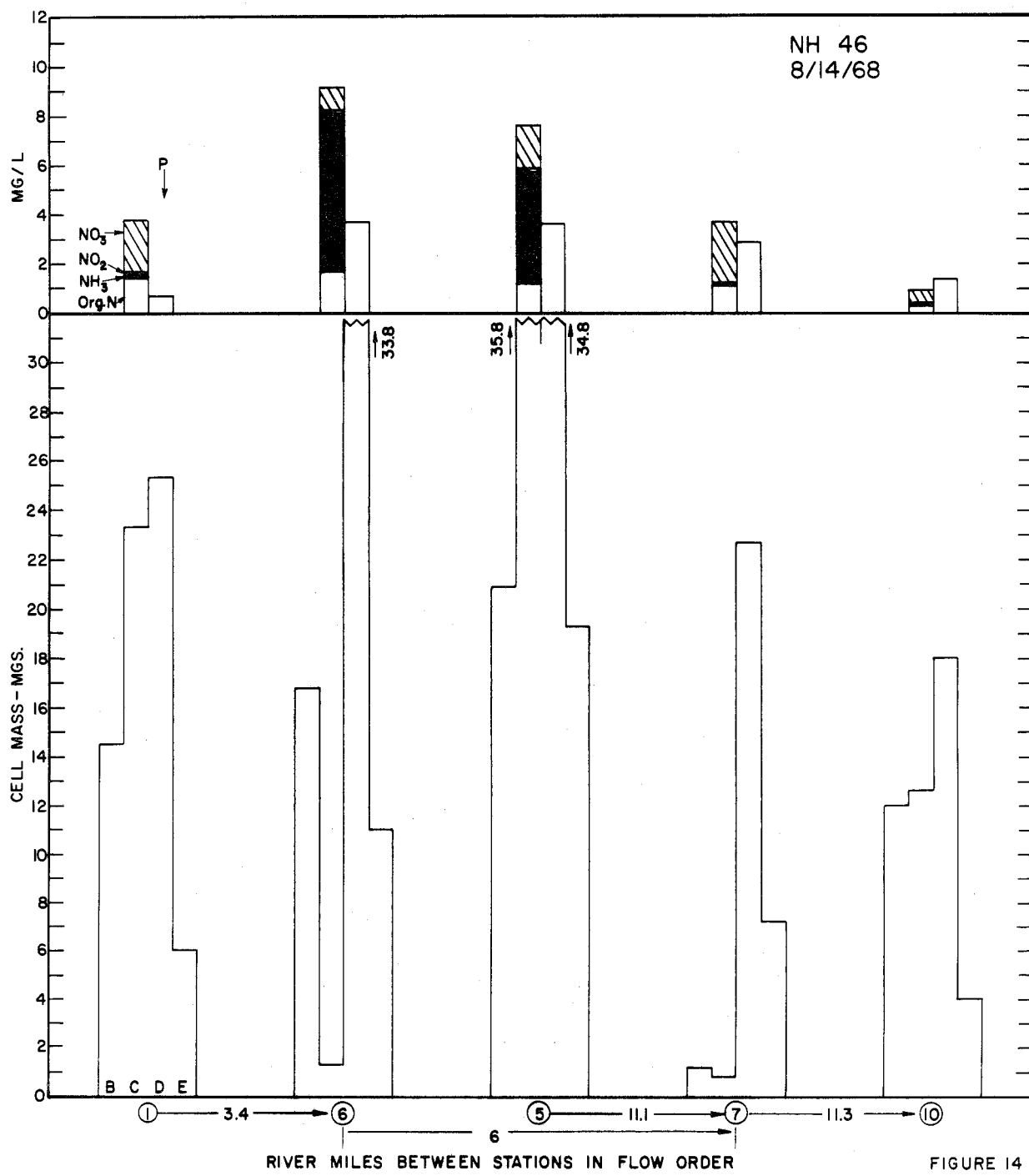
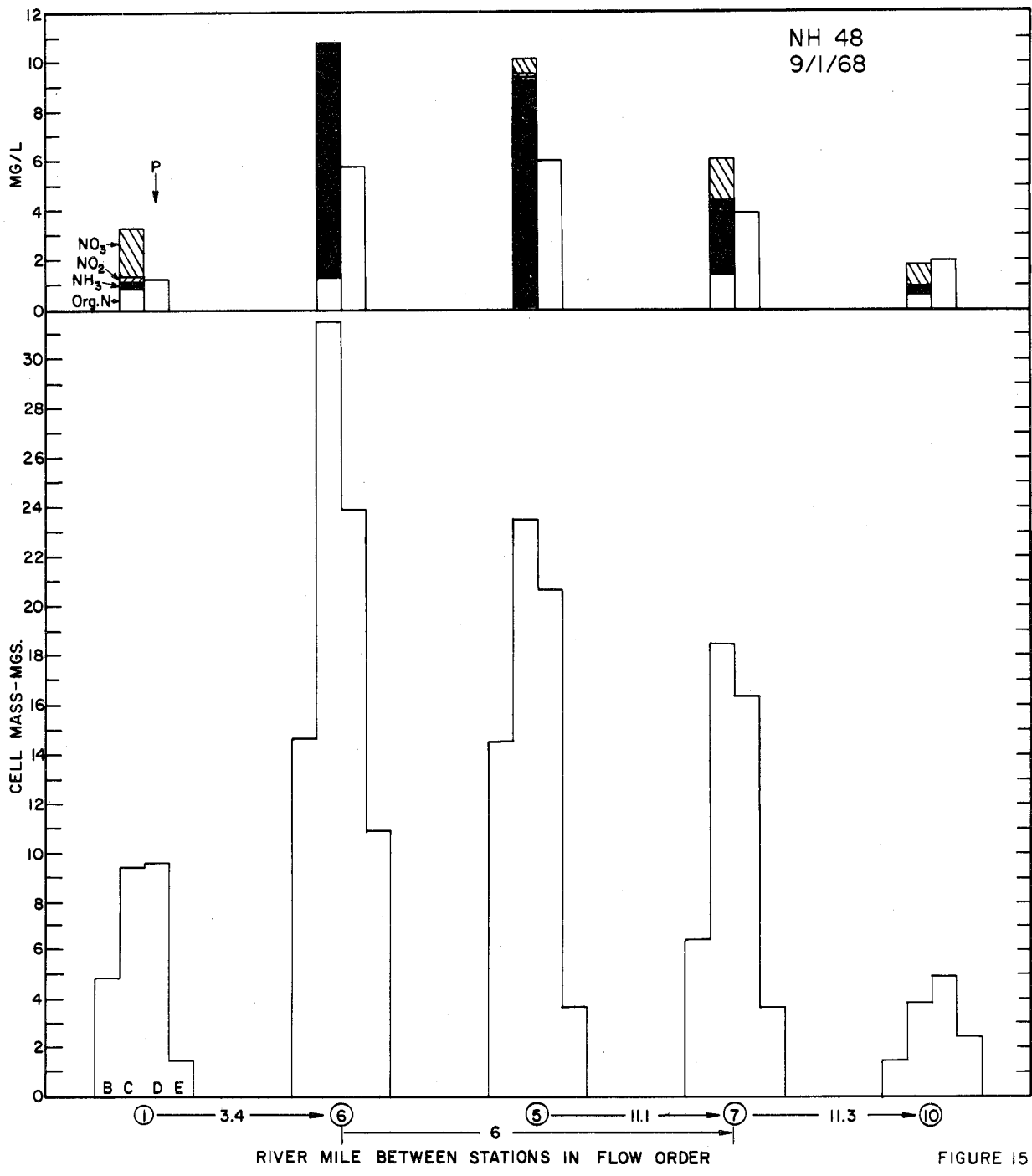


