# Nitrate and nitrite variation in ground water. No. 58 1972

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# NITRATE AND NITRITE VARIATION IN GROUND WATER

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# ABSTRACT

Studies of the magnitude and variation of nitrate and nitrite concentrations in private well water were made in the central Wisconsin farm area of Marathon County, and 55% of the wells contained nitrate concentration of 45 mg/l or more. Among 242 wells investigated, 82 private wells were sampled 2 times/month for a period of 14 months. Nearly 70% of the 82 wells contained nitrate levels of 45 mg/l or greater at one time or another during the sampling period and about 45% of the wells contained nitrate levels of 45 mg/l or more throughout the period. The variations in nitrate concentration were closely related to amount of precipitation and concentration was highest during heavy rainy season and lowest during dry period for the majority of wells examined.

Nitrate and nitrite in well water may be derived from both autotrophic and heterotrophic nitrification following ammonification of organic fertilizer (manure). Kinetics of heterotrophic nitrification by mixed culture system using partially oxidized poultry wastes having high ammonium-N, organic-N and energy source yielded nitrate concentrations ranging from 370 to maximum of 1131 mg/l/day.

Nitrate and nitrite in soil may be lost to atmosphere via denitrification. Aerobic, motile, Gram-negative, rod-shaped bacteria belonging to genus *Pseudomonas* were used as a way of controlling nitrate build-up in aqueous media. Kinetics of dentrification by pure cultures of pseudomonads under ideal conditions ranged from 12 to 83 mg $NO_3^-$/hr/$10^8$ cells. The rate of denitrification is influenced by: history of inoculum; presence or absence of electron acceptor and oxygen; kind of hydrogen donor compounds or C/N ratio of the media used.

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## CONTENTS

# INTRODUCTION

Approximately 80 percent of the earth's atmosphere is nitrogen; about 70 million pounds of nitrogen exist above each acre of soil. Despite the abundance of molecular nitrogen in the atmosphere, it is not a suitable source of the element for most living forms because the nitrogen molecule ($N_2$) is chemically extremely inert, yet the element nitrogen is indispensable in our biosphere.

Small amounts of N-oxides are added to soil through lightning or micro-biological oxidation of ammonium through the process known as nitrification. Naturally occurring nitrogen in forms usable by plants are relatively scarce in soil and water and its concentration can become the limiting factor in development and growth. To meet the increasing demand for food by an expanding population, oxidized soluble forms of nitrogen have been introduced through new technology rather than slow processes of nitrification or symbiotic N-fixation. This effort has resulted in greater use of large amounts of commercial nitrogen, often in addition to heavy applications of manure.

All nitrates are water soluble, and if they are present in ground water in concentrations higher than 45 mg/l, a potential health hazard can exist for domestic animals and human infants. Nitrate is reduced by intestinal bacteria to form nitrite and when combined with oxyhemoglobin in the blood forms methemoglobin. This globin decreases the oxygen-carrying power of the blood and may produce the syndrome known as methemoglobinemia resulting in asphyxia and possible death.

Part I of this study was conducted to determine the magnitude and variation of nitrate and nitrite concentrations in private well waters in the central Wisconsin farm area of Marathon County.

Aside from the application of commercial chemicals, nitrate build-up can result from microbial conversion of organic fertilizer (manure). Part II of this report discusses the kinetics of heterotrophic microorganisms involved in this process.

Microbial denitrification processes as a way of controlling nitrate build-up are discussed in Part III.

# PART I: NITRATE AND NITRITE IN GROUND WATER

## INTRODUCTION

Several studies have been conducted on the occurence of nitrate and nitrite in private water supplies (Nichols, 1920; California State Department of Health, 1963; Smith, 1965; Doneen, 1966; McHarg, 1968; Erwin and Waterworth, 1968 and Murphy and Gosch, 1970). However, practically nothing is known of the extent of fluctuation in nitrate concentration in given wells. This survey was made to determine the variation in nitrate and nitrite content with respect to physical and environmental variables such as well depth, well location and precipitation.

## REVIEW OF LITERATURE

Several authors have found that both man and animals may be affected by nitrate and nitrite if sufficiently high concentrations are ingested: (Mayo, 1895; Comly, 1945; Donahoe, 1949; Chute, 1950; Bosch et al., 1950; Walton, 1951; Campbell, 1952; Orgeron et al., 1957; Werner et al., 1965; and Benarde, 1970).

Toxicologically the salts of nitrite are much more aggressive than are the salts of nitrate (Hanway et al., 1963; Chapen, 1947; Walton, 1951; Bodansky, 1951; Horn, 1958; and Werner et al., 1965). Reports concerning effect of nitrite and nitrate on animals were made by Whitehead and Moxon, 1952; Muhrer et al., 1956; Burden, 1961; Garner, 1961; Sund et al., 1964; Smith, 1965. Nitrate and nitrite have also been shown to inhibit iodine and Vitamin A metabolism in certain experimental animals (Garner et al., 1958; Bloomfield et al., 1961; and Pugh et al., 1962). The effects of nitrite and nitrate on poultry have also been reported (Marret and Sunde, 1967).

### Sources of Nitrogen

The atmosphere is usually considered to contribute from 2 to 6 pounds of nitrogen to an acre of land over the period of a year (Allison, 1957). Various estimates of contribution from atmosphere are found in Public Health Service (1966), Eliassen and Tchobanoglous (1969), and Hutchison and Viets (1969).

Nitrate derived from biological fixation of atmospheric nitrogen and nitrification are discussed in the following reports: Bollen (1951), Schmidt (1954, 1960), Starkey (1958), Eylar and Schmidt (1959), Hirsch et al. (1961), Alexander (1961), Marshall and Alexander (1962), Woldendorp (1963), Krulwich and Funk (1965), USDA (1968), Brezonik (1968), McCoy (1968), Heimbrook (1969), and Thompson (1969).

Nitrate from naturally occurring deposits are discussed in reports by Gale (1917), Mansfield and Boardman (1932), and Ingols and Navarre (1952).

Intrusion of inorganic nitrogen originating from farmland, while it is still controversial as to its contribution, is discussed in the following articles as potential sources of nitrate-nitrogen in both surface and ground water supplies: Allison (1957), Larson and Larson (1957, 1958), California State Department of Health (1963), Lewis (1963), Smith (1965), Nichols (1965), Doneen (1966), Corey et al. (1967), Stewart et al. (1967), Chemical & Engineering News (1968, 1969), Commoner (1968a), Krause and Batsch (1968), Lepkowski (1968), McHarg (1968), Huchthauson and Polkowski (1968), Harmeson and Larson (1969), Wisconsin Department of Natural Resources (1969), Welch (1970), and Kohl et al. (1971).

## Nitrate Standards for Drinking Water

The U.S. Public Health Service (1962), reports that ingestion of water with more than 50 mg/l nitrate may give rise to infantile methemoglobinemia. For this reason, the Public Health Service Standards suggest that water with more than 45 mg/l nitrate is a limit that should not be exceeded.

Various recommendations concerning drinking water standards are cited in the following literature: Nichols, 1920, 1965, Comly, 1945; Gilbert et al., 1946; Cabellero, 1950; Walton, 1951; Campbell, 1952; Whitehead and Moxon, 1952; Sollman, 1957; Taylor, 1958; Lehman, 1958; Mackenthun, 1965.

## STUDY AREA

The wells surveyed were located in a broad valley of Marathon County carved in Pre-Cambrian rock during the Pleistocene age (Fig. 1). The subsoil is derived from the weathering of underlying Pre-Cambrian granite, while the surface horizons are composed of silt loam soil up to 2 feet in depth. Ground water is generally in short supply because of dense crystalline bedrock being close to the surface; however, where bedrock has been weathered to depths of 100 feet or more, ample water reserves do occur. Deep wells are generally recharged from relatively shallow water-producing zones; thus the depths of wells do not necessarily represent the true water-producing zone.

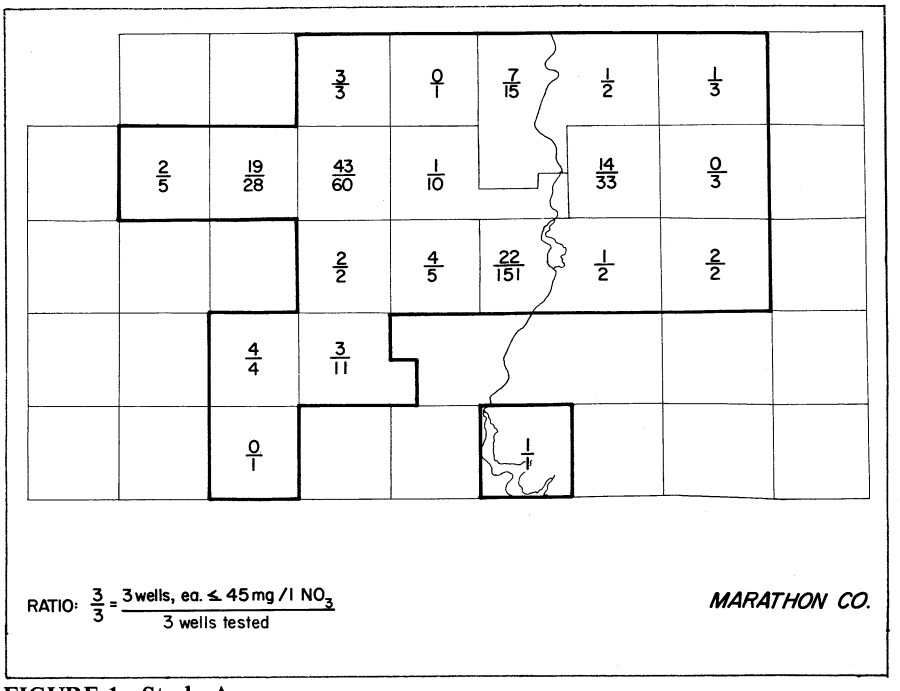


FIGURE 1. Study Area.

## METHODS

From March 1968 through May 1969, 242 private wells representing 20 townships in Marathon County were sampled. A grab sample was collected from each well and when possible the age and type of construction of the well was recorded. The great majority of the wells sampled are old installations constructed prior to the establishment of the state well construction code. Over half of the wells were dug, and dug and drilled types (Table 1).

To obtain a record of nitrate level fluctuation, 82 of the wells were sampled 2 times each month during the sampling period. The water samples were collected in 500 ml. bottles (about two-thirds full) and were aerated by shaking to minimize possible nitrate loss by denitrification. The bottles were placed in an insulated container and returned to the laboratory for analysis within 4 to 6 hours.

Water samples were analyzed in duplicate for ammonium-N, nitrite-N, and nitrate-N content using Keeney's Direct Distillation Method (Keeney and Bremmer, 1965). Several other methods were used to supplement and confirm the validity of each analysis; for example, spectrophotometric micro-determination of nitrate with chromotropic acid reagent (Batten, 1964) and spectrophotometric determination of nitrate ion using rhenium and $\alpha$-furildioxime (Bloomfield et al., 1965). The ammonium-N was tested by Johnson's method (1941) and nitrite-N by a modified method of Tittsler (1930). These two methods were used when concentrations of the above-mentioned nitrogen were less than 0.5 mg/l.

Microbiological analysis of the samples included total bacterial counts which were made on all-purpose media (Nutrient agar: Bacto-peptone, 8 gm; beef extract, 3 gm; Bacto-agar, 15 gm/l; and "plate count" agar; yeast extract, 2.5 gm; tryptone, 5.9 gm; glucose, 1.0 gm; and Bacto-agar, 15 gm/l). The procedure used was spread-plate technique rather than pour-plate technique to promote the growth of both facultative and aerobic organisms. Total coliform counts were made according to the Standard Methods (1962:508-513). In addition to total coliform counts, presence of *Pseudomonas aeruginosa* sp. was determined by Drake's method (1966) to indicate the extent of fecal pollution of the wells, regardless of animal or human origin.

## RESULTS AND DISCUSSION

### Seasonal Variation in Nitrate Level

Of the 242 wells sampled from 20 townships in Marathon County, 55 percent produced water containing nitrates in concentrations of 45 mg/l or greater (Fig. 1).

