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Nitrification activity in an aquatic ecosystem.

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NITRIFICATION ACTIVITY IN AN AQUATIC ECOSYSTEM

A Thesis Presented

By

JOHN EDWARD PERKINS

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To my wife,

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ABSTRACT

NITRIFICATION ACTIVITY IN AN AQUATIC ECOSYSTEM

(May 1978)

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Heterotrophic nitrification has been studied under laboratory conditions for over two decades. However, little is known of the process dynamics in nature. Neither population size nor activity has been studied in any considerable detail. The intent of this thesis was to monitor environmental and chemical factors which may be of significance for evaluating the extent of both autotrophic and heterotrophic nitrification in aquatic ecosystems. The isolation, characterization, and determination of nitrifying activity of specific organisms was also performed.

Temperature, oxygen, nitrate, ammonium, autotrophic nitrifying, and total heterotrophic bacteria were quantitatively determined with respect to depth and annual variation in a hard water eutrophic lake. There was no correlation found between any particular set of conditions and the occurrence of autotrophic nitrifying bacteria. Several heterophic nitrifiers were isolated from the water column. Succinate

was found to facilitate isolation in contrast to acetate. The nitrifying activity of isolates was found to vary when grown with different carbon sources. Of acetate, citrate, pyruvate, and succinate only pyruvate did not generally support nitrification. However, one isolate (S-6) was able to nitrify when grown with this carbon source. Nitrate was consistently the dominant terminal product and appeared to be related to the bound hydroxylamine concentration.

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INTRODUCTION

Nitrification, the oxidation of inorganic nitrogen, has interested students of ecology since it was recognized by Winogradsky in 1891. He determined that autotrophic bacteria were responsible for the transformations involved. This view remains most prominent today. In recent years it has become evident, however, that certain heterotrophic bacteria and fungi can participate in these transformations as well. The question remains as to what significance, if any, these heterotrophs may have in nature.

Nitrification in water bodies is dependent on nitrogen inputs and various ecological factors. Waters are enriched allochthonously by surface runoff, ground water discharge and precipitation: autochthonous sources include fixation by blue green algae and bacteria, or release from sediments. Nitrogen may occur in organic form, as ammonium, and as nitrate nitrogen.

Although there are a number of reports on the effects of single variables on autotrophic nitrification, these have been derived from research performed under controlled laboratory conditions and little is known of the transformations in nature. Heterotrophic nitrification is only now

beginning to attract attention with respect to limiting factors, pathway regulation, and characteristics of the active populations. At this juncture it is premature to attempt to define what relative significance may be attributed to either of the processes. Both occur in nature and in order to have a thorough understanding of the nitrification process and its ecological significance we must have an understanding of the systematics involved.

A review of the literature reveals that our understanding of the role of heterotrophic nitrification in nature is far from complete. The purpose of this thesis therefore, is to determine and evaluate selected variables of significance to heterotrophic nitrification in an aquatic environment.

LITERATURE REVIEW

Fisher et al. (1956) isolated 16 gram negative, heterotrophic bacteria from soil. Some of these produced as much as 10 ppm nitrite-N in an ammonium plus soil extract medium. In an effort to determine what percentage of the heterotrophic microbial population could produce nitrite from peptone, Eylar and Schmidt (1959) isolated 978 organisms, of which 27, 26, and 17 percent of actinomycetes, bacteria, and fungi respectively produced nitrate-N in excess of 0.2 ppm. Two percent of all organisms yielded nitrite in excess of 0.5 ppm and 15 fungi produced nitrate-N in excess of 5.0 ppm. Hirsch et al. (1961) found that 29 percent of actinomycetes and fungi isolated from soil oxidized ammonium to nitrite but the majority of cultures did not yield more than 0.2 ppm. Nitrification by these isolates occurred between pH 5.0 to 9.0 and appeared to be associated with peroxidase activity. Nitrite had been found to accumulate as a result of hydroxylamine oxidation (Castell and Mapplebeck, 1956) when small quantities were detected in fish fillets which had been dipped in hydroxylamine for a preservative study. In those organisms with nitrate reductase, nitrite disappeared. No bacteria had been shown, conclusively, to produce nitrate from ammonium until Gunner (1963) reported

the accumulation of 4.5 ppm nitrate nitrogen by Arthrobacter globiformis. Doxtader and Alexander (1966) grew heterotrophic strains of bacteria, and fungi on various forms of nitrogen; amides, N-alkylhydroxylamines, oximes, hydroxamic acids, and aromatic nitro compounds. All served as oxidizable nitrogen sources.

In studies directed at elucidation of the pathway of heterotrophic nitrification by Pseudomonas aeruginosa, Obaton et al. (1968) found that this organism appears to nitrify by two mechanisms; one an inducible system in which oxime was oxidized to nitrite, and the other a constitutive system in which nitroethane is cleaved into nitrite and acetaldehyde. Neither of these enzyme systems is linked to the growth phase and one can be blocked while the other still operates. An Arthrobacter, isolated from sewage has been found to excrete levels of nitrite and free hydroxylamine in excess of 15 ppm, bound hydroxylamine 10, and nitrate 2 ppm (Verstraete and Alexander, 1972). Nitrification by this organism was maximum between pH 7.5 to 8.0, a C/N ratio of 3/1, and a ferric concentration of 0.1 mg/1. The pathway as postulated by these workers, suggests that ammonium is converted to an organic compound, possibly an amide, which is then oxidized to yield acetohydroxamic acid. It, in turn, is rapidly converted to hydroxylamine. A peroxidase or catalase may then facilitate oxidation to nitrite and nitrate.

Although heterotrophic nitrification is demonstrable in pure culture, there is little evidence of its occurrence in natural or mixed populations. Verstraete and Alexander (1973) and Rho et al. (1977) reported the accumulation of free hydroxylamine, nitrite, and nitrate in samples from natural ecosystems fortified with ammonium and acetate. Results were variable with different habitats. Recently, it has been reported that the accumulation of nitrification products can be influenced by intergeneric relationships (Rho and Gunner, 1976). The concentration of nitrate and nitrite increased approximately 10 fold when a nitrifying Arthrobacter and a Corynebacterium were incubated together, in contrast to individual yields. Such a synergistic relationship could be of importance in regulating the transformations under natural conditions.

Recent reviews of the nitrogen cycle in aquatic environments (Goering, 1972, Keeney, 1972, and Wetzel, 1975) give only cursory mention to heterotrophic nitrification and in the main support the autotrophic concept as the major vehicle of ammonium oxidation. This may in fact be the true situation however, sufficient research has not yet been reported to demonstrate with any accuracy the contributions of both processes under specific environmental conditions.

Nitrification often occurs in environments not favorable to known autotrophs (Alexander et al., 1970). Nor

have autotrophs capable of nitrification at environmental extremes been isolated. Indeed, present techniques are not adequate for definitive enumeration of these organisms. Thus in certain instances it may be that heterotrophs contribute significantly to the reactions involved. This has been proposed in particular situations. Autotrophs are particularly sensitive to light (Reinheimer, 1974), pH, and temperature (Painter, 1970). In aquatic environments temperature is of particular importance. In temperate lakes nitrification activity is high at overturns and in the winter months (Reinheimer, 1974). At these periods, water temperatures are too low for autotrophs and heterotrophic nitrification may assume increased significance.

Much of the current attention on heterotrophic nitrification has focused on aquatic systems. Mcoy (1972) determined that heterotrophs were responsible for in situ nitrification, and Gode and Overbeck (1972) using an MPN procedure for both autotrophs and heterotrophs, determined that at certain periods the latter outnumbered the former by 10,000 to 1.

OBJECTIVES

The objectives of this thesis are to elucidate the role of heterotrophic nitrifying bacteria in an aquatic ecosystem by:

1. evaluating changes in the nitrogen status of the water column;
2. attempting to quantify nitrifying heterotrophs;
3. morphologically and biochemically characterizing specific isolates with particular regard to carbon source.

MATERIALS AND METHODS

Lake Description

Most studies on heterotrophic nitrification in aquatic systems have been performed on hard water, eutrophic lakes. Lake Pontoosuc is of this category. The greater part of its drainage area lies in Lanesborough, Massachusetts (fig. 1) and is underlain by carbonate bedrock. The lake itself has an area of 194 hectare (table 1), a mean depth of 5 m, and a maximum of 11 (fig. 2). It is located between Pittsfield and Lanesborough, Mass. The shoreline area is 90 percent residential with 10 percent highway and municipal park. U. S. route 7 runs along almost the total length of the eastern shore. Its littoral areas are large and covered with extensive growths of Potamogeton crispus, and Myriophylum exalbescens during the summer months. Algal blooms occur throughout the year. Alkalinity in the summer months averages 1.6 meq/l $CaCO_3$ and 1.4 meq/l Ca with a pH of 8.2 (Chesebrough and Screpetis, 1974). Total phosphorous averages 0.03 ppm. Surficial sediments (0-10 cm) at the sampling area had a bulk density of 0.15 g/cm^3.

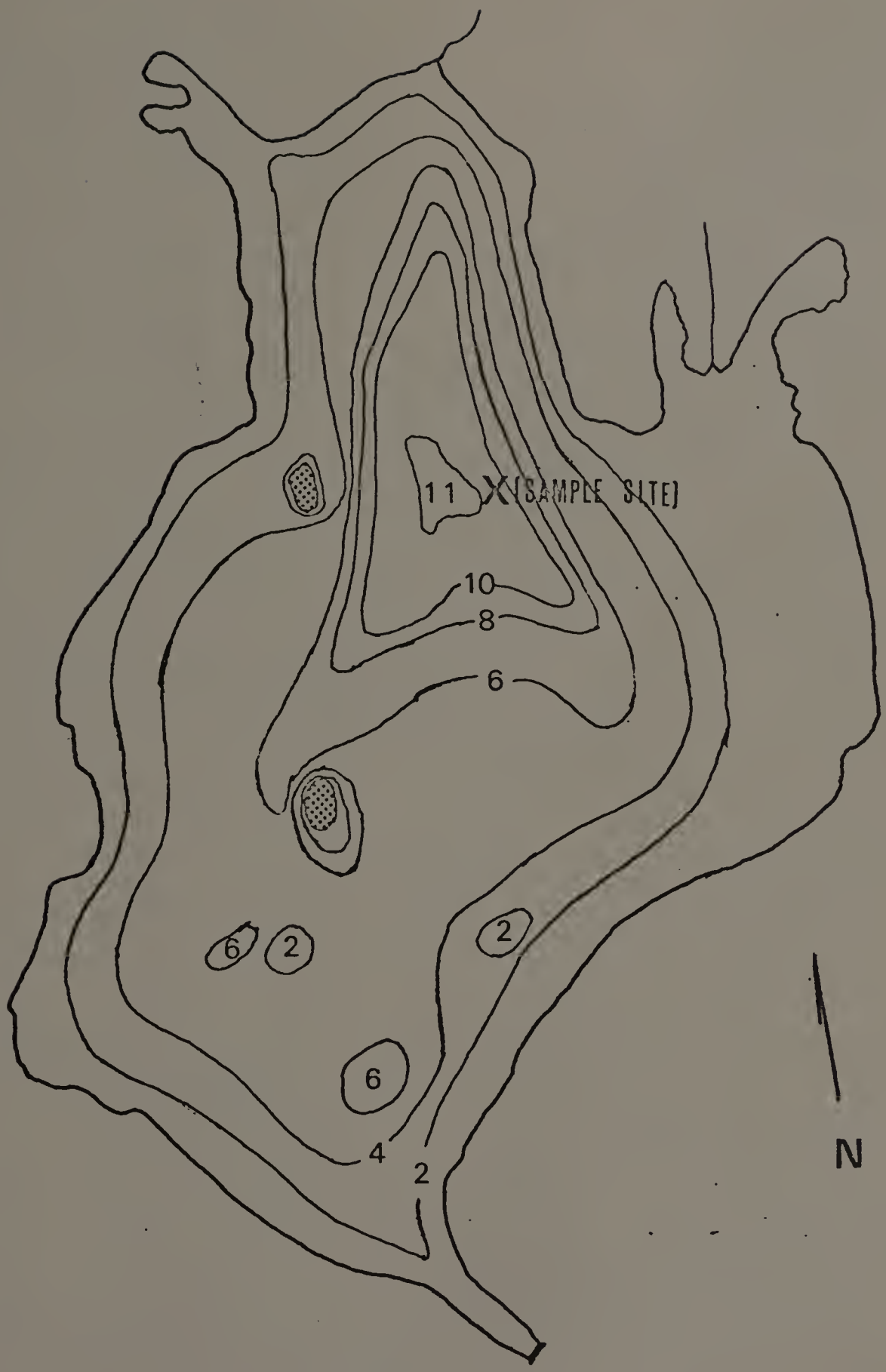
Sampling

Water column and sediment samples were taken in 10 m of water close to the deepest point in the lake. The study

PONTOOSUC LAKE
(DRAINAGE AREA)

Table 1. Morphometric description of Pontoosuc Lake (Adapted from Cheesebrough and Screpetis, 1974)

Maximum Length	2,450 meter
Maximum Effective Length	2,210 m
Maximum Width	1,460 m
Maximum Depth	11 m
Mean Depth	5 m
Area	194 hectare
Volume	$7.23 \times 10^6 m^3$
Shoreline	7.75 kilometer
Shoreline Deveopment	1.59
Drainage Area	55.2 km^2



PONTOOSUC LAKE

DEPTH (m)

period extended from September 1976 to September 1977. A Kemmerer water sampler was employed for the collection of samples for chemical analysis and a JZ sampler with evacuated, sterile, glass bottles was used for the collection of biological materials. One liter, pyrex bottles were used for chemical sample storage.

Sample Analysis

Dissolved oxygen and temperature measurements were made in situ with a YSI model 54A oxygen-temperature meter. Procedures outlines in Standard Methods (American Public Health Association, 1976) were employed for the analysis of all nitrogen containing chemical species. Ammonium and nitrite were determined by the nesslerization and diazotization procedures, respectively. Nitrate was quantified by the phenoldisulphonic acid method for the major portion of the study and was replaced by the cadmium reduction method during the last two months of the sampling period. Autotrophic nitrifying bacteria (Nitrosomonas) numbers were determined with the three tube most probable number method adapted from Standard Methods. Tubes of 1.8 cm ID and 10 ml of Winogradsky medium were used for culture purposes. Cultures were incubated on a rotary shaker at 27 degrees. Total heterotrophic bacteria were enumerated by membrane filtration on 0.45 um, HA, Millipore membrane filters. Difco m-Plate agar was the growth medium and the same incubation temperature (27) was used throughout.

Laboratory Studies

A number of methods for the quantification of heterotrophic nitrifying bacteria were compared. In the first, the Arthrobacter selective media of Hagedorn (1975) was employed according to the method of Tate (1977). In another procedure, the ammonium succinate or acetate agar of Verstraete and Alexander (1972) were utilized. Actidione at a concentration of 0.1 percent was added to retard fungal growth. A 1 m lake water sample (0.1 ml) was spread plated on the agar and colonies subsequently, selected and purified. Isolates were reinoculated into 150 ml flasks, with 70 ml of the medium used for isolation without agar, for confirmation of nitrifying activity. The last procedure involved the utilization of ammonium acetate or succinate as broth in a modification of the three tube MPN method. Flasks of 125 ml with 70 ml of media were used with 10^0, 10^1, and 10^2 ml volumes of 1 m lake water as inoculum.

Heterotrophic nitrifying bacteria were isolated by a two step enrichment-selection procedure. Either 100 or 10 ml of 1, 5, or 10 m water was inoculated into 70 ml of ammonium succinate or pyruvate broth media in 150 ml flasks. Ammonium pyruvate medium consisted of ammonium acetate medium with sodium pyruvate substituted for sodium acetate in a 3/1 carbon/nitrogen ratio. Samples of 10 ml were inoculated directly whereas the 100 ml volumes were concentrated on

Nucleopore 0.2 um membrane filters prior to inoculation. After approximately one week growth, the samples were streaked on ammonium acetate agar. All colonies which produced a smooth opaque surface with a blue periphery were picked as presumptive nitrifiers and inoculated into ammonium succinate for confirmation. Purified nitrifiers were retained for further study.

Eight isolates and a previously identified organism, P-3 (Rho et al., 1977), were assayed for ability to nitrify with acetate, succinate, citrate, and pyruvate as respective carbon sources. The mineral salts medium previously described and the above carbon sources were filter sterilized with Nucleopore 0.2 um filters and added individually to flasks containing the cooled, autoclaved salts solution. Free and bound hydroxylamine, nitrite, nitrate, and growth were assayed periodically for 10 days. Nitrite and nitrate were measured by the diazotoization and cadmium reduction methods mentioned previously. Free hydroxylamine was determined according to the procedure of Magee and Burris (1954) and bound hydroxylamine by the modification of this procedure (Verstraete and Alexander, 1972). Growth was measured by the percent transmission of a one cm cell at 650 um on a Spectronic 20 grating spectrophotometer equipped with red phototube and filter. Flasks of 500 ml with 300 ml of media were used for the culture of P-3 only. In addition to percent transmission, the growth of P-3 was measured by dry

weight determinations. Ten ml of the cell suspension was washed with 0.01 molar phosphate buffer, pH 7.5, and centrifuged at 5,000 rpm, three times. Cells were dried on tared aluminum weighing dishes at 100 degrees for 24 hours. All cultures were aerated on a rotary shaker at 27 degrees. Data was rendered graphically with the assistance of the University of Massachusetts, Cyber computer system with a Tektronix 4013 terminal and copy unit. A copy of the program appears in appendix A.

Isolates were biochemically and morphologically categorized according to the scheme of Skerman (1967).

RESULTS AND DISCUSSION

Analysis of Sampling Parameters

The temperature profile in Pontoosuc Lake exhibited a seasonal variation characteristic of a water body in this temperate climate region. The lake was unstratified between October, 1976 and June, 1977. The summer maximum occurred in late July when surface waters reached 26 degrees. The thermocline extended from 5 to 8 m with hypolimnetic waters stabilizing between 12 and 14 degrees. The minimum temperature occurred at 1 m under February ice where the water registered 0 but rapidly climbed to 3.5 degrees at 2 m. This condition is typical of winter stratification. At this time the lake was overlain by 76 cm of ice.

Temperature appears to be one of the main factors influencing nitrification in lakes. It has been reported that autotrophs are not capable of growth at temperatures below 4 degrees (Painter, 1970), although temperatures to 1 may be tolerated (Alexander, 1965). If one assumes that nitrification by autotrophs is related to growth, little or none would occur in Lake Pontoosuc or more generally, in any of the lakes in this temperate area during the winter months. Conversely, it has been demonstrated that

Table 2. Temperatures (degrees C) at various depths in Pontoosuc Lake between 9-14-76 and 9-27-77.

Depth (m)	9/14	10/2	11/2	11/13	2/9	3/9	4/20	5/4	5/20	6/3
1	19.0	15.0	7.2	6.0	0.0	3.5	12.0	12.0	19.5	18.5
3	18.0	15.0	5.5	4.5	3.5		11.0	12.0	16.0	18.0
5	18.5	15.0	4.5	4.5	3.5		9.0	12.0	13.5	15.0
7	18.0	15.0	4.5	4.5	3.5		8.0	10.0	12.0	13.5
9	16.0	13.2	4.5	4.5	3.5	3.5	8.0	10.0	12.0	11.0
10	15.0	11.2	4.5	4.5	3.5		7.0	9.0	11.0	10.0

Depth (m)	6/15	7/6	7/14	7/21	7/29	8/11	8/18	8/25	9/13	9/27
1	20.0	22.0	22.0	28.0	23.0	24.0	21.0	21.0	19.0	14.0
3	18.5	20.5	22.0	25.5	22.5	24.0	21.0	20.5	19.0	13.5
5	17.0	17.0	18.0	19.0	21.0	21.5	21.0	20.0	19.0	13.0
7	16.0	15.0	15.0	16.0	15.5	16.5	16.0	18.0	18.0	13.0
9	13.0	13.0	14.0	14.0	14.0	13.5	13.0	15.0	14.0	13.0
10	12.0	13.0	13.0	13.0	13.0	13.0	12.0	14.0	14.0	13.0

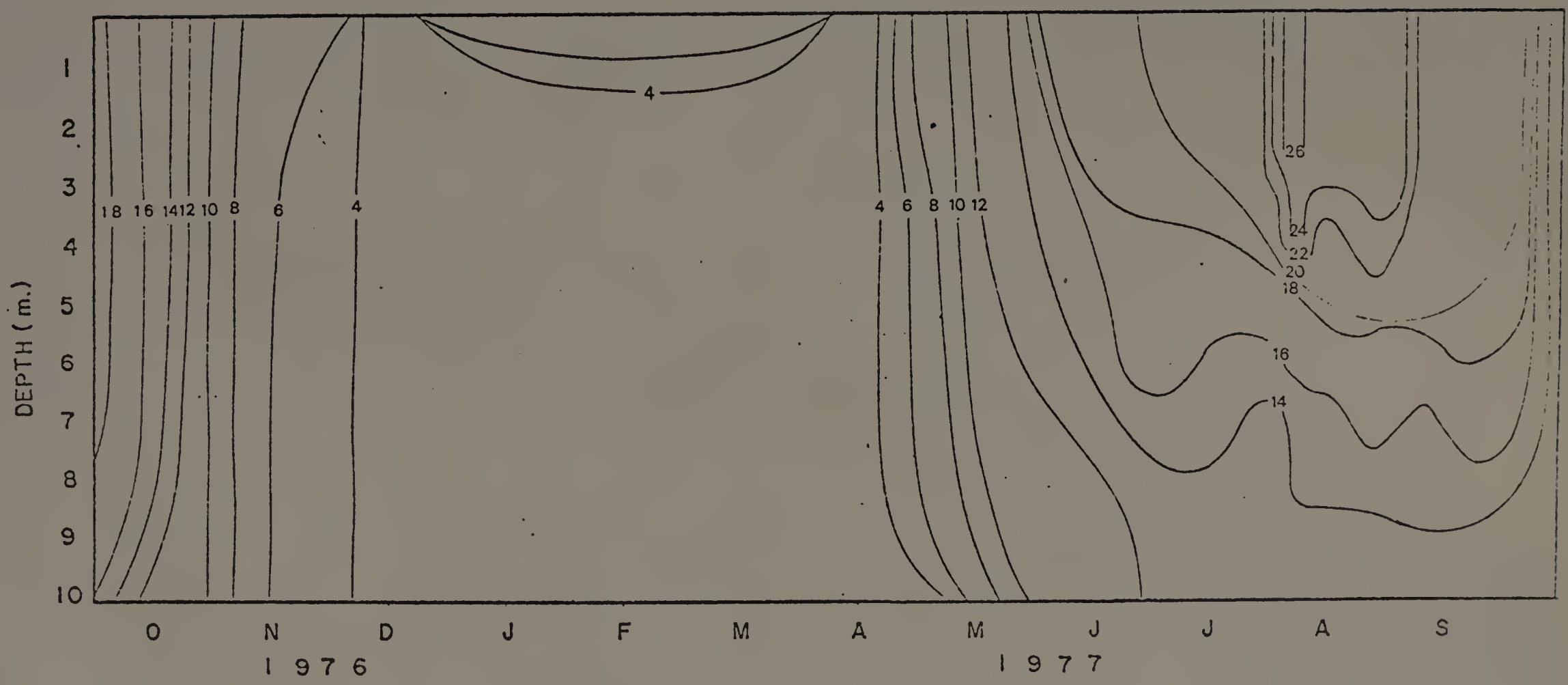


Figure 3. Depth distribution of temperature in Pontoosuc Lake for the period 9-14-76 to 9-27-77.

heterotrophic nitrifying bacteria from this same lake are capable of growth and nitrification at 2 degrees (Rho, 1978, unpublished research).

Oxygen concentrations which occurred during the sampling (table 3, fig. 4) were also typical for a lake of this depth and region. They reflect a moderately eutrophic status. At the beginning of the study period the previous summer's anoxic condition was rapidly coming to a close due to the decline of water temperatures at the surface and mixing with lower layers devoid of oxygen. By the beginning of November water temperatures had been uniform for some time and enough mixing energy had been provided by the wind to create a nearly saturated condition from top to bottom. This uniform condition lasted until winter stratification was firmly established. Turnover could not continue with the initiation of ice cover and as a result, oxygen concentration began to decrease from the bottom up. When ice break-up occurred in late March, oxygen depletion was slowed but rising temperatures resulted in increased respiration and complete spring recovery was not observed. In June, the hypolimnion again became anoxic. This condition lasted until September, 1977.

