THE COMMONALITY OF NUMERICALLY DOMINANT DENITRIFIER STRAINS ISOLATED FROM VARIOUS HABITATS

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ABSTRACT

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Soils, fresh-water lake sediments, and a nitrified poultry manure, were examined for predominant denitrifier species. The samples were from eight countries and included rice, crop, rainforest, desert, acid, organic, and waste-treated environments. Denitrifier populations were generally 10^5 to 10^6 organisms per gram dry weight. The ratio of population densities of denitrifiers to organisms which reduce nitrate only to nitrite to total organisms which can grow anaerobically was fairly constant among samples; the average ratio was 0.26 : 0.68 : 1.

A total of 1500 isolates which grew on nitrate agar incubated in an anaerobic glove box were tested for the ability to denitrify. Following purification, 147 isolates were confirmed as denitrifiers by the production of N_2O and/or N_2 during growth in nitrate broth. The remaining isolates either produced nitrite, ammonia, or could not be maintained in culture. The denitrifier isolates were characterized using 52 properties appropriate for the Pseudomonas - Alcaligenes group. Pseudomonas was the

dominant genus, whereas Alcaligenes faecalis was the most commonly isolated species. Other denitrifiers isolated included: Pseudomonas fluorescens biotype II, Pseudomonas fluorescens biotype IV, Pseudomonas aeruginosa, Pseudomonas stutzeri, Pseudomonas aureofaciens, and Pseudomonas solanacearum. Strains of a denitrifying Flavobacterium species were isolated which is the first report of denitrification by a member of this genus. A few denitrifying bacteria in the genera Corynebacterium and Bacillus were also isolated. About one-third of the isolates do not appear to be closely related to any recognized species though many do conform to the genus Pseudomonas. These were grouped according to common characters into 25 identifiable types. No isolates similar to Pseudomonas denitrificans were recovered. A high correlation between temperature of isolate growth and temperature of habitat was noted. All isolates from tropical areas (mean annual temperature < 20 C) failed to grow at 4 C while 67% grew at 41 C. In comparison 68% of the isolates from temperate soils grew at 4 C and only 9% grew at 41 C.

In conclusion, members of the Pseudomonas and closely related Alcaligenes genera were found to be numerically dominant since they represent 89% of the total denitrifying isolates. The species of greatest importance appear to be Pseudomonas fluorescens biotype II and Alcaligenes faecalis which were found in 58% of the samples and comprised 41% of the isolates.

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to my grandmothers

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INTRODUCTION

Denitrification is one of the most important processes in the nitrogen cycle since it is the means by which combined nitrogen is lost to the biosphere. It is defined as the biological reduction of inorganic forms of nitrogen (nitrate and nitrite) to volatile gases, nitrous oxide and/or molecular nitrogen. It is an enzymatic process accomplished by certain bacteria capable of using nitrate in place of oxygen as the terminal electron acceptor. Payne (1973) states that there are species in 15 genera of bacteria that have been reported to denitrify (32). These include Achromobacter, Alcaligenes, Bacillus, Chromobacterium, Corynebacterium, Halobacterium, Hyphomicrobium, Micrococcus, Moraxella, Nitrosomonas, Propionibacterium, Pseudomonas, Spirillum, Thiobacillus, and Xanthomonas. The most prominent denitrifying bacteria previously reported in soils were Bacillus spp., especially Bacillus cereus. In soils where nitrate fertilizers were added, these forms were replaced by Pseudomonas, Achromobacter, and Bacillus macerans (23).

Denitrification was first recognized in 1886 by Gayon and Dupetit (19). They observed that certain soil bacteria were capable of reducing nitrate to molecular nitrogen and nitrous oxide. With the work of Ferguson and Fred (1908),

it was well established that denitrification in soils was enhanced by the addition of manure (16). Kluyver and Donker (1926) discovered that nitrate served as the hydrogen acceptor in enzymatic dehydrogenation of organic or inorganic substrates (28). However, even with all that was known about denitrification at that time, scientists were not convinced that there was any economic or health significance to the process. Little interest was paid to the subject until 1946. It was then that the astronomer Adel observed that the concentration of nitrous oxide was greater near the surface of the earth than at higher altitudes. He suggested that this was due to a biological decomposition of nitrogen compounds in soil (2,3). Although the enhancement of denitrification is desired for the removal of nitrate from waste treatment systems and from groundwater, present concern centers around the detrimental effects of the process. Denitrification losses of nitrogen from fertilizers added to soils have been reported to vary between 1 and 75% of that applied. However, many soil scientists feel that on the average 10 to 15% of the applied nitrogen is lost due to denitrification (9). With the recent increased cost and world shortage of nitrogen fertilizers, such a loss results in a financial loss to the farmer as well as reduced food production. Furthermore, the denitrification intermediate, N_2O, apparently diffuses into the stratosphere and photo-decomposes to N_2 and small amounts of NO and NO_2, which react with O_3 to

form O_2. Since ozone attentuates the UV light from the sun, such a depletion of O_3 by N_2O means more UV light will reach the Earth's surface and therefore pose a hazard to both plants and animals. Recently, McElroy has hypothesized that the increased global use of nitrogen fertilizer has increased the amount of N_2O eminating from soil thus enhancing ozone destruction. In 1974, almost 40 million metric tons of nitrogen fertilizers were used (11).

There is little known about denitrification in natural ecosystems due to the absence of a sensitive, convenient, and specific assay for the process. Because of such technical problems, one must rely more on ecological information obtained with pure cultures. However, one must have some indication of the importance of the culture in actual denitrification in the environment. Therefore, isolating and quantifying numerically dominant denitrifiers from a wide variety of habitats was a primary objective of this study. From this information, the degree of commonality of the genera and species with apparent ecological significance was determined. Secondary objectives included the development of an effective isolation and identification system for denitrifiers, the determinations of environmental factors which control population density and species composition, and the estimation of general population structure between organisms capable of anaerobic growth, those organisms which convert nitrate only to nitrite, and denitrifiers.

MATERIALS AND METHODS

Description Of Samples

Nineteen soils, three fresh-water sediments, and a poultry manure liquor from a fermenter which was undergoing nitrification and denitrification were used. Samples were collected from locations where loss of nitrogen by denitrification had been measured or was expected. Soils were obtained from seven countries under a permit which allowed entry of untreated samples.

The Connecticut soil, No. 1, was a sample obtained from an experimental soil column which was incubated with glucose and actively denitrifying (39). The Connecticut soil, No. 2, was from the same soil column after the completion of the experiment, at which time denitrification was no longer being measured. Michigan Ponds 1 and 4 are two ponds of a four pond secondary effluent sewage treatment system known as the Water Quality Management Project presently in operation at Michigan State University. Pond 1 is the first pond in the system, thus receives the greatest organic load and Pond 4 is the last pond in the system, and has the lowest organic load. The pond retention times in the summer months are approximately 30 days. Ponds 1 and 4 were both sampled in November 1974 and May 1975.

The nitrified poultry manure liquor was obtained from a nitrified mixed liquor, deoxygenated by bubbling N_2 through it to stimulate denitrification. The incubation temperature of the liquor was 20 C (34).

Contributors of the other samples (all soils) were asked to provide information on crop grown, previous crops, soil type, approximate location where sample was taken, and any other information thought to be useful. A summary of this information is listed in Table 2. Also, a listing of the contributors and their mailing addresses can be found in the Appendix. Contributors were asked to collect and ship the sample as described below.

Sample Collection, Handling, And Storage

Each composited soil sample of approximately 0.5 kg was made up from six subsamples freshly collected from the Ap (plow) horizon in a 10 m^2 homogeneous soil area. The composited sample was then sealed in a plastic bag and placed in a sturdy container for immediate shipment by air. When received, each sample was refrigerated at 1 C until use. No sample was stored more than four weeks before use except for the Minnesota, California, Connecticut, and Kansas samples which were stored until the methods for the isolation and enumeration were standardized.

The three sediments were collected with a plexiglass gravity corer. For Ponds 1 and 4, a composite sample was made from the top 1 cm of each core from three sites for

each pond. The composite samples were stored at 1 C until use.

Sample Characterization

The soil classification is according to the 7th Approximation. For non-U.S. samples, the classification is only approximate. With the help of Dr. E. P. Whiteside, Michigan State University, and using FAO Soil Maps, information supplied by the cooperator, and the soil sample, an approximate equivalent for the 7th Approximation was determined. Soil and sediment pH was obtained by mixing 15.0 g of a sample with 15.0 ml of distilled water. After allowing the suspension to settle for approximately 30 min, the mixture was again stirred and the pH reading taken on an Ionalyzer Model 801/Digital pH Meter (Orion Research Inc., Cambridge, Mass.). The pH meter was calibrated at 4.0, 7.0, and 10.0. A direct pH measurement was taken on the nitrified poultry manure sample. Soil conductivity was done according to the method of the U.S. Salinity Laboratory Staff (6). Soil texture measurements were done according to the manual ribbon method by Dr. D. Mokma, Michigan State University, and by the hygrometer (appendix data only) method. Organic matter composition of the samples was done by the wet combustion method of Allison (7). Approximately 10 g of soil was used for percent moisture determinations. Soils were dried overnight at 110 C and then weighed. Mean annual precipitation and mean annual

temperature of the sample sites were obtained from maps (20,24).

Isolation And Enumeration

After preliminary testing of media and procedures for the recovery of bacteria from soil had yielded a satisfactory procedure, denitrifiers were isolated and enumerated according to the scheme shown in Figure 1. The first dilution was prepared by blending 10.0 g of sample in a Waring blender for 2 min with 90 ml distilled water and a final concentration of 0.1% Tween 80. Isolations were accomplished by spread plating 1.0 ml inocula of appropriate dilutions onto nitrate agar. The agar plates had been pre-dried for 3 to 5 days, thus allowing rapid absorbtion of the inocula. Two dilution series were done for each sample and four plates were prepared for each dilution in the 10^{-4} to 10^{-6} range, unless unusual numbers were anticipated. The plates were then incubated for 3 to 5 days at room temperature in an anaerobic glove box (4), with 90% N_2: 10% H_2 as the atmosphere. After incubation, all of the isolated colonies (approximately 15 to 60) from at least one plate of each series were transferred to nitrate broth (Difco, Detroit, Mi.) tubes containing an inverted (Durham) tube and incubated anaerobically for two weeks. In addition, colonies of different morphology from other plates were also transferred. Gas producers were purified on nitrate agar incubated anaerobically and then transferred

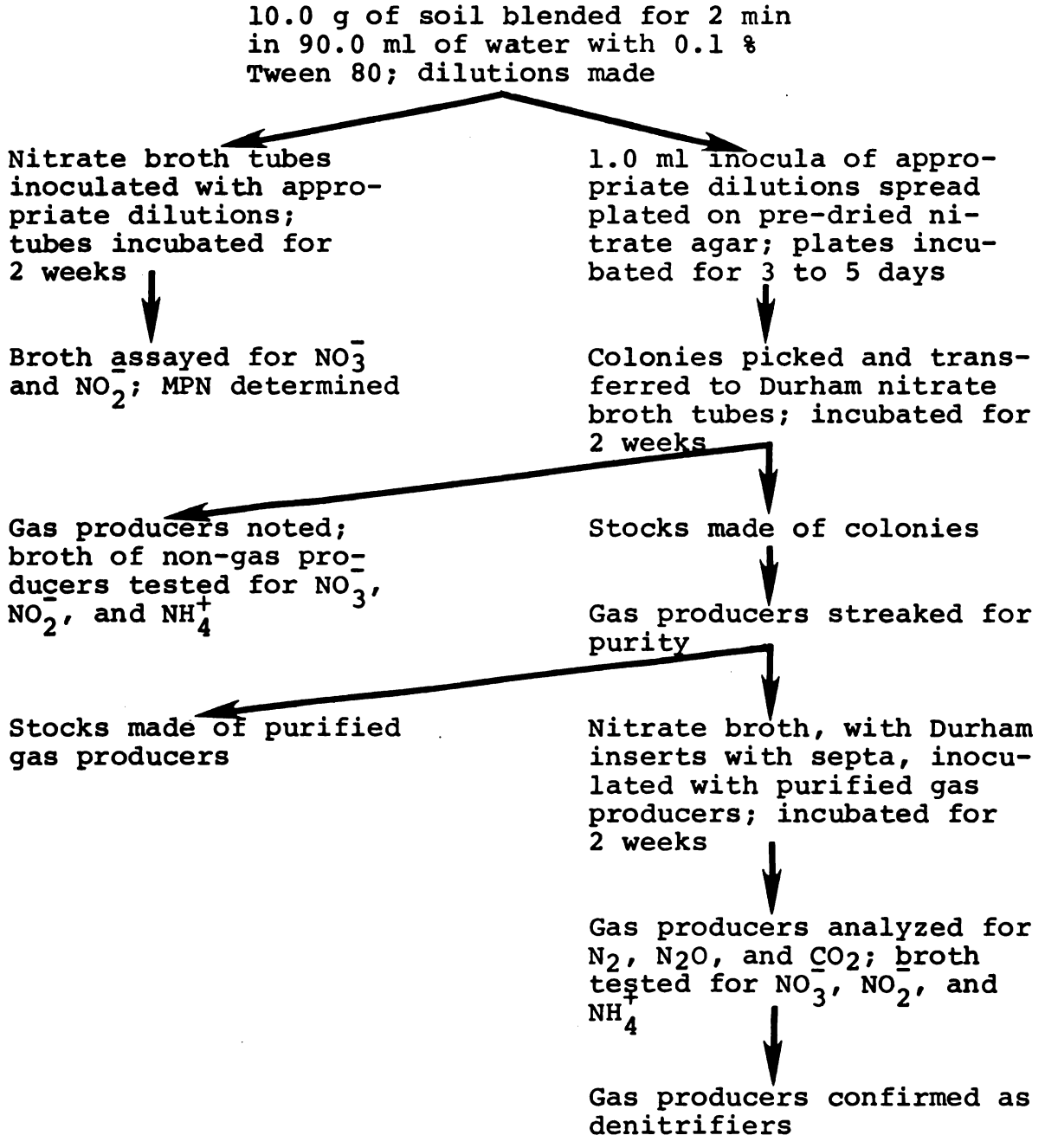

Figure 1. Scheme Utilized for MPN, Isolation, and Confirmation of Denitrifying Bacteria.[1]

[1]All incubations were in an anaerobic glove box, except stocks made of pure gas producers were incubated aerobically.

again to nitrate broth tubes but containing Durham tubes with septa on the upper end. After a two or more week anaerobic incubation, the broth of those that did not produce gas was assayed qualitatively for nitrate, nitrite, and ammonia. Gas produced by the purified isolates was analyzed for N_2, N_2O, and CO_2 by gas chromatography. Confirmed denitrifiers were those organisms able to produce N_2 and/or N_2O after purification.