The relationship between average monthly precipitation and the distribution of nitrate levels of 45 mg/l or greater during the test period is presented in Figure 2. It is generally thought that the concentration of nitrate in ground water is highest following wet periods and lowest during dry periods. With a few exceptions (e.g. Fig. 5, well numbers 45, 55, 59), this pattern was confirmed. However, seasonal variation may be further enhanced by certain factors, such as: geologic structure (i.e., characteristics and depth of soil, subsoil and bedrock formation), degree of fracturing and interconnecting crevices in rock formations, presence of rechargeable aquifer and amount of rechargeable water; well depth; construction of well and depth of casing (Table 1).

In addition to seasonal variation in nitrate levels in the wells, the month in which the highest nitrate concentra-

tion occurred was determined. These data, from 82 wells in 1968 and 80 wells in 1969, are summarized in Figure 3.

## Effect of Well Depth

The relation between well depth and nitrate concentration is shown in Table 2 for all of the wells where depths are known. There was nearly equal distribution of water with concentrations of 45 mg/l $NO_3^-$ or greater and 44 mg/l $NO_3^-$ or less among each depth category. Therefore, there appears to be no correlation between well depth and concentration of $NO_3^-$ for the area investigated.

While the maximum downward migration rate of $NO_3^-$ in silt-loam soil has been reported to be in the range of 1 to 1½ feet per year (Olsen, 1969), this rate may not be applicable to the study area, as was indicated by the frequent fluctuations in $NO_3^-$ and $NO_2^-$ concentrations. A possible explanation for this could be the difference of the soil horizon and underlying bedrock prevalent in the Towns of Rib Falls and Rietbrock. A shallow layer of silt-loam (2 to 4 ft) overlays bedrock which is extensively fractured (Berkman, 1969). These fractures may provide avenues of contamination especially during periods of heavy precipitation, affecting shallow and deep wells alike.

## Nitrate Variation in Individual Wells

The bi-monthly analyses of 82 wells over the 14-month study period revealed fluctuations of nitrate concentration in the ground water throughout the year. These fluctuations were of three types:

(1) Large and frequent increases or decreases of nitrate concentration between monthly samples;
(2) Patterns similar to those of seasonal variation, i.e., usually high in the spring and decreasing during the summer; and
(3) A steady increase in nitrate concentration throughout the year.

Shallow wells (less than 50 ft in depth) are more apt to be associated with fluctuations of the first type. Abrupt change in nitrate concentration was evidenced by an increase as high as 103 mg/l over a month's time (Fig. 5, No. 80). This group of wells also demonstrated an irregular pattern of nitrate concentration fluctuation

**TABLE 1. Types of Wells Sampled and Nitrate Concentrations**

| Well No. | Type of Well | Depth (ft) | Depth Casing or Curbing (ft) | $NO_3$ ≥ 45 mg/l |
|---|---|---|---|---|
| 1 | Dug | 52 | 52 | - |
| 2 | Drilled | 350 | 40 | - |
| 3 | Drilled | 97 | 68 | + |
| 4 | Drilled | 67 | - | + |
| 5 | Dug | 26 | 26 | + |
| 6 | Dug & Drilled | 41 | 27 | + |
| 7 | Drilled | 130 | 30 | - |
| 8 | Drilled | 154 | - | + |
| 9 | Dug & Drilled | 70 | 48 | - |
| 10 | Drilled | 59 | 59 | + |
| 11 | Drilled | - | - | + |
| 12 | Dug | 22 | 22 | - |
| 13 | Drilled | 78 | 13 | - |
| 14 | Dug | 70 | - | + |
| 15 | Dug | 30 | 30 | + |
| 16 | Dug & Drilled | 28 | 28 | + |
| 17 | Dug | 40 | - | + |
| | Drilled | 218 | 40 | - |
| 18 | Drilled | 65 | - | + |
| 19 | Drilled | 63 | 40 | + |
| 20 | Dug & Drilled | 41 | 26½ | - |
| 21 | Dug | 30 | 30 | + |
| 22 | Dug | 36 | 35½ | + |
| 23 | Dug & Drilled | 53 | - | + |
| 24 | Drilled | 50 | 14 | - |
| 25 | Drilled | 205 | 21 | - |
| 26 | Dug | 24 | 24 | + |
| 27 | Drilled | 175 | - | - |
| 28 | Drilled | 25 | - | - |
| 29 | Dug & Drilled | 119 | - | + |
| 30 | Drilled | 190 | 26 | + |
| 31 | Drilled | 78 | - | - |
| 32 | Drilled | 85 | 48 | - |
| 33 | Drilled | 98 | 32 | - |
| 34 | Dug & Drilled | 78 | - | - |
| 35 | Dug | 18 | 18 | - |
| 36 | Dug | 28 | 28 | + |
| 37 | Dug & Drilled | 59 | 22 | + |
| 38 | Drilled | 45 | 45 | + |
| 39 | Dug & Drilled | 70 | - | + |
| 40 | Drilled | 180 | - | - |
| 41 | Drilled | 100 | 27 | + |
| 42 | Dug & Drilled | 56 | 44 | + |
| 43 | Dug | 28 | 26 | + |
| 44 | Drilled | 40 | - | + |
| 45 | Dug & Drilled | 37 | 25 | + |
| 46 | Dug & Drilled | 40 | 40 | + |
| 47 | Drilled | 61 | 50 | - |
| 48 | Drilled | 118 | 38 | + |
| 49 | Dug | 44 | 44 | + |
| 50 | Drilled | 56 | - | + |
| 51 | Drilled | 76 | - | + |
| 52 | Drilled | 76 | - | + |
| 53 | Drilled | 145 | 40 | + |
| 54 | Drilled | 130 | 37 | + |
| 55 | Drilled | 54 | 51 | + |
| | Dug & Drilled | 70 | 35 | - |
| 56 | Dug | 19 | 19 | - |
| 57 | Dug | 35 | 35 | + |
| 58 | Drilled | 72 | 42 | + |
| 59 | Dug | 16 | 16 | + |
| 60 | Dug & Drilled | 72 | 28 | + |
| 61 | Dug & Drilled | 41 | 30 | + |
| 62 | Dug | 27 | 20 | + |
| 63 | Dug | 27 | - | + |
| 64 | Dug | 47 | 47 | + |
| 65 | Drilled | 50 | 32 | + |
| 66 | Dug & Drilled | 40 | 36 | - |
| 67 | Dug | 22 | 15 | - |
| 68 | Dug & Drilled | 44 | 44? | - |
| 69 | Dug & Drilled | 48 | 44? | + |
| 70 | Drilled | 65 | 40 | + |
| 71 | Dug | 27 | 27 | + |
| 72 | Dug | 27 | - | + |
| 73 | Drilled | 185 | 21½ | + |
| 74 | Dug | 17 | 17 | + |
| 75 | Dug & Drilled | 90 | - | + |
| 76 | Dug & Drilled | 90 | 25 | + |
| 77 | Requested to withdraw from sampling program | | | |
| 78 | Dug & Drilled | 70 | 25 | - |
| 79 | Dug & Drilled | 117 | 25 | + |
| 80 | Drilled | 42 | 28½ | + |
| 81 | Dug | - | - | + |
| 82 | Drilled | 72 | 40 | + |

* Bulk of information supplied by Calabresa et al , Department of Natural Resources, Wisconsin.

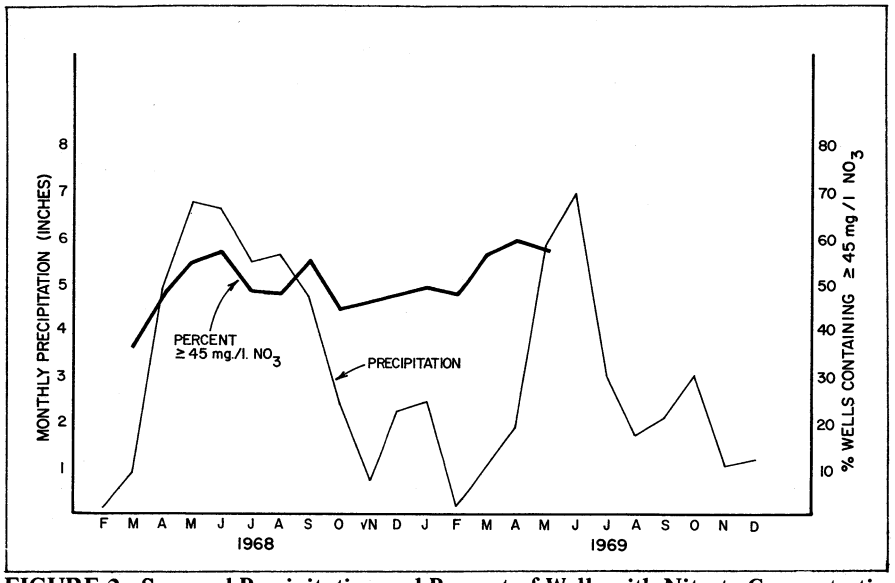
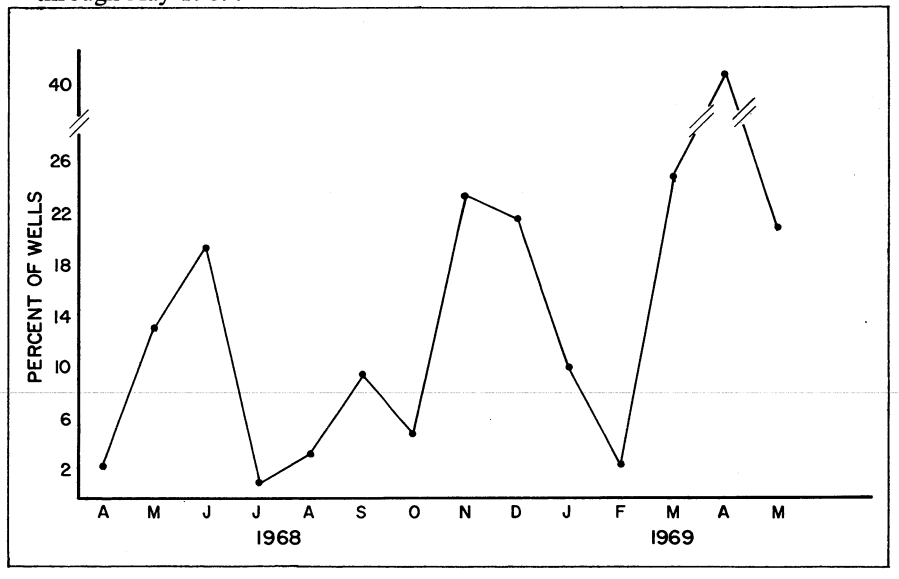



FIGURE 2. Seasonal Precipitation and Percent of Wells with Nitrate Concentration ≥45 mg/l.

FIGURE 3. Occurrence of Highest Nitrate Concentration in Wells, April 1968 through May 1969.




throughout the 14-month period, not conforming to seasonal or precipitation trends (Figs. 5, 8).

Wells of intermediate depth (51 to 100 ft) showed less intensity of fluctuation in nitrate concentration (Fig. 5, No. 55). The largest fluctuation within a monthly sample was 68 mg/l as compared to 103 mg/l for the shallow well group. However, no other fluctuation near this magnitude was observed in any other of the 29 wells surveyed in this category.

Fluctuation of nitrate concentration also occurred in wells deeper than 100 feet (Fig. 4). This occurred irregularly in all wells and the variation was markedly less than in the two groups of shallower wells. The maximum fluctuation in any one month was 28 mg/l $NO_3^-$.

In addition to the fluctuation of nitrate concentrations, certain wells showed a steady increase in concentration over the period of study irrespective of well depth (Figs. 4,6).

The number of wells located within animal yards or near manure pits, with nitrate concentrations of 45 mg/l or greater was not significantly different from wells placed at proper distances (50 ft or more) from these sources.

The depths of casing or curbing were believed to be more significant in preventing intrusion of nitrates, nitrites and coliform contamination into ground water supplies than was the depth of the well. However, analysis of data presented by Calabresa (1970), showed that the depth of casing or curbing did not appear to have an effect on the nitrate concentrations in the well waters. There were no apparent differences in nitrate concentrations based on the depth of the wells studied (Table 1).