Conditions must be aerobic in order for autotrophic, and as far as is known heterotrophic, nitrification to occur. However, oxygen concentrations as low as 0.3 mg/l have been reported to support nitrification in Nitrosomonas (Painter,

Table 3. Oxygen contrations (in ppm) for Pontoosuc Lake between 9-14-76 and 9-27-77.

Depth (m)	9/14	10/2	11/2	11/13	2/9	3/9	4/20	5/4	5/20	6/3
1	9.4	8.2	12.6	12.0	11.0	10.5	8.2	11.0	9.8	9.1
3	8.6	8.1	12.3	12.7	7.5		5.8	11.2	10.0	9.6
5	6.0	7.7	12.6	12.6	5.4		6.0	10.6	10.4	8.0
7	4.2	7.7	12.6	12.6	3.4		4.6	10.0	9.0	6.4
9	0.8	1.3	12.6	12.7	2.7	2.0	4.2	10.0	8.0	1.7
10	0.0	0.0	10.1	12.7	2.0		3.2	10.0	4.9	1.3

Depth (m)	6/15	7/6	7/14	7/21	7/29	8/11	8/18	8/25	9/13	9/27
1	8.8	8.7	7.5	7.6	8.3	7.8	6.9	7.1	7.8	8.6
3	7.5	6.9	6.3	8.4	7.5	7.2	6.7	6.6	7.3	8.7
5	4.8	1.2	0.0	0.0	3.6	0.3	6.3	5.6	6.8	8.5
7	4.2	0.0	0.0	0.0	0.0	0.0	0.5	0.7	0.8	8.2
9	0.3	0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.5	8.0
10	0.3	0.0	0.0	0.0	0.0	0.0	0.4	0.5	0.5	5.6

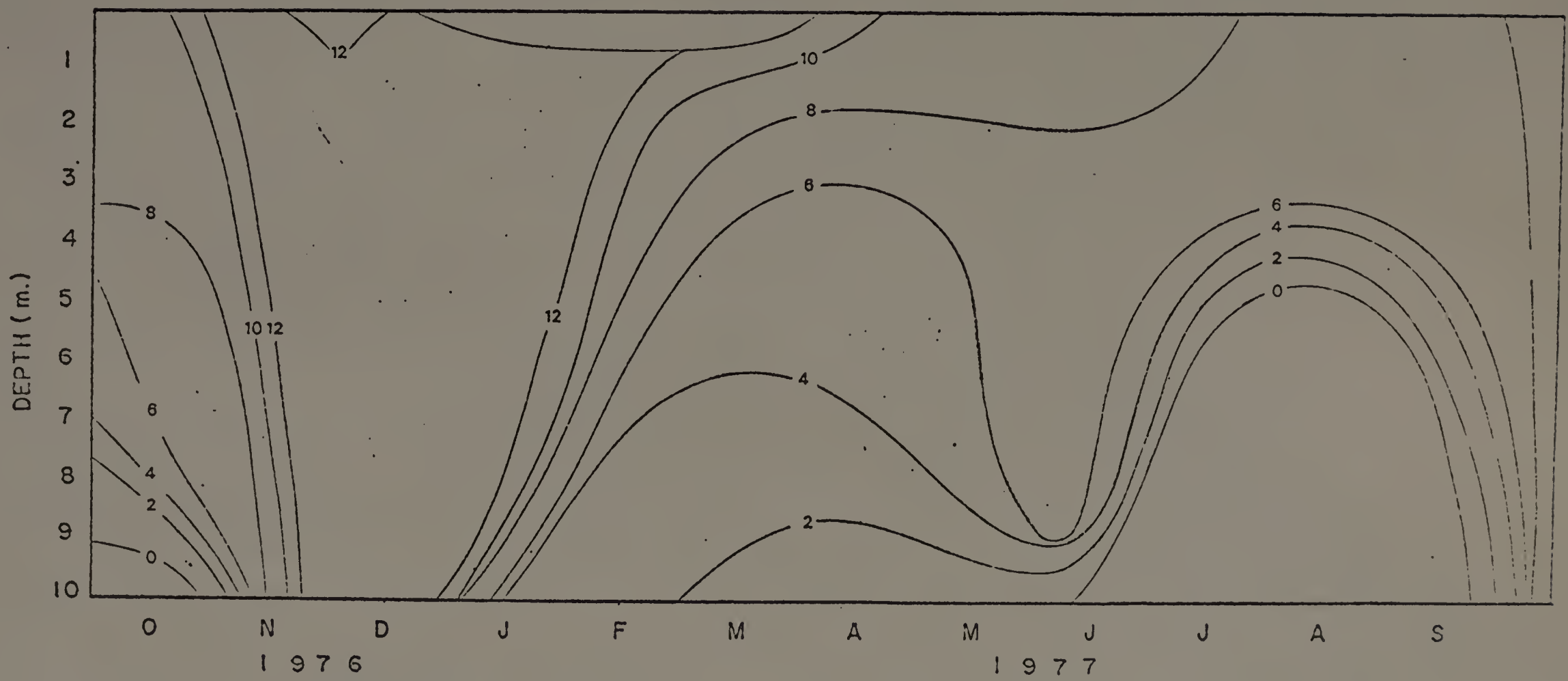


Figure 4. Depth distribution of oxygen concentrations in Pontoosuc Lake for the period 9-14-76 to 9-27-77

1970). It appears that in this lake the only condition under which this parameter would be limiting is in the period of summer stratification. Oxygen is also important for delineating the boundary layer between oxidizing and reducing conditions. In sediments, this area is an active site of nitrification as ammonium diffuses up from anaerobic layers below (Patrick and Reddy, 1976). It is not known however, how the oxidized microzone relates to nitrification at a water-water interface.

The interdependence of oxygen and ammonium concentration is easily seen when its depth-time diagram (table 4, fig. 5) is compared to that for oxygen. An inverse relationship is apparent. Ammonium is the product of protein decomposition by aquatic heterotrophs. When the environment becomes devoid of oxygen, nitrification does not occur and ammonium accumulates. Ammonium may also escape from the sediments as the absorbtive capacity decreases as a result of the reducing conditions (Wetzel, 1975).

Ammonium was lowest in October of 1976 when the decrease in temperature and reoxygenation of the water column began to occur. As the isothermal and uniform oxygen conditions of late 1976 became established, the ammonium concentrations stabilized at 0.4 ppm. It appears this situation remained constant until the spring of 1977 when bottom water concentration began to increase. Again the oxygen

Table 4. Ammonium - nitrogen concentrations (ppm) in Pontoosuc Lake between 9-14-76 and 9-27-77.

Depth (m)	9/14	10/2	11/2	11/13	2/9	3/9	4/20	5/4	5/20	6/3
1	.28	.07	.45	.43	.49	.23	.32	.29	.19	.32
3	.23	.07	.37	.46	.44			.29	.19	.29
5	.25	.07	.39	.49	.58		.58	.30	.21	.37
7	.69	.12	.38	.43	.91			.37	.19	.38
9	5.1	2.0	.39	.44	.84	1.28	.30	.29	.30	.60
10	7.4	6.0						.53	.51	1.97

Depth (m)	6/16	7/6	7/14	7/21	7/29	8/11	8/18	8/25	9/13	9/27
1	.37	.39	.42	.19	.35	.26		.12	.26	.51
3	.35	.44	.56	.23	.42	.32		.12	.32	.28
5	.39	.58	.65	1.25	.65	1.44		.30	.46	.65
7	.58	.67	1.16	1.51	1.86	1.55	.93	2.88	3.60	.30
9	1.39	2.41	3.16	5.57	4.64	6.03	4.06	5.22	7.89	1.48
10	3.25	4.27	3.76	6.50	6.96	6.73	5.02	5.80	9.28	12.53

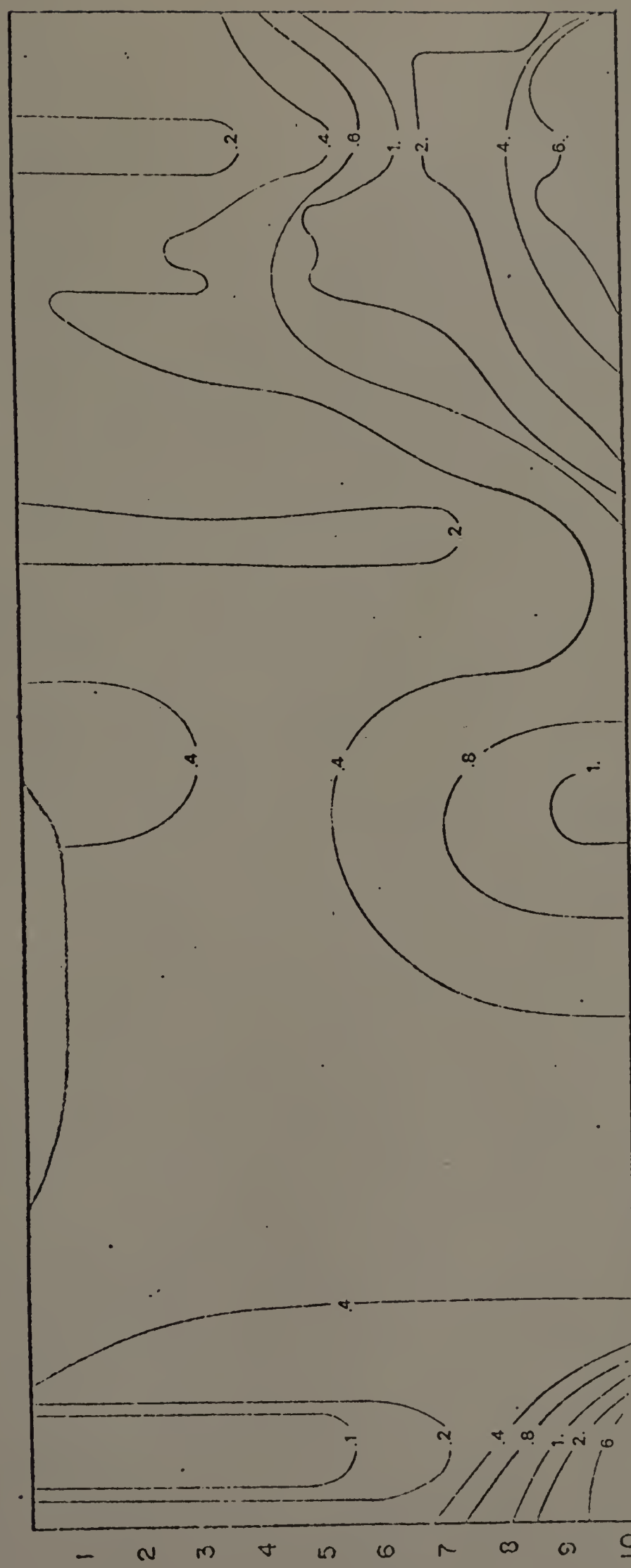


Figure 5. Depth distribution of ammonium-nitrogen in Pontoosuc Lake for the period 9-14-76 to 9-27-77.

increased and the ammonium decreased. With the initiation of anoxic conditions in the summer, ammonium began to increase from the bottom up.

The determination of nitrate concentration was beset by difficulty. The fourteenth edition of Standard Methods did not include the phenoldisulfonic procedure for nitrate determination as did the thirteenth. In late July 1977 this analytic technique was dropped and replaced by the cadmium reduction method. In spite of the limitation of this procedure, some useful conclusions may be drawn (table 5, fig. 6). Very low (.02 ppm) concentrations of nitrate were observed in upper waters at the beginning of the study period. Nitrate concentrations increased ten fold in the area closest to the anaerobic waters. This gain was most probably due to the substantial oxygen concentration together with high ammonium available as a substrate at the oxidized microzone. As the ammonium concentration decreased nitrate levels fell. Concentrations of nitrate increased to a relatively high level in February and remained stable until May. The spring phytoplankton bloom undoubtedly depleted nitrate concentrations by mid June. A slight increase in nitrate was again seen at the end of the study period when the lake once more became oxygenated.

As noted by Verstraete (1975), measures of nitrification by autotrophic bacteria based on current procedures

Table 5. Nitrate-nitrogen concentrations (ppm) in Lake Pontoosuc between 9-14-76 and 9-27-77.

Depth (m)	9/14	10/2	11/2	11/13	2/9	3/9	4/20	5/4	5/20	6/3
1	.02	.04	.03	.02	.19	.21	.09	.05	.05	.08
3	.02	.04	.00	.02	.00			.06	.02	.08
5	.02	.04	.02	.02	.00		.11	.07	.07	.00
7	.00	.04	.03	.03	.24			.10	.05	.03
9	.08	.24	.05	.03	.14	.15	.14	.07	.08	.00
10	.11	.43			.13			.01	.08	.04

Depth (m)	6/15	7/6	7/14	7/21	7/29	8/11	8/18	8/25	9/13	9/27
1	.08	.00	.03	.00	.003	.004	.002	.007	.004	.043
3	.02	.04	.06	.02	.004	.008	.002	.007	.004	.030
5	.02	.00	.03	.09	.012	.004	.004	.021	.007	.06
7	.02	.00	.07	.00	.003	.001		.003	.003	.08
9	.04	.34	.04	.00	.007	.003		.004	.007	.106
10	.16	.04	.04	.02	.006	.008		.014	.009	.016

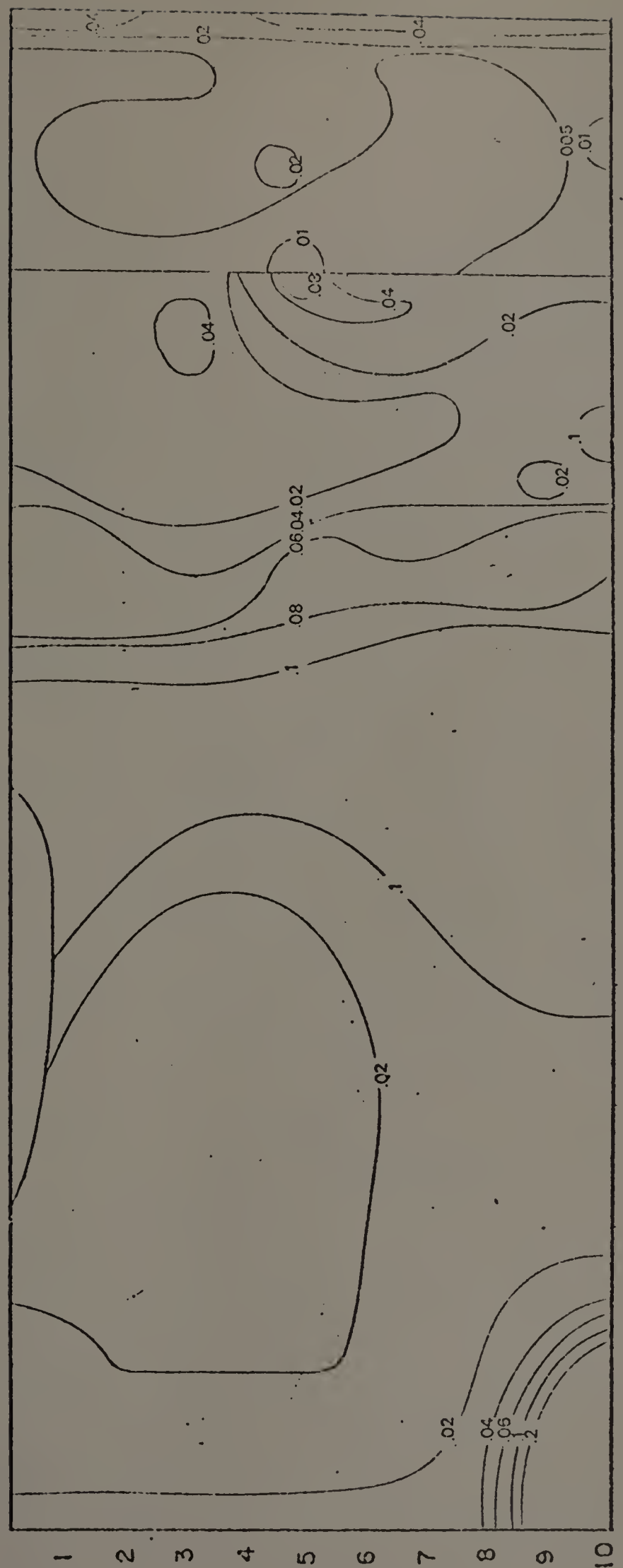


Figure 6. Depth distribution of nitrate-nitrogen in Pontoosuc Lake for the period 9-14-76 to 9-27-77.

may be misleading. The technology employed in isolating and determining the abundance of autotrophs suffers from inaccuracy and reveals only a portion of the in situ population. At the present time, numbers based on the MPN are probably the most efficient way of estimating population and hence, activity.

At no time in the period under study were numbers of autotrophs, as measured by the MPN (table 6), high in relation to total heterotrophs (table 7). The first sample of the period produced the highest numbers of autotrophs found in the water column for any of the sample dates and were highest in the one meter sample. Concentrations of nitrate and ammonium were unrelated to the relatively high numbers of autotrophs. Most probably, the variable operative in determining numbers was the accumulation of ammonium after the anoxic condition of the summer stagnation period. The presence of autotrophs throughout the profile indicates uniform nitrifying activity, discounting the influence of localized conditions such as the influx of storm water runoff. Since enumeration was by MPN, the number recorded is of course only a probability and the confidence limits of all three positive values overlap. Thus, the difference between a value of 4 and one of 0 may not be of great significance. There was little further activity, in terms of detectable autotroph numbers, until May when, again, the presence of nitrifiers was observed on two consecutive sample dates. At this time

Table 6. Numbers of autotrophic nitrifying bacteria (per 100 ml) between 9-14-76 and 9-27-77

Depth (m)	9-14	10-2	11-2	11-13	2-9	5-4	5-20
1	23	0	0	0	0	11	11
5	0	0	0	0	0		
9	4	0	0	4	0	11	11
10	9						
*Sediment		11,000	40,000				2,100

Depth (m)	6-15	7-6	7-14	7-21	8-11	8-18	9-27
1	0	0	0	0	0	0	4
5	0	0	0		0	9	4
9	0	0	0			0	0
10							
*Sediment							230

* Numbers for sediments are denoted in numbers per gram.

nitrate concentrations were decreasing from the highest uniform period of the entire year. Throughout the summer, detectable levels were not encountered until late August when the summer anoxic situation began to recede. In contrast to the water profile, autotrophs were easily detected in sediments. This situation was also reported by Isirimah et al. (1976) indicating the association of autotrophs with particulate matter (Alexander, 1965, Reinheimer, 1972).

The absence of autotrophs has been used as a criterion by researchers to substantiate the importance of heterotrophic nitrification (Ishaque and Cornfield, 1976, Isirimah et al., 1976). Although, this rational has been questioned by Verstraete (1975).

The activity of heterotrophic bacteria in lakes is very difficult to determine accurately. Enumeration by plate count has been the most common of the methods used. Various methods of enumeration in sea water were compared (Jannasch and Jones, 1959). Of agar pour plate, membrane filtration (macro and micro colonies), extinction dilution and direct methods, membrane filtration had the second lowest and direct counting had the highest recoveries per ml. Indeed, direct count techniques often produce readings in excess of 10,000 times those for membrane or pour plate methods (Reinheimer, 1972). The major problem with the direct methods is related to the difficulty in discriminating living organisms from inanimate particulate matter.

Table 7. Total heterotrophic bacteria (per ml) in Pontoosuc Lake between 9-14-76 and 9-27-77.

Depth (m)	9-14	11-2	11-13	2-9	5-4	5-20	6-3	6-15
1	450	1,000	50	375	1,400	650	50	110
3	390	800	90	245	6,740	1,200	95	160
5	600	700	170	185	1,420		75	85
7	550	500	175	315	4,020	400	130	70
9	750	700	100		420	150	130	145
10	11,200	20,000			890	4,000	995	940

Depth (m)	7-6	7-14	7-21	7-29	8-11	8-18	8-25	9-27
1	295	275	120	60	300	1,400	300	500
3	310	410	60	120	1,700	600	200	600
5	80	265	300	120	1,400	5,100	350	1,600
7	85	370	190	150	600	400	550	2,700
9	320	360	210	130	3,100	1,200	800	
10	475	1,300	230	365	7,000	2,900	1,500	2,800

Membrane methods are generally simple and consistent in their result.

Total heterotrophic numbers (table 7) were relatively comparable throughout the whole year, generally ranging from one to five hundred per ml. There were two maxima: one in early May and the other in early August. Lowest numbers were observed on 11-13-76 and values were consistently lower between 6-3-77 and 7-29-77. The increase in numbers in May was probably due to the increase of assimilatory products brought about by the spring algal bloom. This maximum does not agree with that found by Overbeck and Babenzien (Wetzel, 1975). They noted a high point in August of one year and in June and July of the next year. The moderate increase in the numbers during August in Lake Pontoosuc may be related to the same phenomenon. Relatively high levels of ammonium were present at this time, correlating with anoxic conditions. There was an aquatic herbicide application in mid July 1977 and this may have had some effect on the increase in numbers. However, it was not reflected in numbers found on 7-29-77. In the samples from 10 m bacteria were generally higher in number. This may be expected due to the concentration of organic and inorganic material in the sediment reservoir.

The relationship of total numbers of heterotrophs to nitrification remains undefined. Large numbers of individuals of indigenous populations are indeed able to carry out some

part of the process. However, the amounts of products formed are generally present in only trace concentrations.

Thus, no wide ranging conclusion on the dynamics of nitrification in the water column may be drawn from these data. Autotrophs are present in the water column. There was a relatively high concentration of autotrophic nitrifiers in September, 1976, at the end of the summer stagnation period. Oxygen and nitrate concentration were increasing and temperature and ammonium values decreasing. In May, 1977, ammonium and nitrate were decreasing and temperature and oxygen values were increasing. This condition was also associated with detectable concentrations of autotrophic nitrifiers. It is obvious that large amounts of nitrogen are converted to nitrate. If the water body is taken as a whole and the concentration of nitrate present is equal to 0.1 ppm, the lake would contain 10^5 grams of nitrogen as nitrate. However, if we were to use the figure of 70 per 100 ml as representative of the autotrophic nitrifier population, at a steady state level, they would have to oxidize 10^6 times their weight of ammonium-nitrogen to account for the nitrate present in the lake. This calculation yields, approximately, a total lake autotrophic nitrifier biomass of 0.7 g (Roberts, et al., 1957) and a quantity of 723 kg of nitrate nitrogen. There is not enough known about in situ generation or reaction rate to provide an adequate explanation for a transformation of this magnitude.

Enumeration Procedures

This research in part was intended to compare different procedures of heterotrophic nitrifier enumeration with respect to their adaptability in water quality analysis. The procedure of Tate (1977) has been utilized, apparently with some success, for enumeration. In Pontoosuc Lake water, it proved to be inadequate (table 8). Of 30 organisms isolated, 21 of which were Arthrobacter species, only one was found to produce nitrite - N in detectable quantities and then only low levels (less than 0.2 ppm). Although Tate reported high numbers of heterotrophic nitrifiers, 10^5 per gram of soil, it was specified that the quantities of nitrite produced were low. He expressed his findings as a "potential" for heterotrophic nitrification. In order to be valid the numbers should be representative of active nitrifiers, such as those isolated by Rho et al. (1977) or Verstraete and Alexander (1972) where produces were in excess of 10 ppm nitrogen. The Tate process is also quite time consuming.

Other types of agar media were examined with respect to their selectivity for nitrifying heterotrophs. Ammonium acetate and ammonium succinate agar were spread plated with 0.1 ml volumes of water sample. Selected colonies, 38 from acetate and 19 from succinate agar, were purified. When these were inoculated into broth of the same composition as the isolation medium, without agar or Acti Dione, only one

Table 8. Test of Tate Method for heterotrophic nitrifier enumeration.

*Organism	**Gram Stain 2 day	Gram Stain 5 day	Growth Glucose Peptone	Growth Nutrient Broth	***NO_2+NO_3 production
1b	neg c	neg c	+	+	-
1c	neg c	neg c	+	+	+
1h	neg r+c	neg c		+	-
1i		var c		+	-
1j		neg c		+	-
1k		pos r		+	-
1l	neg c	neg c	+	+	-
1m	var c	var c	+	-	-
1n	var c	neg c	+	+	-
1o	pos c	pos c	+	+	-
1p	neg cr	neg c+r	+	+	-
1s	neg cr	neg r	+	+	-
1t	neg c+r	neg r	-	+	-
1w	neg c	neg c	+	+	-
1x	neg c+r	pos c	+	+	-
1y	neg c	neg c	+	+	-
1a'	neg r	neg r	+	+	-
1c'	neg c+r	neg c	+	+	-
1d'	neg r	neg r	+	+	-
5c	neg cr	neg c	+	-	-
5d	neg r	neg r	+	+	-
5f	neg r	neg r	+	+	-
5g	neg r	neg r	+	+	-
5h	neg r	pos c	+	+	-
5i	neg r	neg r	+	+	-
5j	neg c+r	neg c+r	+	+	-
5l	pos c	pos c	+	+	-
5m	neg r	neg c+r	-	+	-
5n	neg r	neg c+r	-	+	-
5o	neg c+r	neg c+r	+	+	-

* 1 designates 1 m sample, 5 designates 5m sample

** neg = gram negative, pos = gram positive, var = gram variable, c = coccus, r = rod, cr = coccal rod, c+r = coccus + rod

*** The only organism to produce any significant ammount of any nitrification product was 1c which accumulated 0.14 ppm on glucose peptone after 7 days.