The method of Focht and Joseph was used for MPN determinations for denitrifiers (18). The method was modified by utilizing an anaerobic glove box for incubations. Five tubes of nitrate broth per dilution were used. In most cases, the dilution range was 10^{-5} to 10^{-8}. For each sample the same two dilution series described above were used to inoculate the MPN broth tubes. After 14 days incubation, the broth of each tube was observed for turbidity and assayed qualitatively for nitrate and nitrite. Tubes in which no nitrate or nitrite was detected were positive for the presence of a denitrifying organism. Tubes in which nitrite was found were positive for the presence of a nitrite accumulating organism. Turbidity indicated the presence of an anaerobe. (In this thesis "anaerobe" is defined as any organism capable of growth under anaerobic incubations.) For the soils and sediments, MPN's and plate counts are expressed as mean organisms per gram dry weight. The population for the nitrified poultry manure is reported as organisms per ml.

Methods

Nitrate and nitrite were determined qualitatively according to the method of Focht and Joseph (18). Quantitative measurements for nitrate were done potentiometrically with a Ionalyzer Model 801/ digital pH meter equipped with a nitrate ion electrode (Orion Research Inc., Cambridge, Mass.). Quantitative measurements for nitrite were done by the Griess-Ilosvay method (7) using a Turner Spectrophotometer Model 350 (G.K. Turner Associates, Palo Alto, Cal.) for the colorimetric readings. Ammonia was detected qualitatively with Nessler's reagent (37).

Nitrogen, nitrous oxide, and carbon dioxide were quantitated using a Carle Model 8000 gas chromatograph (Carle Instruments, Inc., Fullerton, Cal.) equipped with poropak Q and molecular sieve 5A columns and a microthermister detector. The limit for detection of N_2O was 0.5 % of a 50 µl sample. All anaerobic incubations were done at room temperature in an atmosphere of 90% N_2: 10% H_2 contained in a vinyl glove box (Coy Manufacturing, Ann Arbor, Michigan). The methods used in setting up the glove box were those of Aranki et al. (4). The atmosphere of the glove box was sufficient to maintain reduced resazurin, (Eh < -50 mV). The modified Durham inserts consisted of 7.6 cm pieces of glass tubing with an inner diameter of 5 mm. A serum septum (Arthur H. Thomas Company, Philadelphia, Pa.) in one end allowed for the insertion of a 25 gauge needle. Thus, gas collected could be removed and

assayed by gas chromatography.

Denitrifier Characterizations

The confirmed denitrifier isolates were characterized by examining the 52 properties identified in Table 1. All cellular characteristics were examined on early exponential phase cells grown aerobically on nitrate broth or agar at 28 C, unless otherwise indicated. Gram stains were done according to the Kopeloff modification (25). Cellular morphology, cell groupings, and motility were determined by hanging drop observations (1). Cells were measured under phase contrast microscopy after mounting on slides containing a thin film of dried water-agar (12). To obtain the range of dimensions, the smallest and largest cells of several fields were measured. For poly-β-hydroxybutyrate formation, cells were grown on Difco nutrient agar supplimented with 2.0 % glycerol and 1.0 % glucose (filter-sterilized separately). Cells were stained by Burdon's method (13).

Colonial characteristics of size, form, elevation, margin, surface, texture, and light refraction and catalase and oxidase reactions were all observed on 3 day-old cultures grown aerobically on nitrate agar at 28 C (13). Taxo Differentiation Discs for Neisseria and Pseudomonas (BBL, Division of Becton, Dickinson and Company, Cockeysville, Maryland) were used for the oxidase test.

The presence of arginine dihydrolase was determined

Table 1. Characters Examined for Each Denitrifier Isolate.

I. Cellular characteristics:

gram stain
morphology
cell groupings
motility
PHB inclusions
cell length
cell width

II. Colonial characteristics:

size
form
elevation
margin
surface
texture
light refraction

III. Enzyme production:

catalase
oxidase
oxidation of arsenite
arginine dihydrolase

IV. Hydrolytic capabilities:

gelatin
starch
casein

V. Temperature for growth:

4 C
28 C
41 C

VI. Pigment production:

fluorescein (UV)
general pigment (diffusible and non-diffusible)
insoluble blue phenazine pigment

VII. Growth as sole carbon source:

acids
acetate
propionate
citrate
ρ-hydroxybenzoate

alcohols
ethanol
geraniol

amino acids
L-asparagine
DL-arginine
β-alanine
sarcosine

Carbohydrates and sugar derivatives
D-glucose
sucrose
D(+)trehalose
L-arabinose
D-fructose
D-arabinose
D-xylose
D-ribose
maltose
D(+)cellobiose
2-keto gluconate
saccharate

Polyalcohols and glycols
D-sorbitol
meso-inositol
propylene glycol

by the method of Thornley (41). Two tubes of Thornley's medium "2A" were inoculated for each isolate. One of the two tubes was incubated aerobically at 28 C for 4 days. The method was modified by incubating the other tube in an anaerobic glove box, instead of covering with vaseline, at room temperature for 4 days. An alkaline reaction in both tubes was positive for arginine dihydrolase. The ability to oxidize arsenite was determined by the method of Turner (42), after incubating the medium aerobically at 28 C for one week.

The ability to grow at 4 C and 41 C was determined by turbidity after aerobic incubation in nitrate broth. The incubation period for 4 C was 10 days, while that of 41 C was 2 days.

Tests for hydrolytic capabilities, pigment production, and growth on sole carbon sources were performed after aerobic incubations at 28 C for 4 days. Geraniol medium was incubated 7 days.

The media and methods of analysis used to determine the hydrolytic capabilities for starch was that of Colwell and Wiebe (13); for gelatin, Frazier (30); and for casein, Gordon and Mihm (21).

The ability to produce three types of pigment was examined. Medium B (27) was used for the enhancement of fluorescein production. Medium A (27) was used for description of general diffusible and non-diffusible non-fluorescent pigment production. A peptone-glucose medium

(38) was used for the enhancement of the production of an insoluble blue phenazine pigment characteristically produced by Pseudomonas fluorescens biotype IV. For Medium A and the peptone-glucose medium, pigments were examined under white light. For Medium B, plates were examined under long wave ultraviolet light.

Every isolate was tested for the ability to grow at the expense of 25 different organic compounds. The test media were prepared by adding each organic compound, at the appropriate concentration, to the standard mineral base of Colwell and Wiebe (13), with the addition of .001 % phenol red (Sigma Chemical Co., St. Louis, Mo.). The pH indicator aided in evaluation of growth since the metabolism of most of the compounds produced a pH change. The final pH of all media were 7.2. Oxoid Ionagar no. 2 (Colab laboratories, Inc., Chicago Heights, Ill.) was used as the solidifying agent. The media were contained in Quad petri dishes. A control plate, without an added organic compound, was inoculated with each isolate. Growth on plates with the carbon sources compared to the control plates was read as positive for the ability to utilize the sole carbon source. Geraniol media was prepared by adding a drop of the water-insoluble geraniol to 10.0 ml of the mineral base of Tiedje and Mason (40), and adding the phosphate buffer which had been autoclaved separately. Turbidity in excess of the uninoculated control was considered positive for the ability to utilize geraniol as a

sole carbon source. The organic compounds tested as substrates and the concentrations used were:[1]

(a) Acids: 0.1 % acetate (A), 0.1 % propionate (A), 0.1 % citrate (B).

(b) Alcohols: 1.0 % ethanol (G), geraniol (C).

(c) Amino acids: 0.1 % L-asparagine (D), 0.1 % DL-arginine (D), 0.1 % β-alanine (D).

(d) Carbohydrates and sugar derivatives: 1.0 % D-glucose (B), 1.0 % sucrose (B), 1.0 % D(+)trehalose (D), 1.0 % L-arabinose (D), 1.0 % D-fructose (D), 1.0 % D-arabinose (D), 1.0 % D-xylose (D), 1.0 % D-ribose (D), 0.1 % maltose (F), 0.1 % D(+)cellobiose (D), 0.1 % 2-keto gluconate (D), 0.1 % saccharate (D).

(e) Polyalcohols and glycols: 1.0 % D-sorbitol (D), 1.0 % meso-inositol (D), 0.1 % propylene glycol (D).

(f) Miscellaneous: 0.1 % ρ-hydroxybenzoate (C), 0.1 % sarcosine (E).

The following organic compounds were filter-sterilized: ethanol, D-glucose, sucrose, D(+)trehalose, L-arabinose, D-fructose, D-arabinose, D-xylose, D-ribose, maltose,

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[1]The following designations are used to indicate the source of the sole carbon sources: A) J.T. Baker Chemical Co., Phillipsburg, N.J.; B) Mallinckrodt Chemical Works, St. Louis, Mo.; C) Aldrich Chemical Co., Inc., Milwaukee, Wis.; D) Sigma Chemical Co., St. Louis, Mo.; E) Columbia Organic Chemical Co., Inc., Columbia, S.C.; F) Difco, Detroit, Mi.; G) Commercial Solvents Corporation, Terre Haute, Ind.

D(+)cellobiose, 2-keto gluconate, D-sorbitol, and meso-inositol.

In addition to the characterization of the isolated denitrifiers, the same characters were examined for nine known denitrifiers. They were: Pseudomonas denitrificans ATCC 13867, Pseudomonas aureofaciens ATCC 13985, Pseudomonas mendocino ATCC 25411, Alcaligenes faecalis ATCC 8750, Pseudomonas fluorescens II ATCC 17822, Pseudomonas aeruginosa, Paracoccus denitrificans ATCC 2008, Pseudomonas stutzeri ATCC 17588, and Pseudomonas perfectomarinus from Spartina salt marsh (isolated by W.J. Payne).

Effect Of Phosphate In Media

Nitrate broth, autoclaved with the molar concentrations of 0.02, 0.015, 0.01, 0.005, and 0.0 phosphate buffer, pH 7.2, was tested for the ability to support growth and gas production of selected sediment isolates. Twenty-one isolates were obtained from Pond 1 in November. Durham broth tubes containing the five phosphate concentrations were inoculated with each of the 21 isolates. After a two week anaerobic incubation, turbidity and the gas volumes in the Durham tubes were recorded.

The effect of filter-sterilized phosphate buffer on the growth and gas production of known denitrifiers was also observed. Nitrate broth tubes with Durham inserts with septa were prepared with final molarities of 0.025, 0.02, 0.015, 0.01, 0.005, and 0.0 phosphate buffer. A tube of each of

the five phosphate buffer concentrations was inoculated with each of the ten confirmed or known denitrifying bacteria. The denitrifiers were Pseudomonas fluorescens (DMS 19), Pseudomonas perfectomarinus, Hyphomicrobium sp. (WC 24 R), Pseudomonas dentrificans (ATCC 13867), Paracoccus denitrificans (ATCC 2008), Pseudomonas stutzeri (ATCC 17588), Alcaligenes eutrophus,[1] and isolates 4, 15, and 49 (confirmed denitrifiers by the author). After a terminal incubation period, the volume of gas produced in each tube was measured. The gas was analyzed for N_2 and N_2O by gas chromatography.

Effect Of Tween 80

Tween 80 has been used by other workers (36) to aid in the recovery from soil of bacteria imbedded in organic matter films. To test the efficacy of this method, a comparison test was done using three concentrations of Tween 80 SC-15608 (Sargeant-Welch Scientific Co., Skokie, Ill.). Ten grams of Minnesota soil were blended in a Waring blender for 2 min with 90.0 ml distilled water with a final concentration of either 0.1%, 0.05 %, or no Tween 80. Antifoam A spray (Dow Corning Corp., Midland, Mi.) was used after blending. Nitrate broth tubes autoclaved with 0.02 M phosphate buffer, pH 7.2, were inoculated with appropriate

[1]The named denitrifiers were from the collection of W.J Payne. The ATCC numbers were the original numbers and not directly obtained by us from the American Type Culture Collection. Pseudomonas fluorescens was originally from Dr. Clarke Gray, Dartmouth Medical School. Hyphomicrobium sp. was originally from Dr. G.T. Sperl. The Alcaligenes eutrophus (strain H 16) was originally from Dr. H. Kaltwasser.

dilutions. After a two week anaerobic incubation, the broth of each tube was qualitatively assayed for nitrate and nitrite to obtain the MPN of denitrifiers.