## Other Sources

Nitrate concentrations in well water from 59 wells used by Grade A milk suppliers located in areas other than Rib Falls and Rietbrock Townships were spot-checked during April and May, 1969. Of 59 well water samples, 44 percent contained $NO_3^-$ concentration of 45 mg/l or more. However, only one well contained relatively high $NO_2^-$, 975 $\mu$g/l (Table 3).

Unlike the results from other wells in this report, these were obtained from spot testing; consequently, it is not known whether the conditions reported here would prevail throughout the year.

Likewise, during 1968-69, several water samples submitted by individual well owners were analyzed for nitrate content at the University of Wisconsin, Marathon County campus laboratory. The majority of the samples analyzed were from nonfarm wells within Marathon County and the remainder were from nonfarm wells outside the county. Percentage distribution of water with $NO_3^-$ concentrations of 45 mg/l or more in this group is 32 percent (21 of 64 wells analyzed, Table 4). Thus by comparison, this group had a lesser percentage of wells which produced water samples with a $NO_3^-$ concentration of 45 mg/l or more than either the wells in the Rib Falls–Rietbrock area or wells in the other townships included in the study.

Septic tanks are a potential source of nitrate contamination derived from the effluent discharged by means of tile fields and seepage beds (Polta, 1969). Several nitrogenous compounds are found in human waste (protein, amino acids, urea and ammonia), but these are eventually converted to ammonia by action of soil microorganisms. However, many soils reduce possible ammonia contamination by their fixing or adsorption ability. The adsorption alone may remove as much as 10 mg $NH_4^+$-N/100 g basic, negatively charged soil. Under certain conditions, however, adsorbed ammonia may be oxidized to nitrites and sub-

**TABLE 2. Concentration of $NO_3^-$ in the Water at Various Well Depths**

| Well Depth (ft)* | No. of Wells ≥ 45 mg/l $NO_3^-$ | No. of Wells ≤ 44 mg/l $NO_3^-$ |
|---|---|---|
| 10-30 | 12 | 11 |
| 31-50 | 12 | 13 |
| 51-70 | 22 | 18 |
| 71-90 | 16 | 12 |
| 91-110 | 4 | 6 |
| 111-130 | 7 | 2 |
| 131 | 8 | 7 |
| Total** | 81 (54%) | 69 (46%) |

* Well depths in part supplied by Thomas A. Calabresa, Chief, Private Water Supply Section, Bureau of Water & Shoreland Management Wisconsin Dep. Natur. Resour.

** Total refers to 81 wells which were surveyed twice a month and for which the well depth was known.

sequently to nitrates, which are soluble and readily leached into ground water supplies.

To determine the extent of nitrate pollution of drinking water in the community served by septic tanks, the Town of Rib Mountain was selected for study. The Town is located west of Wausau between Rib Mountain and Lake Wausau and the homes are built on pervious sand overlaying weathered granite. Approximately 750 septic tanks served the Town in 1950 and the number has nearly doubled to 1,325 in 1970. The community is devoid of farming, thus nitrate found in drinking water (well water) may be derived from septic tank effluents. Approximately 10 percent of the homes were randomly selected and their drinking water analyzed. Fifteen percent of the samples showed nitrate concentrations of 45 mg/l or more.

**TABLE 3. Spot Check on $NH_4^+$, $NO_2^-$, and $NO_3^-$ Contents in Wells of Grade A Milk Suppliers (April–May, 1969)**

| Date | Well No. | $NH_4^+$ (mg/l) | $NO_2^-$ (µg/l) | $NO_3^-$ (mg/l) | Date | Well No. | $NH_4^+$ (mg/l) | $NO_2^-$ (µg/l) | $NO_3^-$ (mg/l) |
|---|---|---|---|---|---|---|---|---|---|
| April 16 | 1 | 4.0 | 110 | 50 | April 24 | 31 | 2.8 | Tr | 46 |
| | 2 | 2.8 | Tr | 26 | | 32 | 3.6 | 90 | 66 |
| | 3 | 2.6 | 100 | 50 | | 33 | 3.2 | 70 | 57 |
| | 4 | 1.4 | Tr | 56 | May 1 | 34 | 3.2 | Tr | 46 |
| | 5 | 3.2 | Tr | 52 | | 35 | 3.9 | 60 | 45 |
| | 6 | 2.6 | Tr | 75 | | 36 | 3.7 | Tr | 35 |
| | 7 | 2.6 | 100 | 19 | May 14 | 37 | 4.0 | 100 | 49 |
| | 8 | 2.0 | Tr | 109 | | 38 | 2.8 | Tr | 50 |
| | 9 | 2.8 | Tr | 92 | | 39 | 3.2 | Tr | 58 |
| | 10 | 2.8 | 60 | 50 | | 40 | 3.6 | Tr | 41 |
| | 11 | 2.6 | Tr | 45 | | 41 | 2.0 | Tr | 34 |
| | 12 | 2.4 | 90 | 53 | | 42 | 1.6 | Tr | 17 |
| April 24 | 13 | 2.1 | Tr | 52 | | 43 | 2.2 | Tr | 24 |
| | 14 | 1.2 | Tr | 35 | | 44 | 3.0 | Tr | 71 |
| | 15 | 3.6 | Tr | 27 | | 45 | 1.6 | Tr | 33 |
| | 16 | 2.1 | 75 | 27 | | 46 | 2.1 | Tr | 28 |
| | 17 | 2.1 | Tr | 40 | | 47 | 2.2 | Tr | 33 |
| | 18 | 4.0 | Tr | 48 | | 48 | 3.0 | Tr | 35 |
| | 19 | 3.0 | Tr | 36 | | 49 | 2.8 | 90 | 71 |
| | 20 | 4.2 | Tr | 44 | | 50 | 0.8 | Tr | 21 |
| | 21 | 4.8 | 60 | 105 | | 51 | 2.0 | Tr | 84 |
| | 22 | 1.6 | Tr | 22 | | 52 | 1.4 | Tr | 19 |
| | 23 | 6.6 | Tr | 24 | | 53 | 2.0 | 150 | 22 |
| | 24 | 2.1 | 60 | 32 | | 54 | 1.2 | 60 | 22 |
| | 25 | 1.8 | Tr | 21 | | 55 | 1.4 | 975 | 121 |
| | 26 | 3.2 | 90 | 41 | | 56 | 3.2 | Tr | 39 |
| | 27 | 1.0 | 100 | 17 | | 57 | 1.4 | Tr | 22 |
| | 28 | 2.6 | Tr | 32 | | 58 | 1.2 | Tr | 22 |
| | 29 | 2.0 | 50 | 41 | | 59 | 1.4 | Tr | 12 |
| | 30 | 2.4 | 90 | 54 | | | | | |

* Representing twenty townships in Marathon, Clark & Taylor Counties (excluding Rib Falls and Rietbruck townships in Marathon County). 26 out of 59 wells contained 45 mg/l $NO_3^-$ or more (44.0%).

## Variation in Nitrite Concentrations

Comly (1945) has reported that $NO_2^-$ occurrence in well water is uncommon. However, our investigation showed that nearly 80 percent of the wells contained measurable amounts of $NO_2^-$ (10 $\mu$g/l to 400 $\mu$g/l) and that 20 percent of the wells contained $NO_2^-$ ranging from 500 $\mu$g/l to 5,300 $\mu$g/l (Crabtree, 1970). Although there is no clearly defined relationship between the depth of a well and high nitrate concentration for the area, there is a trend existing between increasing $NO_2^-$ concentration and well depth. The highest incidence of $NO_2^-$ concentration occurred most commonly in shallow and dug wells immediately after heavy precipitation. For example, at well depths less than 50 feet, 10 of 37 wells studied (27%) were considered high in nitrite. Figure 9 illustrates the frequency with which $NO_2^-$ fluctuation occurs in representative wells. The highest range of $NO_2^-$ in these wells was 1,225 to 5,000 $\mu$g/l.

In wells of intermediate depth (51 to 100 ft), 17 percent of the wells had high nitrite concentrations. The highest $NO_2^-$ content range was from 975 to 3,500 $\mu$g/l. In deeper wells, ranging in depth from 117 to 175 feet, 16 percent showed high nitrite concentration. The highest $NO_2^-$ content of these three wells ranged from 1,350 to 5,300 $\mu$g/l (Crabtree, 1970).

It is interesting to note that in wells deeper than 180 feet no high concentrations of nitrites were present. The highest range recorded for any one well was from trace amounts to 225 $\mu$g/l (Crabtree, 1970).

## SUMMARY AND CONCLUSIONS

Approximately 242 private wells representing 20 townships in Marathon County were examined for nitrate content and 55% of the wells contained nitrate concentration of 45 mg/l or more. Eighty-two of these wells (Rib Falls-Rietbrock area) were sampled twice each month during the period of investigation to obtain a record of nitrate level fluctuation. Nearly 70% of the 82 wells contained $NO_3^-$ level of 45 mg/l or more at one time or another within the period, and about 45% of the wells contained in excess of 45 mg/l $NO_3^-$ throughout the year. The $NO_3^-$ in ground water is highest following wet periods and lowest during dry periods with a few exceptions. There was no clearly defined relationship between high nitrate

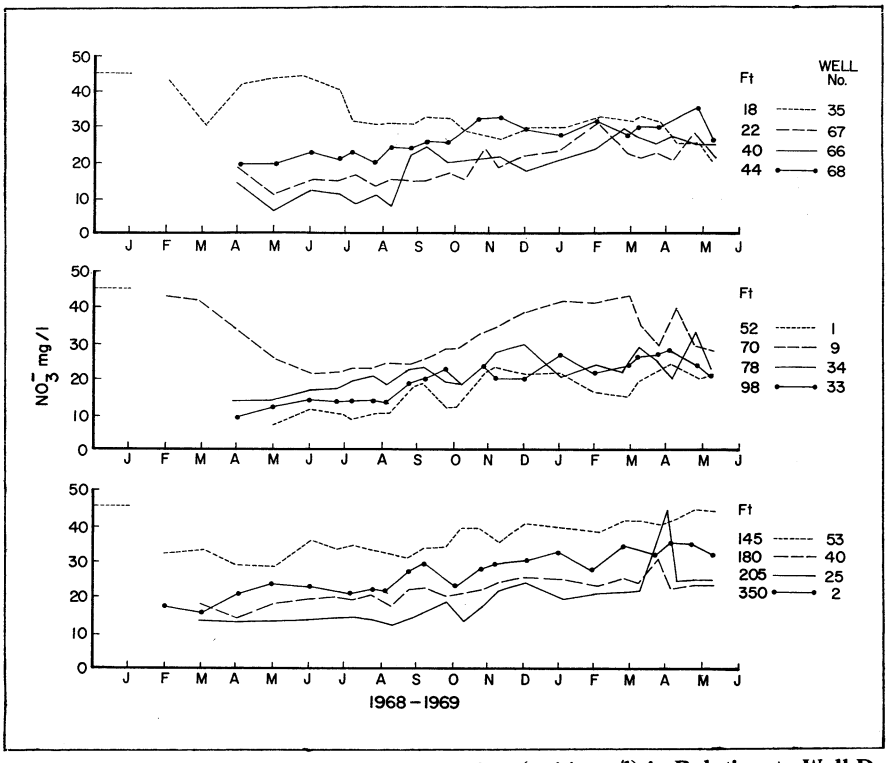
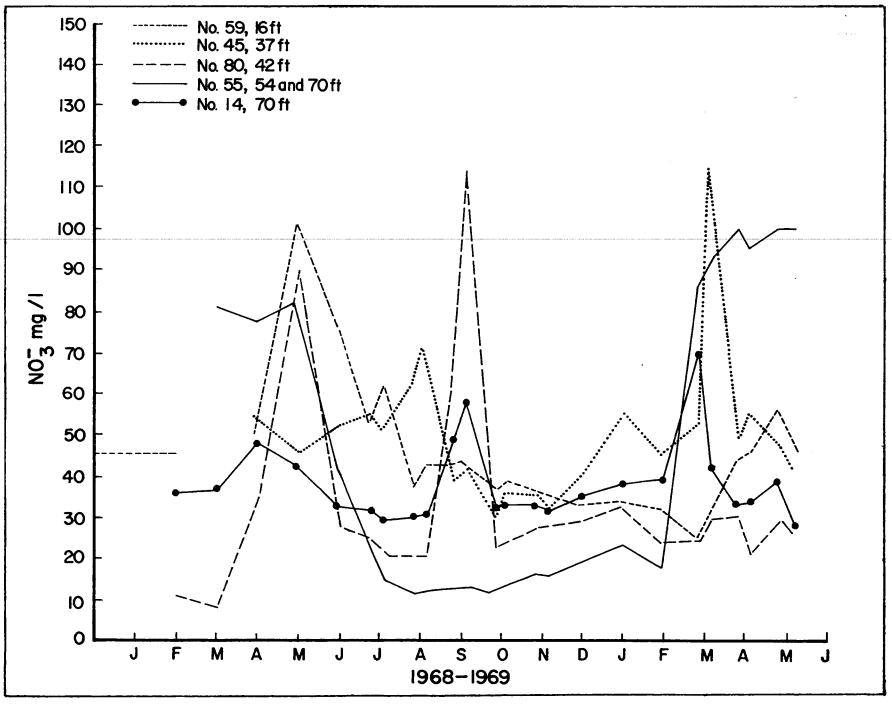



FIGURE 4. Variation in Nitrate Concentration (< 44 mg/l) in Relation to Well Depth.