Table 9. Nitrification by isolates from ammonium acetate and succinate agar media after inoculation into broth media.

Agar medium	Date	# colonies growth positive in broth	* # colonies NO_2^+ NO_3-N positive
Ammonium acetate	7-14-77	18	1
Ammonium acteate	7-21-77	20	0
Ammonium succinate	8-11-77	19	1

* After 7 days the acetate isolate produced 0.08 ppm NO_2-N and the succinate isolate produced 0.13.

of the acetate and one of the succinate isolates produced a positive nitrite reaction (table 9); again, values were less than 0.2 ppm. Even if the quantities were large, one would have to isolate and purify numerous organisms in order to extrapolate the numbers representative of the entire population.

MPN procedures utilizing acetate or succinate as carbon sources were also tested. Lake water dilutions of 10^0, 10^1, and 10^2 were used. Flasks with acetate as carbon source produced no reaction, although succinate fortified flasks did (table 10). However, of the entire series of flasks inoculated, only one of the 100 and one of the 10 ml flasks became positive. Although this is a legitimate recording for an MPN it is not characteristic of a typical reaction. Nonetheless, this procedure, with succinate as a carbon source, appears to be the most promising for future development of an MPN methodology.

Isolation Procedures

Between 5-30-77 and 9-27-77 100ml volumes of water samples taken at 1 m depth were inoculated into ammonium acetate broth medium. On no occasion was a positive nitrification reaction observed. Beginning 8-18-77 a similar 100 ml sample was added to ammonium succinate. These flasks did produce a positive nitrification reaction (table 11).

Table 10. Most probable number test for heterotrophic nitrifier enumeration using ammonium acetate and ammonium succinate broth.

Substrate	Dilution	*Reaction A	B	C
Acetate	10^2	-	-	-
	10^1	-	-	-
	10^0	-	-	-
Succinate	10^2	+	-	-
	10^1	-	-	+
	10^0	-	-	-

* Where a positive reaction was observed, NO_2+NO_3-N accumulated to between 0.5 and 1.0 ppm.

Table 11. Nitrification of 100 ml samples of lake water inoculated into ammonium acetate and ammonium succinate broth.

Medium	Sample depth (m)	Sample date	*Nitrification reaction
Ammonium acetate	1	6-15	-
"	5	"	-
"	1	7-6	-
"	5	"	-
"	1	7-21	-
"	5	"	-
"	1	8-18	-
"	5	"	-
"	1	8-25	-
"	5	"	-
"	1	9-13	-
"	5	"	-
"	1	9-27	-
"	5	"	-
Ammonium succinate	1	8-18	+
"	5	"	+
"	1	8-25	+
"	5	"	-
"	1	9-13	+
"	5	"	-
"	1	9-27	+
"	5	"	+
"	10	"	+

* A positive reaction represents an accumulation of between 0.5 and 1.0 ppm NO_2+NO_3-N.

Nitrate and nitrite were found to be present in low concentrations, unlike the results reported by Verstraete and Alexander (1973). Free and bound hydroxylamine were not detected. When samples from positive succinate flasks were streaked on ammonium acetate agar medium, a characteristic colony, opaque with a blue periphery was produced. This colonial type invariably nitrified. Similarly, one culture which emerged from ammonium pyruvate broth medium was found to be a nitrifying bacterium after culture on ammonium acetate agar. The initial pyruvate flask had no accumulation of nitrification products.

The nature of the carbon source is significant for isolation or determination of characteristics of the nitrifying population. Although no nitrification was detected in the acetate flasks, it is noteworthy that the same volume of water sample, inoculated into succinate flasks, produced active heterotrophic nitrification. A probable explanation is that other, non nitrifiers, are present whose growth rate on acetate is much faster than that of the nitrifiers. These organisms dominate the ecology of the flask, and the growth of nitrifiers is suppressed. When succinate is used as a carbon source the growth rate of the nitrifiers increases, thus effective competition occurs. The presence of synergistic relationships has also been shown to regulate the extent of nitrification in certain instances (Rho and Gunner, 1976).

Organisms isolated from defined, enrichment media have been found to be capable of very active nitrification with the accumulation of greater than 10 ppm of free hydroxylamine, significantly higher than is accumulated when organisms are isolated from protein media. Organic acids used as carbon sources produced variable results when used for detection of nitrification activity and isolation of nitrifying organisms. Clearly, in the lake system under study, acetate was not a carbon source to which the local, heterotrophic nitrifying population responded. It is possible that had inoculum from acetate flasks been subsequently cultured on agar plates with other organic acids, heterotrophic nitrifying bacteria may have appeared.

One actively nitrifying organisms was isolated from a relatively small volume of water, 10 ml, suggesting that large sample volumes may not be necessary.

By use of the procedure, described in the foregoing discussion, 12 active nitrifiers were purified and eight were studied in further detail (table 12).

Characterization and Nitrification of Organisms

The eight nitrifying organisms were categorized biochemically and morphologically according to the scheme outlined in table 13. All appeared to be Arthrobacter types (Skerman, 1967), similar to the culture of Rho et al. (1977) which was isolated from an acetate enriched sample from the

Table 12. Sources of heterotrophic nitrifiers isolated from Lake Pontoosuc.

Organism	Date	Source	Liquid Enrichment
*P-3	9-76	100 ml 1 m water	Ammonium acetate
S-1	8-25-77	"	Ammonium succinate
S-4	"	"	"
S-5	9-27-77	"	"
S-6	"	"	"
S-7	"	100 ml 10 m water	"
S-8	"	10 ml 5 m water	"
S-9	"	100 ml 5 m water	Ammonium pyruvate
S-11	"	"	Ammonium succinate

* P-3 was isolated from Pontoosuc Lake water by Rho et al., 1977.

Table 13. Biochemical and morphological character of *S series of nitrifying heterotrophs.

Test	Reaction
Gram Stain (48 h) (nutrient broth)	gram negative rod
Gram Stain (48 h) (Ammonium succinate broth)	gram negative coccus
Flagella Stain	flagella present, variable arrangement
Motility	(-)
Nitrate reduction	(-)
Indole production	(-)
Starch hydrolysis	(-)
Gelatin liquifaction	(-)
Litmus Milk reaction	alkaline
Enzymes:	
Oxidase	(+)
Catalase	(+)
Peroxidase	(+)
Carbohydrate fermentation:	
Glucose	(-)
Lactose	(-)

* All S-n isolates had similar characterization reactions.

same lake. The 24 and 48 hour cultures grown in nutrient broth at 35° were gram negative rods. However, in mineral salts media the morphology was coccoidal after 72 hours. Flagellar stain gave varied results. All organisms were negative for motility, starch hydrolysis, gelatin liquifaction, nitrate reduction, indol production, and carbohydrate fermentation. All the tested cultures were positive for oxidase and catalase enzymes and the reaction in litmus milk was weakly alkaline after two weeks incubation.

The selective nature of succinate, both for demonstrating nitrification activity and facilitating nitrifier isolation, prompted a new set of experiments. Acetate, citrate, succinate, and pyruvate were used individually to test their respective strengths as a carbon source for heterotrophic nitrification. Pyruvate is excluded in most of the subsequent discussion since it did not generally support nitrification.

A particularly active nitrifier, P-3 (Rho et al., 1977), which had previously been isolated from Pontoosuc Lake, was utilized for the initial carbon substrate experiment. In the production of free hydroxylamine, P-3 is high (table 14-16, fig. 7-9). On all three carbon sources, acetate, citrate, and succinate, free hydroxylamine reached a peak of approximately 40 ppm. The maximum for the different sources varied only in time of appearance. The figure of 40 ppm does not represent the maximum concentration attained.

Table 14. Nitrification and related growth parameters for P-3 when grown with acetate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3^- (ppm)	%T	Dry wt. (ug/ml)
24	0.0	0.0	0.0	0.0	100	
48	0.0	0.0	0.0	0.0	98	0.0
72	0.0	0.0	0.0	0.0	29	3.1
96	40.2	0.0	0.0	0.0	6	7.7
120	30.5	0.8	1.0	0.0	4	8.4
144	0.8	14.5	0.1	0.0	9	7.1
168	0.1	2.6	0.1	2.7	7	7.4
240	0.0	0.0	0.9	2.6		

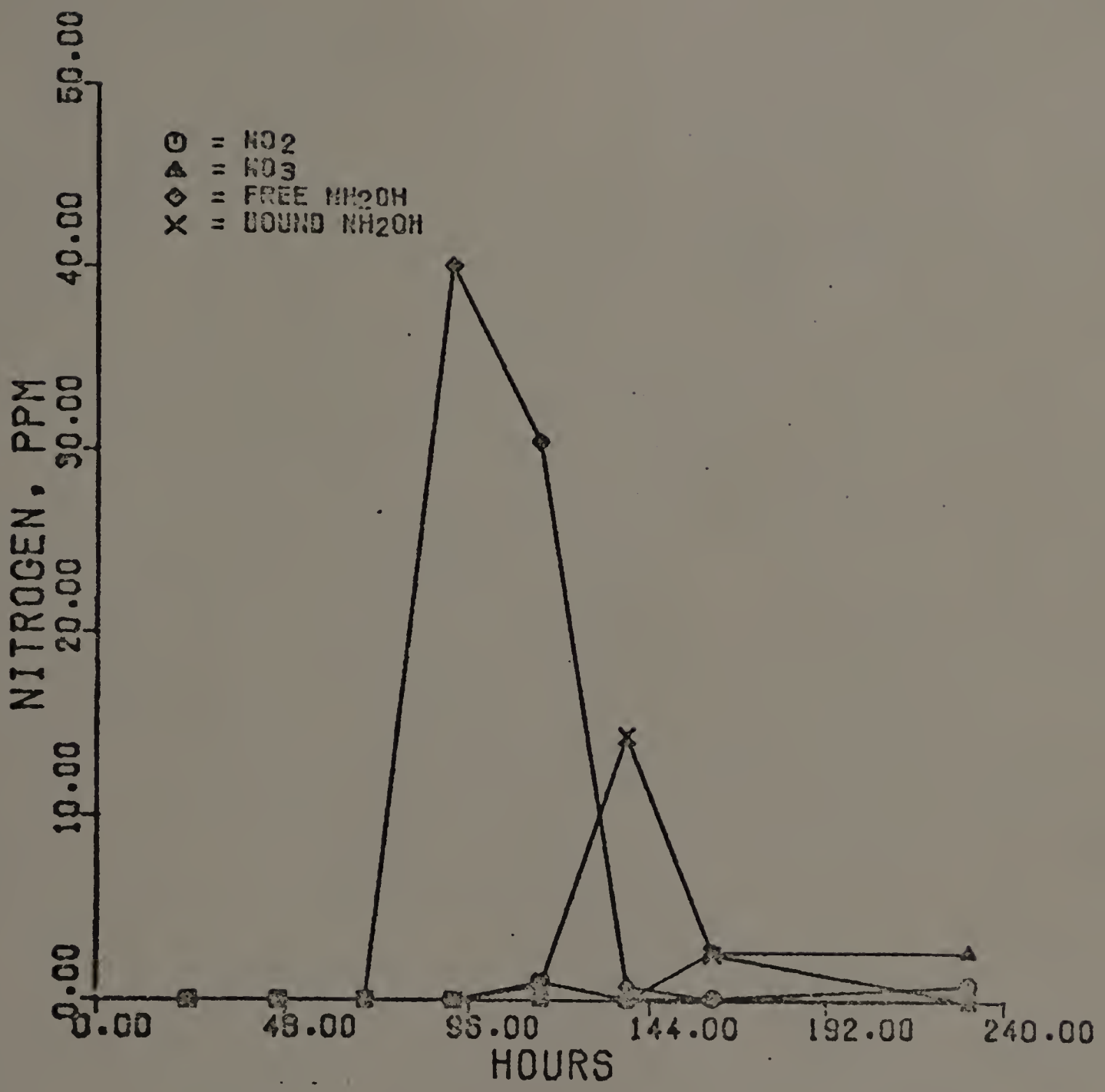


Figure 7. Accumulation of nitrification pruducts by P-3 when grown with acetate as carbon source.

Table 15. Nitrification and related growth parameters for P-3 when grown with succinate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3 (ppm)	%T	Dry wt. (ug/ml)
24	0.0	0.0	0.0	0.0	96	
48	0.0	0.0	0.0	0.0	69	1.5
72	22.8	1.5	0.0	0.0	9	5.9
96	38.6	0.0	0:0	0.0	4	10.4
120	22.5	7.5	0.7	0.0	3	11.4
144	3.4	20.9	0.9	0.0	4	11.3
168	0.1	0.0	0.6	5.7	4	10.8
240	0.0	0.0	0.9	10.5		

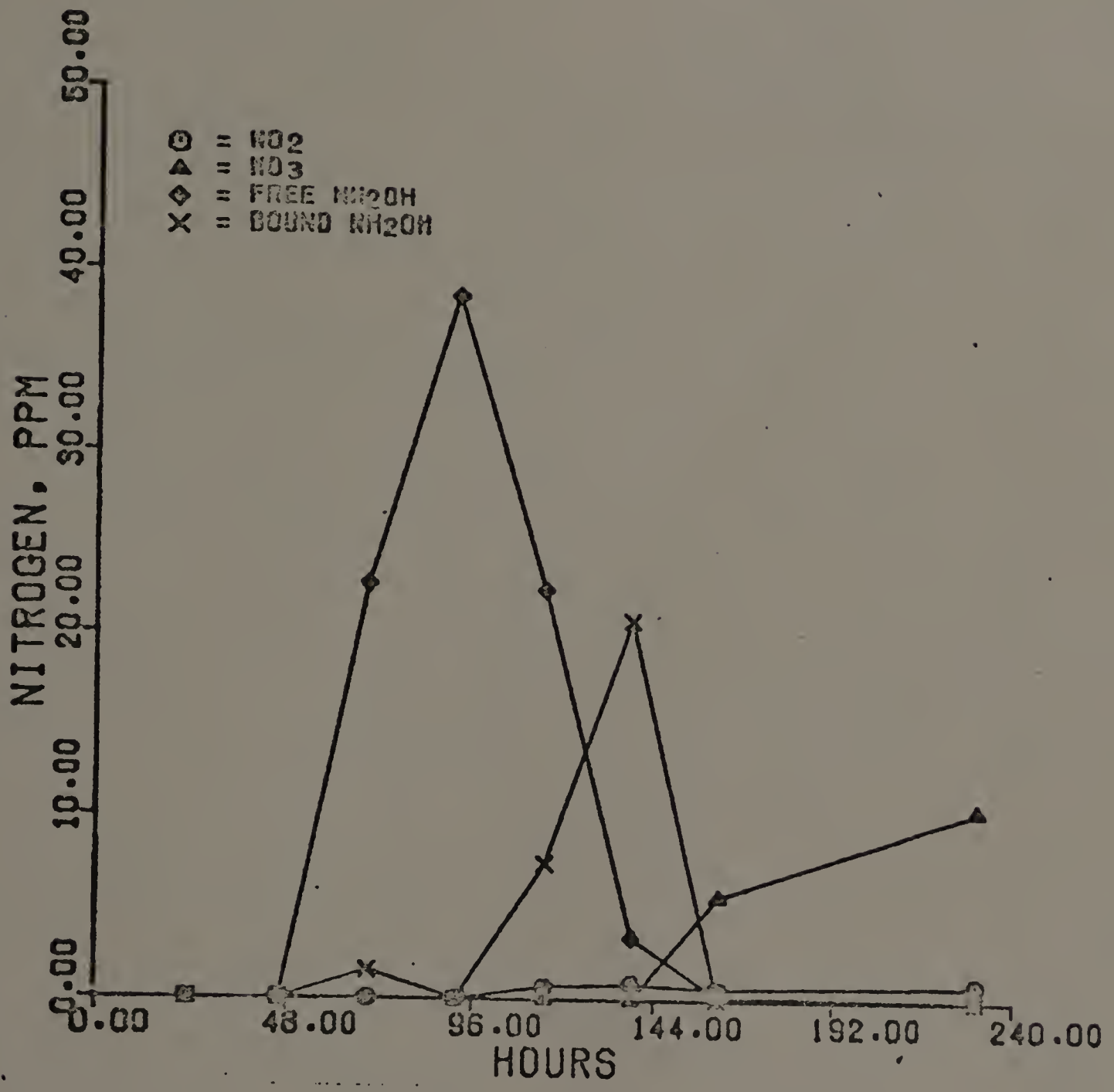


Figure 8. Accumulation of nitrification products by P-3 when grown with succinate as carbon source.

Table 16. Nitrification and related growth parameters for P-3 when grown with citrate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3 (ppm)	%T	Dry wt. (ug/ml)
24	0.0	0.0	0.0	0.0	100	
48	0.0	0.0	0.0	0.0	100	
72	0.0	0.0	0.0	0.0	94	0.4
96	0.0	0.0	0.0	0.0	39	2.2
120	18.6	0.0	0.0	0.0	5	13.7
144	40.2	0.0	0.0	0.0	5	13.7
168	0.1	0.0	10.3	13.0	3	10.6
240	0.0	0.0	10.3	12.4		

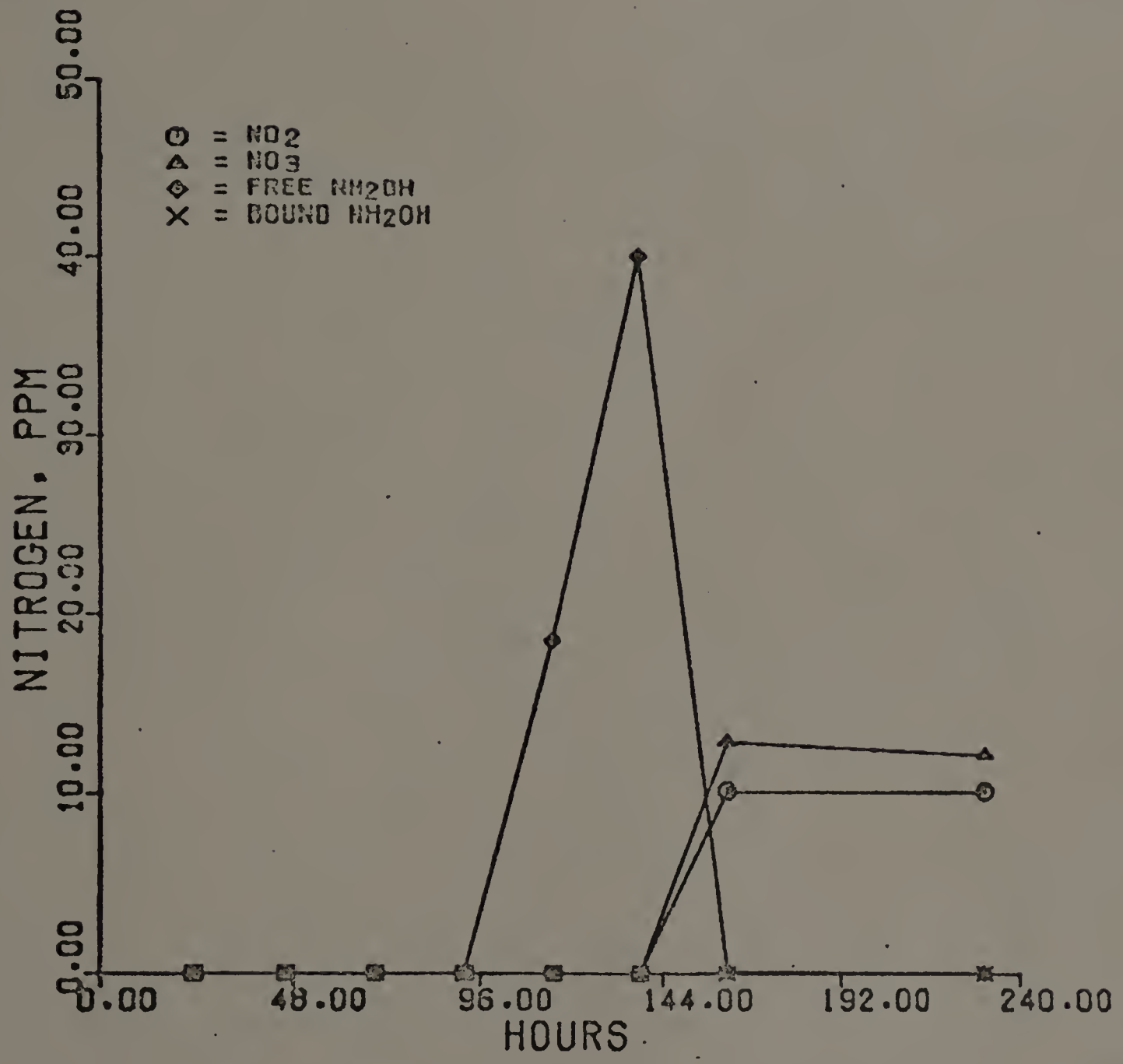


Figure 9. Accumulation of nitrification products by P-3 when grown with citrate as carbon source.