FIGURE 14



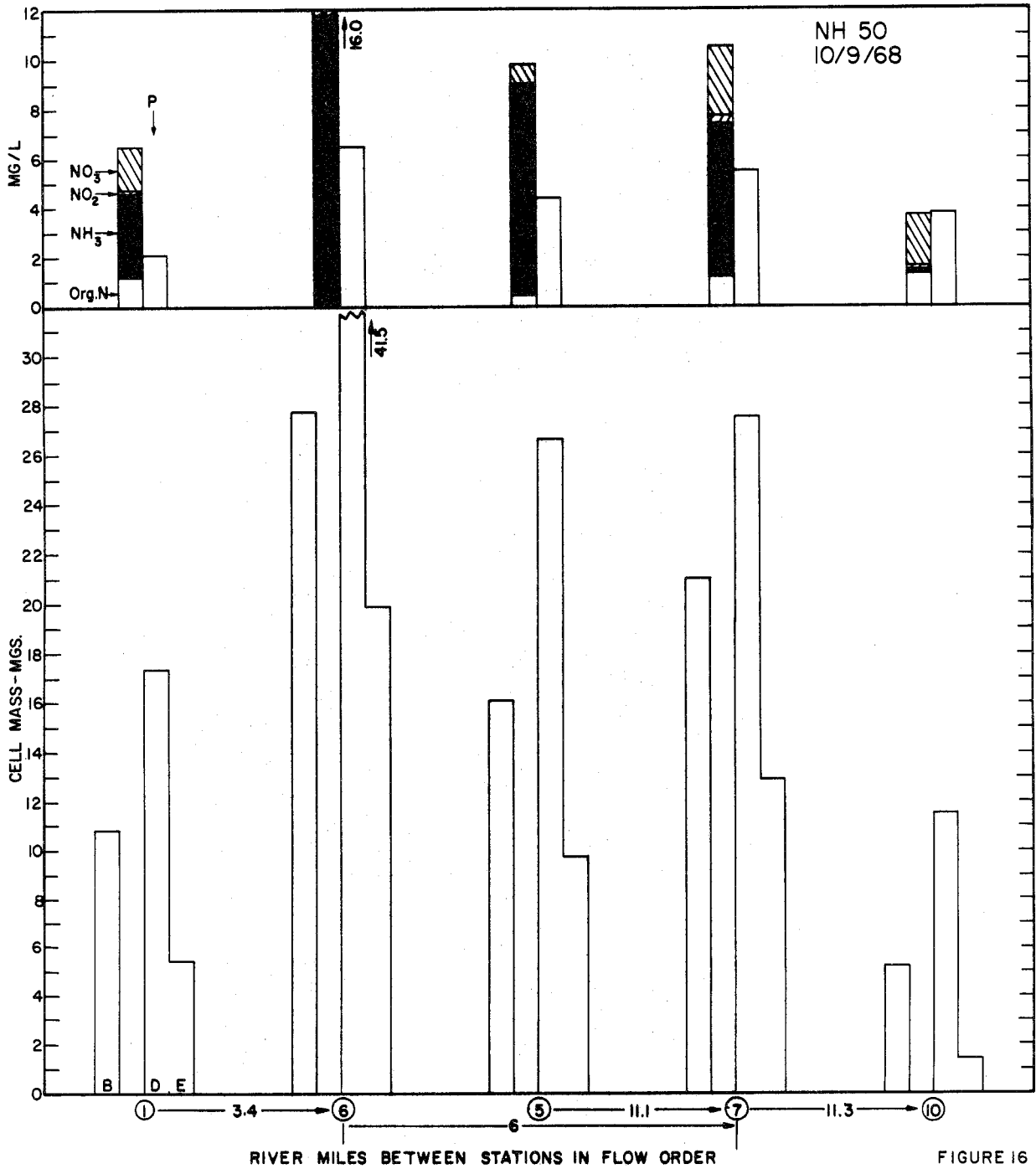