FIGURE 5. Seasonal Variation in Nitrate Concentration in Well Water from Rib Falls–Rietbrock Area.



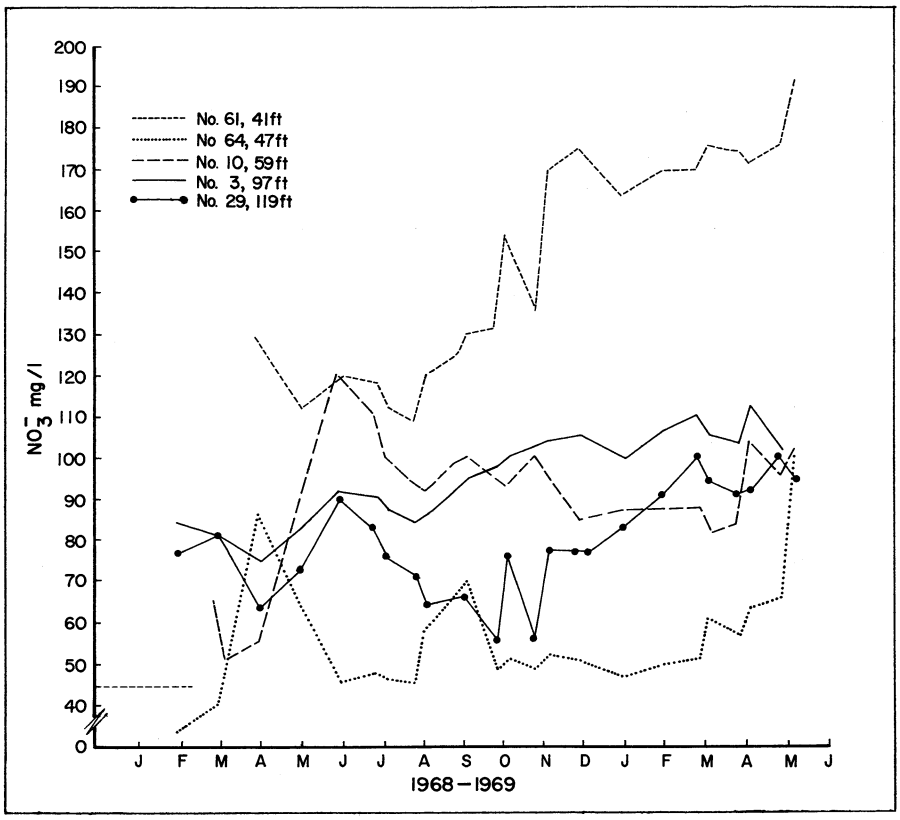



**FIGURE 6. Cumulative Trend of Nitrate Concentration of Well Water from Rib Falls–Rietbrock Area.**

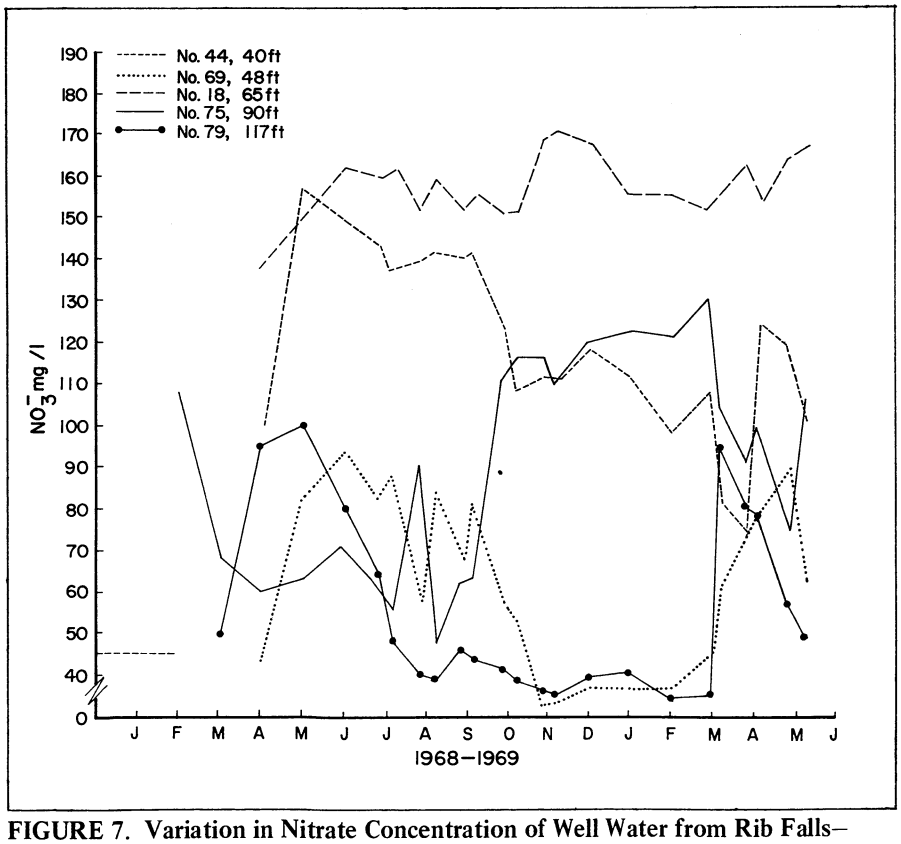



**FIGURE 7. Variation in Nitrate Concentration of Well Water from Rib Falls–Rietbrock Area.**

**TABLE 4. Nitrate Content of Nonfarm Wells, 1968-69***

| Month | Well No. | $NO_3^-$ (mg/l) | Well Depth(ft) | Area | Month | Well No. | $NO_3^-$ (mg/l) | Well Depth(ft) | Area |
|---|---|---|---|---|---|---|---|---|---|
| April | 1 | 17 | 115' | Wausau | September | 33 | 31 | - | Wausau |
| May | 2 | 19 | 85' | Wausau | | 34 | 34 | 42' | Wausau |
| | 3 | 12 | 80' | Wausau | | 35 | 31 | - | Marathon |
| July | 4 | 49 | 70' | Stettin | | 36 | 20 | 14' | Rib Lake |
| | 5 | 34 | - | Stettin | | 37 | 153 | - | Edgar |
| August | 6 | 108 | - | Hamburg | | 38 | 85 | 40' | Cassel |
| | 7 | 69 | - | Hamburg | | 39 | 18 | - | Wausau |
| | 8 | 16 | - | Rib Falls | November | 40 | 18 | - | Athens |
| | 9 | 41 | - | Rib Falls | | 41 | 26 | - | Athens |
| | 10 | 50 | - | Athens | | 42 | 48 | - | Athens |
| | 11 | 65 | - | Stratford | March | 43 | 41 | - | Texas |
| March | 12 | 23 | 175' | Rib Mountain | | 44 | 48 | - | Texas |
| | 13 | 73 | 60' | Maine | | 45 | 35 | - | Athens |
| | 14 | 28 | - | Wausau | | 46 | 46 | - | Merrill |
| April | 15 | 26 | - | Wausau | | 47 | 28 | - | Wausau |
| | 16 | 24 | 34' | Ringle | January | 48 | 179 | 60' | Stratford |
| | 17 | 20 | - | Phillips | | 49 | 192 | 57' | Cleveland |
| May | 18 | 23 | - | Weston | | 50 | 65 | 90' | Emmet |
| | 19 | 28 | - | Weston | | 51 | 44 | 52' | Emmet |
| September | 20 | 16 | - | Stettin | | 52 | 43 | 110' | Emmet |
| | 21 | 27 | - | Stettin | | 53 | 19 | 53' | Emmet |
| October | 22 | 41 | 96' | Wausau | | 54 | 89 | 59' | Cleveland |
| | 23 | 28 | 21' | Wausau | | 55 | 44 | 145' | Emmet |
| | 24 | 47 | 85' | Wausau | | 56 | 32 | 96' | Emmet |
| | 25 | 65 | 80' | Wausau | | 57 | 17 | 325' | Emmet |
| | 26 | 49 | 42' | Wausau | | 58 | 40 | 55' | Emmet |
| | 27 | 51 | 60' | Hamburg | | 59 | <1 | 10' | Emmet |
| | 28 | 35 | 60' | Wausau | | 60 | 21 | 90' | Emmet |
| | 29 | 31 | 76' | Wausau | | 61 | 76 | 60' | Emmet |
| August | 30 | 67 | 60' | Marathon | August | 62 | 10 | - | Antigo |
| | 31 | 34 | - | Wausau | December | 63 | 25 | 135' | Athens |
| September | 32 | 20 | 85' | Wausau | August | 64 | 24 | - | Wausau |

* Water samples were submitted by individual well owners.

concentration and depth of wells examined.

Nitrate concentration of 59 wells used by Grade A milk suppliers located in areas other than Rib Falls-Rietbrock Townships were spot-checked and results showed approximately 44% contained $NO_3^-$ concentration of 45 mg/l or more. Similarly, 32% of nonfarm well water samples submitted by individuals contained nitrate concentration in excess of 45 mg/l. However, only 15% of the samples analyzed from a community served by septic tanks were found to have nitrate concentration in excess of 45 mg/l.

Nitrate variation of individual wells was of three types: large and frequent increases or decreases of nitrate concentration between monthly samples; patterns similar to those of seasonal variation, i.e, usually high in the spring and decreasing during the summer; and a steady increase in nitrate concentration throughout the year.

The majority of wells studied showed a generally rising trend in $NO_3^-$ concentrations throughout the study period and this can be attributed to the cumulative effect of precipitation.

However, unlike nitrate, nitrite variation shows some relationship between high concentration and well depths, with highest incidence of nitrite concentration occurring most commonly in shallow and dug wells immediately after heavy precipitation.

Therefore, it is concluded that the type of well construction (dug, dug and drilled, or drilled) had no significant effect on nitrate concentrations in the wells sampled during the study period, partly because the great majority of wells sampled were old installations constructed prior to the establishment of the state well construction code, and therefore are inadequately cased or curbed for the most part.