Comparison Of Nitrate Broth With Soil Extract-Yeast Extract Broth

Several media were employed to determine which would give the greatest numbers of denitrifying bacteria. Soil extract was prepared according to the method of Lochhead (26). The source of the soil extract was a Brookston loam with an organic matter composition of 3.4 %. After the soil-water mixture was autoclaved and allowed to settle, the supernatant was removed. Lochhead's method was modified by centrifuging (instead of filtering) the supernatant to remove the clay particles. The resulting clear soil extract was used immediately for media preparation. A soil extract broth was prepared by supplementing the soil extract with 0.1 % yeast extract and 0.1 % KNO_3, buffered with potassium phosphate to pH 7.2. The soil extract broth and nitrate broth with 0.02 M potassium phosphate buffer, pH 7.2, were compared for highest MPN's of denitrifying bacteria. The soil used for the inoculations was the Minnesota.

RESULTS

Sample Information And Physical Measurements

Table 2 summarizes information on the samples. The sample range included temperate agricultural, sub-tropical, tropical, rain forest, rice paddy, desert, waste treated soils, a nitrified poultry manure, and freshwater lake sediments. Major soil groups represented included mollisol, histisol, vertisol, entisol, inceptisol, aridisol, and alfisol. Most of the soils did not have crops at sampling time, but those that did had either wheat, corn, or rice. A wide range of pH values (3.8 to 8.2) was represented. Most of the samples had 1 to 5 % organic matter, which is typical of mineral soils. The Venezuelan soil was unusually high in organic matter because of the generally high water table which often reached the surface. Soil texture ranged from very heavy clay soils, eg. vertisols, to very sandy soils. An estimate of the actual percentages of sand, silt, and clay, as determined by the hygrometer method, can be found in Appendix.

Medium Selection

When comparing MPN's of denitrifiers obtained from the Minnesota soil, the nitrate broth autoclaved with 0.02 M phosphate buffer yielded a population estimate (1.66×10^6

Table 2. Summary of Information on the Samples Provided by Contributors or Determined Experimentally.

	Sample	Sampling Site	General Description
1	Minnesota	Lamberton	Agric soil 12" - 18"
2	California	Davis	Agric soil
3	Connecticut 1	Windsor	Agric soil
4	Connecticut 2	Windsor	Agric soil
5	Argentina (SP)	San Pedro	Agric soil
6	Argentina (B)	Balcarce	Agric soil
7	Michigan (muck)	Bath	Organic agric soil
8	Texas	Temple	Agric soil
9	Argentina (P)	Parana	Agric soil
10	Brazil	Mococa	Agric soil
11	Venezuela	San Carlos de Rio Negro	Rain forest
12	Nigeria (C)	Ibadan	Agric soil
13	Nigeria (R)	Ibadan	Rice paddy
14	Columbia	Palmira	Rice paddy
15	Philippines	Los Banos	Rice paddy
16	Taiwan	Taichung	Rice paddy
17	Louisiana	Crowley	Rice paddy
18	Utah	Snowville	Desert
19	Kansas	Pratt	Manured agric soil
20	Poultry waste	-	Poultry waste
21	Michigan (WG)	Hickory Corners	Wintergreen Lake sediment
22	Michigan (P1)	E. Lansing	WQMP sediment, pond 1
23	Michigan (P4)	E. Lansing	WQMP sediment, pond 4

Table 2. (continued)

	Classification (series)	Crop at Sampling Time	Previous Crops	Drainage[1]
1	Typic haplaquoll Webster	-	-	p
2	Typic pelloxerert Clear Lake	-	-	p
3	Entic haplorthod Merrimac	none	tobacco	w
4	Entic haplorthod Merrimac	-	-	w
5	Vertic argiudoll Ramallo	fallow	sweet corn	swp
6	Mollisol Typic argiudoll	none (plowed after wheat)	potato	w
7	Histisol-Typic medisaprist(Carlisle)	-	-	-
8	Typic chromudert Houston	-	-	p
9	Argillic chromudert Febre	fallow	wheat	swp
10	Entisol Tropic fluvaquent	wheat	rice	p
11	Inceptisol Tropaquept	-	-	p
12	Agric hapustalf	corn	corn	swp
13	Agric hapustalf	rice	corn, rice	p
14	Inceptisol Andaquept	rice	rice	swp
15		none	rice	swp
16	Entisol Fluvaquent	rice	rice	swp/p
17	Alfisol-Thermic typic albaqualf(Crowley)	none	rice	swp
18	Calcorthid Thiocal	Artemisia tridentata	none	w
19	Mollisol Aquent	corn	corn	-
20	-	-	-	-
21	Hypereutropic	-	-	-
22	-	-	-	-
23	-	-	-	-

[1]Drainage designations as follows: w - well, p - poor, swp - somewhat poorly.

Table 2. (continued)

	Nitrogen Fertilizer Use in the Last Two Years	Moisture on receipt (%)	pH	Organic Matter (%)
1	-	15.9	7.2	2.34
2	-	20.8	7.34	2.16
3	-	8.7	5.2	1.45
4	-	10.75	5.2	1.45
5	none	22.3	5.92	3.34
6	unknown	23.0	5.69	4.08
7	-	84.5	6.53	81.51
8	-	6.55	7.39	3.61
9	no	28.6	7.84	2.81
10	80 kg N/ha	24.7	4.42	4.94
11	-	56.4	3.84	16.15
12	yes	12.75	5.51	3.21
13	yes	17.2	6.34	1.34
14	125 kg urea/ha	32.0	7.74	3.21
15	no	31.0	6.49	3.34
16	2200 kg $(NH_4)_2SO_4$/ha	19.6	5.09	1.20
17	unknown	17.5	5.66	1.07
18	no	3.7	8.21	2.34
19	320 T/ha/year animal waste	10.9	7.12	4.81
20	-	-	6.44	46.00
21	-	92.0	6.41	26.47
22	-	33.8	-	-
23	-	35.0	6.94	2.50

Table 2. (continued)

	Texture	Conductivity μ mhos	Mean Annual Temperature (C)	Mean Annual Rainfall (mm)	Reference
1	cl	334	5-10	500-1000	(31)
2	sic	618	15-20	400-500	
3	sl	350	10	1000	(39)
4	sl	350	10	1000	(39)
5	sicl	433	15-20	500-1000	
6	sil	687	15-20	500-1000	
7	muck	225	5-10	500-1000	
8	c	2146	20-25	1000-2000	
9	c	315	15-20	500-1000	
10	sicl	303	20-25	1000-2000	
11	l	241	25-30	2000-4000	
12	sl	629	25-30	1000-2000	
13	ls	238	25-30	1000-2000	
14	sicl	398	25-30	1000-2000	
15	sicl	386	25-30	1000-2000	
16	sil	995	20-25	1000-2000	
17	sil	521	20-25	1000-2000	
18	sil	705	10-15	250-500	
19	sil	3623	10-15	500-1000	(43)
20	-	-	-	-	(34)
21	-	579	5-10	500-1000	
22	-	-	5-10	500-1000	
23	-	1031	5-10	500-1000	

org/g dry wt) similar to that of the soil extract broth supplimented with 0.1% yeast extract and 0.1 % KNO_3 (1.12×10^6 org/g dry wt). This suggests that nitrate broth did not lack any growth factors essential for the growth of soil denitrifiers. Since the population estimates were approximately the same and the preparation of nitrate broth less time consuming, all subsequent experiments were done with nitrate broth or agar.

Effect Of Tween 80 As A Soil Dispersing Agent

When using 0.1 % (v/v) Tween 80 as a soil dispersing agent with the Minnesota soil, a mean MPN of 6.07×10^5 org/g dry wt for two dilution series was obtained. The values for 0.05 % and 0.0 % were 2.54×10^5 org/g dry wt and 1.95×10^5 org/g dry wt, respectively. Use of Tween 80 did not appear to have a bacteriocidal effect. Since the three values were within an order of magnitude, I cannot be confident whether Tween 80 aided in the recovery or had a toxic effect on soil organisms. However, it was apparent that the use of Tween 80 with the Waring blender greatly facilitated the mixing of soils high in clay content. For such soils, foaming did occur, but was reduced by the addition of antifoam A. A concentration of 0.1 % Tween 80 was used for all soil preparations.

Effect Of Phosphate

Table 3 summarizes the effect that autoclaved phosphate buffer has on growth and gas production of Pond 1

Table 3. The Effect of Phosphate Buffer on the Growth and Gas Production of Selected Soil and Sediment Organisms.

Phosphate Concentration (M)	Isolates Transferred	Isolates Producing Turbidity	Isolates Producing Gas	Mean Gas Volume per Producer (µl)
autoclaved				
0.000	21[a]	14	11	95
0.005	21	11	7	78
0.010	21	9	9	48
0.015	21	9	8	36
0.020	21	2	1	trace
filter-sterilized				
0.000	10[b]	10	10	133
0.005	10	10	10	128
0.010	10	10	9	146
0.015	10	10	9	157
0.020	10	10	9	155
0.025	10	10	8	145

[a] 21 isolates from Pond 1 (November) sediment.

[b] 7 identified and 3 unidentified cultures of denitrifying bacteria. They were: Pseudomonas fluorescens (DMS 19), Pseudomonas perfectomarinus, Hyphomicrobium (WC 24 R), Pseudomonas dentrificans (ATCC 13867), Paracoccus denitrificans (ATCC 2008), Pseudomonas stutzeri (ATCC 17588), Alcaligenes eutrophus, and isolates 4, 15, and 49.

(November) sediment organisms. Although the organisms were not identified or confirmed as denitrifiers, the data

clearly shows that the presence of autoclaved phosphate buffer is inhibitory to the growth and gas production of the 21 sediment organisms.

The effect of filter-sterilized phosphate buffer on growth and gas production can be seen in Table 3. All ten of the stock denitrifiers grew in all of the phosphate concentrations. However, only in .000 and .005 M buffer did all ten produce gas. The higher concentrations of buffer did affect the gas production of specific organisms. The average volume of gas produced varied insignificantly, with 99 % of the gas N_2 and traces of CO_2 and N_2O. In the broth of all 10 stock denitrifiers, for all of the phosphate molarities, no nitrate was detected. Nitrite was detected in trace quantities in the broth of Alcaligenes eutrophus at 0.01 M, 0.015 M, and 0.025 M buffer; of Hyphomicrobium at 0.02 M buffer; of Pseudomonas stutzeri at 0.015 M buffer; and of isolate 15 at 0.025 M buffer. Alcaligenes eutrophus did not produce gas in broth of phosphate concentrations greater than .005 M and isolate 4 did not produce gas at 0.025 M.

Although the effect of filter-sterilized phosphate buffer was not as pronounced as that of the autoclaved phosphate buffer, it was evident that both treatments exhibited an inhibitory effect on the growth and gas production of certain soil organisms. The media chosen for use in isolating and enumerating denitrifying bacteria was unbuffered nitrate broth and agar.

Quantitation Of Total Anaerobes, Denitrifiers, And Nitrite Accumulators

Table 4 lists the mean population estimates of total anaerobes, denitrifiers, and nitrite accumulators using both MPN and plate count methods. With few exceptions, the MPN and plate counts were in good agreement. Of the 25 samples done for MPN of denitrifiers, 72% of the values fell in the range of 10^5 to 10^7 organisms$\cdot g^{-1}$. The exceptions which fell below 10^5 organisms$\cdot g^{-1}$ were samples from Pond 4 sediment (November), Utah, Venezuela, and Texas. The Pond 1 sediment (November), nitrified poultry manure, and Connecticut 1 samples had values above 10^7 organisms$\cdot g^{-1}$ or $\cdot ml^{-1}$. The MPN values of nitrite accumulators were greater than those of the denitrifiers. Of the 22 samples examined for nitrite accumulators, 59 % of the MPN's were between 10^6 and 10^7 organisms$\cdot g^{-1}$ and 91 % ranged from 10^6 to 10^8. Only the Venezuelan soil had an MPN of less than 10^6 nitrite accumulators per gram dry weight. For the 25 samples examined for MPN of total organisms capable of growth under anaerobic incubations, 76 % were in the range of 10^6 to 10^8 organisms$\cdot g^{-1}$. An outstanding exception was more than 10^{10} organisms$\cdot ml^{-1}$ for the nitrified poultry manure. In some cases, nitrite accumulator numbers were read to be higher than those of total anaerobes. This was presumably due to the greater sensitivity of the nitrite detection method compared to visual detection of turbidity.

Table 4 also illustrates numerical relationships of

Table 4. Population Densities and Ratios of Denitrifiers, Nitrite Accumulators, and Total Anaerobes.