FIGURE 16

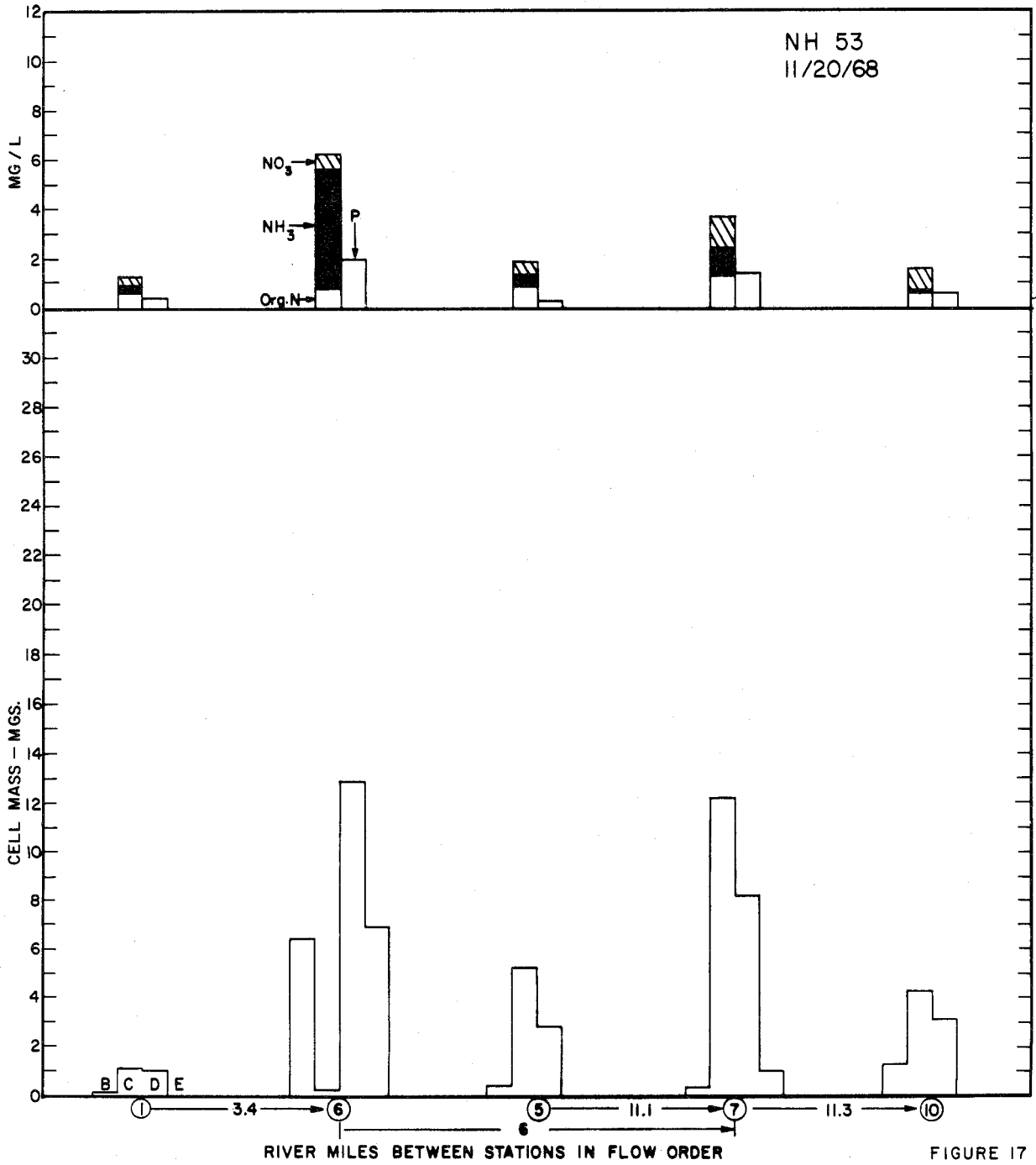


FIGURE 17