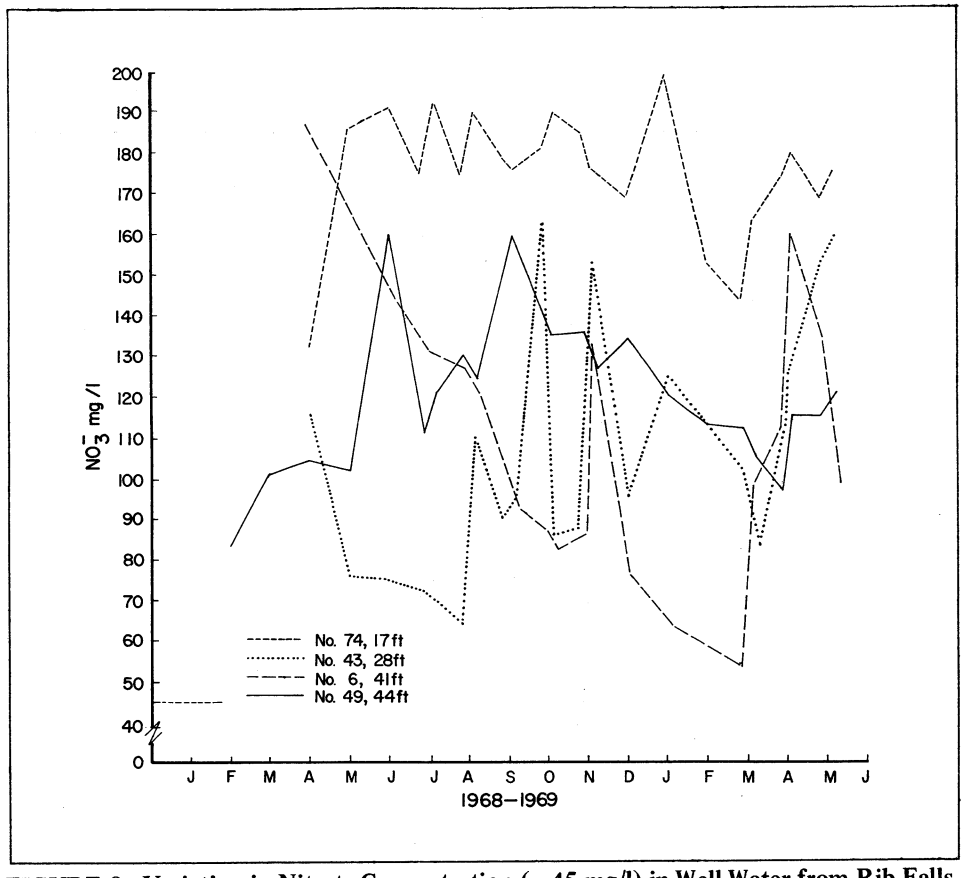



**FIGURE 8. Variation in Nitrate Concentration (> 45 mg/l) in Well Water from Rib Falls–Rietbrock Area.**

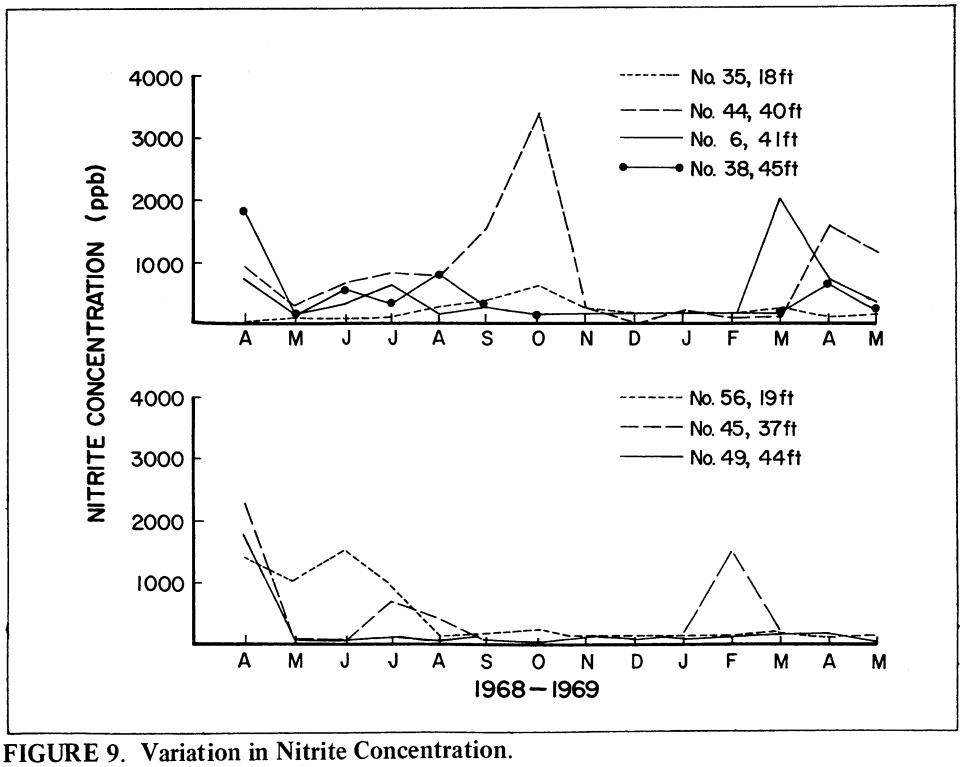



**FIGURE 9. Variation in Nitrite Concentration.**

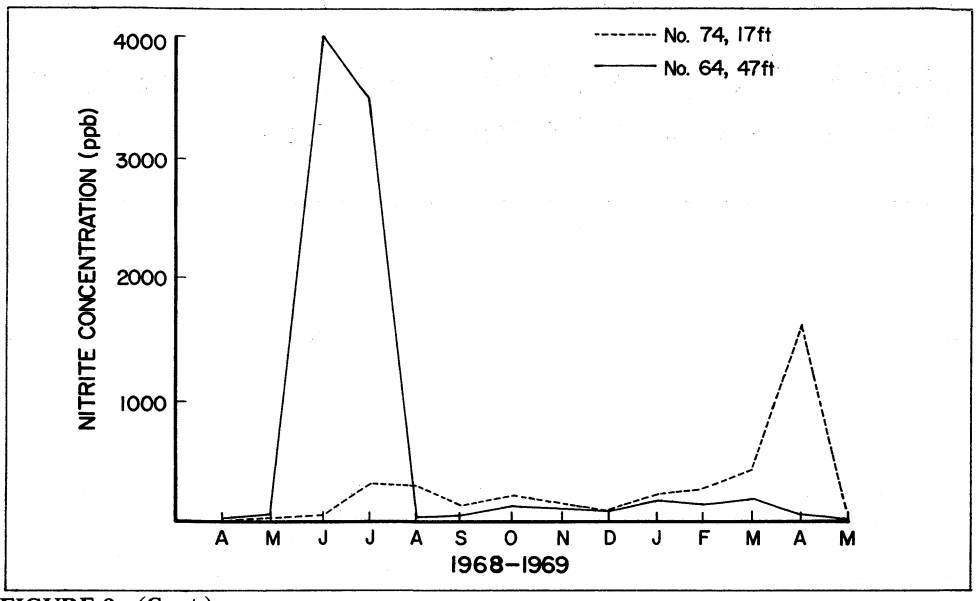



FIGURE 9. (Cont.)

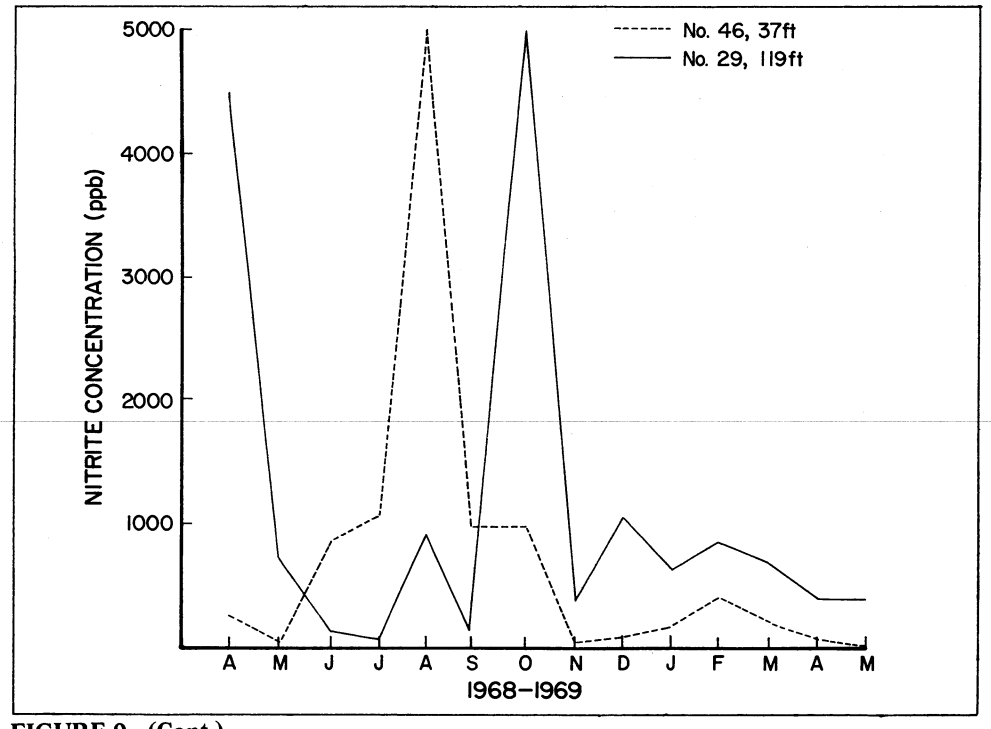



FIGURE 9. (Cont.)

# PART II: KINETICS OF HETEROTROPHIC NITRIFICATION BY MIXED CULTURE SYSTEM

## INTRODUCTION

The nitrate in soil and ground water may originate from microbial transformation of the manure that is returned to the soil. Traditionally, nitrification was considered the work of autotrophs *(Nitrosomonas* and *Nitrosococcus)* in which ammonium is oxidized to nitrite and subsequently the nitrite to nitrate *(Nitrobacter).* However, the possibility of heterotrophic nitrification, especially by a certain group of fungi, has been recently recognized as important in nitrate formation. The heterotrophic nitrifiers, particularly *Aspergillus flavus,* have been reported as potential nitrate formers (Schmidt, 1954 and 1960; Eylar and Schmidt, 1959; Hirsch et al., 1961; and Marshall and Alexander, 1962). Thus, the compounds important in nitrification are no longer limited to inorganic nitrogen but include a series of amino acids or peptides which may be converted to nitrates. Based on these findings, attempts were made to enumerate the distribution of both autotrophs and heterotrophs within farm environs.

Nitrification by 130 cultures of *Aspergillus* (six morphological groups) has been investigated and nitrifying species were found in all groups (Heimbrook, 1969). Approximately 70 percent of cultures belonging to *Aspergillus flavus-oryzae* and *A. wentii* are capable of producing nitrate from amino-N. *A. flavus* group yielded 65-100 mg/l $NO_3^-$-N. Similar observations are reported in a study on the occurrence of nitrifying micro organisms in aquatic environments (Thompson, 1969). In view of these findings, it is reasonable to assume that nitrate pollution of subsurface water may be attributed, at least in part, to the work of heterotrophic nitrifiers where large amounts of waste are returned to the soil.

## METHODS

The media employed in determining the autotrophs were as follows: Medium for nitrification (Medium A, oxidation of $NH_4^+$ to $NO_2^-$) contained: $NH_4Cl$, 0.975; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2$, 0.01; $K_2HPO_4$, 0.5; $CaCO_3$, 5.0; $KH_2PO_4$, 0.5 g/l.

Medium for second step nitrification (Medium B, oxidation of $NO_2^-$ to $NO_3^-$) contained: $NaNO_2$, 0.495; $MgSO_4 \cdot 7H_2O$, 0.20; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2$, 0.01; $K_2HPO_4$, 1.0; $KH_2PO_4$, 0.5; $CaCO_3$, 5.0 g/l.

The heterotrophic nitrification media (Medium C) contained the following ingredients in g/l : Bacto-Peptone, 5.0; malt extract, 1.0; yeast extract, 1.0; glucose, 2.0; $K_2HPO_4$, 1.0; $KH_2PO_4$, 0.25; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 4H_2O$, 0.01.

The medium (D) for kinetics of heterotrophic nitrification by mixed culture system was made by partially oxidized (about 10%) poultry wastes supernatant* composed of the following components: COD, 32.5; volatile acid, 7.12; total solid, 29.38; ash, 10.58; total P, 0.20; total N, 2.27; $NH_4^+$-N, 1.36, and BOD, 14.7 g/l.