	Sample	Denitrifiers[a]		Nitrite Accumulators	
		MPN	Plate	MPN	Plate
1	Minnesota	2.88×10^6	1.38×10^6	6.48×10^6	1.38×10^6
2	California	2.38×10^6	2.08×10^6	1.58×10^7	2.92×10^6
3	Connecticut 1	1.37×10^7	1.08×10^6	1.37×10^7	2.98×10^6
4	Connecticut 2	1.42×10^5	$<1.11 \times 10^6$	1.31×10^6	4.16×10^6
5	Argentina(SP)	1.40×10^6	1.47×10^6	7.58×10^6	1.72×10^6
6	Argentina(B)	3.20×10^5	$<1.29 \times 10^5$	8.79×10^6	2.06×10^6
7	Michigan(muck)	7.03×10^6	1.94×10^6	1.44×10^7	1.61×10^7
8	Texas	1.24×10^4	5.35×10^4	1.35×10^6	1.02×10^6
9	Argentina(P)	1.59×10^6	1.56×10^6	2.55×10^6	2.69×10^6
10	Brazil	6.17×10^5	4.02×10^5	4.22×10^6	1.41×10^6
11	Venezuela	$<4.54 \times 10^4$	$<2.27 \times 10^5$	8.51×10^5	9.08×10^5
12	Nigeria(C)	3.12×10^5	4.54×10^5	3.35×10^6	1.70×10^6
13	Nigeria(R)	5.86×10^5	4.27×10^5	1.49×10^6	1.22×10^6
14	Columbia	2.48×10^5	3.68×10^6	3.64×10^6	1.47×10^7
15	Philippines	2.19×10^6	8.78×10^5	2.93×10^7	2.56×10^6
16	Taiwan	5.67×10^5	2.46×10^5	1.70×10^7	8.00×10^5
17	Louisiana	1.67×10^6	4.78×10^5	7.11×10^6	1.31×10^6
18	Utah	8.12×10^2	$<1.03 \times 10^4$	$>1.10 \times 10^5$	2.00×10^5
19	Kansas	2.28×10^5	2.22×10^5	1.04×10^7	1.00×10^6
20	Poultry waste	3.50×10^{10}	2.00×10^8	-	1.38×10^8

[a]Numbers expressed as organisms$\cdot g^{-1}$ except for poultry waste which is expressd as organisms$\cdot ml^{-1}$.

Table 4. (continued)

	Sample	Denitrifiers		Nitrite Accumulators	
		MPN	Plate	MPN	Plate
21	Michigan(WG)	3.44×10^5	3.75×10^5	1.17×10^6	1.56×10^6
22	Michigan(P1) November	3.45×10^7	2.05×10^7	-	-
23	Michigan(P1) May	3.70×10^6	1.81×10^6	1.19×10^7	1.43×10^6
24	Michigan(P4) November	5.23×10^4	1.92×10^5	-	7.68×10^4
25	Michigan(P4) May	3.77×10^6	7.55×10^5	3.16×10^6	7.55×10^5

	Anaerobes (MPN)[a]	N : A[b]	D : A[b]	D : N[b]
1	6.36×10^6	1.00 : 1[c]	.44 : 1[c]	.44 : 1
2	3.69×10^7	.43 : 1	.06 : 1	.15 : 1
3	$>1.37 \times 10^7$	<1.00 : 1	<1.00 : 1	1.00 : 1
4	7.10×10^6	.18 : 1	.02 : 1	.11 : 1
5	1.01×10^7	.75 : 1	.14 : 1	.18 : 1
6	8.15×10^6	1.00 : 1[c]	.03 : 1[c]	.03 : 1
7	1.83×10^7	.79 : 1	.38 : 1	.49 : 1
8	7.81×10^5	1.00 : 1[c]	.009 : 1[c]	.009 : 1
9	8.25×10^6	.31 : 1	.19 : 1	.62 : 1
10	3.22×10^6	1.00 : 1[c]	.15 : 1[c]	.15 : 1
11	1.35×10^6	.63 : 1	<.03 : 1	<.05 : 1

[b] N-nitrite accumulators, A-anaerobes, D-dentrifiers

[c] N counts used as A counts because of greater number

[d] D counts used as A counts because of greater number

Table 4. (continued)

	Anaerobes (MPN)[a]	N : A[b]	D : A[b]	D : N[b]
12	4.71×10^5	1.00 : 1[c]	.09 : 1[c]	.09 : 1
13	1.79×10^6	.83 : 1	.33 : 1	.39 : 1
14	6.78×10^6	.54 : 1	.04 : 1	.07 : 1
15	8.32×10^6	1.00 : 1[c]	.07 : 1[c]	.07 : 1
16	1.13×10^7	1.00 : 1[c]	.03 : 1[c]	.03 : 1
17	6.52×10^6	1.00 : 1[c]	.23 : 1[c]	.23 : 1
18	$>1.30 \times 10^5$	>.85 : 1	<.006 : 1	.007 : 1
19	1.20×10^7	.87 : 1	.02 : 1	.02 : 1
20	4.45×10^{10}	-	.79 : 1	-
21	2.88×10^6	.41 : 1	.12 : 1	.29 : 1
22	1.11×10^8	-	.31 : 1	-
23	1.43×10^7	.83 : 1	.26 : 1	.31 : 1
24	3.53×10^5	-	.15 : 1	-
25	2.00×10^6	.84 : 1[d]	1.00 : 1[d]	1.19 : 1[d]

nitrite accumulators and denitrifiers to anaerobes and denitrifiers to nitrite accumulators. In most cases, nitrite accumulators were within an order of magnitude of total anaerobes. In some instances, nitrite accumulator values were greater than those of anaerobes. Since this is not possible, when calculating ratios in these instances, the nitrite accumulator numbers were used as total anaerobe numbers. Denitrifier numbers were generally within an

order of magnitude of both the anaerobe and nitrite accumulator counts. The Utah soil had the lowest number of denitrifiers relative to nitrite accumulators of all of the soils - .007 : 1. Whereas, Pond 4 sediment (May) had a greater than 1 : 1 ratio of denitrifiers to nitrite accumulators. Excluding the Utah and Venezuela soils because of their extreme pH and the poultry manure sample and Connecticut soils because of their laboratory incubations, a mean population relationship for denitrifiers to nitrite accumulators to anaerobes of soils and sediments was calculated to be 0.24 : 0.68 : 1.

The Ability Of Isolates To Utilize Nitrate

From the 25 samples, 1553 isolates were tested for denitrification and 16.2 % of these were gas producers (Table 5). For each sample, the range for isolates producing gas was from 0.0 % to 50.0 %. Samples from which no gas producers were isolated were Connecticut No. 2, Utah, and the Venezuela soils. Pond 1 sediment (November) and Minnesota soil had the highest percentages of gas producers. The Pond 1 sediment (May) was next with 39.1 % of its isolates producing gas. Those organisms able to reduce nitrate to nitrite comprised 38.5 % of the 1531 isolates from 24 samples. The range of those reducing nitrate to nitrite was 4.5 % for the Pond 4 sediment (November) to 71.8 % for the Argentina(B) soil.

After observing that the spent broth of certain

Table 5. Percent of Isolates Utilizing Nitrate and Producing the Following Nitrogen Compounds.

	Sample	Number Tested	Gas%	NO_2^-%	NH_4^+%	Nothing From NO_3^-%	No N detected%
1	Minnesota	87	40.2	29.9	16.1	20.7	2.3
2	California	75	12.0	17.3	12.0	65.3	1.3
3	Connecticut 1	81	7.4	14.8	-	-	-
4	Connecticut 2	73	0.0	35.6	26.0	58.9	1.4
5	Argentina(SP)	87	26.4	32.2	24.1	31.0	4.6
6	Argentina(B)	39	2.6	71.8	41.0	20.5	0.0
7	Michigan(muck)	84	9.5	61.9	8.3	25.0	1.2
8	Texas	84	2.4	22.6	5.9	75.0	0.0
9	Argentina(P)	69	15.9	27.5	11.6	43.5	8.7
10	Brazil	54	20.4	37.0	31.5	22.2	0.0
11	Venezuela	45	0.0	33.3	26.7	66.7	0.0
12	Nigeria(C)	79	12.7	43.0	25.3	43.0	0.0
13	Nigeria(R)	48	18.8	50.0	45.8	27.1	0.0
14	Columbia	41	17.1	58.1	36.6	19.5	4.9
15	Philippines	124	9.8	30.6	41.1	25.8	1.6
16	Taiwan	40	15.0	50.0	37.5	35.0	0.0
17	Louisiana	42	16.7	47.6	23.8	42.9	0.0
18	Utah	66	0.0	69.7	10.6	30.3	0.0
19	Kansas	22	9.1	40.9	31.8	50.0	0.0
20	Poultry waste	67	34.3	17.9	-	-	-
21	Michigan(WG)	67	8.9	40.3	14.9	47.8	0.0
22	Michigan(P1) November	22	50.0	-	-	-	-
23	Michigan(P1) May	69	39.1	33.3	11.6	18.8	7.2
24	Michigan(P4) November	66	9.1	4.5	-	-	-
25	Michigan(P4) May	22	27.3	54.5	40.9	27.3	0.0

isolates did not contain nitrate or nitrite, or produced gas, assays were done for ammonia. From 21 samples with a

total of 1317 isolates, 24.9 % showed increased ammonia in the medium. For the individual samples, the range was from 5.9 % for the Texas sample to 45.8 % for the Nigeria(R) soil. Even after assaying for ammonia, certain isolates were discovered that were able to deplete the broth of nitrate without subsequent gas, nitrite, or ammonia formation. Although their numbers were few, they did represent 1.6 % of 1317 isolates tested. From the 21 soils, they were found in 9. The Argentine(P) soil had the highest percentage of this group with 8.7 %.

The next most prevalent group of isolates were those unable to utilize nitrate. That is, nitrate was detected without the formation of gas, nitrite, or ammonia. These isolates represented 37.9 % of 1317 tested from 21 samples and ranged from 18.8 % of the isolates for Pond 1 sediment (May) to 75.0 % for the Texas soil. Many of these organisms did not appear to grow in the nitrate broth after transfer from agar.

Identification Of Isolated Denitrifying Bacteria

Over 250 organisms were isolated that initially produced gas (Table 5). However, many of these isolates were no longer viable after short anaerobic incubation periods or else lost the ability to produce gas after purification. Of the original gas producers, 147 denitrified after purification; they were isolated from 19 of the 25 samples.

These 147 confirmed denitrifiers, as well as nine stock

cultures were characterized by examining 52 properties for each. The results are recorded in the Appendix. Many of the organisms were identified to the species level by the current taxonomic criteria of Bergey's Manual of Determinative Bacteriology, 8th edition (10), appropriate supplimentary literature (38, 14, 33)), and comparison to known denitrifiers. Approximately one-third of the isolates were not identified to the species level and several were not identified to the genus level. Such isolates which appeared to be related based on the 52 properties tested, were given type numbers in the order in which they were isolated. Figure 2 illustrates a simplified characterization scheme used for the identification of the denitrifiers. Table 6 lists the identity of the organisms and their numbers for each sample.

Gram negative rods comprised 93.2% of the 147 denitrifiers and gram negative motile rods, mostly oxidase and catalase positive, represented 86.4% of the total. Other groups were: gram variable rods, 4.8 % pleomorphic strains 1.4 %; and gram positive cocci, < 1.0 %. Some samples were represented by a single species, such as the nitrified poultry manure, and the Brazilian, Kansan, and Michigan (muck) soils. However, from most soils several species were isolated. The greatest diversity was found in the Argentina (SP) soil where 10 different species were recovered. The species composition and number of species