This sequence takes less than 72 hours from beginning to end and the high value attained represents only one point on a bell shaped curve. Sample aliquots would have to be taken at short intervals in order to adequately extrapolate the peak of the curve. Bound hydroxylamine was observed only in media with acetate or succinate as the carbon source. Nitrate and nitrite were present in nearly equal proportions in the citrate flask, a maximum of between 10 and 12 ppm each. In the acetate and succinate flasks nitrate reached much higher quantities in relation to nitrite 2.7 : 1.0 and 10.5 : 0.9 respectively. That this organism produced quantities of nitrate in excess of nitrite, may prove of importance in accounting for the accumulation of nitrate in natural ecosystems. The percent transmission and dry weight were also determined for this organism. The most rapid growth occurred on pyruvate, succinate, acetate and citrate respectively (figure 10). It appears that there is a correlation between growth rate and nitrification in the presence of pyruvate, acetate, and succinate. Pyruvate did not support nitrification; however, growth was most rapid. When succinate or acetate was used as a carbon source, growth was slower and nitrification was higher. Citrate produced the lowest growth rate. However, nitrification in this medium took longer to begin. It has been observed that when Q-1, another heterotrophic nitrifier, is cultured with citrate, no growth

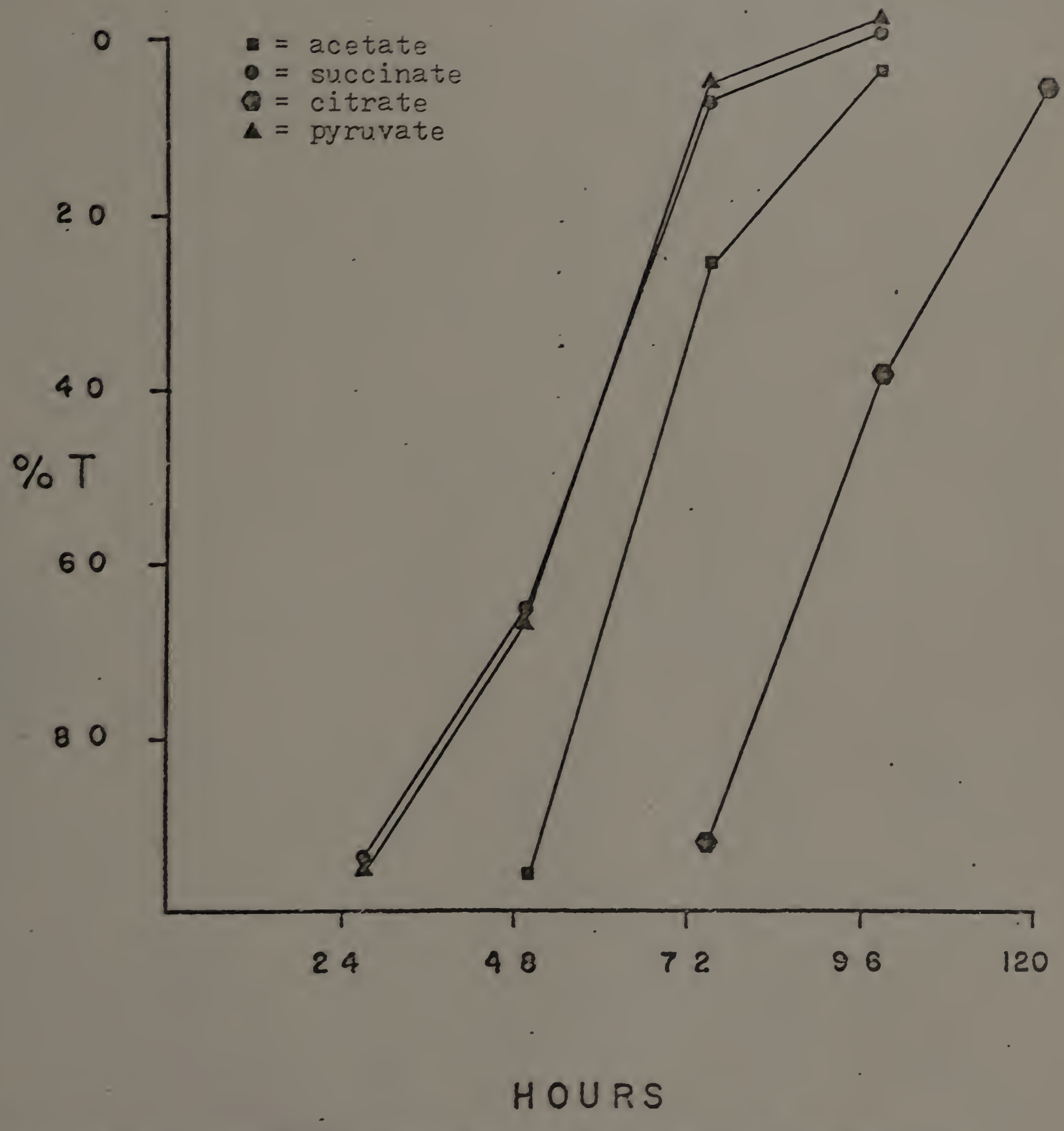


Figure 10. Growth curves of P-3 when grown with various carbon substrates.

or nitrification is observed (Rho, 1978, unpublished). The characteristic common to all three carbon sources which supported nitrification, is their presence as intermediates in the TCA cycle. This characteristic was also noted by Verstraete and Alexander (1972). An explanation of this occurrence might be found in how carbon sources affect catabolic and anabolic cell processes. It has been demonstrated that when glucose or other glycolytic components are supplied to the cell, nitrification does not occur (Verstraete and Alexander, 1972). Glucose can be easily oxidized or pyruvate reduced to any of the glycolytic, biosynthetic precursors. Pyruvate can be decarboxylated to yield acetate for direct entrance into the TCA cycle. However, when acetate is used as a carbon source, it cannot be reduced directly to pyruvate, and must go through the glyoxylate cycle in order to produce glycolytic intermediates (Stanier et al., 1976). When this occurs the alpha keto glutarate step is inhibited. Alpha keto glutarate is the skeleton for glutamate which combines with ammonium and enters into the cell as protein material. If this were the case, there would be insufficient carbon skeleton for ammonium uptake and a build up of ammonium would occur. Nitrification may be a way to detoxify the ammonium excess. This hypothesis could be tested with glutamate synthetase and internal ammonium concentration assays.

Table 17. Nitrification and related growth parameters for S-6 when grown with acetate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3 (ppm)	%T
24	0.0	0.0	0.0	0.0	99
40	0.0	0.0	0.1	0.0	33
64	12.4	0.0	0.2	0.9	4
88	24.1	2.5	6.7	1.4	
112	0.0	4.1	1.8	11.6	
136	0.0	3.5	0.7	10.6	
160	0.0	2.9	0.4	10.1	
231	0.0	3.6	0.8	2.3	

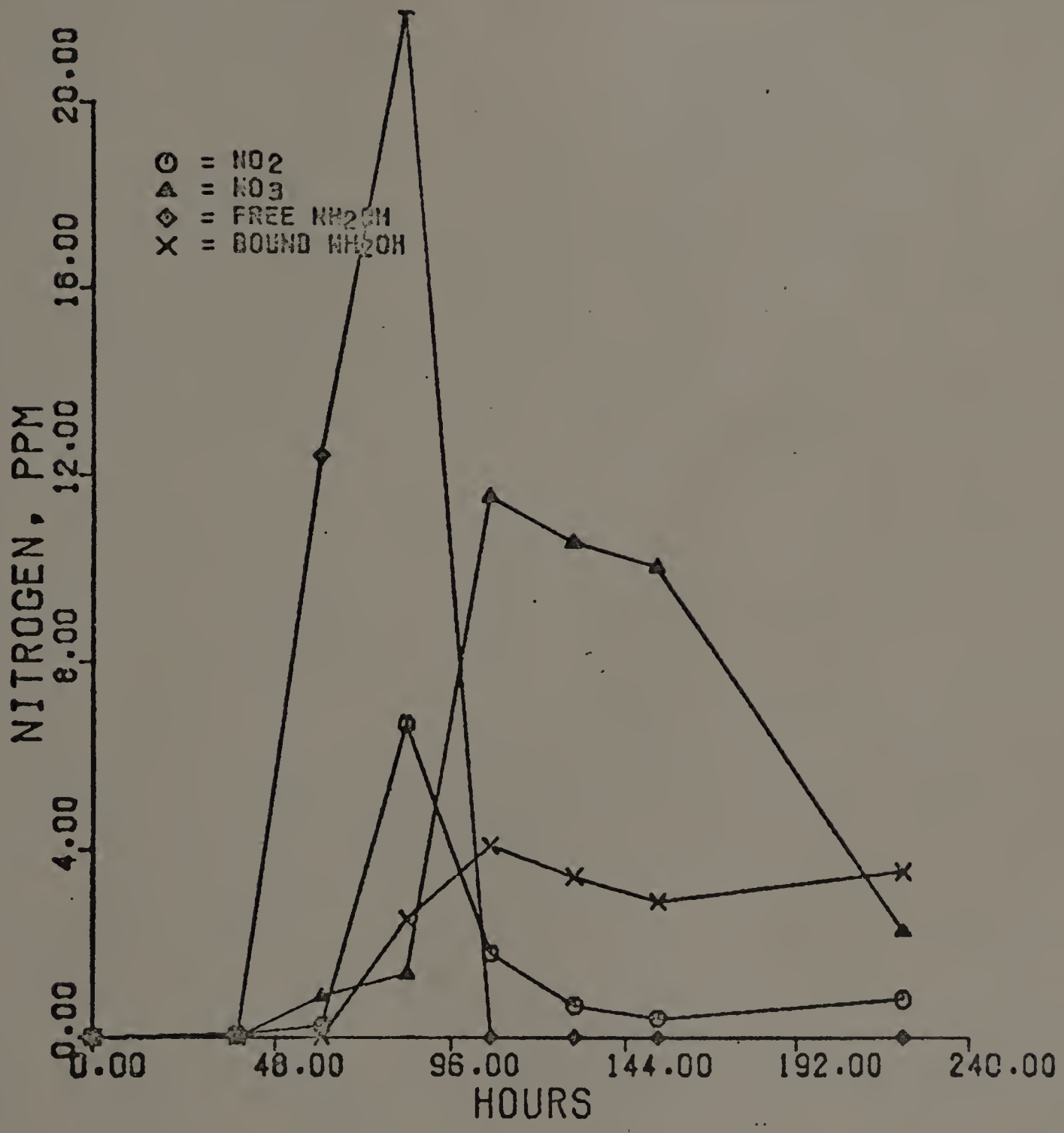


Figure 11. Accumulation of nitrification products by S-6 when grown with acetate as carbon source.

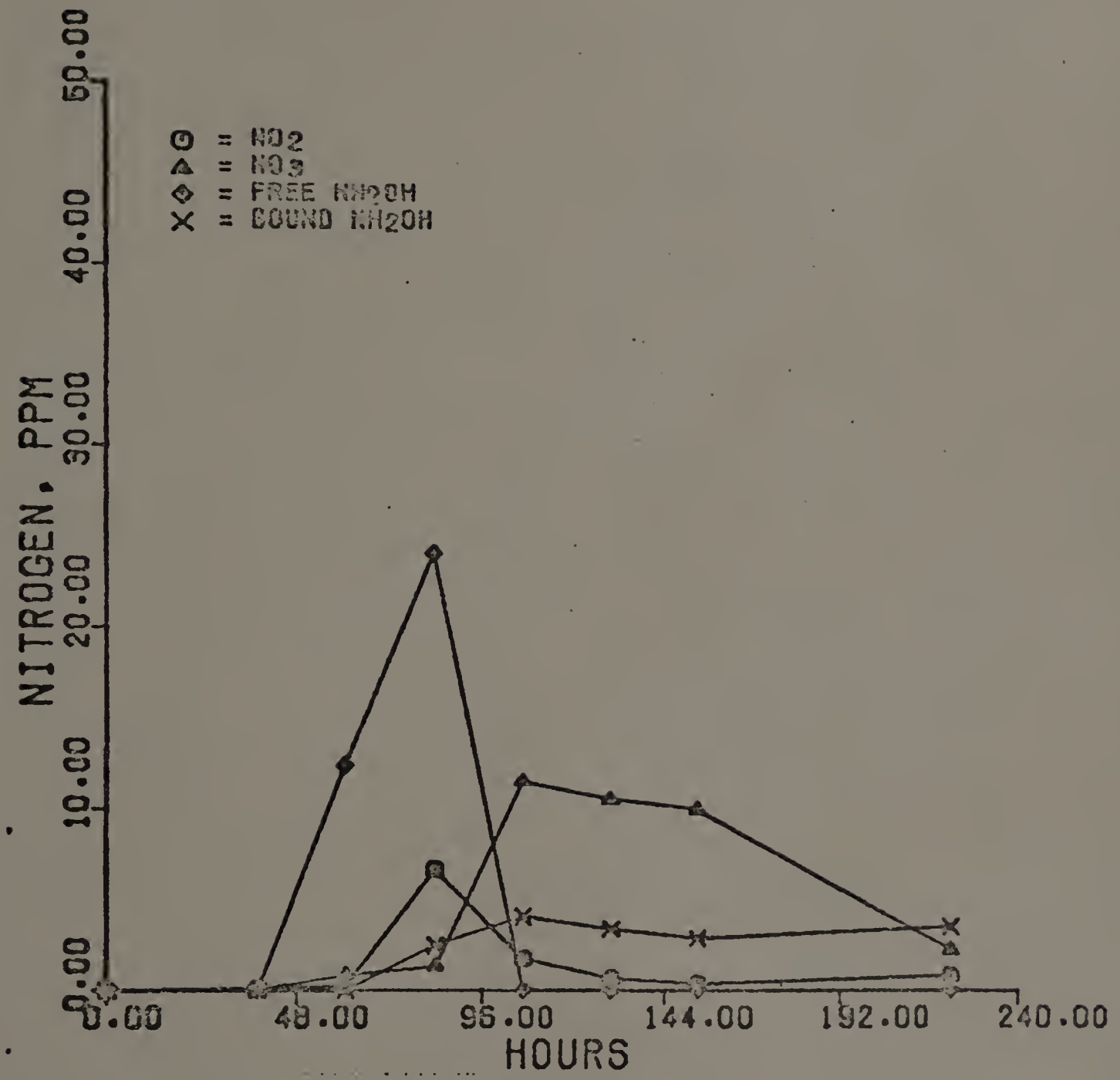


Figure 12. Same as fig. 11 with expanded Y axis.

Table 18. Nitrification and related growth parameters for S-6 when grown with succinate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3 (ppm)	%T
24	0.0	0.0	0.0	0.0	34
40	1.7	0.0	0.0	0.0	8
64	22.0	0.0	0.4	3.1	3
88	5.0	3.7	9.6	15.8	
112	0.0	11.3	0.3	10.4	
136	0.0	25.6	0.3	15.6	
160	0.0	5.6	0.3	10.6	
231	0.0	4.7	0.6	7.8	

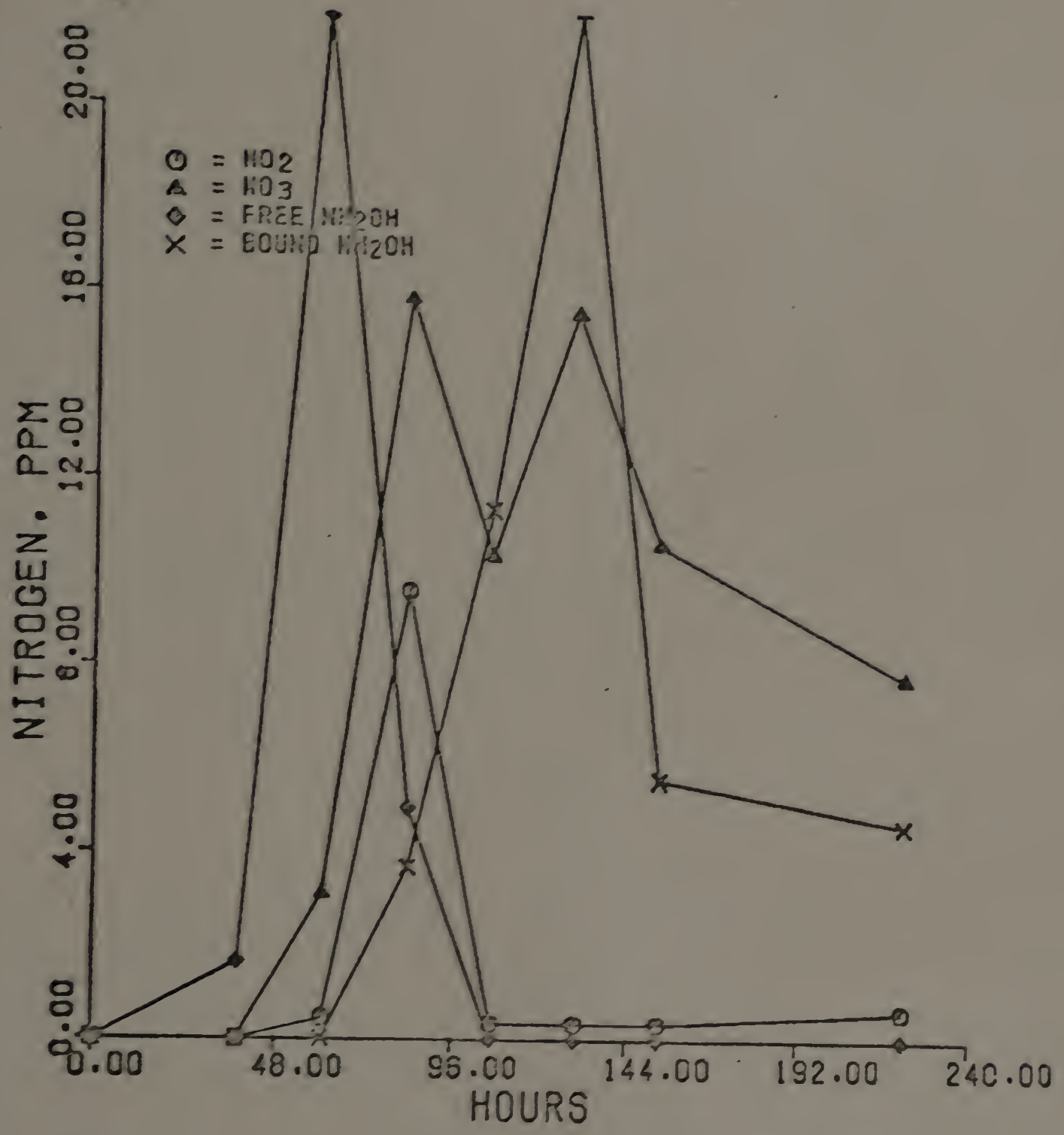


Figure 13. Accumulation of nitrification products by S-6 when grown with succinate as carbon source.

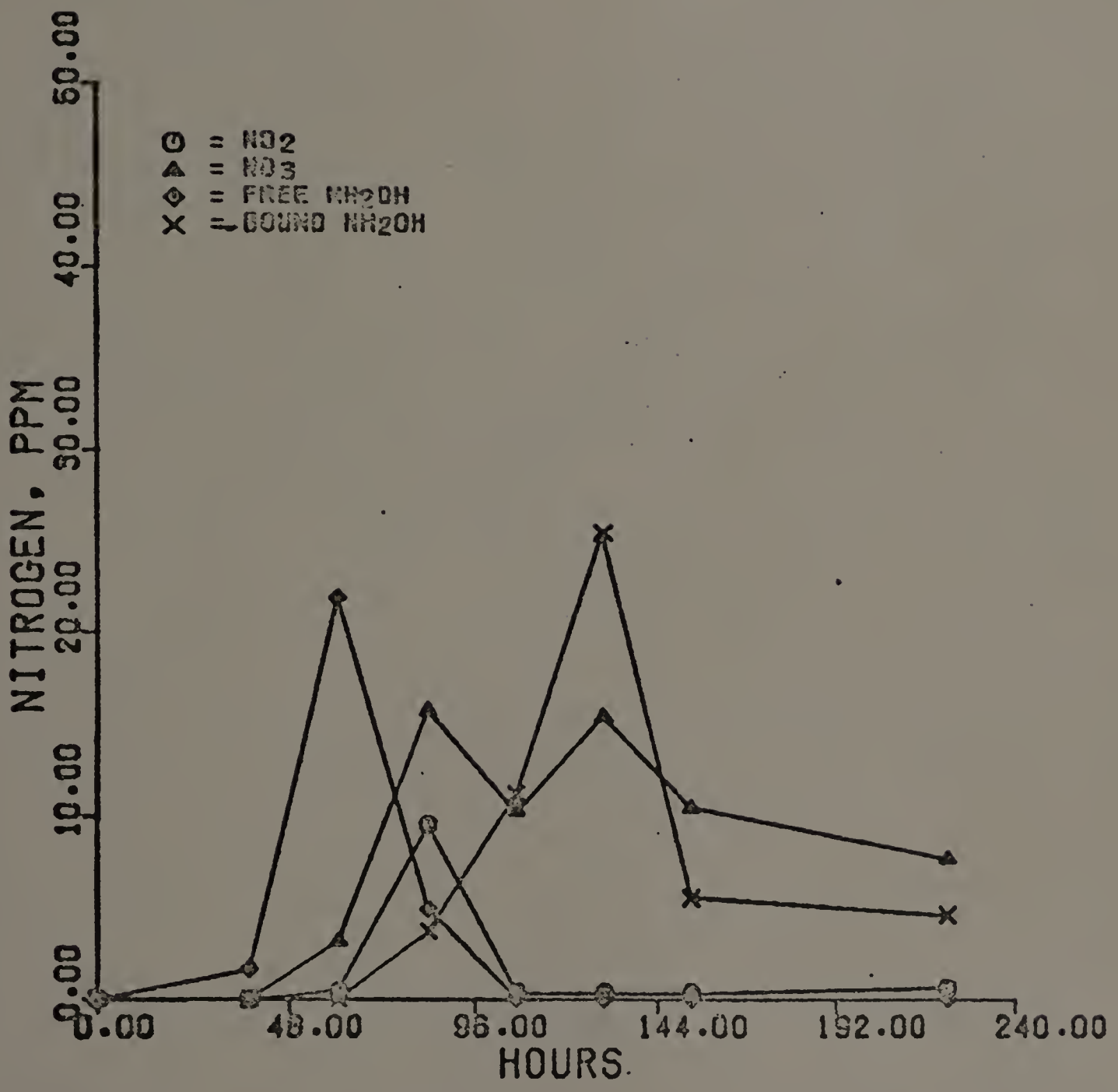


Figure 14. Same as fig. 14 with expanded Y axis.

Table 19. Nitrification and related growth parameters for S-6 when grown with citrate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3 (ppm)	%T
20	0.0	0.0	0.0	0.0	89
37	10.7	4.9	1.2	1.4	25
61	9.4		0.4	0.2	3
88	0.0	3.7	0.2	2.6	
115	0.0	3.7	0.4	2.2	
133	0.0	3.2	0.4	1.3	
229	0.0	2.1	0.6	2.0	

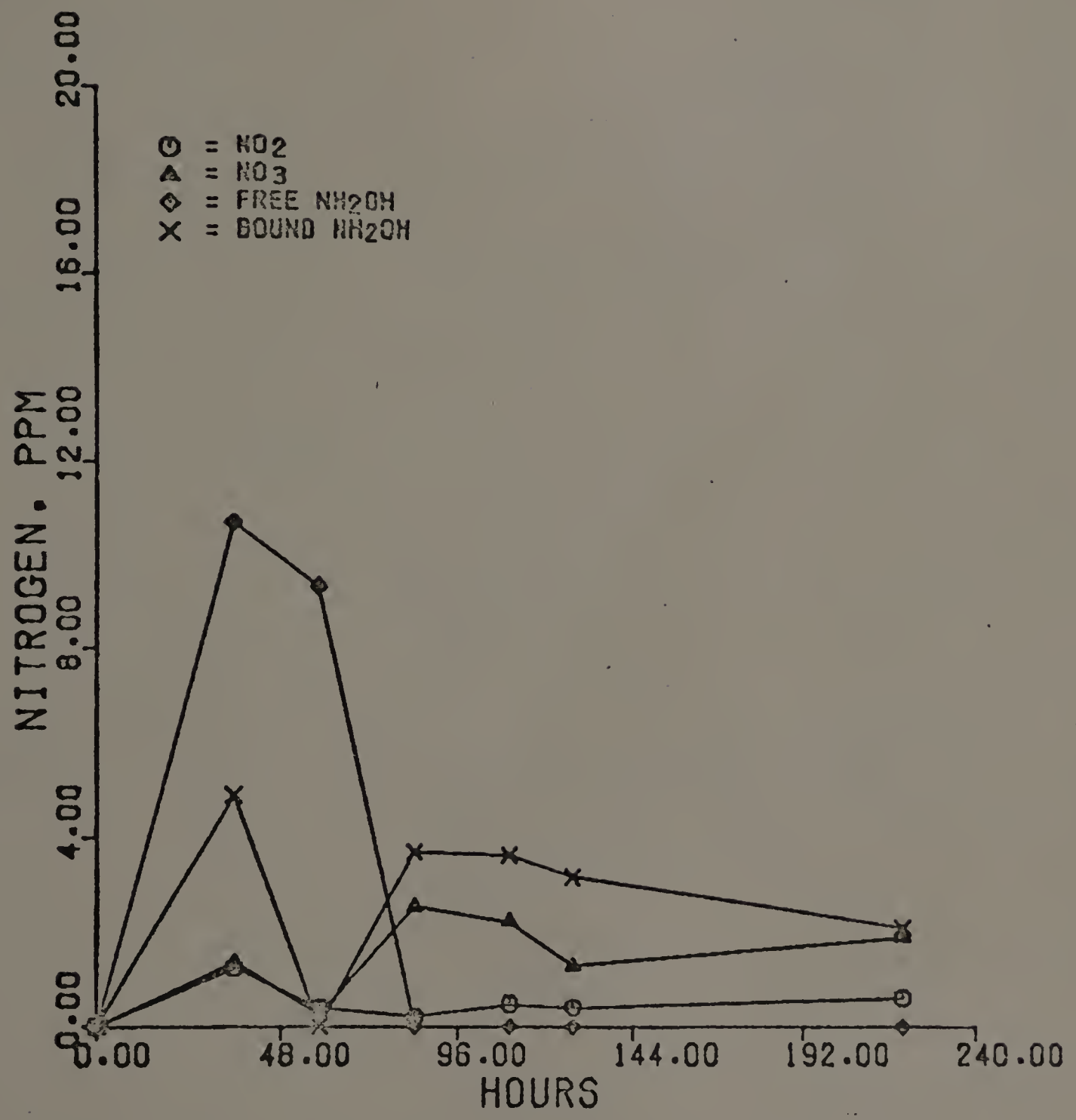


Figure 15. Accumulation of nitrification products by S-6 when grown with citrate as carbon source.