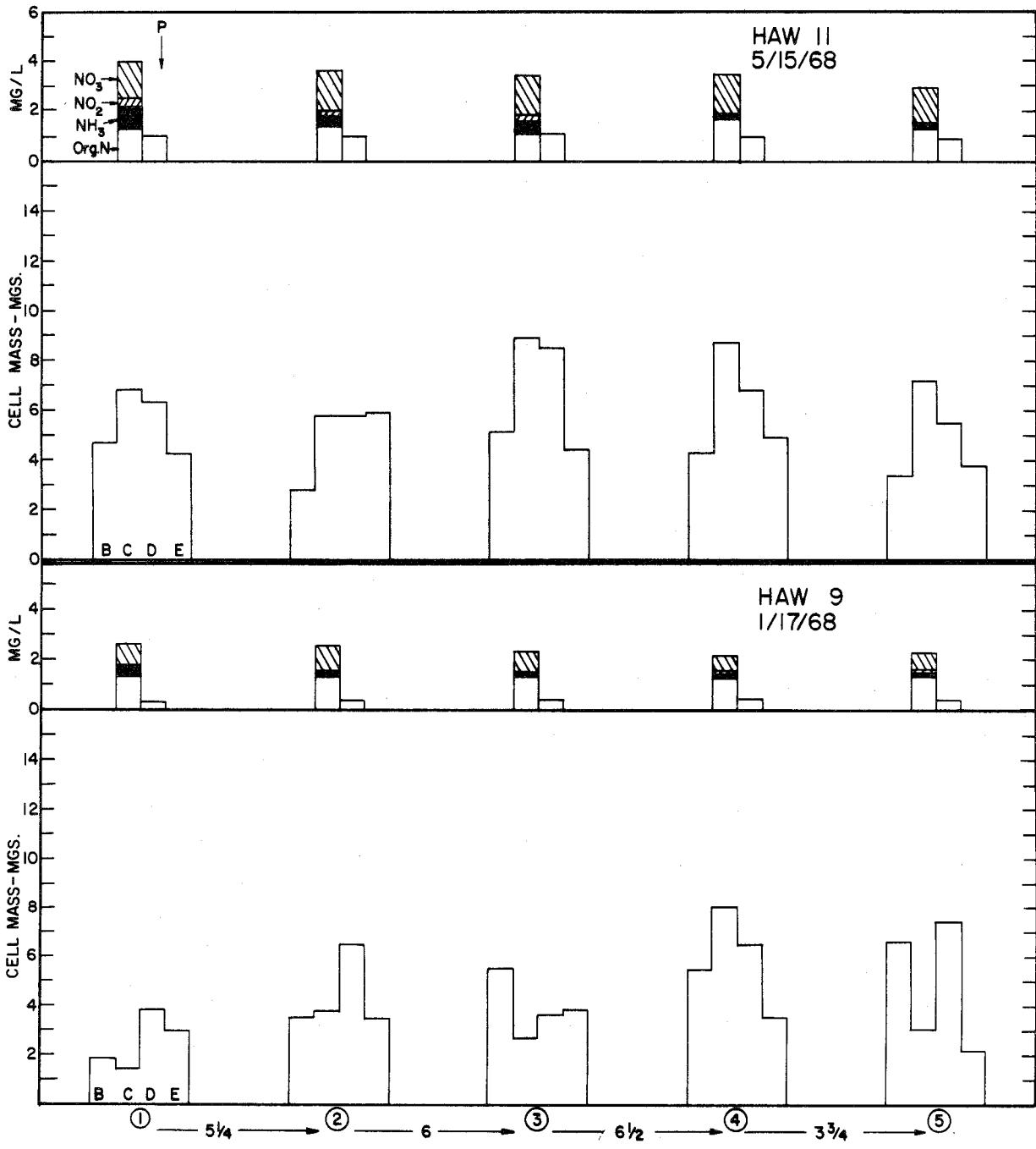


FIGURE 18