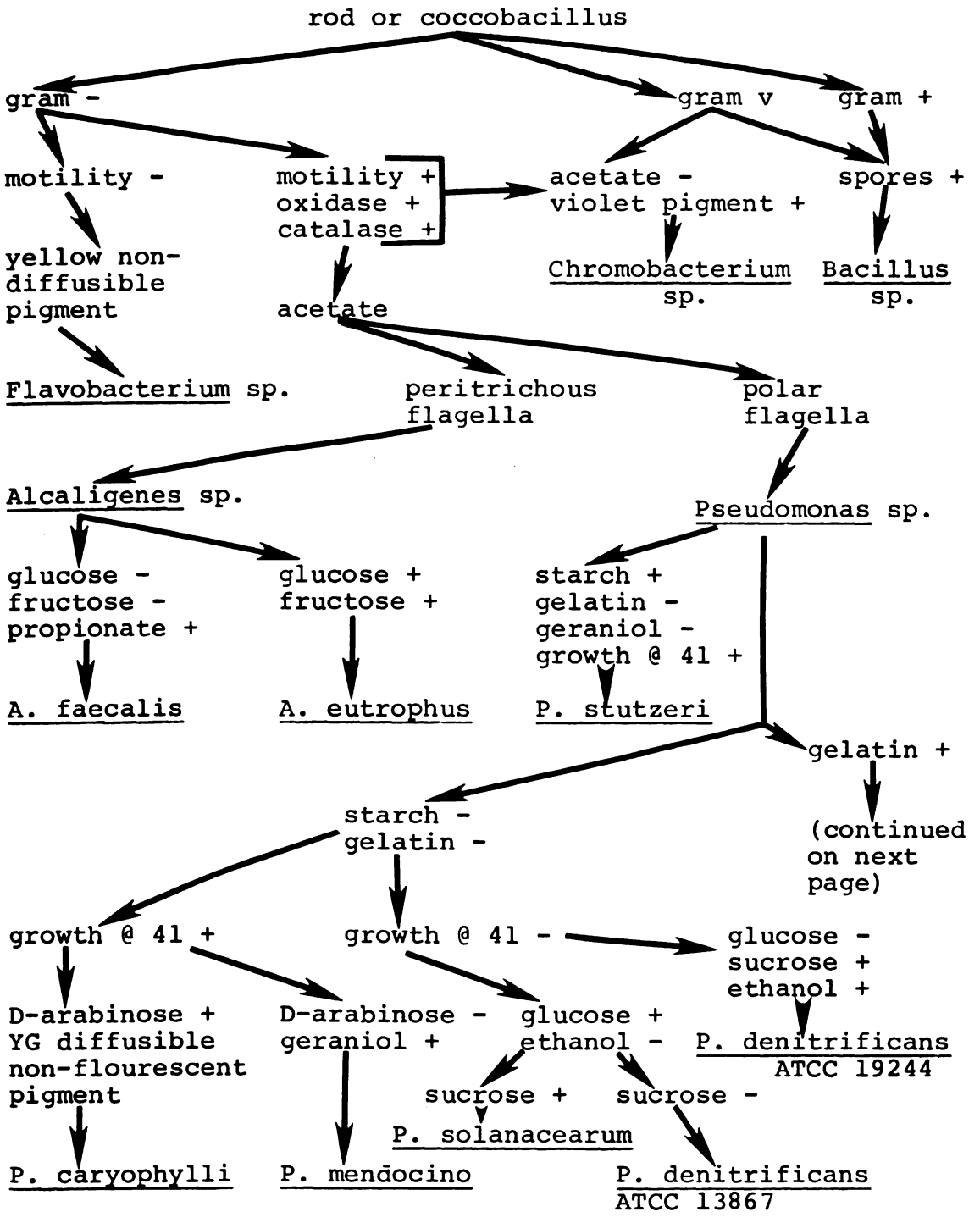
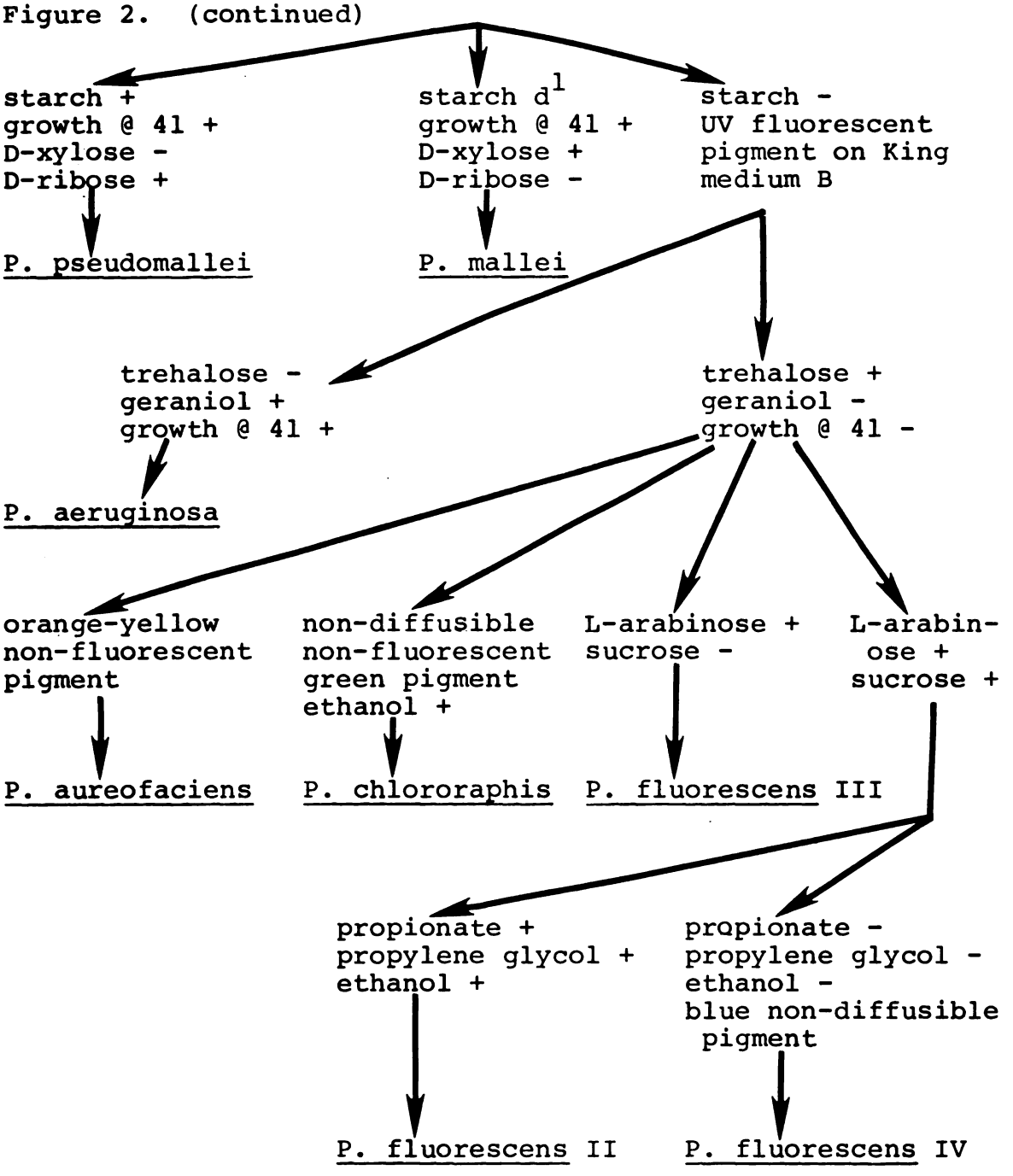


Figure 2. Characterization Scheme for the Identification of Soil and Sediment Denitrifiers.

Figure 2. (continued)



[1]d = positive for more than 10 % but less than 90 % of all strains studied (10).

varied among samples (Table 6). No two samples were identical in both.

At least five genera were represented in the total number of denitrifiers, as shown in Table 7. Pseudomonas spp. dominated by comprising 65.3 % of the total isolated. There appeared to be 37 distinct species or strains among the 147 isolates characterized. A complete list of these can be found in Appendix. Table 8 lists the major species represented along with their occurrence in the 19 samples. Although Pseudomonas was the predominant genera, Alcaligenes faecalis was the predominant species, and occurred in the most samples. Pseudomonas fluorescens II also occurred in high numbers. Identified species found in fewer numbers than those listed in Table 8 included Pseudomonas aureofaciens, and Pseudomonas solanacearum.

Temperature Relationship

From Table 2, soil and sediment samples obtained directly from the environment can be divided into two main groups based on mean annual temperature. One group is described as temperate, representing samples with a mean annual temperature of 20 C and below, and the other tropical, representing samples with a mean annual temperature of 20 C and above (Table 9). By observing the ability of denitrifying isolates to grow at 4 C and 41 C, a relationship was determined between the mean annual temperature of the sampling location and the temperature at which the

Table 6. Denitrifiers Isolated from Samples.

Sample	Organisms Isolated	Number that Denitrified After Purification
Michigan(P4) November	*Alcaligenes faecalis*	1
	Pseudomonas fluorescens II	2
	Pseudomonas fluorescens(?)	1
	Pseudomonas sp. type 1	1
	Pseudomonas sp. type 2	2
		7
Poultry manure	*Alcaligenes faecalis*	14
Connecticut 1	*Alcaligenes faecalis*	3
	unknown type 3	2
		5
California	*Alcaligenes faecalis*	1
	Pseudomonas fluorescens II	5
	Corynebacterium sp.	1
	Flavobacterium sp.	1
		8
Minnesota	*Alcaligenes faecalis*	1
	Pseudomonas aureofaciens	3
	Pseudomonas fluorescens II	16
	Pseudomonas sp. type 2	5
	Pseudomonas sp. type 4	1
	Pseudomonas sp. type 5	4
	Pseudomonas sp. type 6	2
	Pseudomonas sp. type 7	1
		33
Brazil	*Pseudomonas* sp. type 18	4
Kansas	*Pseudomonas fluorescens*	1
Argentina(P)	*Pseudomonas fluorescens* IV	3
	Flavobacterium sp.	5
	Pseudomonas sp. type 19	1
		9

Table 6. (continued)

Sample	Organisms Isolated	Number that Denitrified After Purification
Argentina(SP)	Alcaligenes faecalis	1
	Pseudomonas fluorescens II	1
	Pseudomonas fluorescens(?)	2
	Pseudomonas stutzeri	1
	Bacillus sp.	2
	Pseudomonas sp. type 11	1
	Pseudomonas sp. type 23	1
	unknown type 21	1
	unknown type 22	1
	unknown type 24	1
		12
Michigan(P1) May	Alcaligenes faecalis	9
	Pseudomonas fluorescens II	2
	Pseudomonas fluorescens IV	1
	Pseudomonas sp. type 8	1
	Pseudomonas sp. type 9	1
	Pseudomonas sp. type 10	1
	Pseudomonas sp. type 11	1
		16
Philippine	Pseudomonas aeruginosa	6
	Pseudomonas sp. type 11	1
		7
Michigan(muck)	Pseudomonas stutzeri	2
Michigan(P4) May	Pseudomonas fluorescens(?)	1
	Pseudomonas sp. type 12	1
	Pseudomonas sp. type 13	1
	unknown type 3	1
		4
Nigeria(C)	Alcaligenes faecalis	1
	Alcaligenes eutrophus	1
	Corynebacterium sp.	1
	Pseudomonas sp. type 11	1
	Pseudomonas sp. type 19	2
	Pseudomonas sp. type 20	1
		7

Table 6. (continued)

Sample	Organisms Isolated	Number that Denitrified After Purification
Nigeria(R)	Alcaligenes faecalis	1
	Pseudomonas solanacearum	1
	Pseudomonas sp. type 11	1
	Pseudomonas sp. type 16	1
	Pseudomonas sp. type 17	1
		5
Taiwan	Alcaligenes faecalis	1
	Pseudomonas sp. type 16	2
	Pseudomonas sp. type 14	1
		4
Michigan(WG)	Pseudomonas stutzeri	2
	Alcaligenes faecalis	1
		3
Texas	Pseudomonas sp. type 11	1
	Pseudomonas sp. type 25	2
		3
Louisiana	Pseudomonas sp. 14	1
	Pseudomonas sp. 16	1
	unknown type 15	1
		3

Table 7. Major Genera Recovered.

Genera	Percent of Total
Pseudomonas	65.3
Alcaligenes	23.8
Flavobacterium	4.1
Bacillus	1.4
Corynebacterium	1.4
unknown others	4.1

Table 8. Major Species Recovered.

Species	Percent of Total	Occurrence in 19 Samples
Alcaligenes faecalis	23.1	11
Pseudomonas fluorescens II	17.7	5
Pseudomonas sp. type 2	4.8	2
Pseudomonas sp. type 11	4.1	6
Flavobacterium sp.	4.1	2
Pseudomonas aeruginosa	4.1	1
Pseudomonas fluorescens IV	3.4	3
Pseudomonas stutzeri	3.4	3
Pseudomonas fluorescens(?)	2.7	3
Pseudomonas sp. type 16	2.7	3
Pseudomonas sp. type 5	2.7	1
Pseudomonas sp. type 18	2.7	1

Table 9. Samples from Tropical and Temperate Locations.[1]

Soils From Locations With Mean Annual Temperatures of 20 C And Above	Soils From Locations With Mean Annual Temperatures of 20 C and Below
Nigeria(R)	Argentina(SP)
Nigeria(C)	Argentina(P)
Philippines	Minnesota
Louisiana	California
Brazil	Michigan(muck)
Taiwan	Michigan(WG)
Texas	Michigan(P1) May
	Michigan(P4) May
	Michigan(P4) November
	Kansas

[1]Samples were excluded which were incubated under unnatural conditions or of which denitrifiers were not isolated.

isolates from the samples can grow. Of the 95 denitrifiers isolated from the temperate samples, 68.4 % grew at 4 C, 9.5 % grew at 41 C, and 22.1 % grew only at 28 C. Of the 33 denitrifiers isolated from the tropical soils, none grew at 4 C, 66.7 % grew at 41 C, and 33.3 % grew only at 28 C. No denitrifiers were isolated that could grow at both 4 C and 41 C.

A species relationship can also be observed with Pseudomonas fluorescens II and Alcaligenes faecalis, which grow at 4 C and 28 C, but not at 41 C. Of the total number of both species isolated from the two temperature groups, all of the Pseudomonas fluorescens II isolates and 85.0 % of the Alcaligenes faecalis isolates were from the temperate samples.

DISCUSSION

Development Of A Medium And Method For The Isolation And Enumeration Of Denitrifying Bacteria

Before actual isolations were attempted, it was desirable to know that the medium chosen for use would give the highest possible counts and yet be as selective as possible for denitrifying bacteria. The importance of soil extract for the enumeration of soil bacteria has been emphasized by many soil microbiologists (29). A broad range of heat-stable soil nutrients is provided in small amounts by soil extract. Yet the amount of carbon is low enough to prevent perceptible antibiotic and organic acid production and the growth of spreaders. However, the use of soil extract has been criticized by many. Küster found the greatest numbers appeared on soil extract prepared from the soil that was being examined (22). Thus, a soil extract medium prepared from one soil may not be suitable for the growth of bacteria from another soil. Bacteriocidal substances have also been reported in extracts of soil (22). In this study the soil extract-yeast extract media did not yield as high a population of denitrifiers as did nitrate broth and thus it was not used.

On agar, diffusibility of metabolites away from colonial growth is limited. The metabolism of an organism

may produce a pH change that is toxic and lowers the organisms viability. The process of denitrification generates OH^- ions. In unbuffered broth cultures of denitrifying bacteria, I observed pH increases from 7.2 to 8.9 for certain isolates, which resulted in their death. The need for a buffer to neutralize such an effect is evident. However, my testing on the use of phosphate buffer demonstrated that it had an inhibitory effect on denitrification. When autoclaved, the buffer may have precipitated trace divalent cations, needed by the nitrate reductase (32).