Table 20. Nitrification and related growth parameters for S-6 when grown with pyruvate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2^- (ppm)	NO_3^- (ppm)	%T
20	0.0	0.0	0.0	0.0	55
37	0.0	0.0	0.0	0.0	9
61	0.0	0.0	0.0	0.0	2
88	1.5	0.0	0.0	0.0	
115	0.0	1.3	0.8	4.8	
133	0.0	1.2	0.7	4.3	
229	0.0	0.7	0.8	4.3	

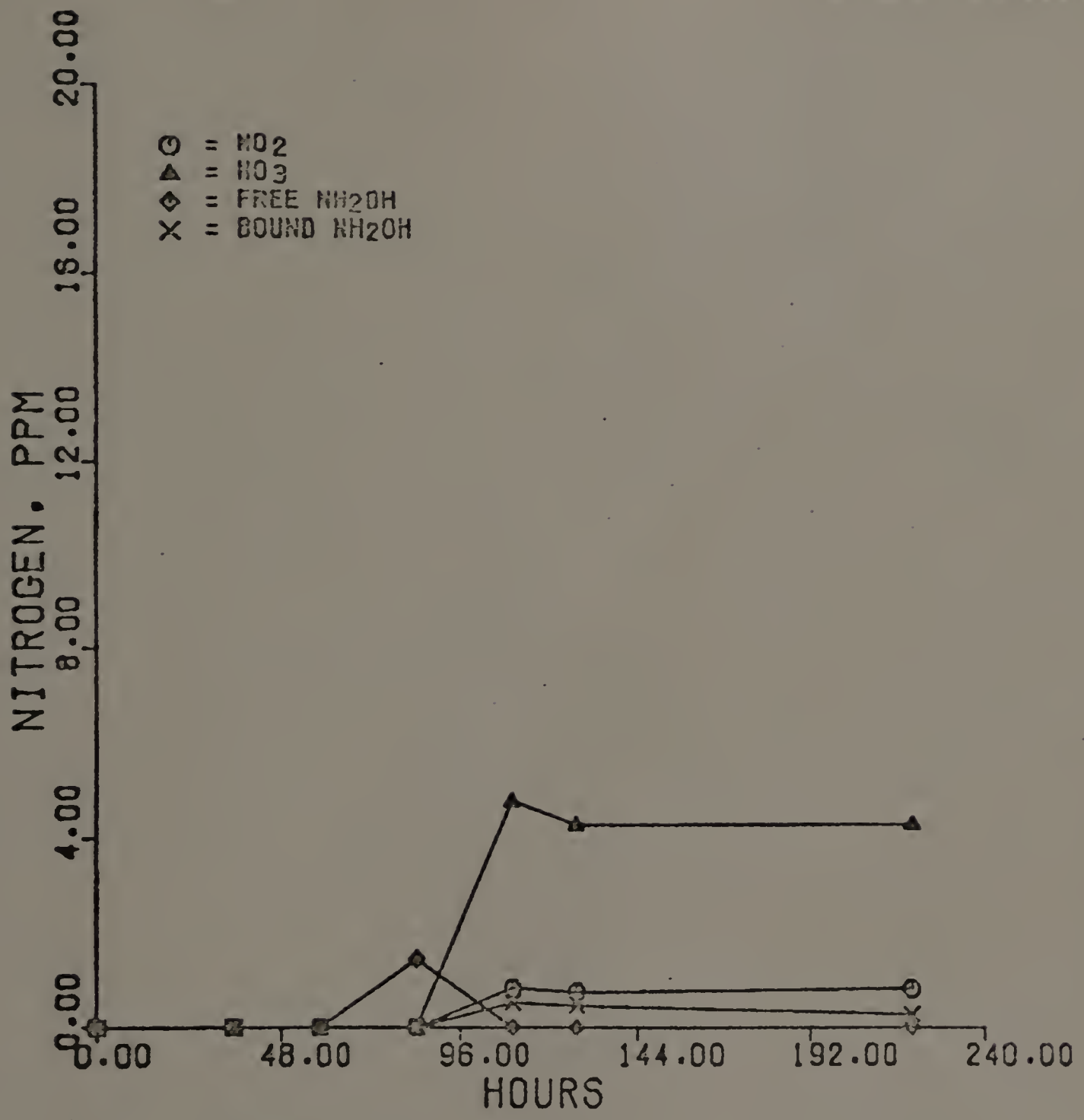


Figure 16. Accumulation of nitrification products by S-6 when grown with pyruvate as carbon source.

The most active organism isolated (table 17-30, fig. 11-16) was designated S-6. Nitrification with acetate, citrate, and succinate as carbon source was high, and alone among the isolates it demonstrated the ability to nitrify with pyruvate as a carbon substrate. With acetate, free hydroxylamine concentration attained a peak of 24.1 ppm in a 48 hour pulse beginning at 48 hours. Nitrite reached an early peak of 6.7 ppm and dropped off rapidly to a low level. Nitrate accumulated to 11.8 ppm and remained at this level for three days dropping sharply at the last sample period. Bound hydroxylamine climbed to 4.1 ppm and remained close to that concentration. Activity with succinate as a carbon source was not appreciably different from that with acetate, however concentrations of most products, except hydroxylamine, accumulated to high levels. Free hydroxylamine rose to 22.0 ppm and nitrate to 15.8 ppm. The major difference came when, at 96 hours, the concentration of nitrate fell to nearly 10 ppm and bound hydroxylamine rose to 25.6 ppm. Nitrite exhibited an early pulse to 9.6 ppm at 72 hours and again fell to very low levels. The graph of S-6 with citrate is different than those of the succinate and acetate grown cultures. The early peak of free hydroxylamine is only half the concentration of succinate and acetate. Bound hydroxylamine was second to free hydroxylamine in concentration (5.0 ppm). The yield of nitrate was similar but lower in concentration. An unexplained drop in the bound hydroxylamine

concentration to 0 ppm was noted at 61 hours. At this time the bound hydroxylamine test turned orange instead of green and could not be read. At the next sample period it rose again to the expected level. Nitrate concentration also dropped conspicuously at this time. Nitrite as usual was low. An atypical nitrification reaction was observed with pyruvate. A trace amount of free hydroxylamine was present at 88 hours dropping to 0 ppm at the next period with a concomitant rise in the other three products. Bound hydroxylamine and nitrite were present in low concentration with more nitrite present than bound hydroxylamine. This was not typical in that nitrate was the dominant product at a level of 4.5 ppm. Although this is a moderate concentration for nitrate accumulation, it is quite high in relation to that obtained by other workers. The lag phase prior to any accumulation was also atypical.

The majority of the nitrifying isolates were typified by S-7 (table 21-23, fig. 17-19). Free hydroxylamine reached a peak of 6.0 ppm at 63 hours and receded rapidly with concomitant rise in nitrate and bound hydroxylamine with traces of nitrite. With this organism, nitrate concentration was highest for the longest period of time and bound hydroxylamine was present in lower concentration. The occurrence of the two intermediates appears to be related. The concentration of each remained stable after the 111 hour sample period. When this organism was grown with citrate as the

Table 21. Nitrification and related growth parameters for S-7 when grown with acetate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3 (ppm)	%T
24	0.0	0.0	0.0	0.0	99
40	0.0	0.0	0.1	0.0	33
64	6.0	0.0	0.1	0.7	12
88	2.5	1.3	0.7	2.7	3
112	0.0	2.7	0.0	3.4	
136	0.0	2.6	0.2	4.4	
160	0.0	2.5	0.2	3.0	
231	0.0	2.3	0.5	4.0	

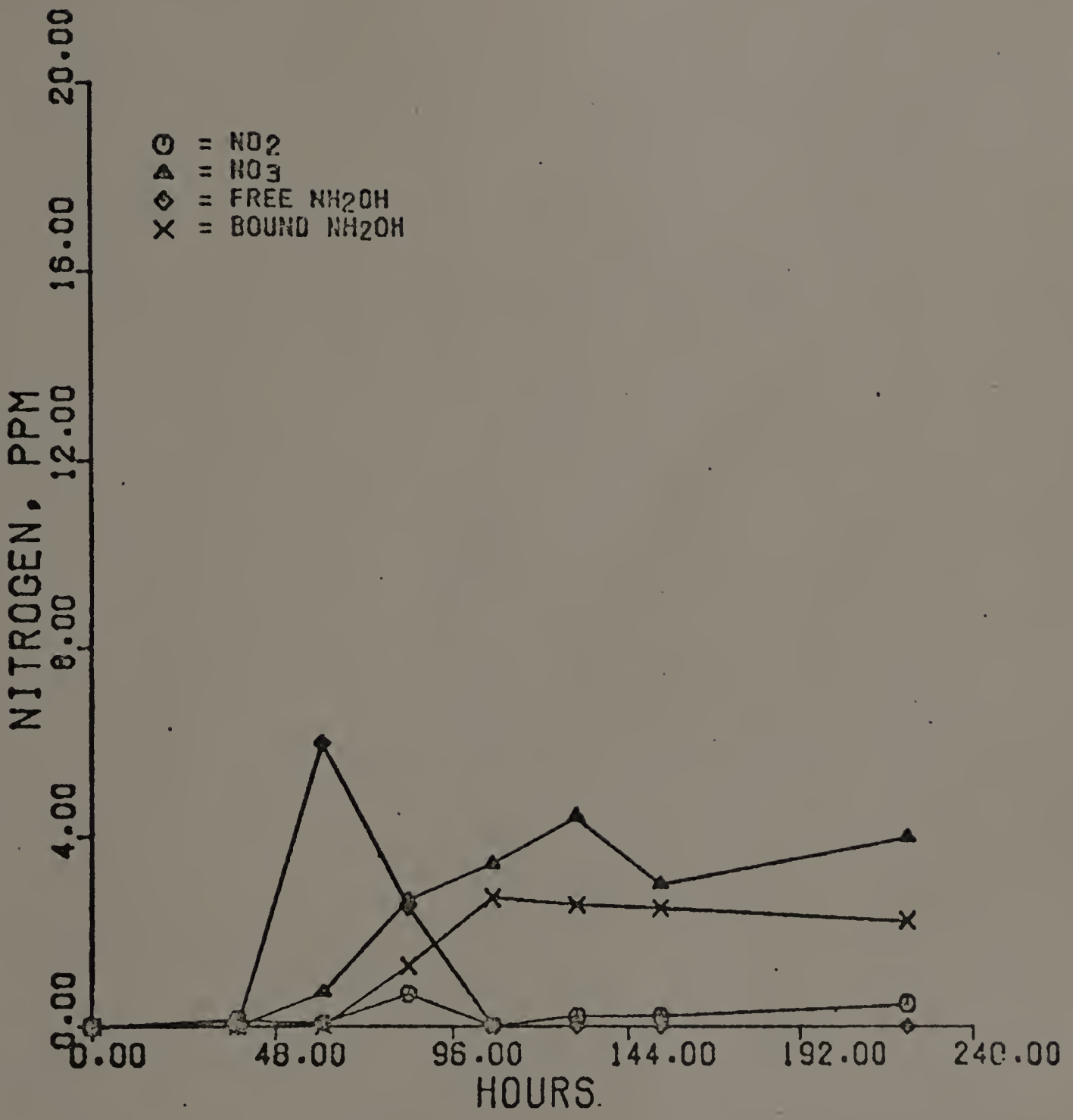


Figure 17. Accumulation of nitrification products by S-7 when grown with acetate as carbon source.

Table 22. Nitrification and related growth parameters for S-7 when grown with succinate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2^- (ppm)	NO_3^- (ppm)	%T
24	0.0	0.0	0.0	0.0	51
40	1.0	0.0	0.0	0.0	8
64	15.5	0.0	0.2	1.0	3
88	2.0	3.3	0.5	6.6	
112	0.0	4.1	0.3	5.1	
136	0.0	4.5	0.3	7.4	
160	0.0	3.8	0.3	5.3	
231	0.0	3.2	0.5	4.3	

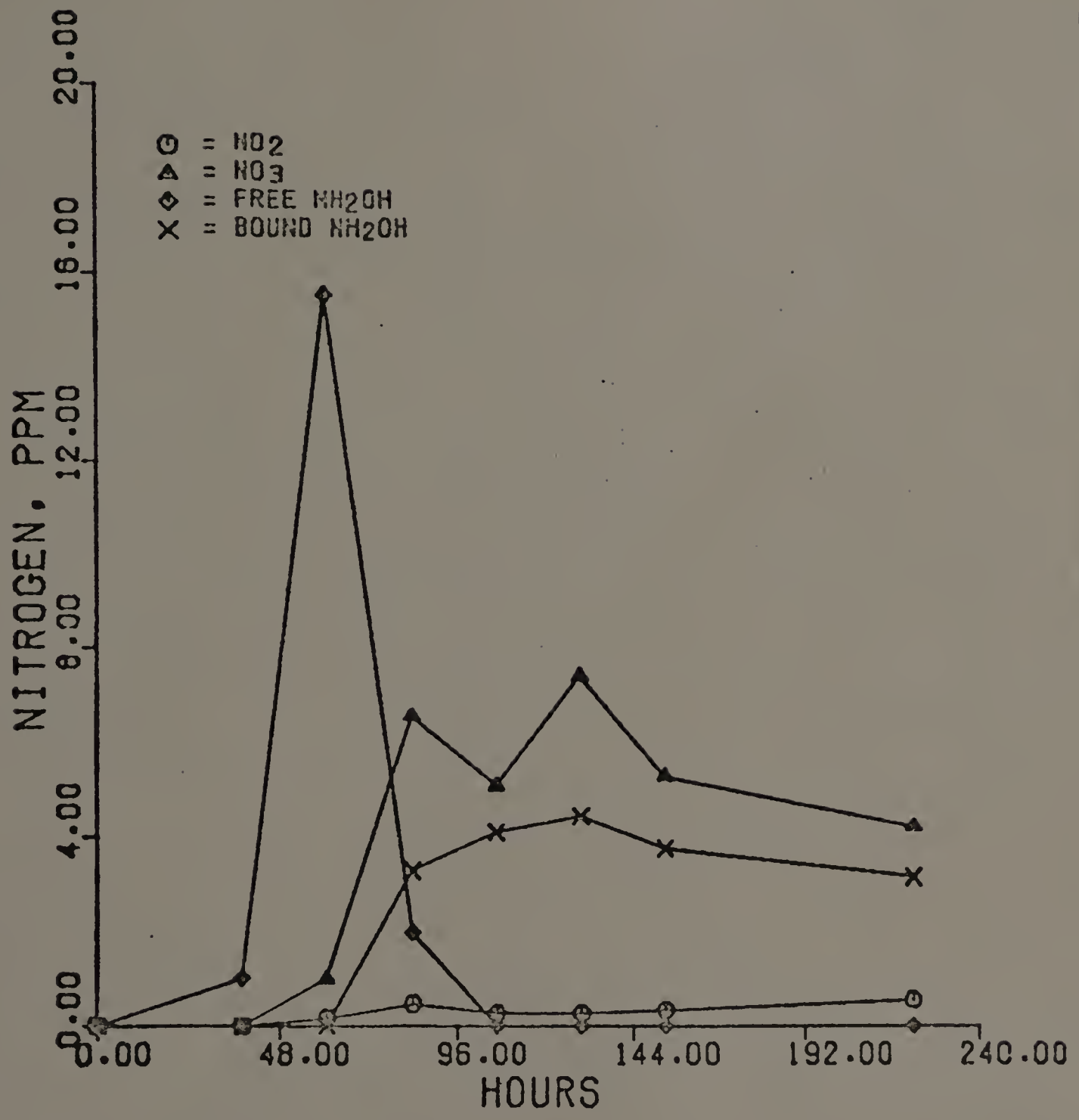


Figure 18. Accumulation of nitrification products by S-7 when grown with succinate as carbon source.

Table 23. Nitrification and related growth parameters for S-7 when grown with citrate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3 (ppm)	%T
20	0.0	0.0	0.0	0.0	97
37	5.6	0.7	0.0	0.0	25
61	0.7	0.7	0.0	0.0	2
88	0.0	0.9	0.0	0.0	
115	0.0	0.8	0.0	0.0	
133	0.0	0.7	0.0	0.0	
229	0.0	0.6	0.0	0.0	

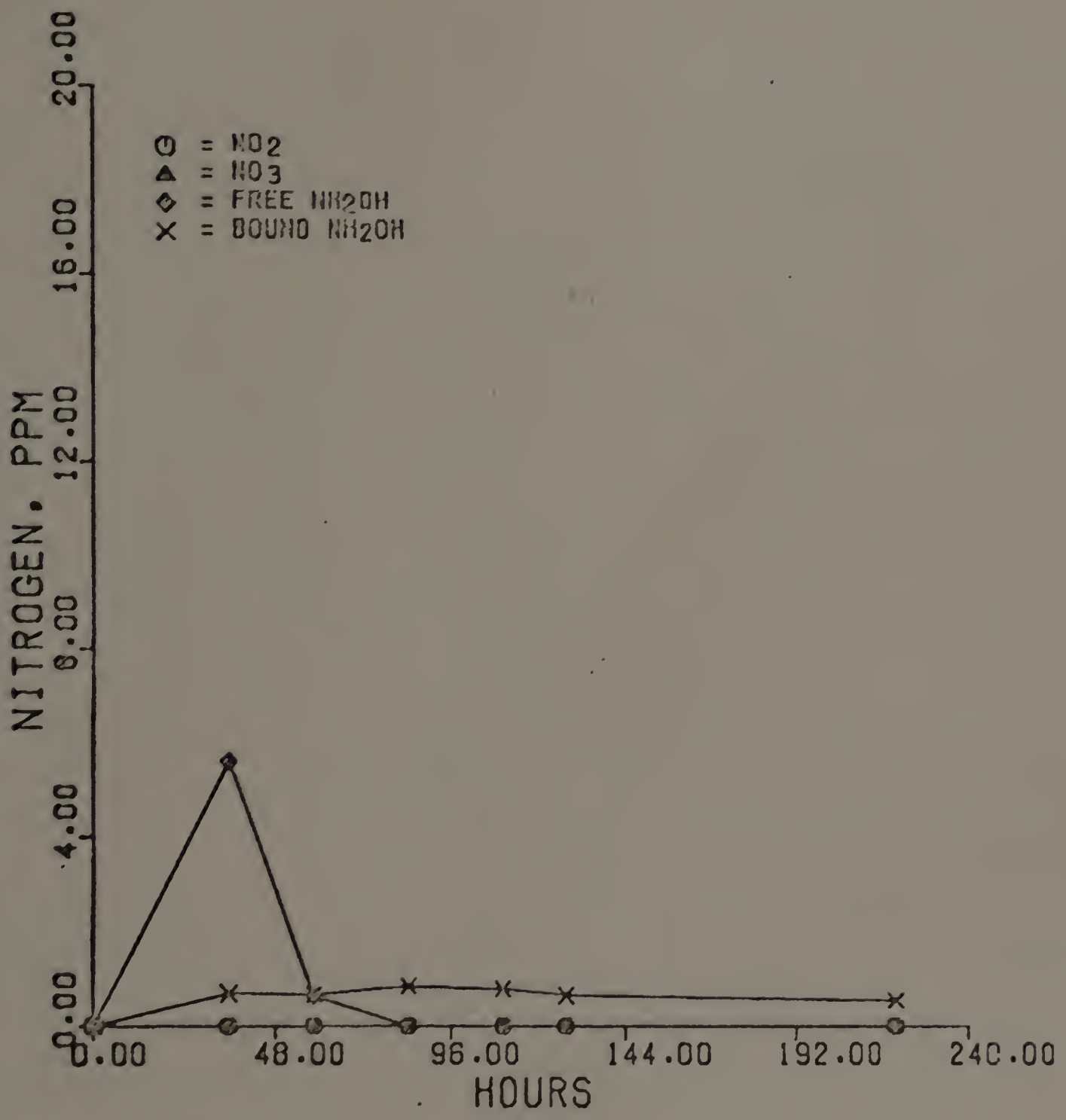


Figure 19. Accumulation of nitrification products by S-7 when grown with citrate as carbon source.

Table 24. Nitrification and related growth parameters for S-9 when grown with acetate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3 (ppm)	%T
24	0.0	0.0	0.0	0.0	99
40	0.0	0.0	0.2	0.0	33
64	4.8	0.0	0.0	1.1	14
88	5.8	0.6	0.7	4.4	4
112	0.0	2.5	0.3	4.1	
136	0.0	2.4	0.2	4.7	
160	0.0	2.4	0.3	4.3	
231	0.0	2.1	0.8	10.9	

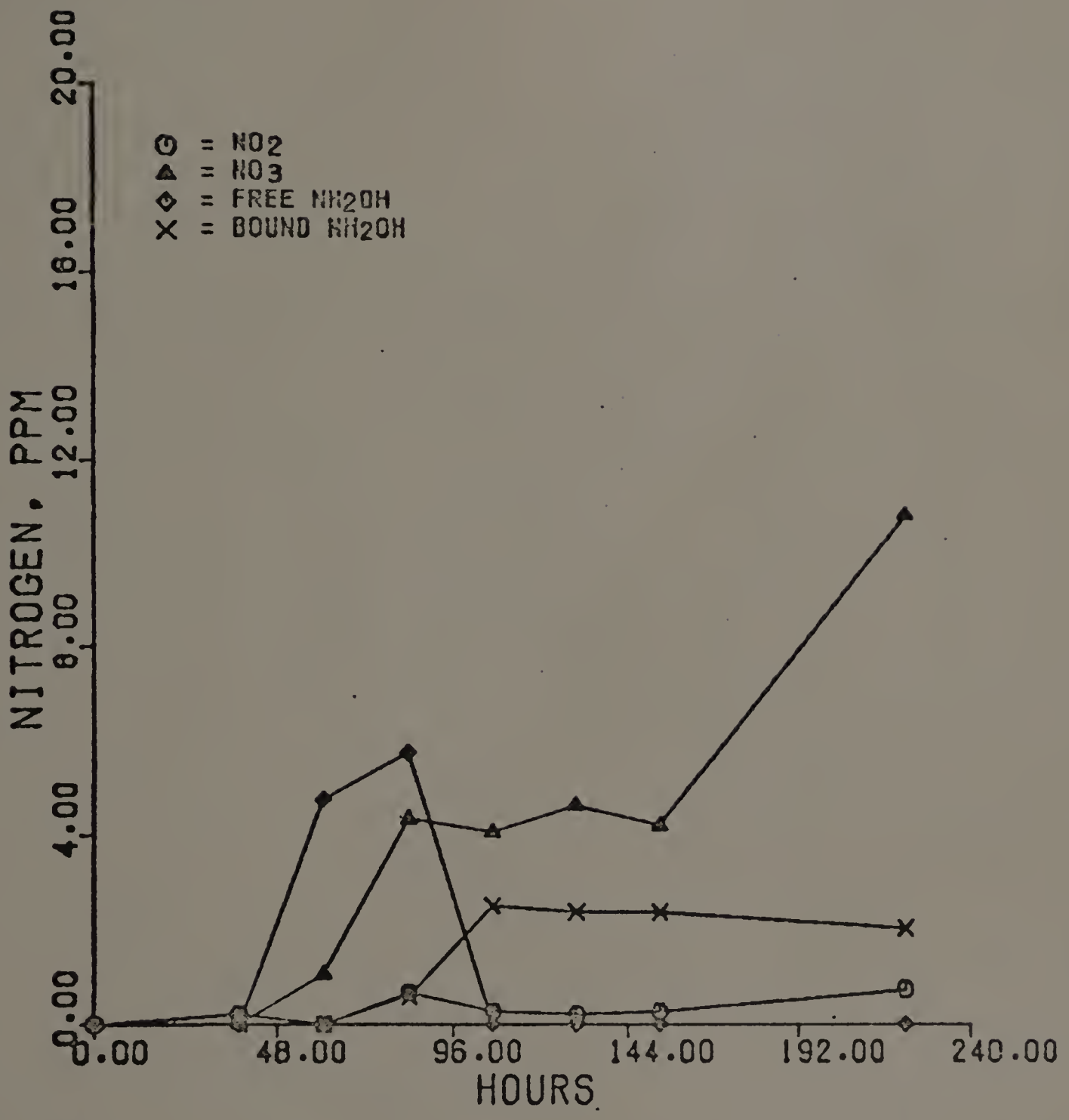


Figure 20. Accumulation of nitrification products by S-9 when grown with acetate as carbon source.