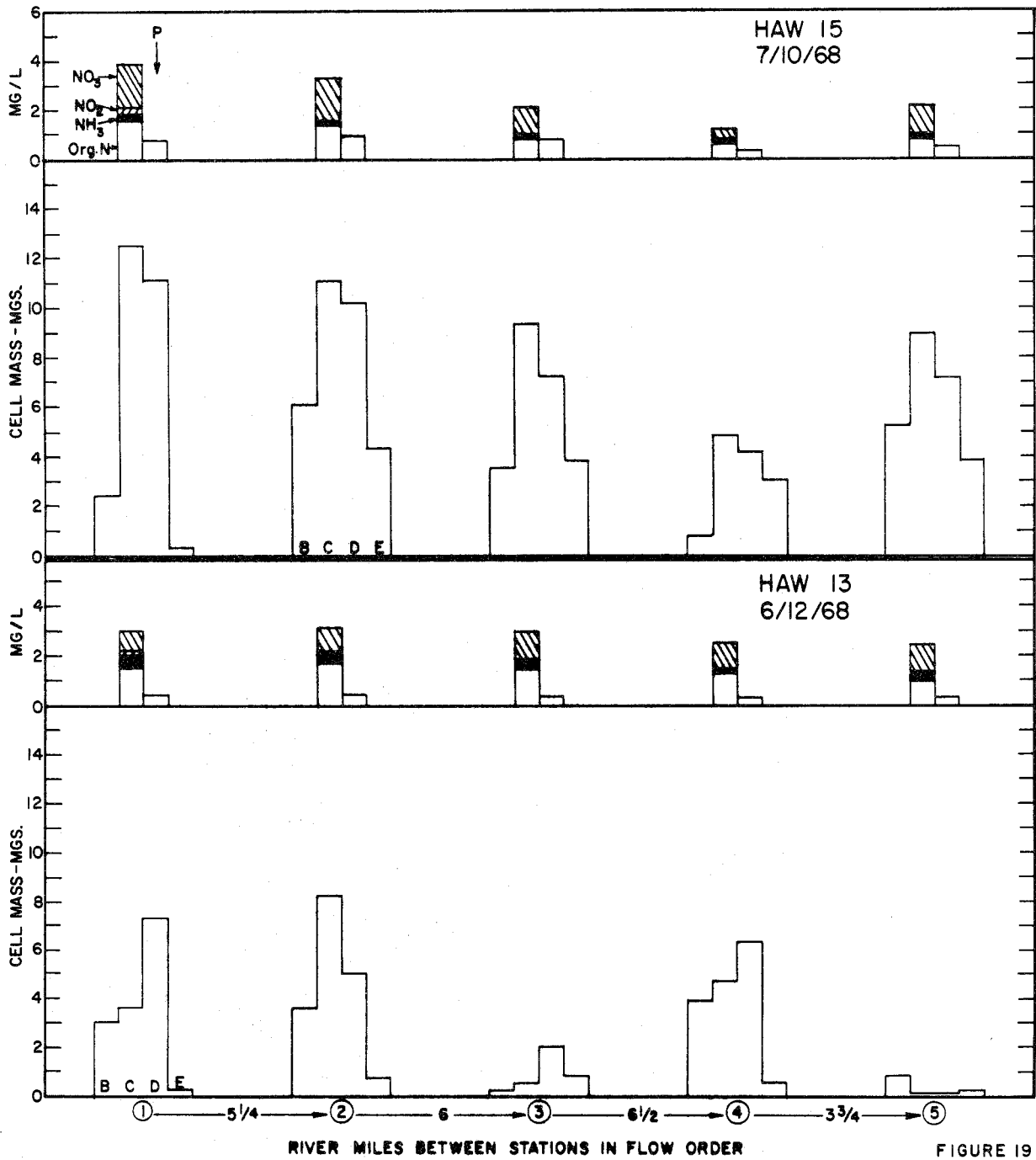
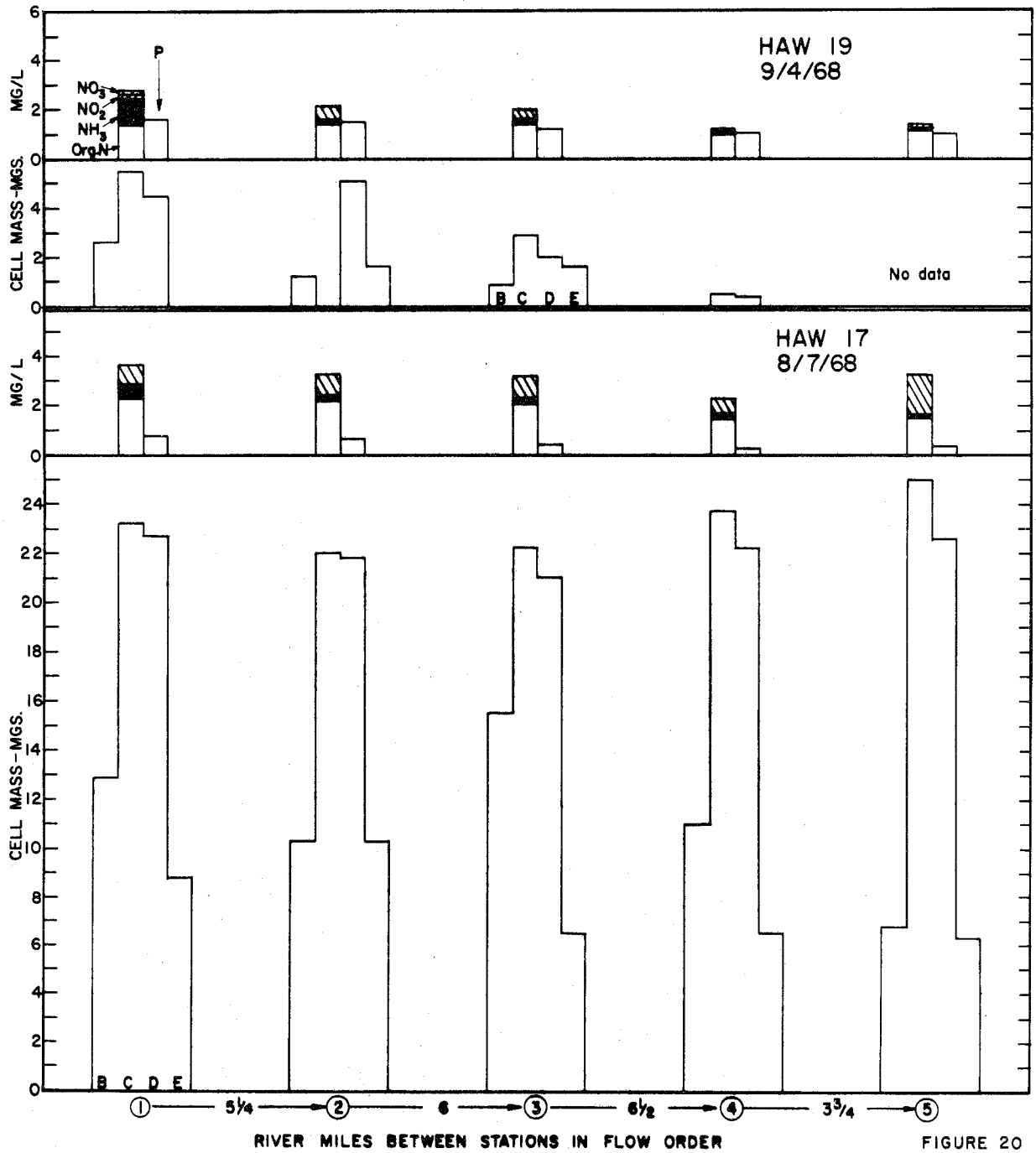