The medium and method of inoculation excluded growth of many unwanted microorganisms. Since inoculations were carried out under aerobic conditions, the death of some non-sporeforming obligate anaerobes would occur. Incubations under anaerobic conditions would not permit the growth of aerobes incapable of anaerobic growth with nitrate. Since the medium was carbohydrate free, carbohydrate fermenters could not grow. Because no reducing agent was added to the medium, the redox would not be low enough to permit the growth of many spore-forming obligate anaerobes. Therefore, the medium was considered selective for organisms capable of respiring with nitrate. However, 37.9 % of the isolates tested were unable to utilize the nitrate in the broth after isolation on agar. Many of these produced only pinpoint colonies on agar and were thought possibly to be microaerophiles capable of growing on trace

amounts of O_2 trapped in the agar.

Although the medium and method for denitrifier isolations seemed satisfactory, there are problems. The incubation period was limited to a five day maximum because of the over-growth of certain organisms. Yet, the possibility does exist that slow-growing organisms would not be visible in five days. Such denitrifiers would be missed. Because of their slow growth, their contribution to denitrification would likely be minimal. Many organisms that grew on agar would not grow in broth. Such organisms could have been microaerophilic, as discussed earlier. It is also possible that such organisms which were able to grow well on agar but not in broth, were inhibited by volatile fatty amines released from the polyurethane foam stoppers during autoclaving (5) or needed H_2 (present in glove box atmosphere), which is insoluble in broth.

An unresolved troublesome problem was the loss of the denitrifying ability by certain isolates after purification. Most of the organisms were still able to grow anaerobically with nitrate, reducing it to nitrite, but were unable to form gas. This suggests a lack of nitrite reductase. Another possibility is an alternate dissimilatory pathway after nitrite formation. Fewson and Nicholas (17) state that hydroxylamine and ammonia can be formed during nitrate dissimilation. This could be an explanation for the observation that certain organisms depleted the broth of nitrate, yet produced neither nitrite, ammonia, or gas. Isotopic

studies with ^{15}N nitrate could prove the existence of such an alternate pathway. An unsuitable medium or an unstable genetic character could also explain the loss of denitrifying ability.

Commonality Of Denitrifier Isolates

Four of the 14 known genera of denitrifying bacteria (32) were identified among the 147 types isolated and characterized from 25 samples examined. Since Propionibacterium spp. are only found in dairy products or the skin and intestinal tract of man and animals, Moraxella spp. are parasitic on the mucous membranes of warm-blooded animals and man, Halobacterium spp. require very high salt concentrations for survival, and Achromobacter spp. are now in other genera, these genera were not expected to be isolated. Also, since culturing conditions eliminated the possibility of autotrophs, Thiobacillus spp. were not expected. Therefore, the following genera were possibilities for the identification of denitrifiers isolated: Alcaligenes, Bacillus,Chromobacterium, Corynebacterium, Hyphomicrobium, Micrococcus, Pseudomonas, Spirillum, and Xanthomonas. However, only Alcaligenes, Bacillus, Corynebacterium, and Pseudomonas were identified of those known genera of denitrifiers. A Flavobacterium sp. was also identified as a denitrifier, which has not previously been reported.

Flavobacterium spp., however, have been reported to reduce nitrate to nitrite (32). The high proportion of

the isolates being Pseudomonas spp. was not surprising because of their ability to utilize such a great number of carbon sources and their well-known prevalence in soils. The prevalence of Alcaligenes was surprising since members of this genus had not been thought to be common soil denitrifiers. Although they are defined as separate genera, Alcaligenes and Pseudomonas are very similar. Almost all members of both genera are gram negative rods, obligate respirers, oxidase and catalase positive, able to use acetate as a sole carbon source, motile, and have a G + C content of DNA from 58 to 70 moles % (10). They are differentiated on the number and location of flagella - for Pseudomonas, single polar, and for Alcaligenes, peritrichous. However, Bergey's Manual (10) describes Alcaligenes as possessing one to four (occasionally up to eight) peritrichous or degenerate flagella. The possibility of an Alcaligenes sp. having a single subpolar flagella would make it virtually impossible to distinguish from a pseudomonad.

Almost two-thirds of the denitrifier isolates were identified to the species level. Nine separate species were identified. Most of the 16 described strains of denitrifiers recognized in Bergey's Manual (10) in the genera of Pseudomonas and Alcaligenes were represented. However, Pseudomonas fluorescens biotype II and Alcaligenes faecalis were numerically dominant and were most frequently observed from the samples. Pseudomonas fluorescens II is

found in soil and water and generally considered to be saprophytic (10). Some strains of Pseudomonas fluorescens are known to be common inhabitants of the rhizosphere (35). The presence of denitrifying strains in anaerobic microsites of the rhizosphere may greatly affect plant productivity by causing a localized depletion of nitrogen.

The Alcaligenes faecalis strains isolated were very similar to the strain described by Pichinoty et al. (33). The major differences observed were the inability of my strains to utilize D-saccharate, ethanol, and citrate as sole carbon sources. The high occurrence of Alcaligenes faecalis leads one to question its source. It is a species of a genus widely distributed in decomposing organic matter and also found in the intestinal tract of vertebrates. Its origin could have been fecal because of its wide distribution in these agricultural and natural soils. However, it is more likely an indigenous soil inhabitant.

Many of the most commonly studied denitrifying bacteria were not isolated or isolated in very small numbers. Paracoccus (formerly Micrococcus) denitrificans and Pseudomonas denitrificans (ATCC 19244) and Pseudomonas denitrificans (ATCC 13867) were not identified from any of the 25 samples. Such an observation leads one to believe that they play a minor role in the environment.

Douderoff et. al. (14) examined many previously isolated strains of pseudomonads to determine the validity of P. denitrificans as a species and found no other

organisms related to either of these two dissimilar species. Due to the absence of other related strains, he proposed that the name Pseudomonas denitrificans be abandoned. Since no new isolates from diverse samples were found in this study which were related to either of the P. denitrificans, the proposal of Douderoff (14) is supported.

Correlations Between Samples, And MPN's And Isolates

There appears to be very little correlation between sample environmental parameters, population densities, and identity of organisms isolated. Only the environmental extremes--desert, acid rain forest--had any effect on population densities. Almost all of the MPN's for denitrifiers ranged from 10^5 to 10^6 organisms$\cdot g^{-1}$ when pH values were between 4.42 and 7.84. Only at 3.84 and 8.21 were MPN's less than 10^4 organisms$\cdot g^{-1}$. Certain isolates did appear only in particular pH ranges, e.g. Pseudomonas fluorescens II was mainly isolated in samples with a pH range of 6.94 to 7.34. Alcaligenes faecalis was isolated from samples representing a broad pH range.

The most dramatic correlation observed was between the mean annual temperatures of the sample locations and the ability of isolates to grow at certain temperatures. Either the temperatures of the environment select for certain organisms or the organisms themselves adapt to a particular temperature range. The fact that none of the isolates from the tropical samples were able to grow at

4 C is reasonable, since there is no advantage in adapting to a temperature the organism will never encounter. Whereas, there is a competitive advantage for temperate isolates to be able to grow at 4 C, since they are exposed to 4 C but not 41 C.

Improvements

It is probable that the media and isolation procedure used in this study could be further improved. For higher counts and possibly more representative isolates, a soil extract medium made from the soil in which enumerations and isolations are to be done could be employed. Also, adjusting the pH of the medium to the same pH as the soil would best mimic the actual environment, therefore allowing the growth of those denitrifiers unable to grow at neutral pH. However, the incubation period would have to be much longer, since growth would be much slower under such circumstances. The use of nitrite, instead of nitrate, in the initial isolation medium, would eliminate the organisms able to reduce nitrate to nitrite but no further. This approach would only work if a concentration of nitrite could be found that would not be toxic to the soil denitrifiers. One might also be able to use N_2O as the electron acceptor and thus only recover denitrifiers.

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APPENDIX

Table 10. Percentages of Sand, Silt, and Clay in the Soil Samples as Determined by the Hygrometer Method.*

Soil	% Sand	% Silt	% Clay
Minnesota	30	55	15
California	35	47	18
Argentina(SP)	42	39	19
Argentina(B)	33	54.5	12.5
Texas	43	27	30
Argentina(P)	38	41	21
Brazil	40	29.5	30.5
Nigeria(C)	15	76	9
Nigeria(R)	10	85	5
Columbia	36	42	22
Philippines	27	58	15
Taiwan	32	57	11
Louisiana	41	44	15
Utah	37	50.5	12.5
Kansas	28	64	8

*The hygrometer method depends on complete dispersion of soil particles. Due to the extreme diversity of the soils studied, this could not be achieved by a standard method for all cases. Therefore, some of the above percentages are probably incorrect.

Explanation of Table 11

Table 11 contains the data of the 52 properties examined for the identification of the denitrifying isolates. Positive and negative designations were + and 0 respectively; 3 means the test was not done. Test were coded by number as follows:

1 gram reaction
2 motility
3 oxidase
4 catalase
5 acetate
6 gelatin hydrolysis
7 starch hydrolysis
8 casein hydrolysis
9 fluorescein (medium B)
10 blue phenazine (peptone-glucose)
11 D-glucose
12 sucrose
13 D(+)trehalose
14 L-arabinose
15 D-fructose
16 D-arabinose
17 ethanol
18 growth at 41 C
19 propionate
20 propylene glycol
21 L-asparagine
22 D-sorbitol
23 geraniol
24 D-xylose
25 DL-arginine
26 D-ribose
27 maltose
28 D(+)cellobiose
29 meso-inositol
30 sarcosine
31 β-alanine
32 ρ-hydroxybenzoate (base)
33 ρ-hydroxybenzoate (acid)
34 2-keto-gluconate
35 saccharate
36 growth at 4 C
37 arginine dihydrolase
38 arsenite oxidation
39 PHB granules
40 citrate

Pigment color abbreviations were: unpig - unpigmented, pale yel - pale yellow, YG - yellow-green, BG - blue-green, and d - diffusible. The pigments without the d designation were not diffusible. Cell dimension were in μm. For colonial characteristics, column 1 was size in mm, with p for pinpoint; 2, form, where c - circular and i - irregular; 3, elevation, where c - convex, u - umbonate, p - pulvinate, r - raised, and f - flat; 4, margin, where u - undulate, en - entire, * - not describable, and er - erose; 5, surface, where s - smooth, ru - ruffled, and ro - rough; 6, texture, where b - buttery, d - dry, and m - mucoid; 7, light refraction, where g - glistening, t - translucent, d - dense, and o - opaque. The number 8 designates cell groupings, with s - singular and c - chains.

Table 11. Results of 52 Properties Tested.

Sample	Isolate Number	Tests 1111111111222222222233333333334 12345678901234567890123456789012345678890
Lake 4		
November	4	0++++0000000000000000+000000000+0000+00+0
	6	0++++00000+00++0+0+0++0+++00+++00+++00++
	12	0++++00000+00++0+0+0++0+++00+++00+++000+
	13	0+++++0++0+++++++0++++++++0++0++0++++00+
	14	0+++++0+00+00++0+0+0+00+0+0000+0+0++00+0
	15	0+++++0++0+00+++00+0+00+++0+++++0++++00+
	16	0+++++0++0+++++++0++++++++0++0++0++++00+
Nitrified	17	0++++0000000000000000000000000000+0000
poultry	18	0++++000000000000+000000000000000+0000
manure	19	0++++0000000000000000000000000000+0000
	20	0++++000000000000++000000000000+0000
	21	0++++000000000000++000000000000+0000
	22	0++++0000000000000000000000000000+0000
	24	0++++0000000000000000000000000000+0000
	25	0++++0000000000000000000000000000+0000
	26	0++++0000000000000000000000000000+0000
	27	0++++0000000000000000000000000000+0000
	28	0++++000000000000+0000000000000000000
	29	0++++0000000000000000000000000000+0000
	30	0++++000000000000+000000000000000+0000
	31	0++++000000000000+000000000000000000
Connecticut		
1	36	v+++0+00000000000+0000000000000000000+0
	37	0+++0000000000000000000000000000000000
	39	v++0000000000000000000000000000000000+0
	40	0+++000000+0000000000000+00000000000000
	41	0++++00000000+0+0000000000000000000000
California	42	0+++++0++++++++++0++++++++0+++++0++++00+
	43	0++++000000000000+0+000000000000++000+
	44	0+++++0++++++++++0++++++++0+++++0++++00+
	45	0+++++00+++++++++0++++++++0+++++0++++00+
	46	00+++++000+++++0000000++00++0000000+0000
	47	0+++++0++0+++++++0++++0+++00+0++0++++00+
	48	000++00000+00+0+0000+0000000000000000+0
	49	0+++++0++0+++++++0++++0+++0+++++0++++00+
Minnesota	51	0++++00000+0+++0+0++++0+++00+++00+++00++
	52	0+++++0++0+++++++0++++++++00+0++0++++00+
	53	0+++++0++0+++++0+0++++++++0+++++0++++00+
	54	0++++00000+00++0+0++++++++00+++00+++000+

Table 11. (continued)