Table 25. Nitrification and related growth parameters for S-9 when grown with succinate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2^- (ppm)	NO_3 (ppm)	%T
24	0.0	0.0	0.0	0.0	36
40	0.9	0.0	0.0	0.0	7
64	0.5	0.0	0.0	0.0	2
88	0.4	0.4	0.0	0.0	
112	0.0	0.6	0.0	0.0	
136	0.0	0.7	0.0	0.0	
160	0.0	0.5	0.0	0.0	
231	0.0	0.5	0.0	0.0	

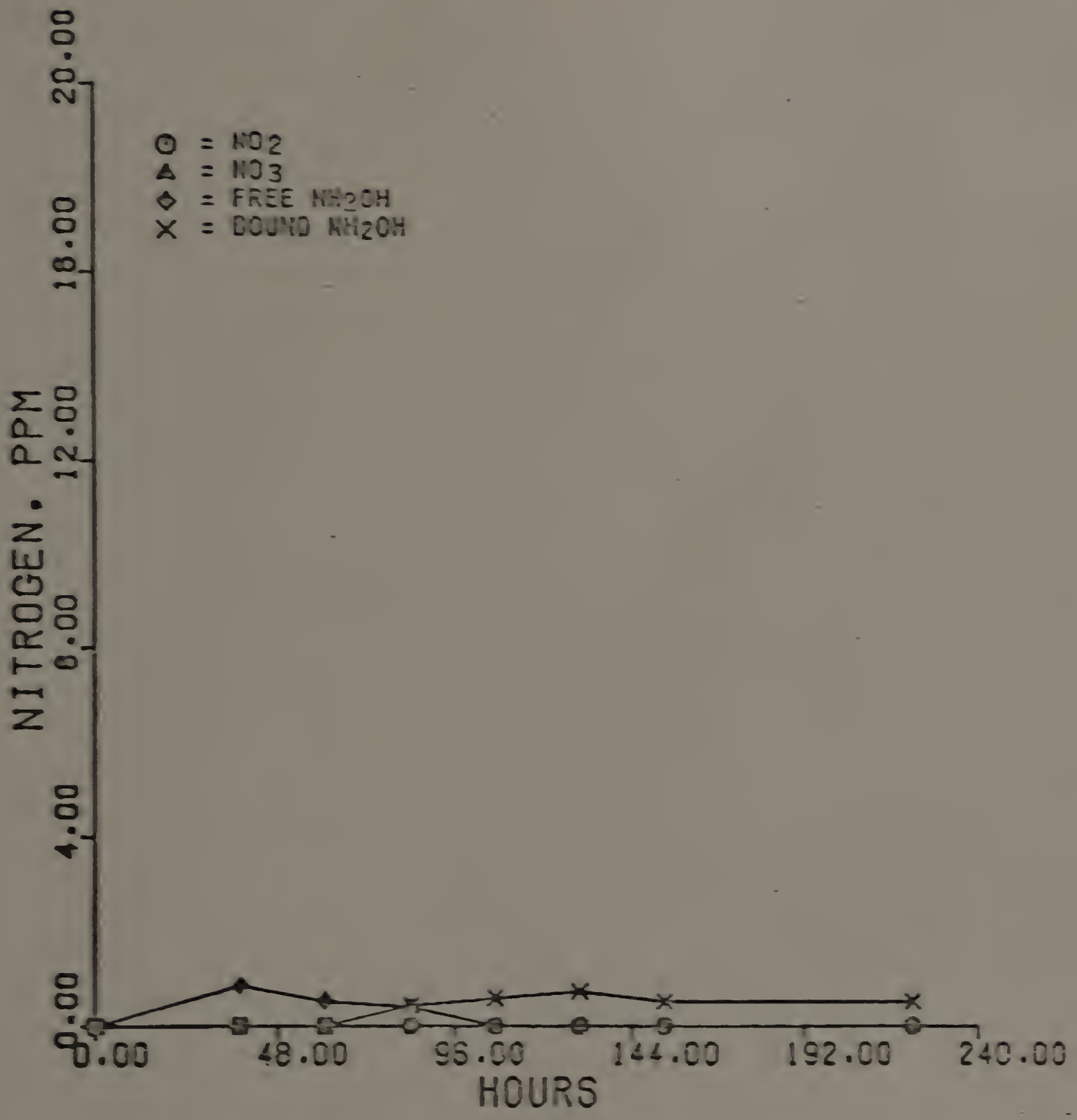


Figure 21. Accumulation of nitrification products by S-9 when grown with succinate as carbon source.

Table 26. Nitrification and related growth parameters for S-9 when grown with citrate as carbon source.

Hours	f NH_2OH (ppm)	b NH_2OH (ppm)	NO_2 (ppm)	NO_3 (ppm)	%T
20	0.0	0.0	0.0	0.0	92
37	13.1	0.0	0.5	0.7	26
61	11.5	1.8	0.1	0.5	2
88	0.0	3.1	0.1	1.9	
115	0.0	2.8	0.5	3.4	
133	0.0	2.4	0.4	2.7	
229	0.0	1.4	0.8	3.0	

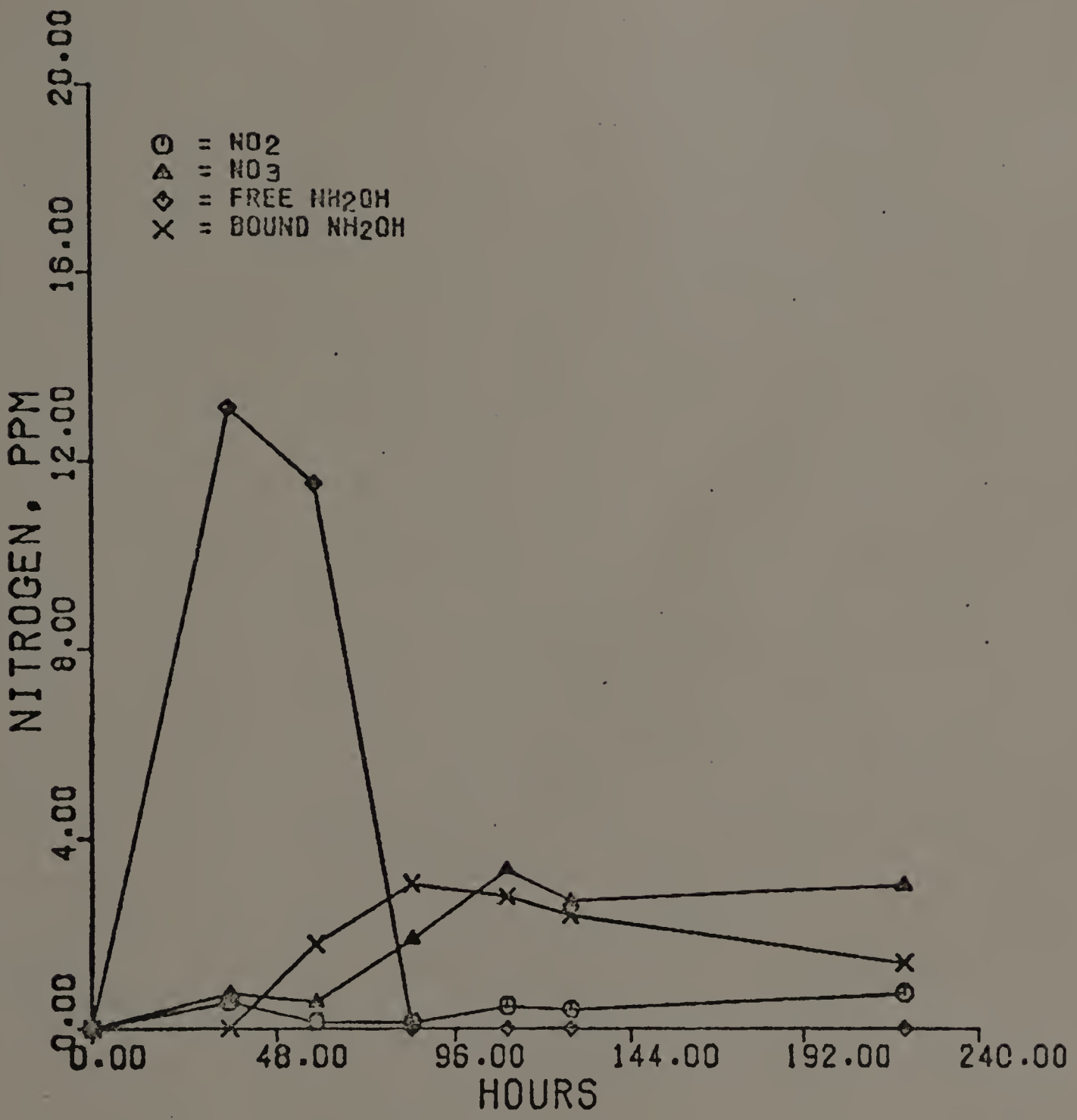


Figure 22. Accumulation of nitrification products by S-9 when grown with citrate as carbon source.

carbon source the two forms of hydroxylamine were the only two products which accumulated to any extent. As mentioned, this pattern was common to the bulk of the isolates. This may be described as a "citrate" effect and does not appear to fit in the same category as acetate or succinate.

Isolation of S-9 was performed with pyruvate as the carbon source enrichment medium (table 24-26, fig. 20-22). This organism did not nitrify substantially in the presence of succinate in comparison with acetate or citrate. Nitrification data for the activity of S-1, 4, 5, 8, and 11 are included in appendix B.

If heterotrophic nitrification is operative under natural conditions, the carbon sources available may significantly affect the nature of the process. Vallentyne (1956) compiled a review of organic matter in waters and sediments. It was reported that glucose, sucrose, citric, and malic acids had been found in waters. However, analytical techniques were not direct enough for adequate substantiation. Since that time, the development of radioisotope techniques has led to a greater understanding of the status of the carbon nutrient fraction (Hobbie, 1972). In a Manitoba lake (Robinson et al., 1973), the velocity of uptake on a variety of organic acids was shown to be correlated with bacterial plate counts made on a mineral salts medium with individual organic acids added. Succinic acid supported the highest proportion of

bacteria, acetic and citric, and pyruvic acids correlated with bacterial counts but that for succinic did not. Appropriately, the V(max) of acetate and succinate were very close, 0.031 and 0.028 respectively, while pyruvate and citrate were at opposite ends of the scale with 0.077 and 0.008. Berland et al. (1976) also determined that succinate and pyruvate were the most acceptable of organic acids screened on 220 bacteria isolated from surface waters.

CONCLUSIONS AND RECOMMENDATIONS

Significant levels of nitrate and ammonium were present in Pontoosuc Lake during the study period. The concentrations of ammonium were inversely related to oxygen concentration and were highest in the anoxic waters of summer stagnation. No observable pattern in annual nitrate variation was apparent. Autotrophic nitrifying bacteria (Nitrosomonas sp.) were not detected in the majority of the samples. There was conspicuous activity on two occasions however. One occurred in September of 1976 and the other in May of 1977. If the activity of autotrophic nitrifying bacteria is based on the occurrence of high numbers, the autotrophs are not responsible for in situ nitrification in this lake.

Heterotrophic nitrifying bacteria were also present in the lake water. However, a quantitative value for the population was not determined. Succinate, when used as a carbon source, was effective for detection of heterotrophic nitrifier activity whereas acetate was not. Enrichment of lake water with ammonium succinate broth, and subsequent culture on ammonium acetate agar, was proven as a simplified method of isolation for this water body. Upon biochemical and morphological examination, all organisms appeared to

be Arthrobacter sp. varying only in nitrification pattern or quantity when grown in broth media with acetate, citrate, pyruvate, or succinate as carbon sources. One organism, S-6, was particularly active in accumulating high concentrations of free and bound hydroxylamine, and nitrate. Its activity was highest when grown with acetate or succinate. Pyruvate was acceptable as a carbon source for nitrification by this organism alone, with nitrate being the dominant product. The general pattern of nitrification by all isolates consisted of an early pulse of free hydroxylamine accumulation a comparatively insignificant accumulation of nitrite, and a concomitant rise in nitrate and bound hydroxylamine as the final phase, corresponding to the stationary phase of the growth curve. The concentration of nitrate generally exceeded that of bound hydroxylamine by two to one.

In order to further investigate the role of heterotrophic nitrifying bacteria, studies must be performed to develop a standardized, efficient, enumeration procedure. Only then will population dynamics as they relate to fluctuations in environmental variables become possible to assess. The activity of heterotrophic nitrifier pure cultures under the activity of controlled environmental parameters including carbon and nitrogen sources also deserve further consideration.

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APPENDIX A

```
00100 PROGRAM TEC(INPUT-OUTPUT,TAPE1,TAPE9-OUTPUT,TAPE8=INPUT)
00110 CALL(PLOTTER)
00120 DEF(PLOTTER)
00130 DIMENSION X(12),Y(12,5),YY(8)
00140 CALL PLOTS(XDUM,YDUM)
00150 CALL PLOT(0.5,0.5,-3)
00160 INPUTS=8
00170 READ (1,9)(X(I),I=1,INPUTS),((Y(I,J),I=1,INPUTS),J=1,5)
00180 REWIND 1
00190 DO 1 I=1,INPUTS
00200 PRINT9,X(I)
00210 1 CONTINUE
00220 DO 2 K=1,INPUTS
00230 Y(K,1)=Y(K,1)*0.137
00240 PRINT9,Y(K,1)
00250 2 CONTINUE
00260 DO 3 L=1,INPUTS
00270 IF(Y(L,2) .EQ. 0) GO TO 111
00280 Y(L,2)=0.20/Y(L,2)
00290 111 CONTINUE
00300 3 CONTINUE
00310 DO 4 M=1,INPUTS
00320 Y(M,3)=(Y(M,3)*(Y(M,2)/2.21))-Y(M,1)
00330 IF(Y(M,3).LT.0.) Y(M,3)=0.
00340 PRINT9,Y(M,3)
00350 4 CONTINUE
00360 DO 5 N=1,INPUTS
00330 Y(N,4)=Y(N,4)*1.34
00390 PRINT9,Y(N,4)
00410 5 CONTINUE
00420 DO 6 I=1,INPUTS
00430 Y(I,5)=Y(I,5)*3.33-Y(I,4)
00440 IF(Y(I,5).LT.0)Y(I,5)=0
00460 PRINT9,Y(I,5)
00480 6 CONTINUE
00510 9 FORMAT(8F7.3)
00520 Y(INPUTS+1,1)=0.0
00530 Y(INPUTS+2,1)=4.0
00540 Y(INPUTS+1,3)=0.0
00550 Y(INPUTS+2,3)=4.0
00560 Y(INPUTS+1,4)=0.0
00570 Y(INPUTS+2,4)=4.0
00580 Y(INPUTS+1,5)=0.0
00590 Y(INPUTS+2,5)=4.0
00600 CALL PAGE
00610 CALL SYMBOL(0.6,4.65,0.09,5H= NO ,0.0,5)
00620 CALL SYMBOL(.98,4.33,0.09,1H2,0.0,1)
00630 CALL SYMBOL(0.6,4.50,0.09,5H= NO ,0.0,5)
00640 CALL SYMBOL(.98,4.18,0.09,1H3,0.0,1)
00650 CALL SYMBOL(0.6,4.35,0.09,12H= FREE NH OH,0.0,12)
00660 CALL SYMBOL(1.4,4.33,0.09,1H2,0.0,1)
00670 CALL SYMBOL(0.6,4.20,0.09,13H= BOUND NH OH,0.0,13)
00680 CALL SYMBOL(1.5,4.18,0.09,1H2,0.0,1)
00690 CALL SYMBOL(0.4,4.675,0.09,1,0.0,-1)
00700 CALL SYMBOL(0.4,4.525,0.09,2,0.0,-1)
00710 CALL SYMBOL(0.4,4.375,0.09,5,0.0,-1)
00720 CALL SYMBOL(0.4,4.225,0.09,4,0.0,-1)
00730 CALL SCALE(X,5.0,INPUTS,1)
00740 CALL AXIS(0.0,0.0,5HHOURS,-5,5.0,0.0,0.0,48.0)
00750 CALL AXIS(0.0,0.0,13HNITROGEN, PPM,13,5.0,90.0,0.0,4.0)
00820 CALL LINE(X,Y,INPUTS,1,1,1)
00830 DO 10 I=1,INPUTS
00840 Y(I,1)=Y(I,3)
00850 10 CONTINUE
00860 CALL LINE(X,Y,INPUTS,1,1,2)
00870 DO 11 I=1,INPUTS
00880 Y(I,1)=Y(I,4)
00890 11 CONTINUE
00900 CALL LINE(X,Y,INPUTS,1,1,5)
00910 DO 12 I=1,INPUTS
00920 Y(I,1)=Y(I,5)
00930 12 CONTINUE
00940 CALL LINE(X,Y,INPUTS,1,1,4)
00950 CALL PLOT(8.,0.,999)
00960 STOP
00970 END
```

Figure 23. Computer program utilized for data analysis.

APPENDIX B

Nitrification Activity of Additional Isolates

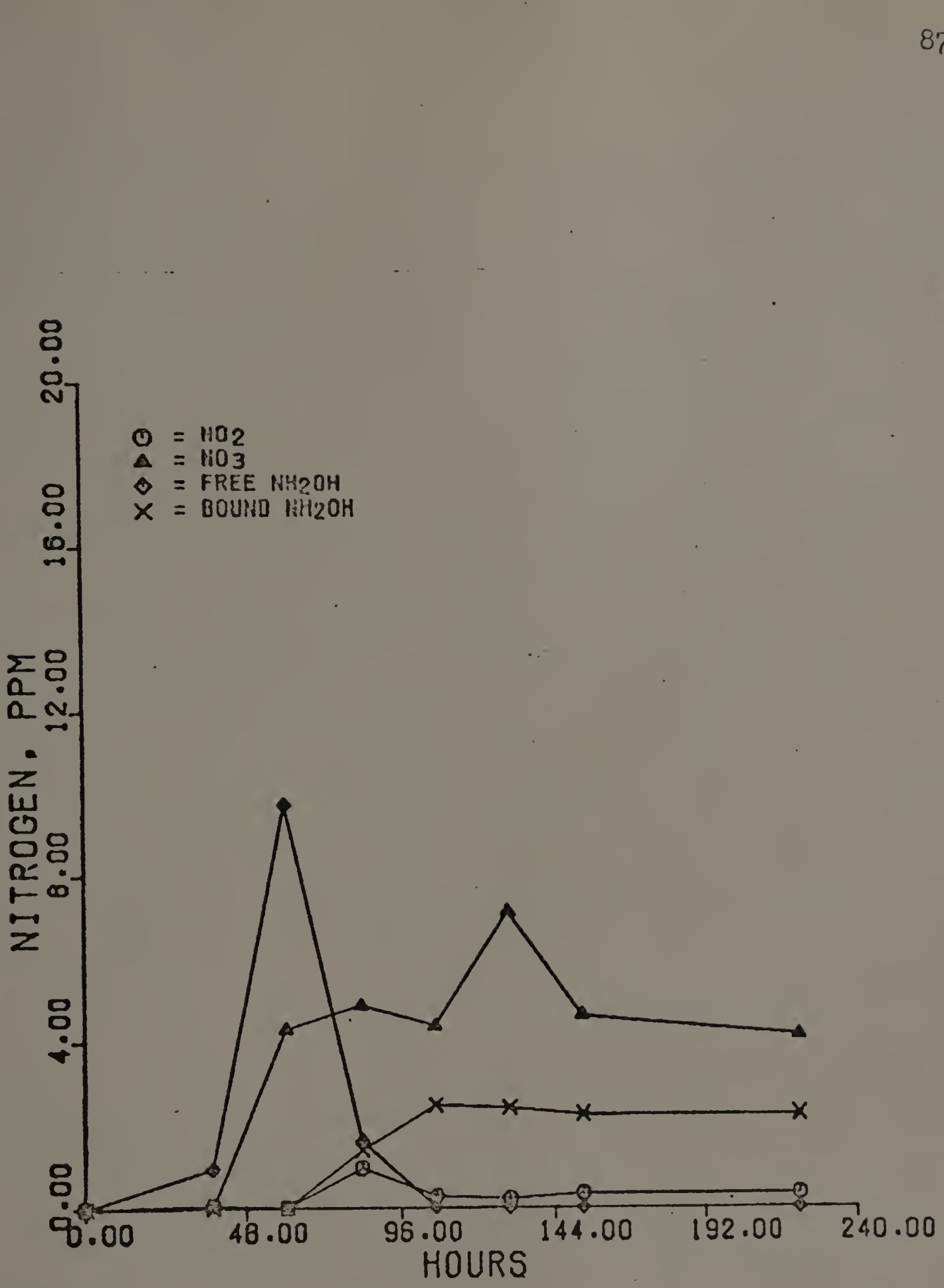

Figure 24. Accumulation of nitrification products by S-1 when grown with acetate as carbon source.

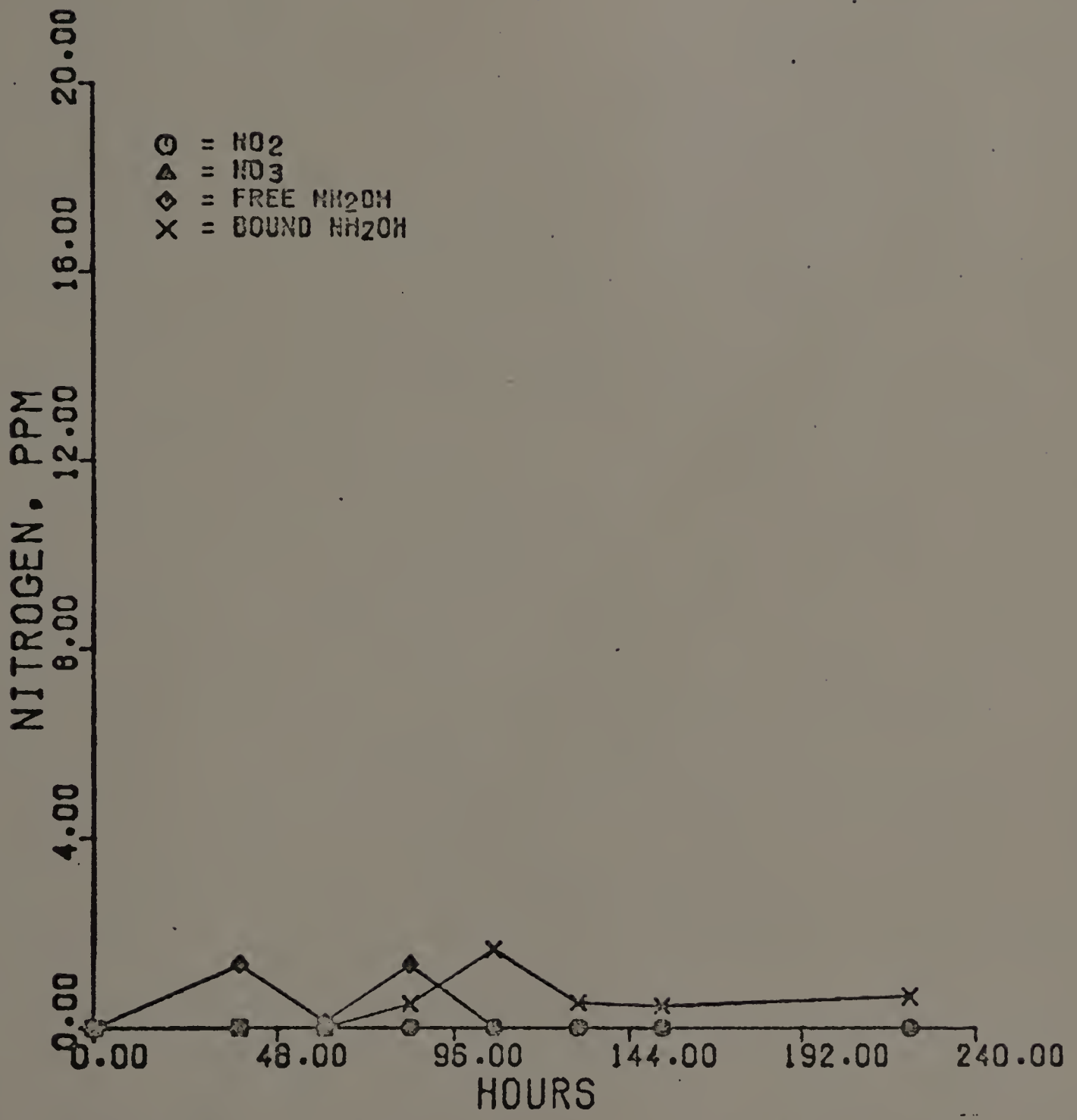

Figure 25. Accumulation of nitrification products by S-1 when grown with succinate as carbon source.