All of the above media were supplemented with biotin to have a final concentration of 100 $\mu$g/l. The pH of Media A and B was adjusted to 7.5, and Medium C to 5.0. The low pH of Medium C was to discourage the growth of bacteria, but after 1 week of incubation, the pH of the cultures rose to approximately 7.3.

All media were dispensed in 4 oz bottles. The bottles were inoculated with the serially diluted soil suspension. The 3 bottles for autotrophic growth were incubated on a reciprocal shaker at 60 strokes per minute, whereas the bottles for heterotrophic growth were kept quiescent on their sides to promote aeration. Enumeration of autotrophic nitrifiers was begun at the end of 2, 3 and 4 weeks by testing either the presence of $NO_2^-$ in Medium A or $NO_3^-$ in Medium B. When the tests were positive, then the results were further confirmed by measuring residual $NH_4^+$ and $NO_2^-$ in the media. The enumeration of heterotrophic nitrifiers was made at the end of 2 and 3 weeks of the incubation period. Due to the interference from a heavy growth of organisms accompanied by various pigmentation of the media, the colorimetric methods used above (see Part I) were abandoned. Thus, Keeney's Direct Distillation Method (Keeney and Bremmer, 1965) was used to determine the presence of $NO_2^-$ or $NO_3^-$ as positive evidence of heterotrophic nitrification. Determination of $NH_4^+$, $NO_2^-$, $NO_3^-$ by other methods is presented in Part I of this report, and the methods concerning kinetics of nitrification are presented in the experimental section.

*Supplies by ZIMPRO-Division of Sterling Drug, Inc., Rothschild, Wisconsin

## RESULTS AND DISCUSSION

The numbers of autotrophic and heterotrophic nitrifiers in soils which had received varied amounts of bovine manure are shown in Table 5. It is interesting to find that the heterotrophic nitrifiers outnumbered the autotrophs in the soils. However, the importance of the autotrophs is not questioned here, but rather they are used in evaluating the nitrification potential of the heterotrophs in the soil enriched with organic materials, especially when one considers the slowness of autotrophic growth and the fastidious growth requirements as they are known in laboratory cultures. They are strict aerobes and they would be able to function only if the soil environs are aerobic. Likewise, regardless of their diverse physiological types, autotrophs are inhibited or retarded whenever subjected to media composed of highly complex organic matter. Consequently, one may question whether in "Nature" other organisms must be involved, either in setting up a suitable microenvironment for autotrophs or whether the heterotrophs themselves are involved in the nitrification process. This possibility is supported by experimentation which showed that in the presence of biotin

**TABLE 5. Most Probable Numbers of Autotrophic and Heterotrophic Nitrifiers from the Farm Environs (County x $10^3$/g Soil)**

| Bovine Manure Application rate | Autotrophs: Medium A ($NH_4^+ - NO_2^-$) | Autotrophs: Medium B ($NO_2^- - NO_3^-$) | Heterotrophs: Medium C |
|---|---|---|---|
| 0 T/A | 1.2 | 1.5 | 9.3 |
| 5 T/A | 9.0 | 12.0 | 15.0 |
| 10 T/A | 43 | 126 | 42 |
| 20 T/A | 22 | 540 | 75 |
| 30 T/A | 14 | 170 | 110 |
| 60 T/A | 11 | 13 | 120 |

| Soil Description | | Sample Site 1 | Sample Site 2 | Sample Site 3 |
|---|---|---|---|---|
| Soil receiving Silage juice | Autotrophs: (A) | 0.4 | 0.62 | 0.47 |
| | Autotrophs: (B) | 0.2 | 1.7 | 1.4 |
| | Heterotrophs: (C) | 3.6 | 3.4 | 2.4 |
| Septic tank runoff | Autotrophs: (A) | 0.22 | 0.38 | 0.36 |
| | " (B) | 0.96 | 1.4 | 0.8 |
| | Heterotrophs: (C) | 0.46 | 0.53 | 0.39 |
| Feed lot runoff | Autotrophs: (A) | 1.1 | 0.76 | 0.84 |
| | " (B) | 3.1 | 0.98 | 2.6 |
| | Heterotrophs: (C) | 24 | 13 | 28 |

(150 $\mu$g/ml) the rate of oxidation of nitrite to nitrate by *Nitrobacter agilis* is increased about threefold (Krulwich and Funk, 1965). Although little is known of the biotin level in manured soil, it is doubtful if it is present in the concentration mentioned above, unless furnished by heterotrophs growing in a microenvironment.

For the study of heterotrophic nitrification, Medium D was diluted with distilled water to make possible the maintenance of dissolved oxygen content of the system greater than 2 mg/l for the duration of the experiment. Medium D-1 contained 8 parts Medium D and 2 parts distilled water; D-2 contained 4 parts Medium D and 6 parts water; and Medium D-3 contained 2 parts Medium D and 8 parts water. The 3 flasks, containing respectively, D-1, D-2 and D-3 were inoculated with 1 gram of soil obtained from a manure-dressed field. They were incubated in a rotary shaker (170 rpm) and 10 ml samples were withdrawn from each flask daily for duration of 1 to 3 weeks.

According to Figure 10 (D-1), there was rapid nitrification after 3 days which then tapered off after a 5-day incubation. Maximum nitrification rate (K) is approximately 1,131 mg of $NO_3^-$/day and some 72 percent of $NH_4^+$N in the medium was converted to $NO_3^-$-N at the end of 7 days.

With Medium D-2 (Fig. 11), however, rapid nitrification did not occur until 4 days after incubation. Nitrification continued for a period of 4 days, and ultimately a maximum nitrification rate of approximately 370 mg of $NO_3^-$-N/day was obtained. Increase in $NO_3^-$-N after 11 days (at which time $NH_4^+$-N tapered off) is attributable to the conversion of either intermediate $NO_2^-$ or organic-N to $NO_3^-$-N.

Maximum rate of nitrification in Medium D-3 (Fig. 12) was approximately 510mg $NO_3^-$-N/day and it began after about 4 days. However, apparent conversion of $NH_4^+$-N to $NO_3^-$-N (87%) was completed within 7 days. At 26 days $NO_3^-$-N exceeded that of the initial $NH_4^+$ content which indicates conversion of intermediate $NO_2^-$-N or organic-N to $NO_3^-$-N.

In all experiments, rapid initial oxidation of $NH_4^+$-N did not coincide with increase of $NO_3^-$-N, which indicates possible formation of cell-N or intermediate $NO_2^-$-N. However, the second decline in $NH_4^+$-N coincides with a rise in $NO_3^-$-N. It should be mentioned here that BOD of Medium D is primarily composed of acetate and

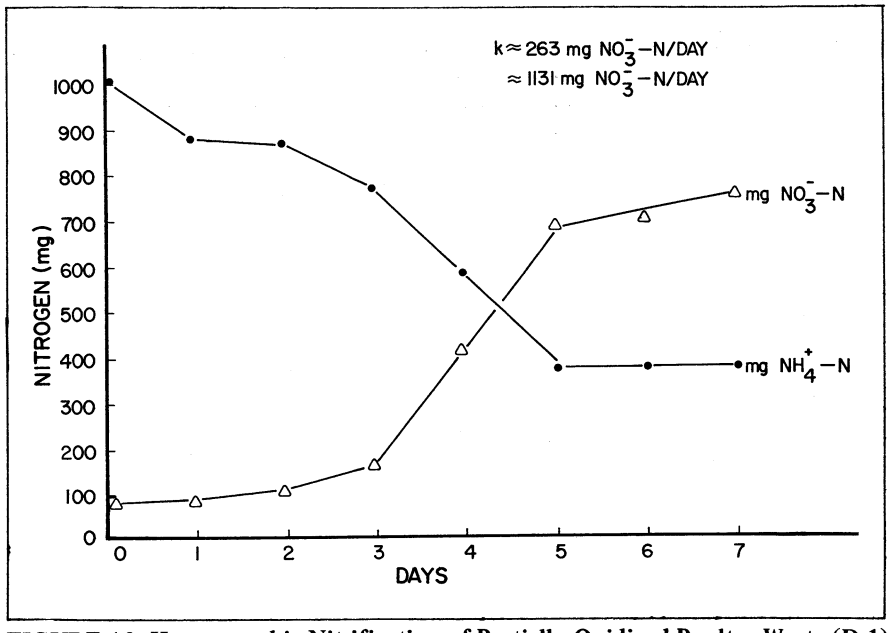



FIGURE 10. Heterotrophic Nitrification of Partially Oxidized Poultry Waste (D-1).

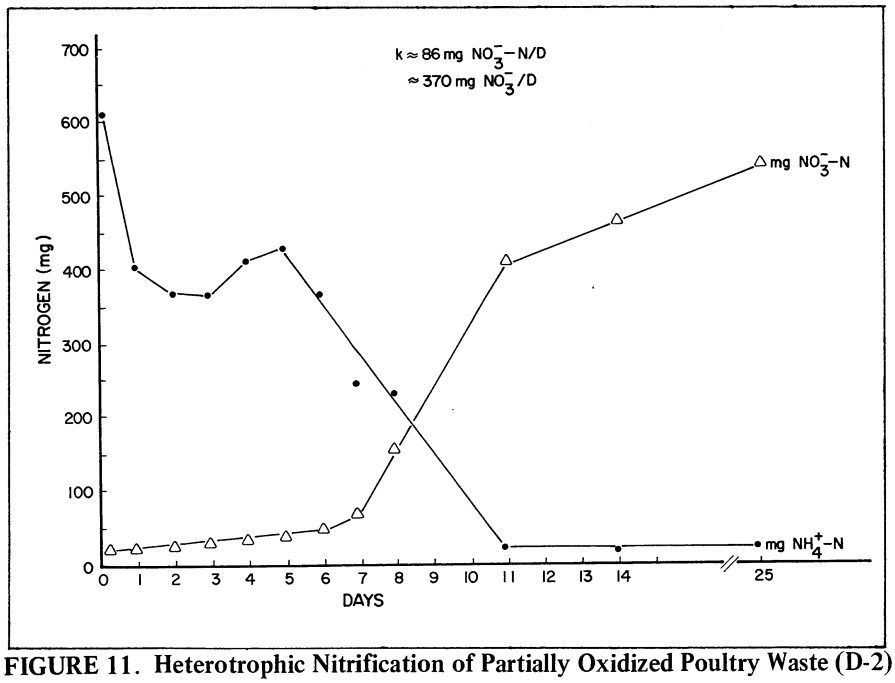



FIGURE 11. Heterotrophic Nitrification of Partially Oxidized Poultry Waste (D-2).

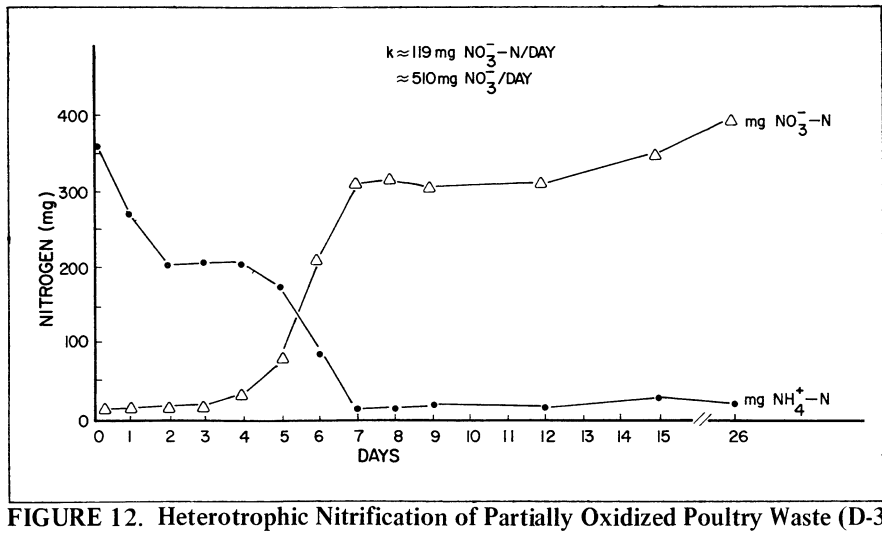



FIGURE 12. Heterotrophic Nitrification of Partially Oxidized Poultry Waste (D-3).

the N difference [(total-N) - ($NH_4^+$-N)] is considered an exceedingly complex and rather heat stable organic-N. Thus it is apparent that nitrification by heterotrophic organisms can occur in conditions suitable for autotrophic bacterial nitrification. Heterotrophic nitrifying organisms can use either $NH_4^+$-N, organic-N, or $NO_2^-$-N as intermediate compounds.