Sample	Isolate Number	Tests 1111111111222222222233333333334 12345678901234567890123456789012345678890
Minnesota (continued)	55	0++++000+0+0+++++0+++00+++00+0+00++++00+
	56	0++++00000+0+++0+0+0++0+++00+++00+++00++
	57	0++++000+0+0+++++0+++00+++00+0+00++++00+
	58	0++++000+0+0+++++0+++0++++000++00++++00+
	59	0+++++0++0+++++0+0++++++++00+0++0++++00+
	60	0+++++0++0+++++0+0++++++++00+0++0++++00+
	61	0+++++00+0+++++000++++++++00+0++0++++00+
	62	0+++++0++0+++++++0++++++++00+0++0++++00+
	63	0+++++0++0+++++0+0++++++++00+0++0++++00+
	64	0+++++0++0+++++++0++++0+++00+0++0++++00+
	65	0++++000000000000000+000000000000000000++
	66	0+++++0++0+++++0+0++++0+++00+0++0++++00+
	67	0+++++0++0+++++++0++++0+++0+++++0++++0++
	68	0+++++0++0+++++0+0++++++++0+++++0++++0++
	69	0+++++0+00+++++0+0+++0++++00+0++0+++00++
	70	0+++++0++0+++++++0++++++++00+0++0++++00+
	71	0+++++0+00+++++0+0++++++++00+0++0+++000+
	72	0+++++0++0+++++++0++++0+++00+0++0++++00+
	73	0+++++0++0+++++++0++++++++0+++++0++++00+
	74	0+++++0+00+++++0+0++++++++00+0++0++++0++
	75	0+++++00+0+++++0+0++++++++0+++++0++++00+
	78	0++++00000+0+++0+0+++0++++0++0+00++++00+
	79	0+++++0++0+++++++0++++++++0++0++0++++00+
	80	0++++000+0+00++++0+++0++++0+++++0+++000+
	81	0++++00000++0++0+0++++++++0++++00+++00++
	82	0+++++0+0++++++0+0++++++++00+0++00+++0++
	83	0++++00000+0+++0+0++++++++0+++00+++00++
	84	0+++++0++0+++++++0+++++++++++++0++++00+
	85	0+++++0+00+0+++++0+++0++++++++++0+++00++
Lake 1 May	86	0++0+0000000000000000000000000000000+0
	87	0++++00000+00+00+00++++0+00000+0000++000
	89	0+++++00+0++0++++0++++++++00+0++0++++00+
	90	0++0+0000000000000000000000000000000+0
	91	0+++0000000000000000000000000000000030
	97	0+0+0+0+000+00000+00+0000000000000000000
	98	0++++000+0+++++++0++++++0++000000+00000+
	99	0++++0000000000000000000000000000000+0
	101	0++++000000000000+00+0+00000000+000000+0
	102	0++++0000000000000+0000000000000000+0

Table 11. (continued)

Sample	Isolate Number	Tests 1111111111222222222233333333334 12345678901234567890123456789012345678890
Lake 1 (continued)	103	0++0+00000000+00+00++0+0+00++0+0000+0000
	104	0+++0000000000000000000000000000000000+0
	105	0+++++0++++++++++0++++++++0++0++0+++00++
	106	0++++000000000000000+000000000000+0000+0
	107	0++++00000+++++++0++++0+++++++++000000++
	881	03++0000000000000000000000000000000000+0
Lake 4 May	108	v+0+0++0000000000+00+000000000000000000+0
	110	0+++++0+00+++++000+0+00+++00+0++0+++00++
	111	0+++++0++0+0+++000++++0+++00+0++0+0++00+
	882	0+++++0000++0++000+0++0++++++0000+0+00++
Louisiana	114	0++0+00000+++0+00++0+000+0+000000++000+0
	115	+00+0000000000000+00000000000000000000000
	1181	0+++++0000+0+++00++0+00+000000++0++000++
Brazil	126	0+++++0000+0+++00+00++0+000+0+++0++000++
	129	0+++++0000+0+++00+00+00+000+0+++0++0000+
	133	0+++++0000+0+++00+00++0+000+00++0++000++
	135	0++0++0000+0+++00++0++0+000+00++0++000++
Nigeria(R)	137	000++000000000+0+0+++0++++000+++0++000++
	141	0+00+00000+0+++00++0+0++000+0+++0++0000+
	143	0++++00000++++++00++++0++++++0+0+00000+0
	144	0+++0000000000000000000000000000000000++
	145	03+3000000++00+00+0030+00000000000003333
Nigeria(C)	1471	00000++000++0000000000+++++++0000++000+0
	148	0+0++00000000000+0+0+00000000+0+00+000++
	149	0++++00000++++++0000++0++++++0++000000+0
	151	0++0++0000+0000000+0000000++000000000000
	153	0++0++000000000000+0000000++000000000000
	154	0++++00000+00++000+++0+0+00000++0000+00+
	155	0++++00000+000+0+++++000000000+0+00000+0
Philippines	156	0+++++0++0+0+++++++++00+++000+++0+00+00+
	162	0+++++0++0+0+++++++++00+++000+++0+00+00+
	163	0++++00000+++++++0++++0+++++++++000000++

Table 11. (continued)

Sample	Isolate Number	Tests 1111111111222222222233333333334 12345678901234567890123456789012345678890
Philippines (continued)		
	164	0+0+++0++0+0+++++++++00+++000+++0+00+00+
	165	0+++++0++0+0+++++++++00+++000+++0+00+00+
	166	0+++++0++0+0+++++++++00+++000+++0+00+00+
	167	0+++++0++0+0+++++++++00+++000+++0+00000+
Taiwan	171	00000++0000000000+00000000000000000000+0
	172	0++++00000+0+++00++0+0+++0000+++0++000++
	173	0++0000000+++0+00000+000+033333333333333
	174	0++0++0000+0+++00++0+00+00000+++0++000++
Argentina(P)		
	175	00++0++000++0++0000000++00++0000000+0000
	176	00++0++000++0++0000000++00++0000000+0000
	177	00++0++000++0++00000000+00++0000000+0000
	178	00++0++000++0++00000000+00++0000000+0000
	179	0++++00000000+000000000000++000+00+00000
	180	00++0++000++0++00000000+00++0000000+0000
	183	0+0+++0++++++++000+0++++++00+0++0++++0++
	184	0+0+++0++++++++000+0++++++00+0++0++++0++
	185	0+0+++0++++++++++0+0+++++++++0++0++++0++
Argentina(SP)		
	188	0++++00000+++++++0++++0++++++0++000000+0
	189	00000++0000000+000000+000000000000000+0+0
	190	0++++000+0+00++++0++++++++0000+00++++0++
	191	0+++000000000000000000000000000000+000++
	192	v000000000+++++000000+0+0++0+000000000+0
	193	v0000+00000000+000000+0+0+00+000000000+0
	195	0++++0+000+00++0++00000++0+00+0+0000000+
	196	0++++000+0+00++++0+++0++++0000+00++++0++
	199	v00+0++000++0++00000+++00++0+00000+000+0
	202	00000++000++0++00000++00++000000000000+0
	204	0+0000+000+++++00+00000+00+0000000000000
	205	0+++++0++0+++++0+0++++++++00+0++0++++00+
Kansas	206	0+++++0+++++0++++0++++++++++++++00+++0++
Michigan(WG)		
	220	0++++0+000+00++0++0++00+0++00+0+0000000+
	221	0++++0+000+00++0++0++00+0++00+0+0000000+
	223	0+++++000000000000+00000000000000000000+0

Table 11. (continued)

Sample	Isolate Number	Tests 1111111111222222222233333333334 12345678901234567890123456789012345678890
Michigan (muck)	224	0++++0+000+00++0++00+0++0++00+0+0000000+
	2312	0++++0+000+00++0++0++00+0++00+0+0000000+
Texas	232	0+0++000000000+00++0+00000000++000+000++
	233	0+0++000000000+00++0+00000000++000+000++
	234	0++++00000+++++++000++0++++++0+0+00000+0
Stock Cultures		
	991	0++++00000+000++00+0+0+0+00000++0000+00+
	992	0+0+++0++0+++++000+0+00+++00+0++0++++00+
	993	0++0+0+000+00++0+++++00++0000++00000000+
	994	0++++00000000000+000+00000000+000000000+
	995	0+++++00+0+++++++0+++++++++00+0++0++++00+
	996	0+++++00+0+00+++++++++0+++000+++0+0000++
	997	00+++00000+++++0+0++++00+++0+0++0+000000
	998	0++++0+000+000+00+0000++00+00+000000000+
	999	0++++0+000+00000+00++00+00+00+000000000+

Table 11. (continued)

Isolate Number	Cell Morphology	Pigment Color	Cell Dimensions	Colonial Characteristics 1 2 3 4 5 6 7 8
4	rod	unpig	.8 x. 2.5	1 c c en s b g s
6	rod	white	.8 x 4.0	2 c c en s b g s
12	rod	cream	.8-1.2x2.5-3.3	2 c c en s b g s
13	rod	cream	.8 x 1.7-3.3	1 c c en s b g s
14	rod	cream	.8 x 3.3-4.1	4 c c en s b g s
15	rod	cream	.8 x 2.5	5 i c en s b g s
16	rod	cream	.8 x 1.7-3.3	4 i c en s b g s
17	rod	unpig	.8 x 2.0-2.9	3 c u er ru b t s
18	rod	unpig	.8 x 2.5	1 c c en s b g s
19	rod	unpig	.8 x 2.0-2.5	2 c u er ru b t s
20	rod	unpig	.8 x 1.7-2.9	1 c c er s b g s
21	rod	unpig	.8 x 2.0-2.9	1 c c en s b g s
22	rod	unpig	.8 x 1.7-3.3	3 c u er ru b t s
24	rod	unpig	.8 x 1.7-2.0	3 c u er ru b t s
25	rod	unpig	.8 x 3.3	3 c u er ru b t s
26	rod	unpig	<8-.8x1.7-2.5	3 c u er ru b t s
27	rod	unpig	.8 x 2.5	3 c u er ru b t s
28	rod	unpig	.8 x 2.9-3.3	1 c c en s b g s
29	rod	unpig	.8 x 1.7-2.5	2 c c en s b g s
30	rod	unpig	1.2 x 2.9	1 c c er s b g s
31	rod	unpig	.8-1.2x2.5-3.0	3 c c en s b g s
36	rod	cream	.8-1.2x2.5-3.0	2 c c en s b g s
37	rod	unpig	1.2x2.5-2.9	2 c c er ro b o s
39	rod	unpig	.8x2.9-4.1	1 c c en ro b g s
40	rod	unpig	<8-.8x2.5-2.9	1 c c en s b g s
41	rod	unpig	.8 x 2.0	p c c en s b g s
42	rod	cream	.8 x 2.0-3.7	2 c c en s b g s
43	rod	unpig	.8 x 1.7-2.5	3 c c en s b g s
44	rod	pale yel	.8 x 2.0-3.3	2 c c en s b g s
45	rod	pale yel	<8-.8x2.5-3.3	1 c c en s b g s
46	rod	yel	.4 x 3.7-4.1	1 c c en s b g s
47	rod	pale yel d	.8 x 1.7-4.1	4 c c en s b g s
48	pleomorph	cream	-	p c c en s b g -
49	rod	white	.8-1.2x2.9-4.1	3 c c en s b g s
51	rod	gray	1.7 x 3.7	2 c c en s b g s
52	rod	tan	.8-1.2x2.0-4.1	4 i c en s b g s
53	rod	pale yel	.8x2.5-3.3	2 c c en s b g s
54	rod	cream	1.2x2.0-3.3	2 c c en s b g s

Table 11. (continued)

Isolate Number	Cell Morphology	Pigment Color	Cell Dimensions	Colonial Characteristics 1	2	3	4	5	6	7	8
55	rod	tan	.8x1.7-2.9	4	c	c	en	s	b	g	s
56	rod	gray	1.2-1.7x4.1-4.5	2	c	c	en	s	b	g	s
57	rod	tan	.8x1.7-2.9	2	c	c	en	s	b	g	s
58	rod	gray	.8-1.2x1.7-3.7	5	c	c	en	s	b	g	s
59	rod	orange d	.8x2.0-3.3	2	c	c	en	s	b	g	s
60	rod	orange d	.8x2.0-3.3	3	c	c	en	s	b	g	s
61	rod	tan	1.2x2.0-3.7	7	c	c	en	s	b	g	s
62	rod	orange d	.8 x 2.5	2	c	c	en	s	b	g	s
63	rod	cream	.8x2.0-3.3	2	c	c	en	s	b	g	s
64	rod	pale yel	.8-1.2x2.5-3.3	3	c	c	en	s	b	g	s
65	rod	unpig	.8 x 2.0	2	c	c	en	s	b	g	s
66	rod	tan	1.2x2.0-2.5	7	c	c	en	s	b	g	s
67	rod	YG d	.8-1.2x2.0-2.9	4	c	c	en	s	b	g	s
68	rod	YG d	.8x2.5-3.7	3	c	c	en	s	b	g	s
69	rod	white	.8x 2.0-2.9	3	c	c	en	s	b	g	s
70	rod	YG d	.8x2.5-3.3	3	c	c	en	s	b	g	s
71	rod	white	<8-.8x1.7-2.0	4	c	c	en	s	b	g	s
72	rod	YG d	.8x1.7-2.5	3	c	c	en	s	b	g	s
73	rod	YG d	<8-.8x2.0-4.1	3	c	c	en	s	b	g	s
74	rod	white	1.2x2.5-3.7	5	c	c	en	s	b	g	s
75	rod	YG d	.8x1.7-3.3	3	c	c	en	s	b	g	s
78	rod	gray	.8 x 2.9	5	c	c	en	s	b	g	s
79	rod	YG d	.8x2.9-3.7	4	c	c	en	s	b	g	s
80	rod	white	1.2x2.9-4.1	6	c	c	en	s	m	g	s
81	rod	white	1.2x2.9-3.3	3	c	c	en	s	b	g	s
82	rod	white	.8 x 2.5	4	c	c	en	s	b	g	s
83	rod	white	.8-1.2x2.0-3.7	2	c	c	en	s	b	g	s
84	rod	YG d	.8x2.9-3.3	3	c	c	en	s	b	g	s
85	rod	white	.8-1.2x2.0-4.5	3	c	c	en	s	b	g	s
86	rod	cream	.8-1.2x2.5	1	c	p	en	s	b	g	s
87	rod	white	.8x2.0-2.9	2	c	c	en	s	b	g	s
89	rod	orange	.8x2.0-3.7	2	c	c	en	s	b	g	s
90	rod	white	.8 x 2.5	1	c	c	en	s	b	g	s
91	rod	white	-	-	-	-	-	-	-	-	-
97	rod	yellow	<8-.8x2.5-3.3	2	c	c	en	ro	b	g	s
98	rod	gray	<8-.8x1.7-2.5	3	c	c	en	s	b	g	s
99	rod	white	.8 x 2.5	2	c	c	en	s	b	g	s
101	rod	cream	.8x2.5-2.9	1	c	c	en	s	b	g	s
102	rod	cream	1.2 x 2.5	1	c	c	en	s	b	g	s
103	rod	cream	.8 x 2.5	2	c	c	en	s	b	g	s
104	rod	cream	-	2	c	c	en	s	b	g	-
105	rod	BG d	.8x2.0-2.9	7	c	c	en	s	b	g	s