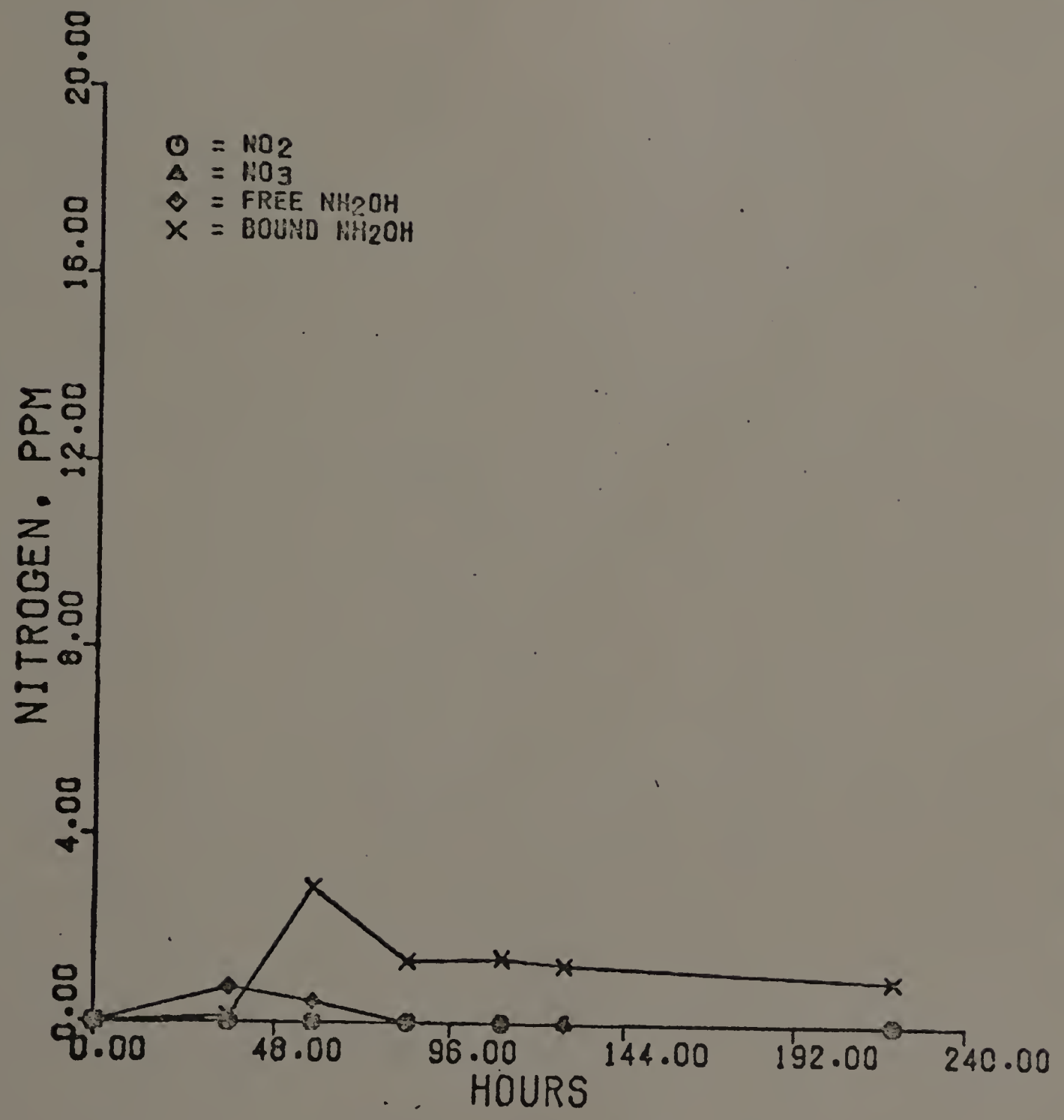

Figure 26. Accumulation of nitrification products by S-1 when grown with citrate as carbon source.

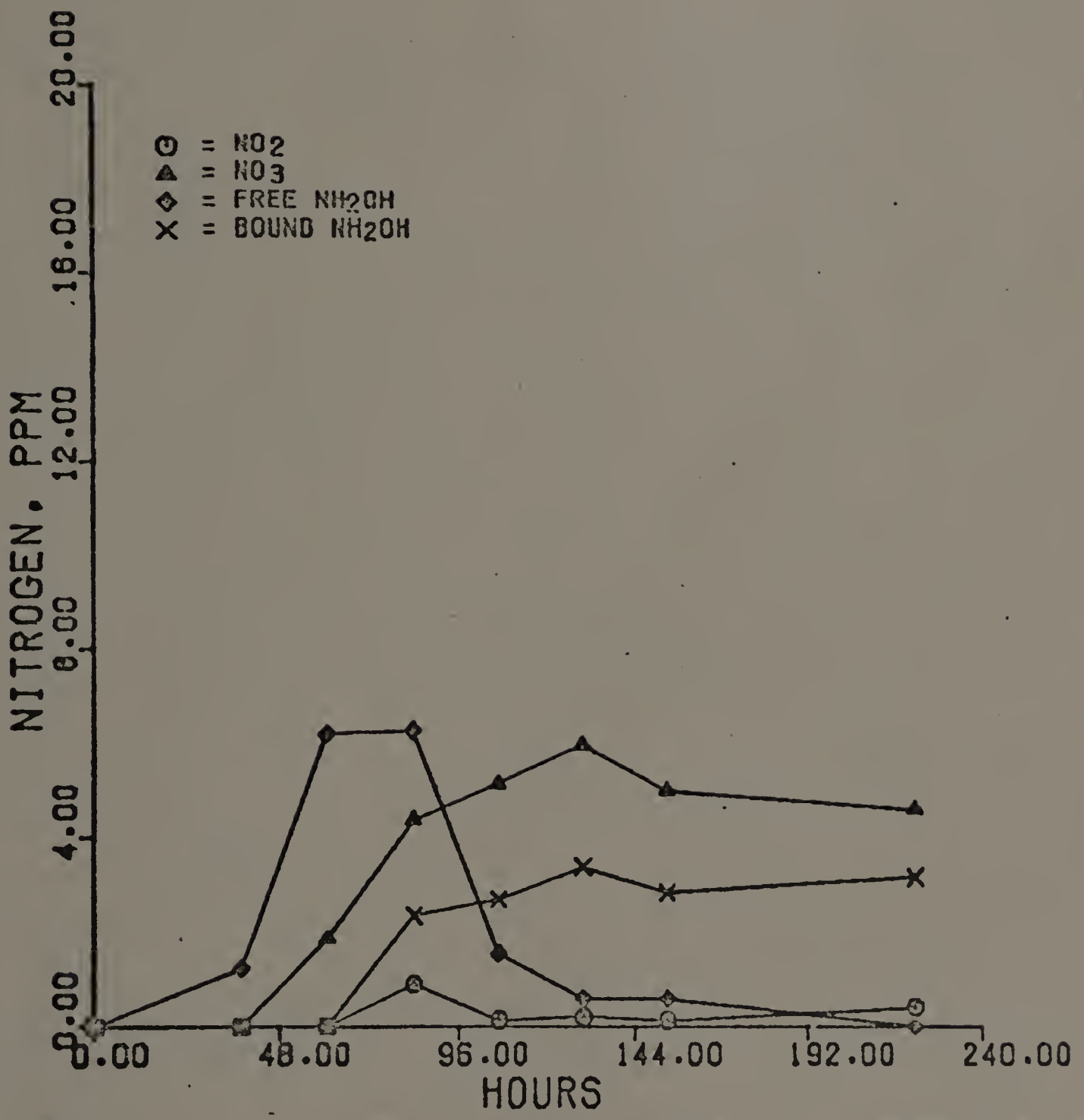

Figure 27. Accumulation of nitrification products by S-4 when grown with acetate as carbon source.

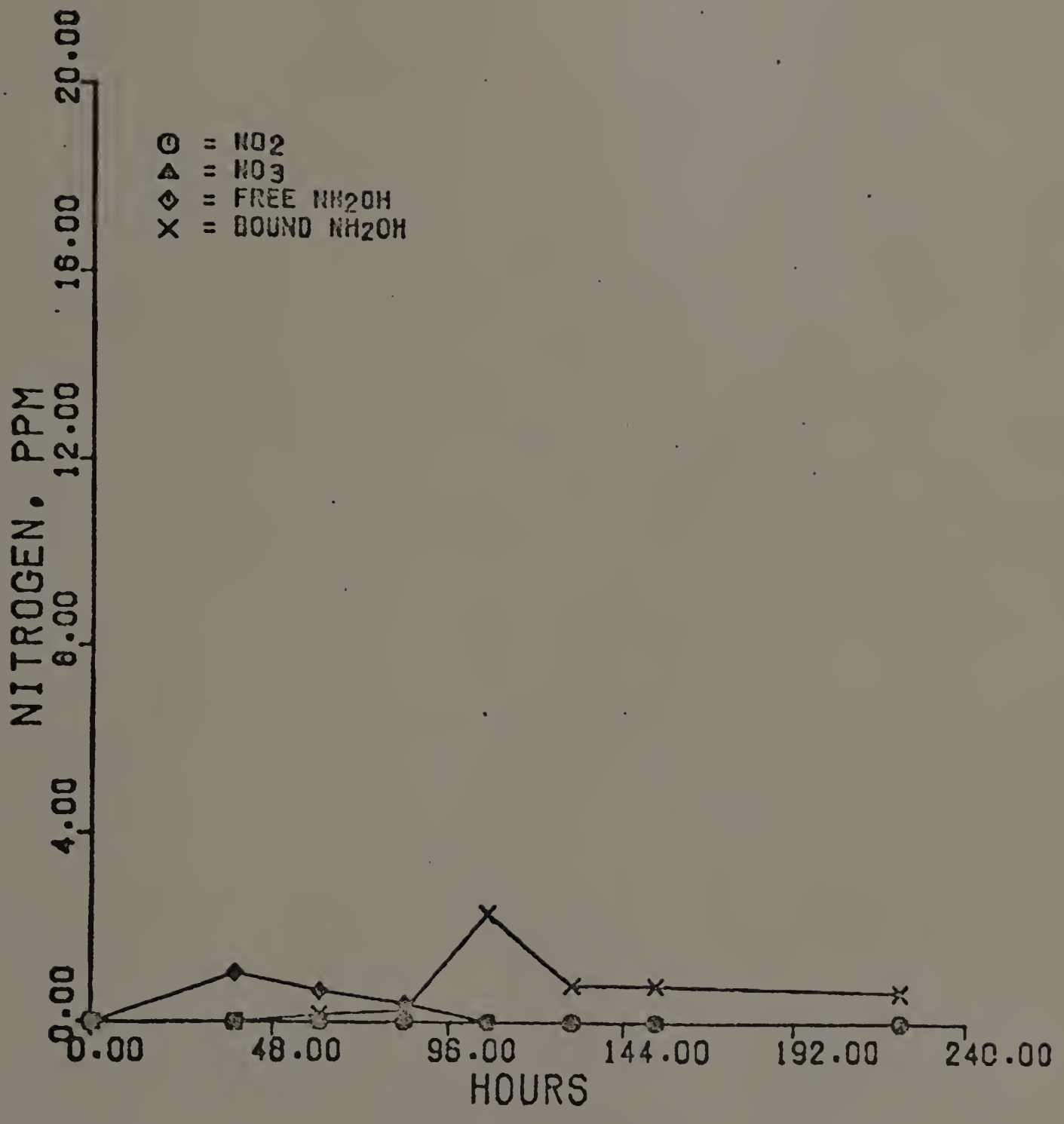

Figure 28. Accumulation of nitrification products by S-4 when grown with succinate as carbon source.

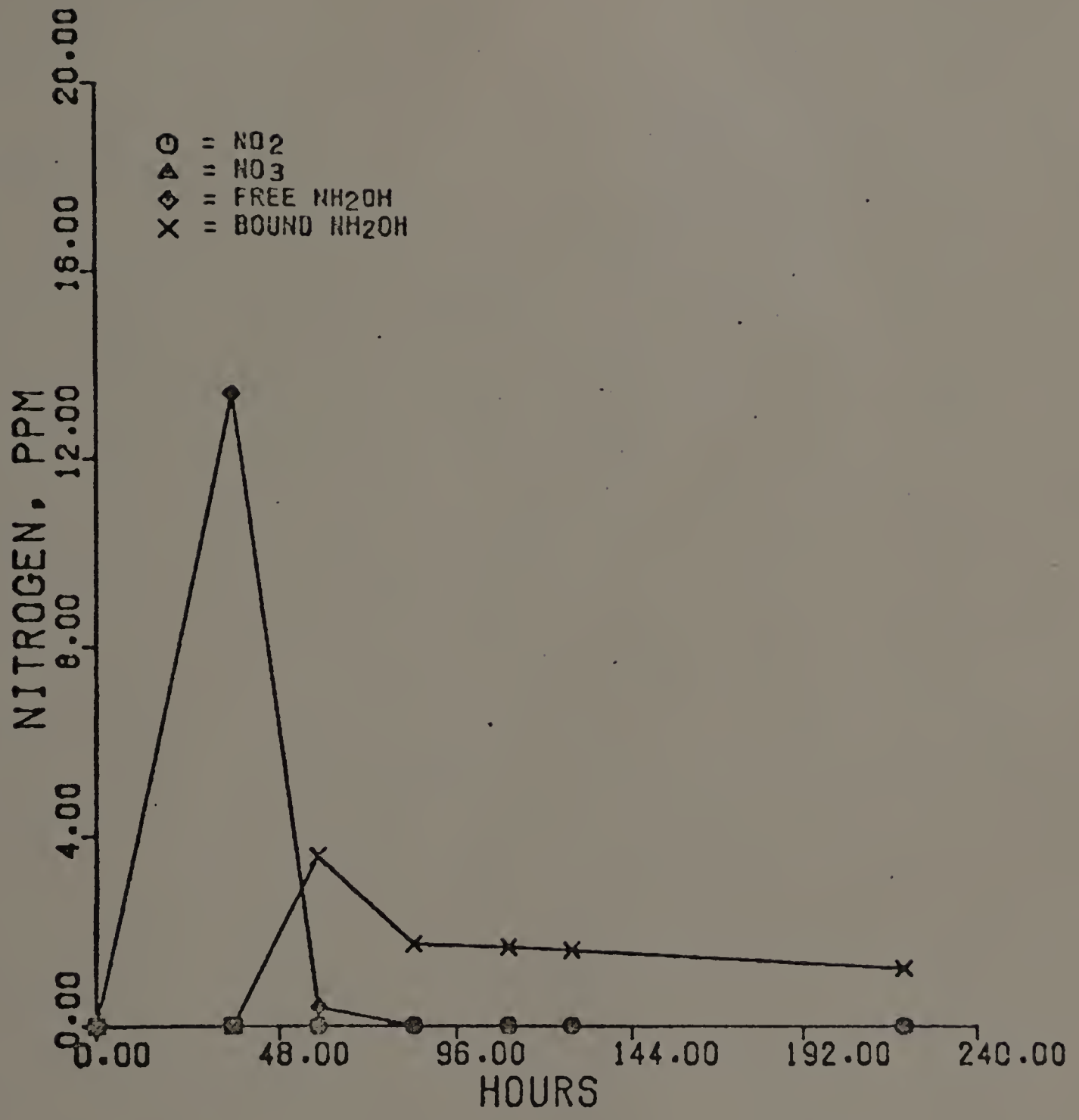

Figure 29. Accumulation of nitrification products by S-4 when grown with citrate as carbon source.

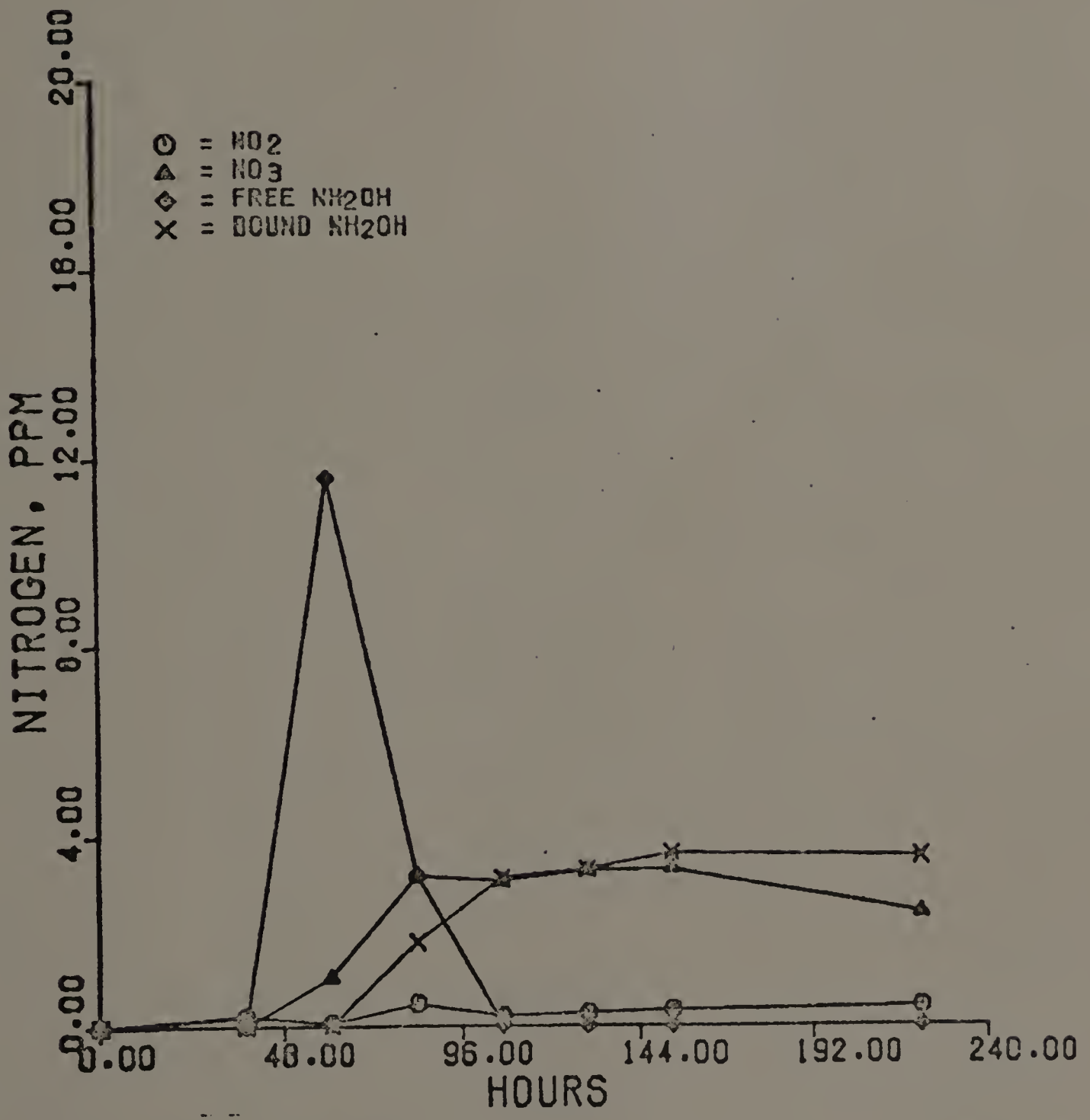

Figure 30. Accumulation of nitrification products by S-5 when grown with acetate as carbon source.

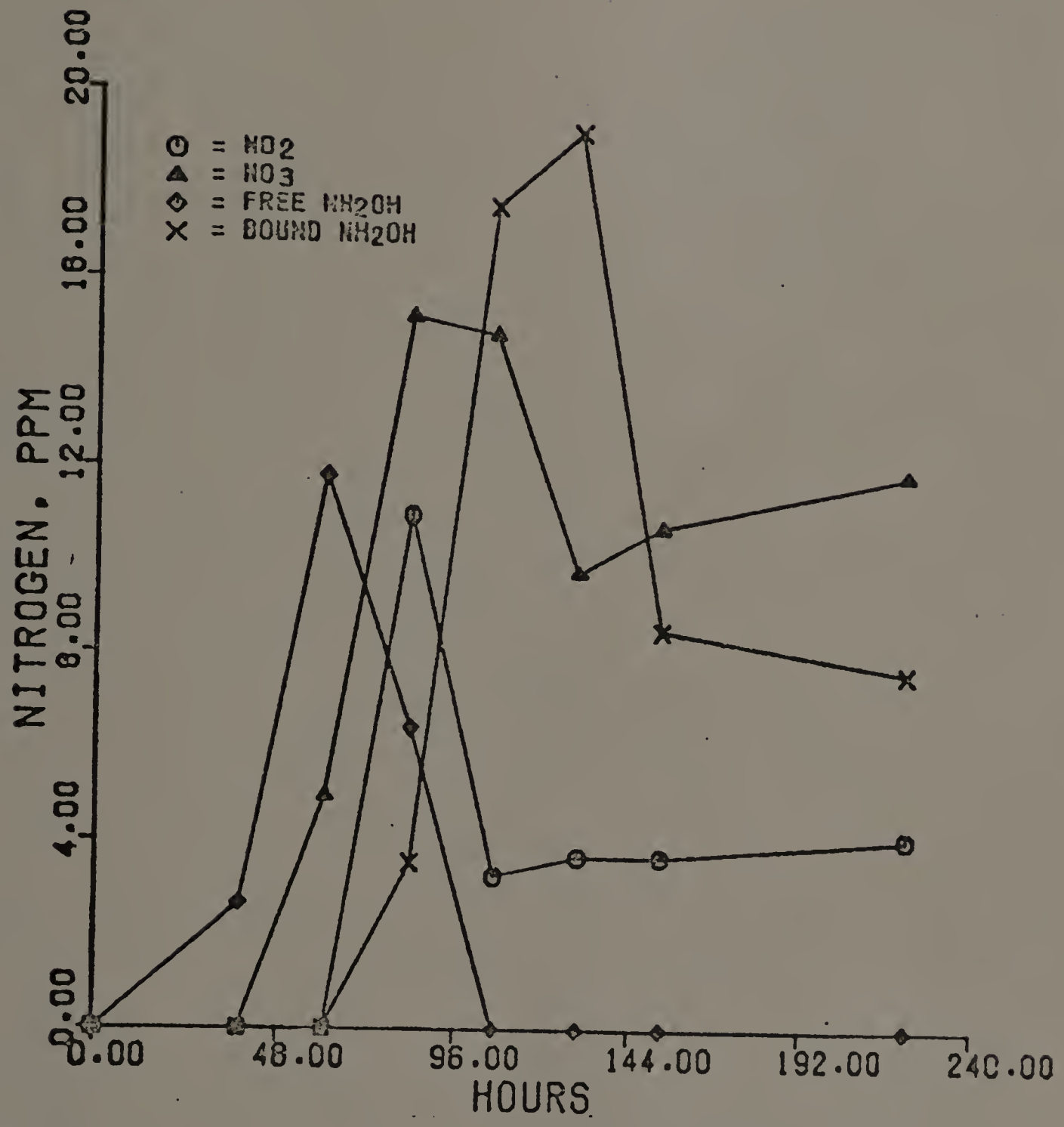

Figure 31. Accumulation of nitrification products by S-5 when grown with succinate as carbon source.

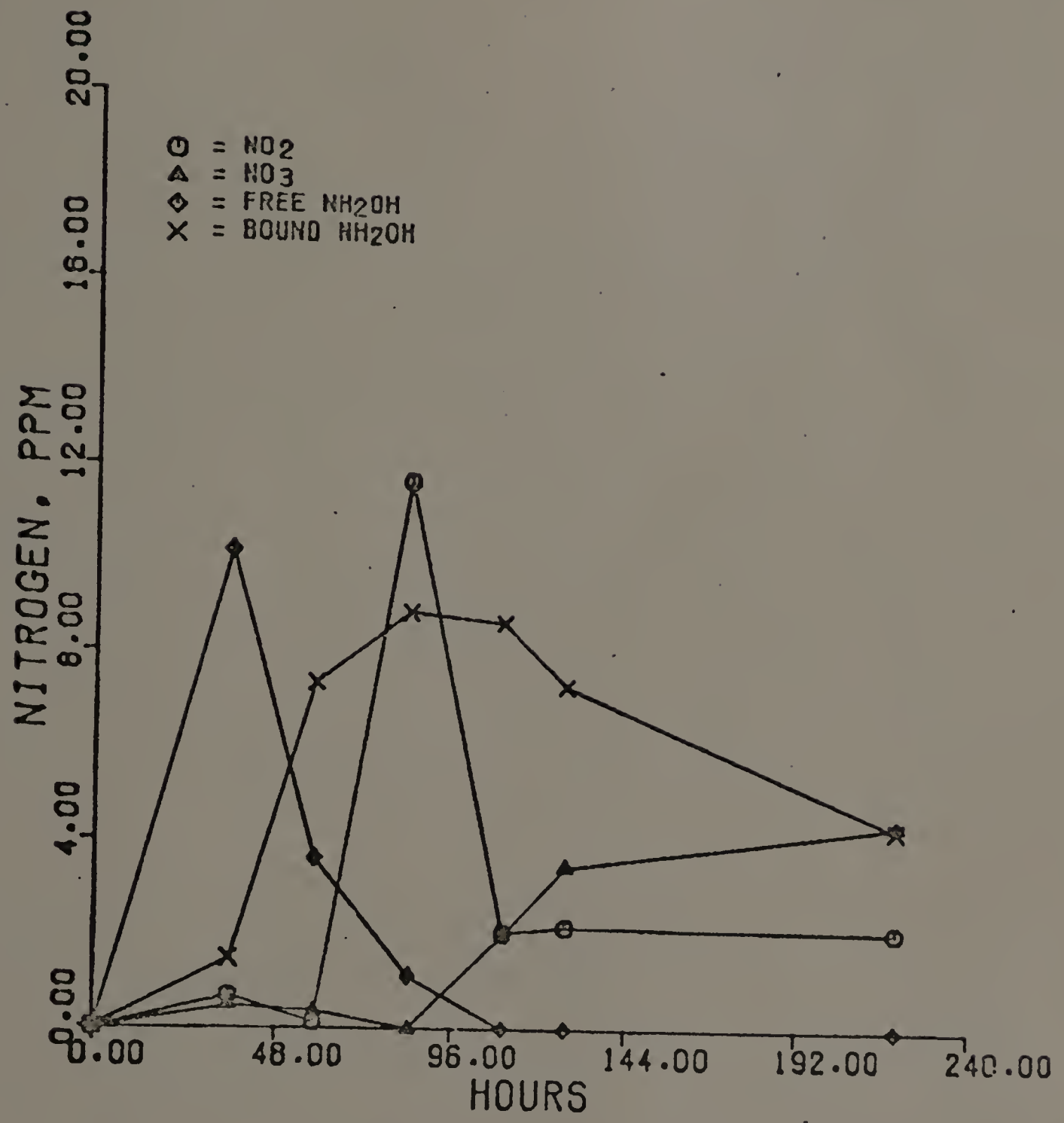

Figure 32. Accumulation of nitrification products by S-5 when grown with citrate as carbon source.