FIGURE 19



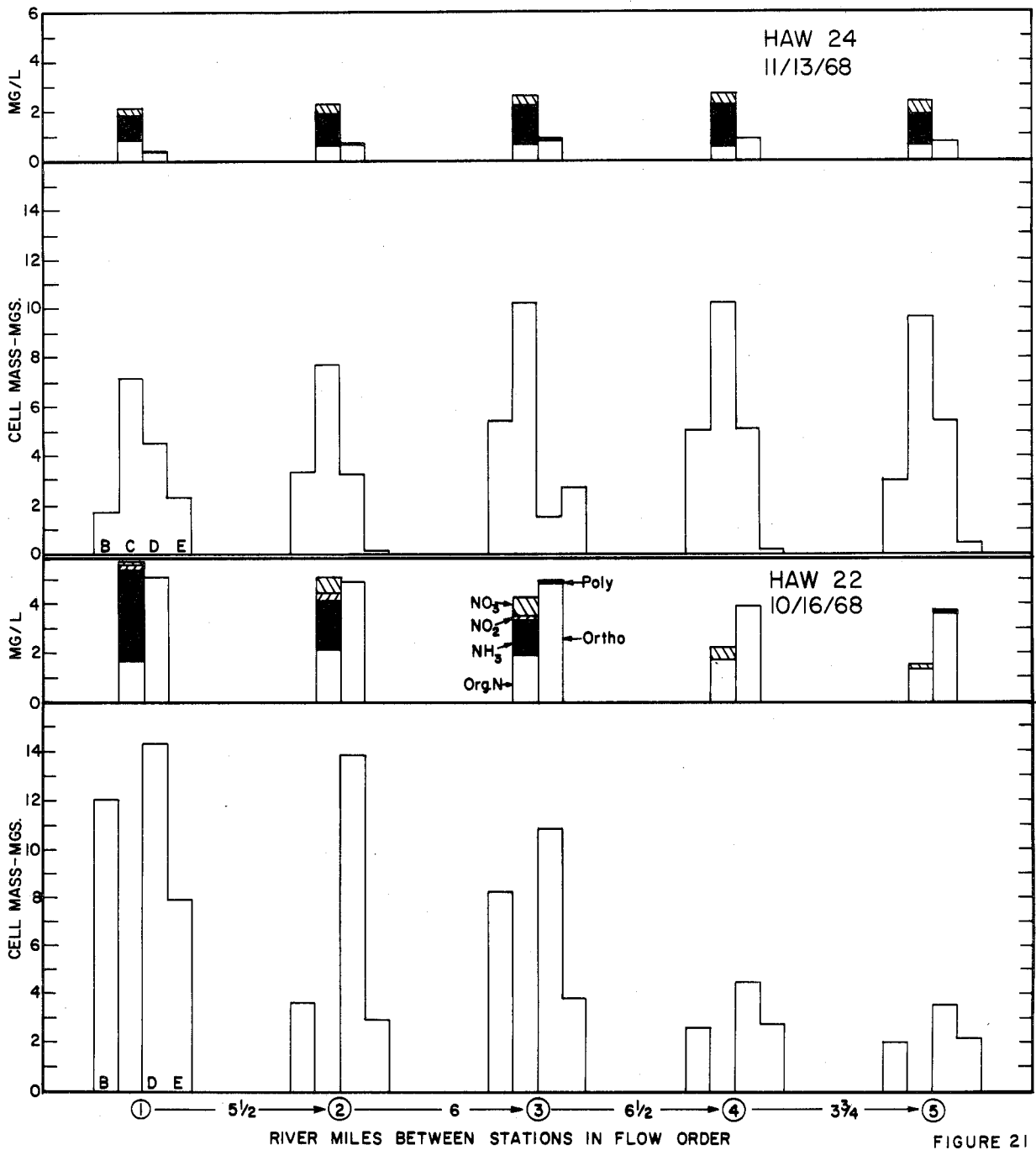