Table 11. (continued)

Isolate Number	Cell Morphology	Pigment Color	Cell Dimensions	Colonial Characteristics 1 2 3 4 5 6 7 8
106	rod	yellow	.8x1.7-2.5	p c u u s d g s
107	rod	gray	.8x2.0-2.5	3 c c en s m g s
881	rod	white	-	p c c en s b g -
108	rod	unpig	.8x1.7-2.9	1 c c u s b g s
110	rod	cream d	1.2x2.0-2.9	3 c c en s b g s
111	rod	gray	<8-.8x1.7-2.5	3 c c en s b g s
882	rod	cream	1.2x2.5-3.7	2 c c u ro b g s
114	rod	-	.8x1.7-2.5	2 c c en s b g s
115	coccus	unpig	1.2	p c c en s b g d
1181	rod	cream	.8 x 1.7	1 c c er s b g s
126	rod	cream	<8-.8x1.7-2.0	2 c c er s b g s
129	rod	cream	.8 x 2.0	2 c c en s b g s
133	rod	cream	.8x1.7-2.0	2 c c en s b g s
135	rod	pale yel	.8x1.7-2.0	2 c c en s b g s
137	rod	yellow	1.2 x 2.5	3 c c en s b g s
141	rod	cream	.8x1.7-2.0	2 c c en s b g s
143	rod	gray	.8 x 2.9	2 c p en s m g s
144	rod	cream	.8x2.0-2.5	2 c c en s b g s
145	rod	unpig	-	- - - - - - - -
1471	pleomorph	cream	-	1 c u er ro b g -
148	rod	cream	.8 x 2.5	2 c c en s b g s
149	rod	white	.8 x 2.0	4 c c en s m g s
151	rod	cream	.8x2.0-3.3	1 c c en s b g s
153	rod	pale yel	.8 x 3.7	1 c c en s b g s
154	rod	cream	.8 x 2.0	5 c r er ro b g s
155	rod	white	.8-1.2x1.7-3.3	1 c c en s b g s
156	rod	blue d	.8 x 2.5	5 c u er ro b g s
162	rod	blue d	<8-.8x2.5-3.3	5 c u er ro b g s
163	rod	white	.8x1.7-2.5	4 c p en s m g s
164	rod	blue d	.8x1.7-2.5	5 c u er ro b g s
165	rod	blue d	<8-.8x2.0-2.5	4 c u er ro b g s
166	rod	blue d	.8x2.0-2.5	3 i u er ro b g s
167	rod	blue d	1.2x2.9-3.7	4 c u er ro b g s
171	rod	cream	1.7x3.3-5.0	1 c c u ro b t c
172	rod	cream	.8 x 2.5	1 c c en s b g s
173	rod	unpig	1.2x2.9-3.3	2 c p en ru b g s
174	rod	cream	.8 x 1.7	3 c c en s b g s

Table 11. (continued)

Isolate Number	Cell Morphology	Pigment Color	Cell Dimensions	Colonial Characteristics 1	2	3	4	5	6	7	8
175	rod	yellow	.8x4.1-6.6	2	c	c	u	ro	b	o	s
176	rod	yellow	.8 x 4.1	2	c	c	u	ro	b	o	s
177	rod	yellow	.8 x 3.7	3	c	c	u	ro	b	o	s
178	rod	yellow	.8x3.3-5.8	1	c	c	u	ro	b	o	s
179	rod	cream	.8x2.5-2.9	1	c	c	en	s	b	g	s
180	rod	yellow	.8x2.5-4.1	2	c	c	u	ro	b	g	s
183	rod	cream	.8x2.0x2.5	5	c	c	en	s	b	d	s
184	rod	cream	.8x2.5-4.1	4	c	c	en	s	b	d	s
185	rod	cream	.8-1.2x2.9	5	c	c	en	s	b	d	s
188	rod	cream	.8x1.7-2.5	2	c	c	en	s	b	o	s
189	rod	cream	.8 x 3.3	2	c	c	en	s	b	o	c
190	rod	cream	.8x2.5-2.9	2	c	c	en	s	b	o	s
191	rod	cream	1.2 x 2.9	2	c	c	en	s	b	g	s
192	rod	cream	.8-1.2x2.9-3.3	2	c	c	en	s	b	g	c
193	rod	cream	.8-1.2x5.0-6.6	2	c	c	en	s	b	g	c
195	rod	yellow	.8x1.7-2.0	2	i	c	en	s	b	g	s
196	rod	cream	.8-1.2x2.5-3.7	2	c	c	u	s	b	g	s
199	rod	cream	1.2 x 5.3	2	c	c	en	s	b	g	c
202	rod	cream	.8 x 2.0	3	c	c	en	ru	b	g	c
204	rod	white	.8 x 2.9	10	c	f	*	ro	b	c	s
205	rod	cream	.8x2.0-2.9	2	c	c	en	s	b	g	s
206	rod	cream	.8x1.7-2.9	3	i	u	u	s	b	d	s
220	rod	yellow	.8x2.0-2.9	2	c	c	u	s	b	g	s
221	rod	yellow	.8x2.9-4.1	2	c	c	en	s	b	g	s
223	rod	-	.8 x 2.0	1	c	c	en	s	b	g	s
224	rod	yellow	.8 x 2.5	2	c	c	u	ro	b	g	s
2312	rod	gray	.8 x 3.3	2	c	u	er	ro	d	g	s
232	rod	gray	.8 x 3.3	3	c	c	en	s	b	g	s
233	rod	gray	.8-1.2x1.7-2.5	3	i	c	en	s	b	g	s
234	rod	gray	.8-1.2x2.0-2.9	4	c	c	en	s	m	g	s
991	rod	white	.8x2.5-3.7	3	c	c	er	ro	b	o	s
992	rod	orange d	.8x3.7-4.1	4	c	c	en	s	b	g	s
993	rod	yellow	.8x2.0-3.3	4	c	c	u	s	b	o	s
994	rod	white	.8-1.2x1.7-2.0	7	c	u	er	ro	b	o	s
995	rod	gray	.8x2.5-4.1	3	c	c	en	s	b	g	s
996	rod	blue d	.8x1.7-2.5	3	c	c	er	ro	b	o	s
997	coccus	cream	.8 x 1.2	1	c	p	en	s	b	g	s
998	rod	yellow	.8x2.5-3.3	2	c	c	u	s	b	g	s
999	rod	yellow	.8x1.7-2.9	3	c	c	en	s	b	g	s

Table 12. Names of the Denitrifiers.

Isolate Number	Isolate Name	Isolate Number	Isolate Name
4	A. faecalis	61	P. fluorescens II
6	P. type 2	62	P. aureofaciens
12	P. type 2	63	P. fluorescens II
13	P. fluorescens II	64	P. fluorescens II
14	P. type 1	65	A. faecalis
15	P. fluorescens (?)	66	P. fluorescens II
16	P. fluorescens II	67	P. fluorescens II
17	A. faecalis	68	P. fluorescens II
18	A. faecalis	69	P. type 5
19	A. faecalis	70	P. fluorescens II
20	A. faecalis	71	P. type 5
21	A. faecalis	72	P. fluorescens II
22	A. faecalis	73	P. fluorescens II
24	A. faecalis	74	P. type 5
25	A. faecalis	75	P. fluorescens II
26	A. faecalis	78	P. type 6
27	A. faecalis	79	P. fluorescens II
28	A. faecalis	80	P. fluorescens II
29	A. faecalis	81	P. type 2
30	A. faecalis	82	P. type 5
31	A. faecalis	83	P. type 2
36	unknown type 3	84	P. type 6
37	A. faecalis	85	P. type 7
39	unknown type 3	86	A. faecalis
40	A. faecalis	87	P. type 8
41	A. faecalis	89	P. fluorescens II
42	P. fluorescens II	90	A. faecalis
43	A. faecalis	91	A. faecalis
44	P. fluorescens II	97	P. type 9
45	P. fluorescens II	98	P. fluorescens II
46	Flavobacterium sp.	99	A. faecalis
47	P. fluorescens II	101	A. faecalis
48	Corynebacterium sp.	102	A. faecalis
49	P. fluorescens II	103	P. type 10
51	P. type 2	104	A. faecalis
52	P. fluorescens II	105	P. fluorescens II
53	P. fluorescens II	106	A. faecalis
54	P. type 2	107	P. type 11
55	P. type 4	881	A. faecalis
56	P. type 2	108	unknown type 3
57	P. fluorescens II	110	P. type 12
58	P. fluorescens II	111	P. fluorescens(?)
59	P. aureofaciens	882	P. type 13
60	P. aureofaciens	114	P. type 14

Table 12. (continued)

Isolate Number	Isolate Name	Isolate Number	Isolate Name
115	unknown type 15	195	P. stutzeri
1181	P. type 16	196	P. fluorescens(?)
126	P. type 18	199	unknown type 22
129	P. type 18	202	P. type 23
133	P. type 18	204	unknown type 24
135	P. type 18	205	P. fluorescens II
137	P. type 17	206	P. fluorescens IV
141	P. type 16	220	P. stutzeri
143	P. type 11	221	P. stutzeri
144	A. faecalis	223	A. faecalis
145	P. solanacearum	224	P. stutzeri
1471	Corynebacterium sp.	2312	P. stutzeri
148	A. faecalis	232	P. type 25
149	P. type 11	233	P. type 25
151	P. type 19	234	P. type 11
153	P. type 19	991	P. denitrificans ATCC 13867
154	A. eutrophus	992	P. aureofaciens ATCC 13985
155	P. type 20	993	P. mendocino ATCC 25411
156	P. aeruginosa	994	A. faecalis ATCC 8750
162	P. aeruginosa	995	P. fluorescens II ATCC 17822
163	P. type 11	996	P. aeruginosa
164	P. aeruginosa	997	Pa.denitrificans ATCC 2008
165	P. aeruginosa	998	P. stutzeri ATCC 17588
166	P. aeruginosa	999	P. perfectomarinus
167	P. aeruginosa		
171	A. faecalis		
172	P. type 16		
173	P. type 14		
174	P. type 16		
175	Flavobacterium sp.		
176	Flavobacterium sp.		
177	Flavobacterium sp.		
178	Flavobacterium sp.		
179	P. type 19		
180	Flavobacterium sp.		
183	P. fluorescens IV		
184	P. fluorescens IV		
185	P. fluorescens IV		
188	P. type 11		
189	unknown type 21		
190	P. fluorescens(?)		
191	A. faecalis		
192	Bacillus sp.		
193	Bacillus sp.		

Table 13. Number and Insertion of Flagella of Selected Denitrifiers.

Isolate	Number of Flagella	Insertion of Flagella
4	1	polar or subpolar (based on 1 cell)
17	1	polar
21	1, some 2	polar
43	3 - 4	side attachment
62	1 and 2	polar
65	3 - 4	side attachment
107	1	polar or subpolar (based on 2 cells)
154	1	polar
156	1	polar
174	1	polar
191	1, some more	side attachment
Alcaligenes faecalis ATCC 8750	1	side attachment (based on 1 cell)
Pseudomonas fluorescens II ATCC 17822	up to 4	polar
Pseudomonas perfectomarinus	1	polar or subpolar

Table 14. Samples, Contributors, and Their Addresses.

Sample	Contributors and Their Addresses
Minnesota	Dr. Robert G. Gast Dept. of Soil Science Univ. of Minnesota St. Paul, Minn. 55101
California	Dr. Francis E. Broadbent Dept. of Soils and Plant Nutrition College of Agriculture Davis, Cal. 95616
Connecticut 1 and Connecticut 2	Dr. James L. Starr Dept. of Soil and Water, Connecticut Agricultural Experiment Station Box 1106 New Haven, Conn. 06504
Argentina(SP)	Ing. Arg. Adolfo Amma INTA Est. Exp. San Pedro Bs.As. Argentina
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Table 14. (continued)

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