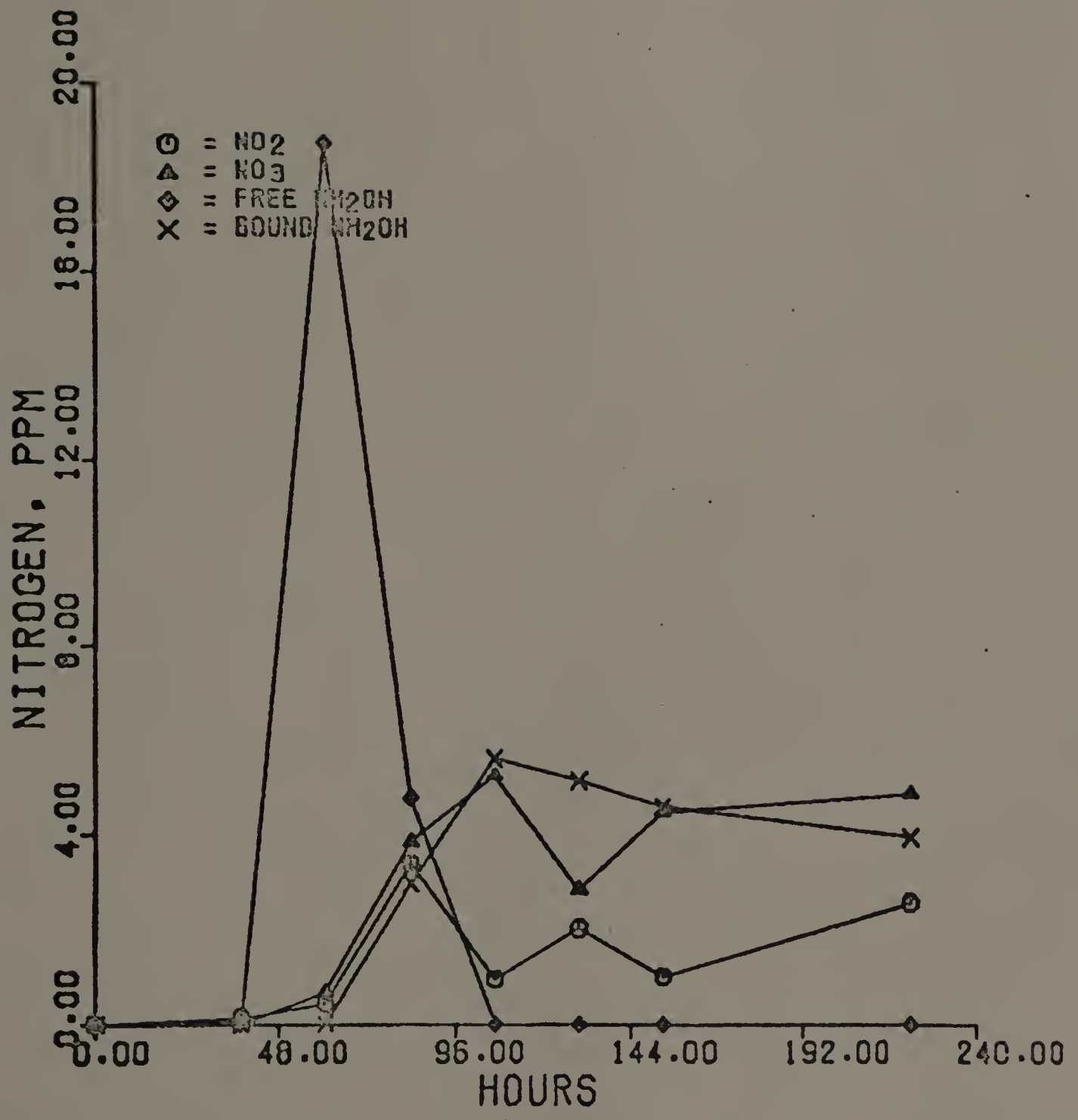

Figure 33. Accumulation of nitrification products by S-8 when grown with acetate as carbon source.

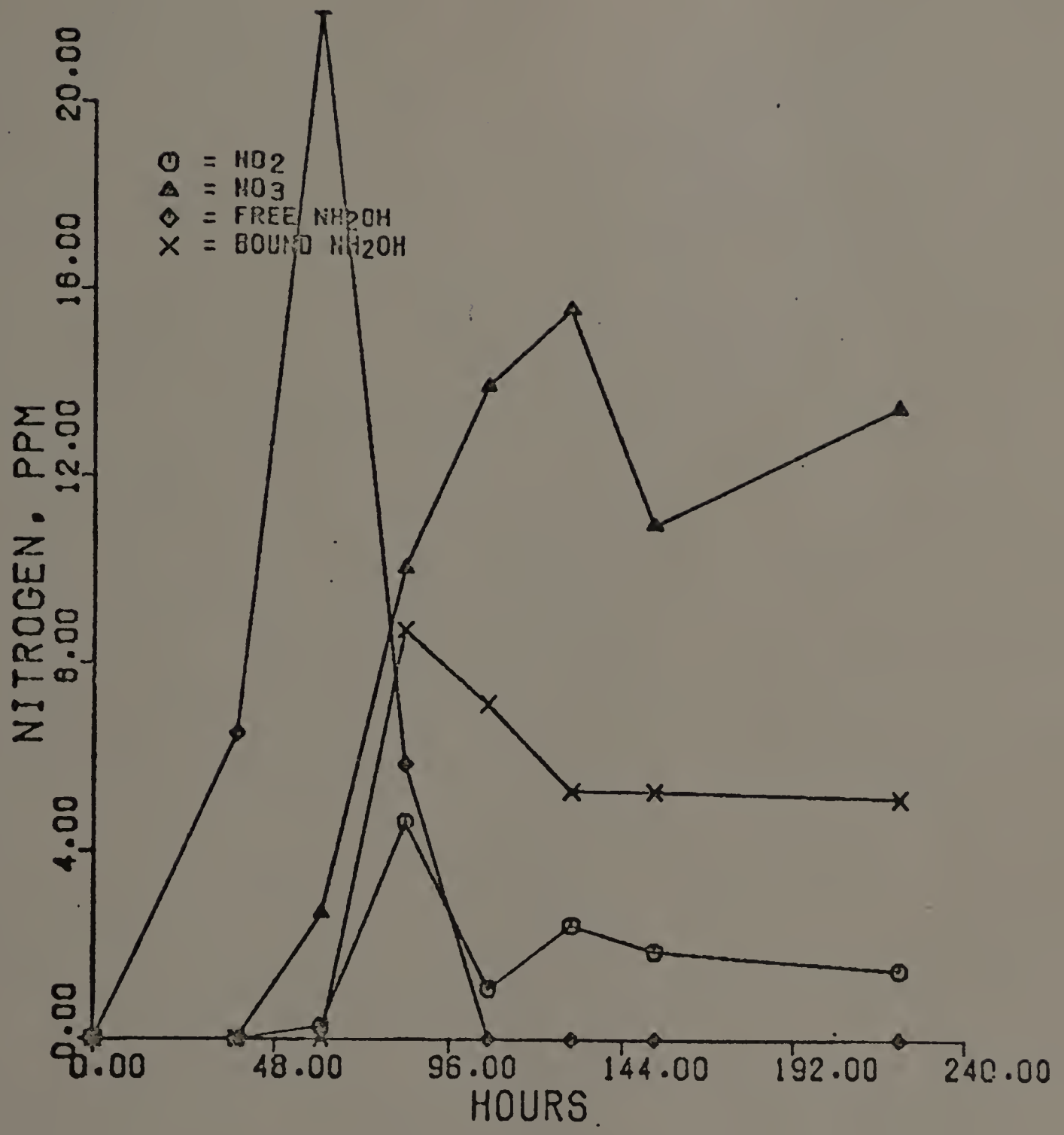

Figure 34. Accumulation of nitrification products by S-8 when grown with succinate as carbon source.

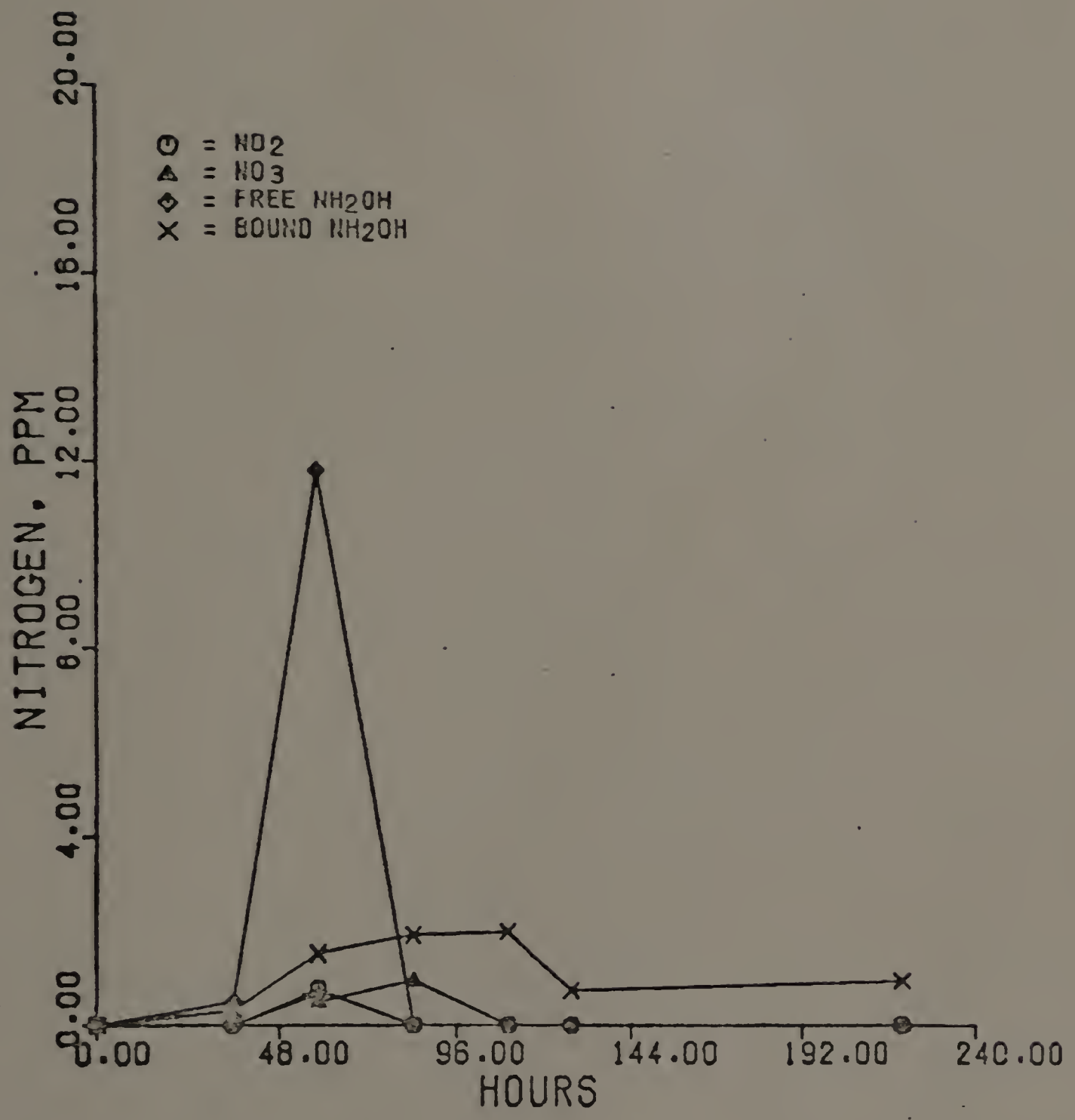

Figure 35. Accumulation of nitrification products by S-8 when grown with citrate as carbon source.

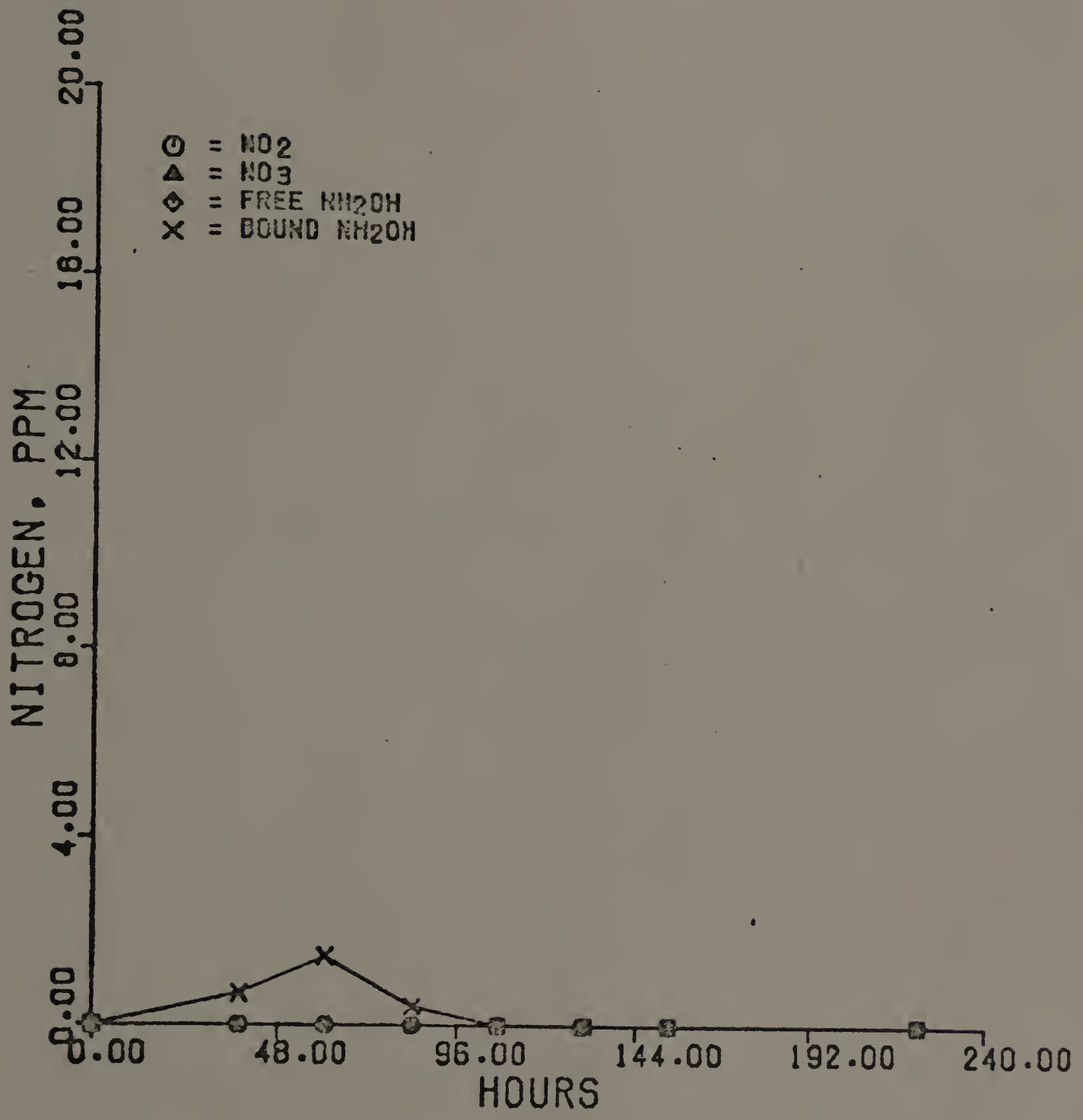

Figure 36. Accumulation of nitrification products by S-11 when grown with acetate as carbon source.

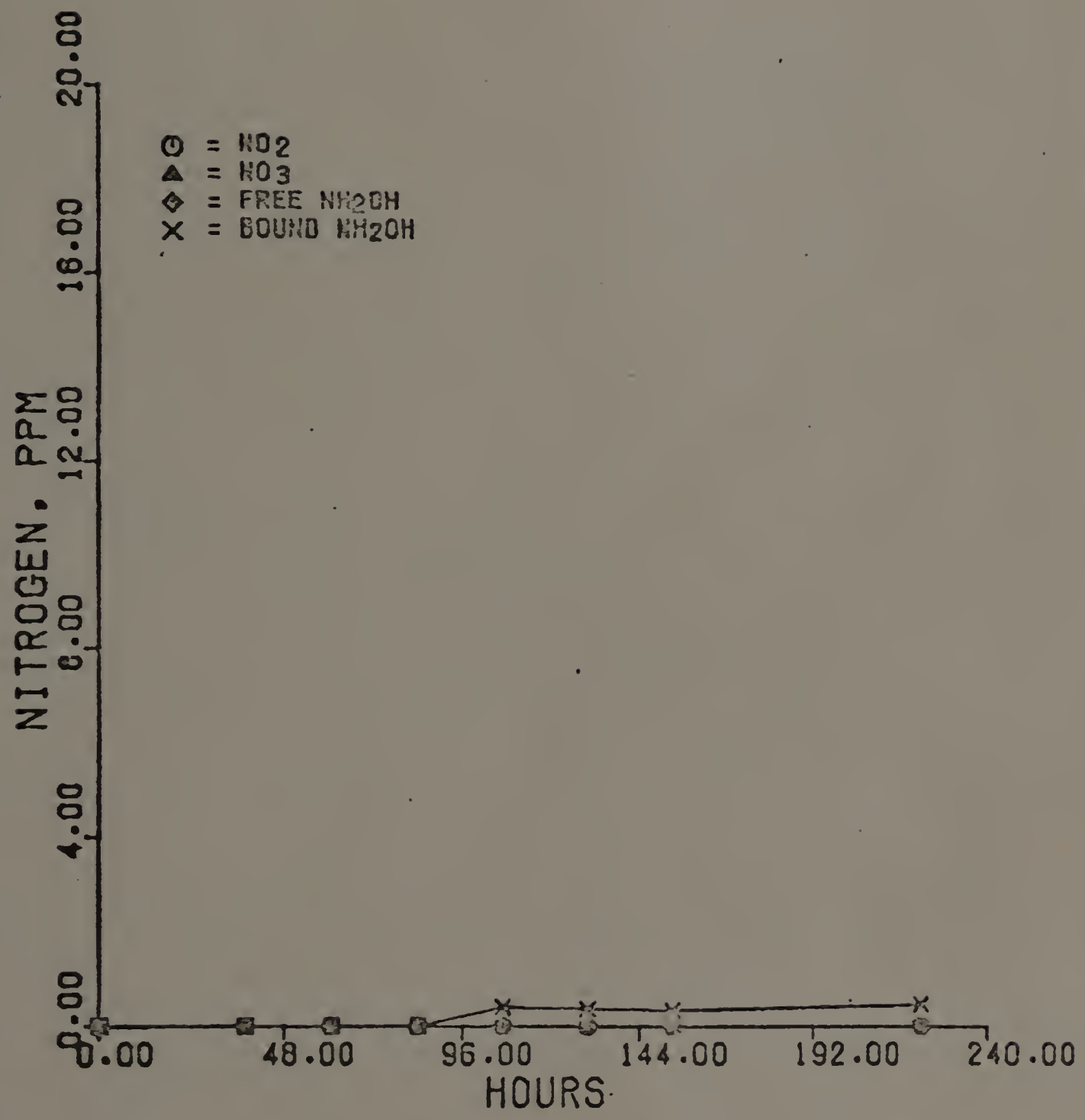

Figure 37. Accumulation of nitrification products by S-11 when grown with succinate as carbon source.

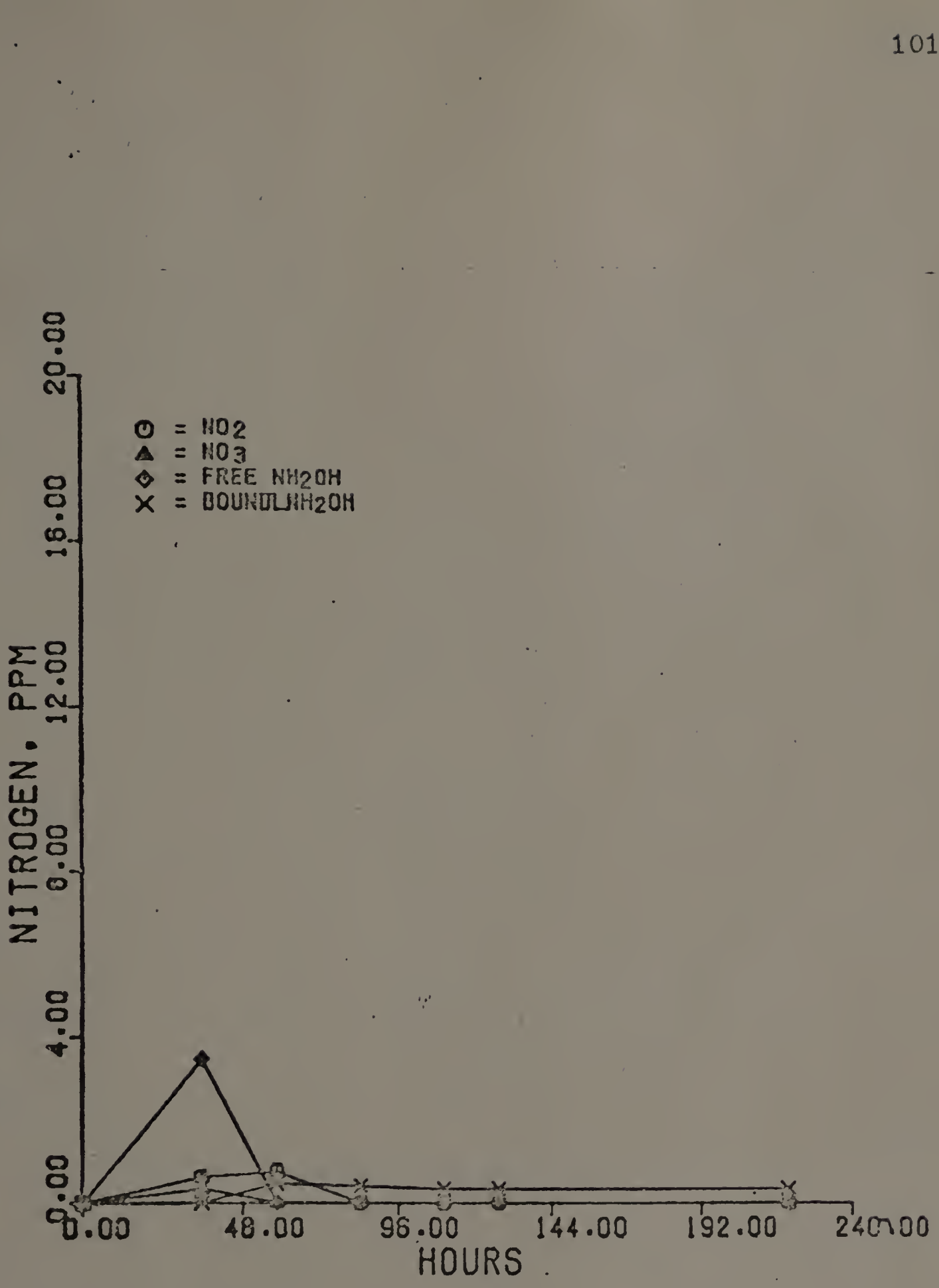

Figure 38. Accumulation of nitrification products by S-11 when grown on citrate as carbon source.