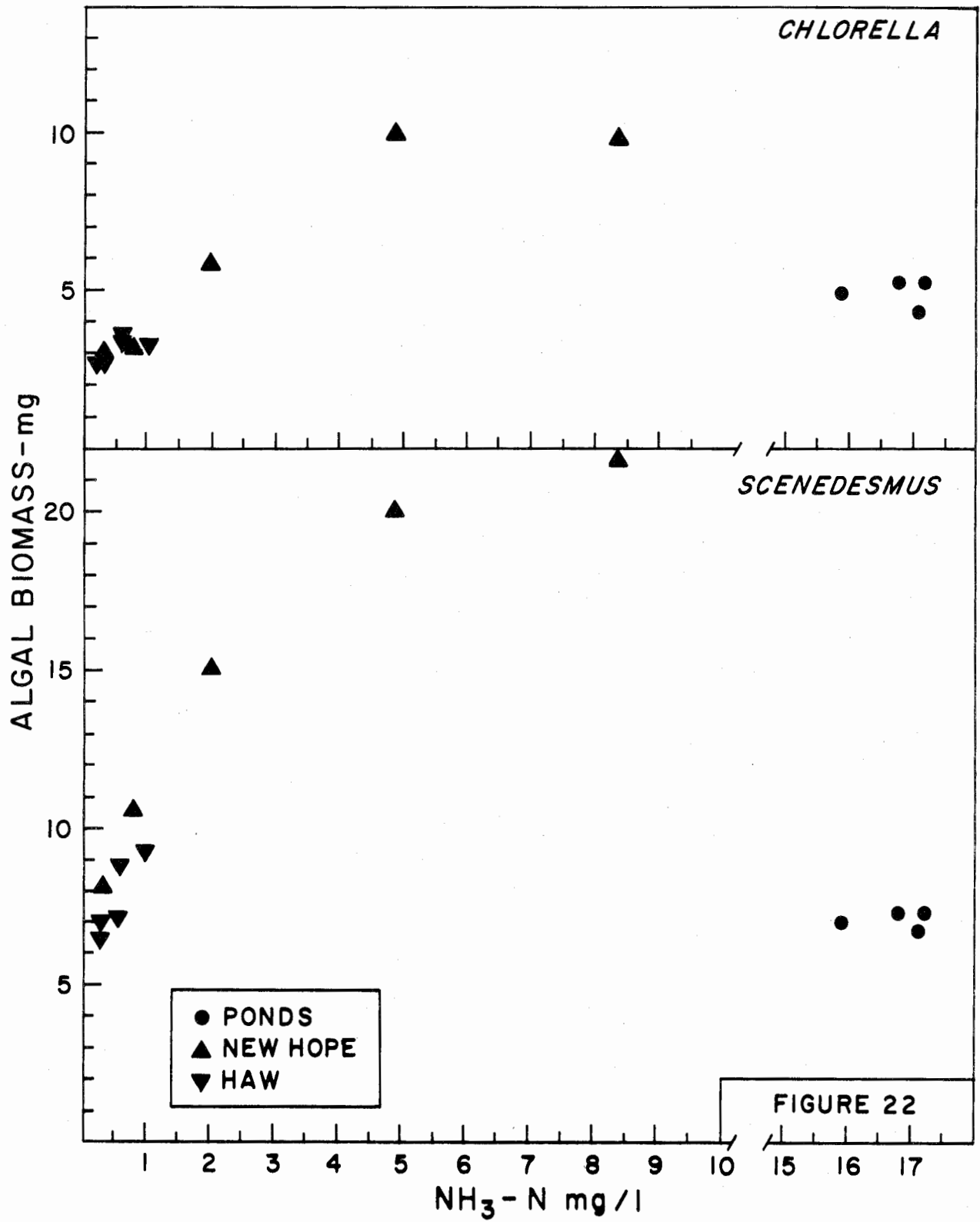
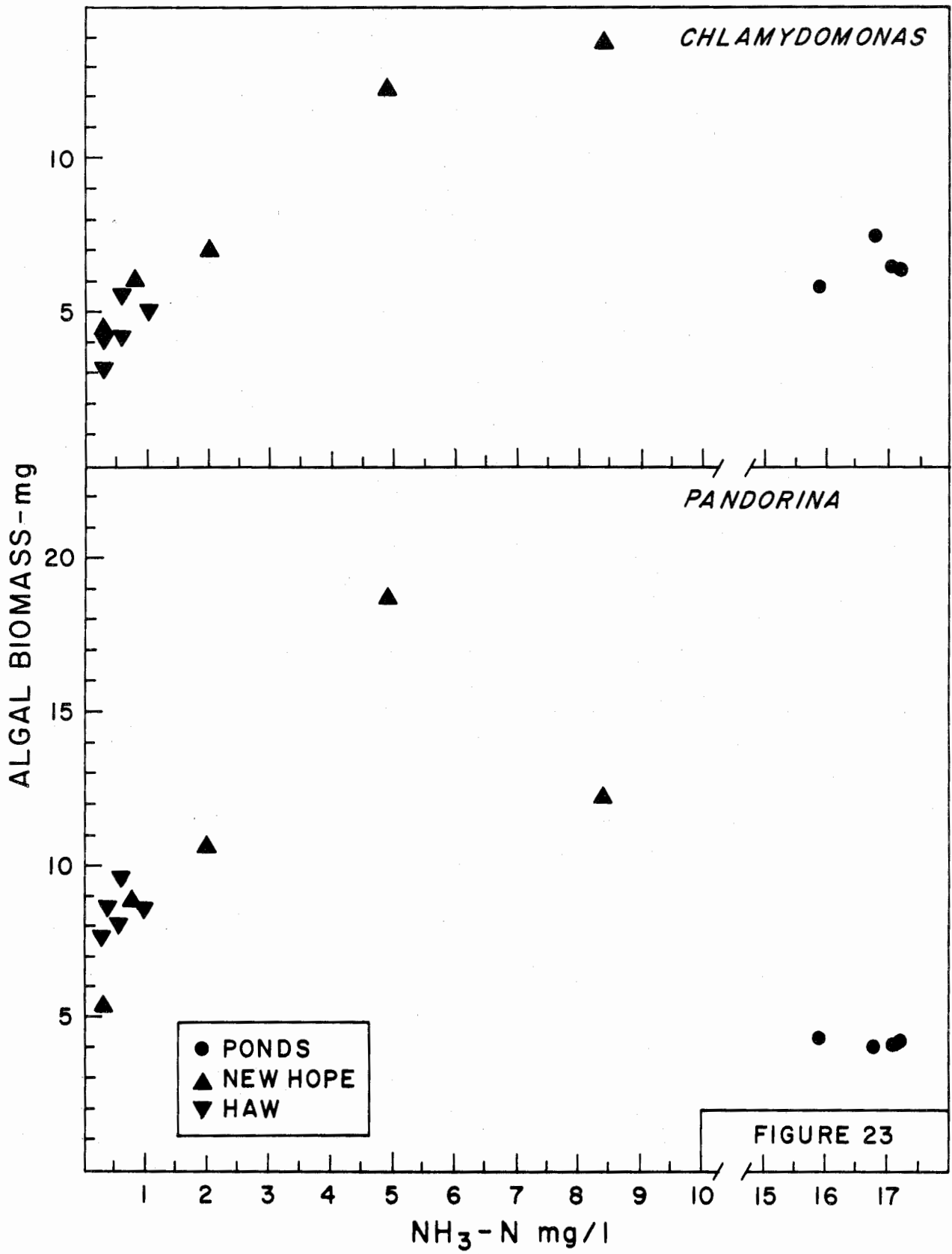