The experiment showed that N in poultry waste can be converted to $NO_3^-$-N at a rate ranging somewhere between 300 and 1,100 mg of $NO_3^-$-N/day by various heterotrophic bacteria and fungi. Consequently, if manure is applied properly (early in the season), most of the organic-N can be recovered as nitrate utilizable by crops within a growing season. However, with improper application of manure, such as large amounts spread on the field in late summer, there is sufficient time for heterotrophic nitrification before freezing. Nitrate formed during such period is unlikely to be used either by plant or by bacterial denitrification; consequently, it may be leached to underground water supplies, especially if the area is subjected to heavy rainfall before the ground is frozen.

# PART III: ENUMERATION OF DENITRIFYING ORGANISMS AND KINETICS OF DENITRIFICATION

## INTRODUCTION

Present farming practices often create situations in which nitrates accumulate through heavy application of commercial fertilizers for nitrification faster than they are removed by either crops or by denitrification. Therefore the distribution of denitrifiers from the sources previously mentioned was investigated.

There has been an attempt to remove nitrate from aerobically treated sewage (tertiary treatment) by bacterial denitrification. Thus a second purpose of this part of the study is to determine the feasibility of nitrate removal by denitrification process.

## METHODS

Because there are no precise and practical methods for enumerating the denitrifiers, the Most Probable Number method was used. The liquid medium used (Medium E) contained the following ingredients in g/1: casein hydrolysate, 0.5; Bacto-peptone, 0.2; Bacto-tryptone, 0.2; Starch (soluble), 0.2; $KNO_3$, 1.0; $K_2HPO_4$, 0.5; $KH_2PO_4$, 0.25; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.01, and $Na_2MoO_2 \cdot 2H_2O$, 0.001 g/1.

The medium was heated to 80C and cooled to 37C in a water bath to drive off all the dissolved oxygen. This process was necessary just prior to use as the denitrification process is favored in an absence of dissolved oxygen. The tubes were inoculated with the serially diluted manured soil suspension. The inoculated tubes were incubated at 30C and observed daily for gas ($N_2$) production. Tubes with gas bubbles were considered to be positive, and these were further confirmed by testing for the presence or absence of the intermediate, $NO_2^-$, by the colorimetric analysis and residual $NO_3^-$ by Keeney's Distillation Method. At the end of two weeks, those tubes without gas formation were tested for nitrate reduction, i.e., for $NO_2^-$.

Denitrifying bacteria were isolated: the highest dilution tubes with gas ($N_2$) were used as the source material. They were serially diluted and plated on nutrient agar. The plates with the colony number ranging between 100 - 200 were used. The isolated colonies on the plates were picked randomly and inoculated into Medium D and incubated at 30C for a week. The tubes with the most rapid gas production were used for further purification by repeated dilution and plating. The gas produced by the purified isolates was analyzed by a gas chromatograph (F&M Model 700) to confirm the $N_2$ to be the end product of dentrification.

## RESULTS AND DISCUSSION

The numbers of nitrate reducers and denitrifiers in the soil which received varied amounts of bovine manure are presented in Table 6.

The total number of denitrifiers increased as the rate of manure application increased. However, the denitrifying bacteria were found regardless of aerated or water-logged soil, with or without the presence of detectable nitrites or nitrates. Nevertheless, denitrification and nitrate reduction by heterotrophs may occur only when a sufficient amount of nitrite or nitrate is present in a given microbial environment lacking in other more suitable hydrogen acceptors.

The question inevitably raised is why the accumulation of such high nitrate content in the ground water supply exists in the presence of exceedingly large numbers of denitrifiers. A reasonable explanation is that the denitrifying bacteria found under the described field conditions may not be active in the denitrification process. The soils may be sufficiently aerobic or lacking in hydrogen donor compounds. Under aerobic conditions, the ecosystem would inhibit the denitrification process regardless of the presence of nitrates.

**TABLE 6. Most Probable Numbers of Nitrate Reducers and Denitrifiers from the Farm Environs***

| Samples Bovine Manure (Application Rate) | Nitrate Reducers | Denitrifiers | |
|---|---|---|---|
| | $NO_3^- \rightarrow NO_2^-$ | $NO_2^- \rightarrow N_2^-$ | $NO_3^- \rightarrow N_2^-$ |
| 0 T/A | 320 | 0.7 | 2.5 |
| 5 T/A | 540 | 1.1 | 3.7 |
| 10 T/A | 1,200 | 1.6 | 7.8 |
| 20 T/A | 3,600 | 2.7 | 25 |
| 30 T/A | 4,900 | 7.8 | 32 |
| 60 T/A | 9,200 | 12.8 | 57 |
| Soil receiving | | | |
| Silage juice | 370 | 0.5 | 3.8 |
| Septic tank runoff | 90 | 0.2 | 1.5 |
| Feedlot runoff | 5,100 | 2.8 | 34 |

* (Count x $10^4$ Cells/g Soil)

Likewise, aerobic environments in manured soils would promote the rapid metabolism of both the nitrifiers and the denitrifiers; both involve a mineralization of the manure. If, then, anoxic conditions occur after the nitrification, denitrification processes still may be inhibited by the lack of suitable hydrogen donor compounds. Thus, nitrification and denitrification may be sequential, or denitrification may fail for the lack of hydrogen donor.

## Denitrification (Endogenous) by Resting Cells of the Isolates

Four cultures, P-1, P-2, P-3, P-4 (all Gram-negative, motile, polar flagellates belonging to the genus *Pseudomonas)* representing isolates from the manure-dressed soil, were selected for denitrification studies. Other results such as nutritional requirements, effects of various substrates on the denitrification, oxygen demands, are being obtained from these cultures through experimentation presently in progress at the University of Wisconsin, Marathon County campus.

Since denitrification may occur more prominently in the rhizosphere, owing to the availability of hydrogen donor compounds (excreted by roots) in oxygen-deficient environments, the experiments were designed to simulate the rhizosphere.

Medium F for denitrification contained the following ingredients: Trypticase, 0.3; Phytone, 0.05; NaCl, 0.03; $K_2HPO_4$, 0.03; $Na_2MoO_2 \cdot 4H_2O$, 0.0001 g/l. Tenfold concentration of this medium in the form of agar slopes was used in maintaining the stock cultures. The medium was dispensed into 4 specially designed Erlenmeyer flasks and steam sterilized and cooled to 37C in a water bath to drive off any dissolved oxygen. Any residual oxygen in the flasks was removed by sparging with high purity dry nitrogen gas.

The flasks were inoculated with 24-hour cultures prepared in liquid Medium F and incubated at 28C. The cultures were mixed by both magnetic stirrers and the sparging nitrogen gas. After 18 hours incubation (at which time the medium is considered anoxic), nitrate was added to the flasks to have a final concentration of 100 mg of $NO_3^-$/ml. It should be mentioned that both the inoculum and the hydrogen acceptor ($NO_3^-$) were added with hypodermic syringes via the serum cap to avoid introduction of air.

Immediately after the addition of nitrate, samples of about 15 ml each were withdrawn from each flask and analyzed. The viable cell population was determined by dilution and plating method. A portion of the samples was filtered through a millipore membrane and the filtrates were analyzed for $NO_2^-$ and $NO_3^-$. Nitrite, if formed by the reduction of nitrate, was determined by the colorimetric method and residual nitrate by Keeney's Direct Distillation Method. Sampling was made at half hour to hour intervals. A summary of the denitrification by the four isolates is shown in Figure 13.

All cultures were able to produce $N_2$ gas within a 1- to 2-hour period except for the culture P-4, for which the lag period lasted 2 to 4 hours. The observed lag period may be attributed to the time required for the cells to synthesize enzymes necessary for nitrate dissimilation. The comparison of plate counts made at zero hour and at the end of the experiment showed no significant increase or decrease in the number of viable cells. This indicates either that the nutrient was being depleted during the 18-hour incubation period preceding the experiment or that the cultures were in early stationary phase. If the above observation is true, the values obtained from this experiment may be derived from endogenous respiration, with nitrate being the ultimate hydrogen acceptor.

Considerable quantities (3 to 6 mg/l) of nitrite were produced by the cultures P-1, P-2, and P-3 during the log phases of denitrification. However, the culture P-4 rarely accumulated nitrite in its reductive dissimilation of nitrate to gaseous nitrogen. Table 7 shows the rate of endogenous denitrification by the isolates.

## Effects of Hydrogen Donors on the Dissimilatory Nitrate Reduction by the Denitrifiers

The presence of a hydrogen donor during denitrification should accelerate the reductive processes. On this assumption, three hydrogen donor compounds, glucose, glutamic acid, and glycerol were selected. All the procedures involved in this experiment were the same as in the preceding

except for the incubation period prior to the addition of nitrate, which was reduced from 18 to 8 hours, and the hydrogen donor compounds which were added (0.5 g/l) with the nitrate. One flask without the hydrogen donor was kept as a control. Samples were withdrawn more frequently as denitrification did proceed much faster, owing to the presence of hydrogen donors. As the nitrate was depleted in the flask, additions were made until depletion of the hydrogen donors. Depending upon the cultures and the hydrogen donor compounds, three to six denitrification experiments were made. The summarized data are in Figure 14 and maximum denitrification rates calculated from the curves are presented in Table 8.

Viable cell counts showed slight increases in the population during the experiments and this increase may be attributed in part to utilization of hydrogen donor compounds for cell reproduction in presence of hydrogen acceptor, $NO_3^-$.

A slight lag period observed in the first run may be attributed to lack of necessary enzyme systems. Although the responsible dehydrogenases for glucose, glutamic acid, and glycerol may be considered as constituative enzymes, the enzyme involved in the hydrogen transport from the substrate to nitrate required induction. Nitrate reduction started without a lag period in the second run, because the cells were already adapted during the first run.

Comparison of the 4 denitrification systems: control, glucose, glutamic acid and glycerol, showed different efficiencies in regard to denitrification rates. In the controls, denitrification was not detectable after the first run owing to depletion of hydrogen donor of the basal medium. With all isolates except P-4, the rate of denitrification using glutamic acid as a hydrogen donor was considerably higher than it was using either glucose or glycerol. This observation is in accordance with the findings of Woldendorp (1963) in which the denitrification activity of certain pseudomonads isolated from grassland soil was stimulated by the addition of glutamic acid as hydrogen donor.

Curiously the effect of glumatic acid on the denitrification by P-4 was exceptional in being slower than with glucose and glycerol, and on all three substrates denitrification was slow. This slowness may be attributed to a physiology peculiar to this organism in which the cells accumulate considerable amounts (30% of cell dry weight) of poly-B-hydroxybutyrate. It is assumed that large amounts of the hydrogen donor compounds are thus being converted to the intracellular polymer rather than oxidized to $CO_2$ and $H_2O$ via Kreb's Cycle. The polymer is considered as an endogenous metabolite, and is used only after the depletion of externally available nutrients. Also, endogenous mobilization of the polymer is dependent upon depolymerase activity and this activity may not be optimum when nitrate is the ultimate hydrogen acceptor.

The maximum denitrification rate of 83 mg of $NO_3^-$ per hour per 122 x 106 cells per ml is in a range to be of considerable importance, but the practical application of the process to nitrate-polluted water requires further investigation because of the inherent disadvantage that it requires added hydrogen donor not natural to a ground water supply. It might, however, be applicable to the polluted waters of municipal or industrial sewage where a natural hydrogen donor is present or may be added.