FIGURE 21





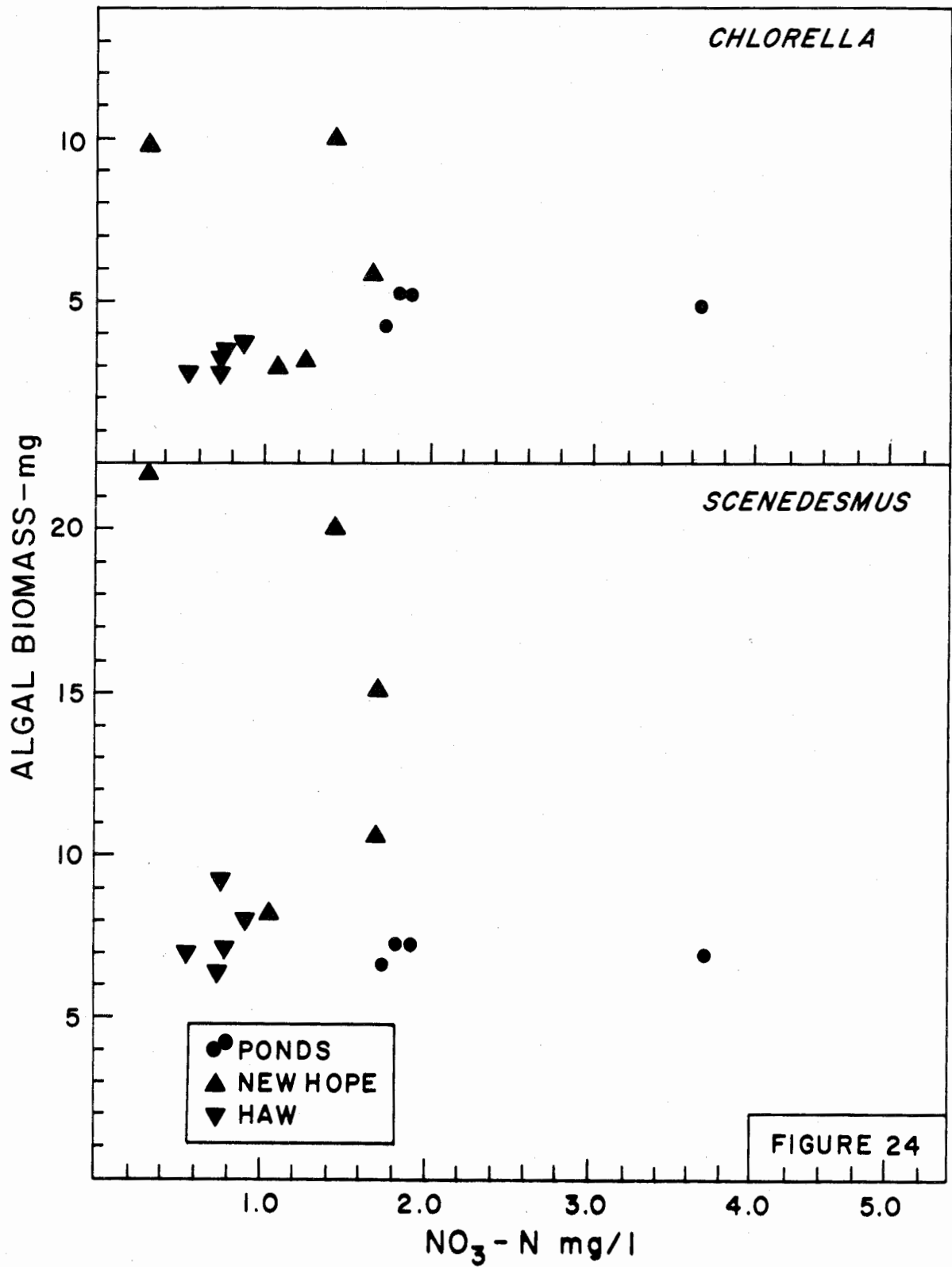


FIGURE 24