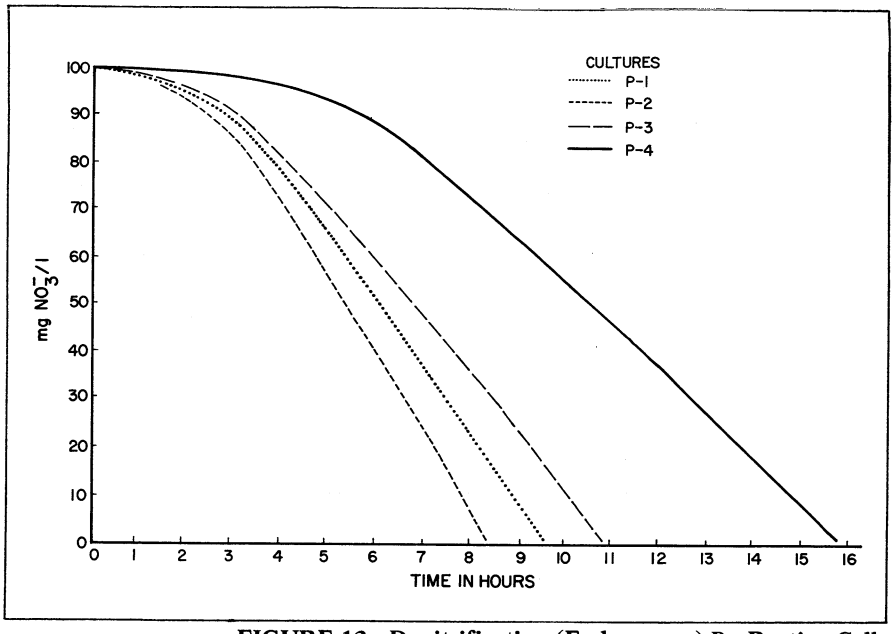



**FIGURE 13. Denitrification (Endogenous) By Resting Cells**

**TABLE 7. Rates of Dissimilatory Nitrate Reduction to Gaseous Nitrogen**

TABLE 7. Rates of Dissimilatory Nitrate Reduction to Gaseous Nitrogen

| Culture | $NO_3^-$/hr (mg/l) | Cells/ml ($10^6$) |
|---|---|---|
| P-1 | 14.0 | 86* to 89** |
| P-2 | 16.3 | 79 to 81 |
| P-3 | 12.0 | 96 to 93 |
| P-4 | 9.2 | 84 to 92 |

* Average of triplicate plate counts at 0 hr.
** Average of triplicate plate counts at the end of the experiment

FIGURE 14. The Effect of Hydrogen Donors on the Rate of Denitrification (Culture P-1).

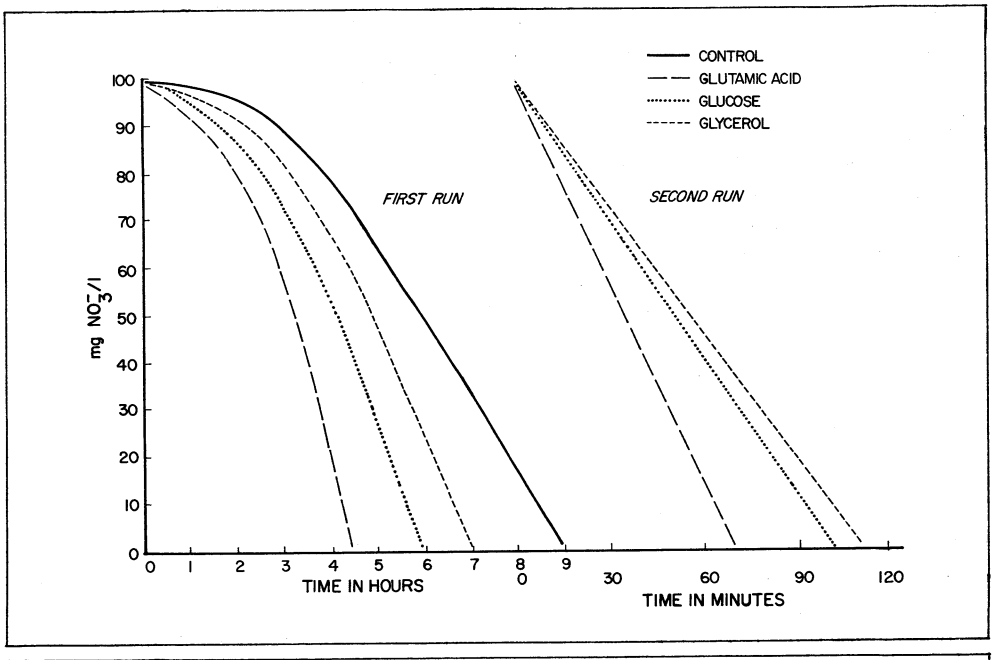



FIGURE 14. (Cont.) Culture P-2

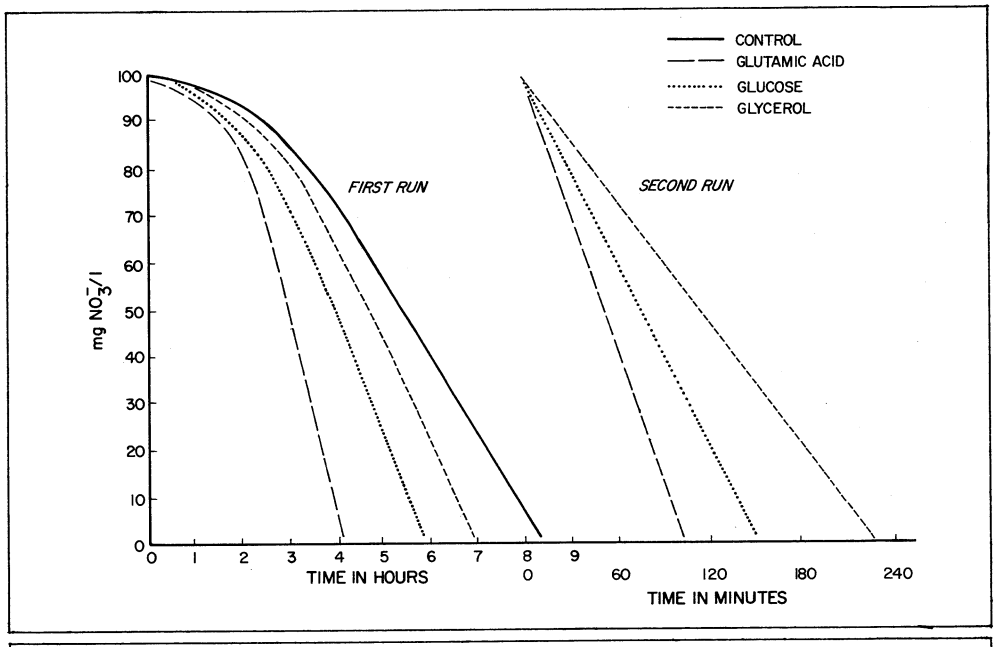
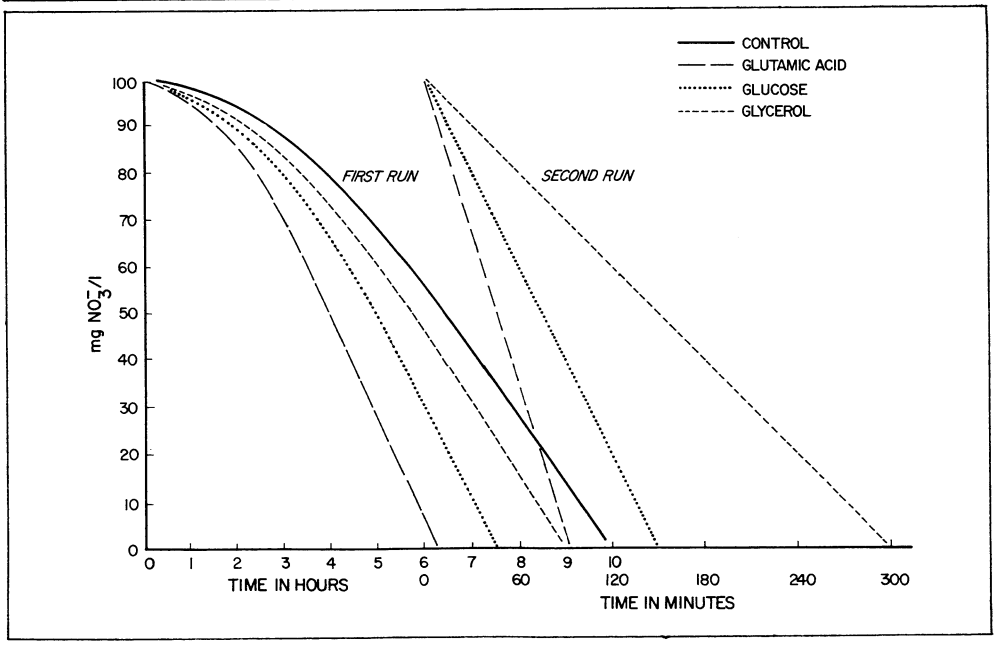



FIGURE 14. (Cont.) Culture P-3



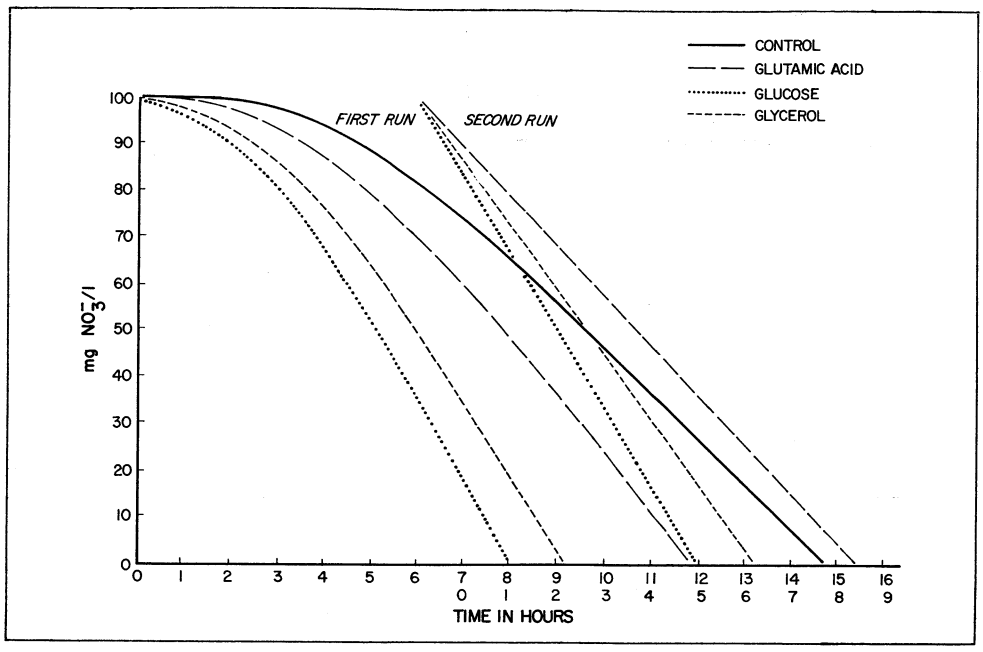



**FIGURE 14 (Cont.)**
**Culture P-4**

**TABLE 8. The Effect of Different Hydrogen Donors on the Rate of Denitrification**

| Cultures | Hydrogen Donors | Maximum Rates $NO_3^-$/hr. (mg/l) | Maximum Rates Cells/ml ($10^6$) |
|---|---|---|---|
| P-1 | control | 15.7 | 86 to 122 |
| | glucose | 57.0 | 69 to 178 |
| | glutamic acid | 83.0 | 65 to 284 |
| | glycerol | 52.0 | 49 to 174 |
| P-2 | control | 16.2 | 89 to 162 |
| | glucose | 39.5 | 72 to 280 |
| | glutamic acid | 57.0 | 78 to 430 |
| | glycerol | 25.6 | 98 to 290 |
| P-3 | control | 12 | 82 to 158 |
| | glucose | 40 | 96 to 180 |
| | glutamic acid | 63 | 78 to 40 |
| | glycerol | 20 | 89 to 177 |
| P-4 | control | 11.0 | 71 to 124 |
| | glucose | 19.5 | 34 to 84 |
| | glutamic acid | 12.0 | 39 to 85 |
| | glycerol | 16.0 | 59 to 91 |

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