UTILIZATION AND PRODUCTION OF N_2O BY DENITRIFIERS ISOLATED FROM DIFFERENT SOIL ENVIRONMENTS AND EFFECT OF pH ON THE RATES AND PRODUCTS OF DENITRIFICATION

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Crop and Soil Sciences

1978

ABSTRACT

UTILIZATION AND PRODUCTION OF N_2O
BY DENITRIFIERS ISOLATED FROM
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A total of 88 strains which grew by denitrification in tryptic soy broth (TSB) were examined for their ability to grow on N_2O as their electron acceptor as well as for their tendancy to produce N_2O from NO_3^- in the absence and presence of acetylene. Eight strains did not grow with oxygen as the electron acceptor and three more did not grow with NO_3^-. Thus 77 were confirmed as active denitrifiers for the survey. Sixty-four or 83% of the 77 strains reduced N_2O, while 13 strains produced but could not use N_2O. One strain, 204, exhibited reduction of NO_3^- to N_2 but could not produce or use N_2O. Strains [Nos. 42, 44, 69, 110, 151] reduced NO_3^- to N_2 but apparently did not have the capacity to grow on N_2O. For most taxonomic groups 2/3 or more of the strains reduced N_2O. However, none of the strains which clustered as Pseudomonas aeruginosa grew on N_2O. All strains of P. stutzeri studied utilized N_2O as a terminal electron acceptor. No strain of Pseudomonas sp. type 2 utilized N_2O. A high proportion of P. fluorescens biotype II reduced N_2O. This was also the most commonly encountered denitrifier in the world survey of new isolates by Gamble, suggesting that the capacity for N_2O reduction commonly exists in soils. The accumulation of N_2O from NO_3^- in the presence of acetylene by all but one of the isolates provides strong evidence that N_2O is

generally an intermediate in denitrification as well as provides additional support for the usefulness of this chemical as a general inhibitor of N_2O reduction.

Tryptic soy broth was found to be superior to nutrient broth as the medium base for denitrifier growth. Cell yield was linearly related to concentration of N_2O (0.1 to 1 atm) in the incubation vessel for the four strains tested; thus high concentrations of N_2O are not toxic. Cellular growth yields on N_2O in batch culture ranged from 5.6 g cells/e^- transferred for the fastest growing strain tested to 2.2 for slower growing strains. N_2O when used in most probable number tubes as the only electron acceptor was not consumed at dilutions down to 10^{-3} per gram. Additions of fresh carbon source and NO_3^- after growth did not stimulate N_2O reduction. Since pure cultures of denitrifiers grew well under the same conditions this result was unexpected; the explanation has not been found.

Limited studies were done on phase II denitrification rates in soils of different pH using the acetylene inhibition method in an anaerobic assay. Two of three very acid (pH 4 to 5) Nigerian soils showed significant denitrification in natural but not in autoclaved samples. This indicates presence of denitrifying enzymes in these soils. In contrast, four Michigan soils which had been decreasing in pH due to addition of different N fertilizer carriers showed little denitrification activity. The same soils which had recently been limed showed greater activity. The high activity in the pH 4.4 and 4.5 Nigerian samples suggest that acid tolerant denitrifying populations may have developed in these soils which had been acid for a very long period. The Michigan soils had become acid only recently.

To my son Chinaedu and my father Ukaobasi.

ACKNOWLEDGEMENTS

I am very grateful to Dr. J. M. Tiedje, Chairman of my guidance committee, for this opportunity and for his support, understanding, guidance and encouragement throughout the duration of this study.

I have to emphasize that I was very lucky to work in a laboratory where fellow graduate students were very friendly and always ready to help each other. Mary Firestone, Mike Betlach and Scott Smith are due special thanks for sharing their knowledge and experiences with me.

I would also like to extend a warm thanks to my wife Victoria Ifeoma Okereke, my daughter Jenete Okereke and my little son Chinaedu Echezona Okereke for their patience, endurance and understanding.

I thank the members of my guidance committee; Drs. B. G. Ellis and F. Dazzo for their encouragement.

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CHAPTER I

UTILIZATION AND PRODUCTION OF N_2O BY DENITRIFIERS ISOLATED FROM DIFFERENT SOIL ENVIRONMENTS

INTRODUCTION

Nitrogen and nitrous oxide are considered the major products of denitrification (2, 13, 17). Whether nitrous oxide is produced and/or utilized by most denitrifiers is however a question to which no definite answer has been given. This subject has been of considerable debate ever since nitrous oxide was recognized by Gayon and Duppet (14, 15, 16) as a gaseous product of the denitrification reaction. Several hypothetical pathways have been proposed, some treat nitrous oxide as an obligatory intermediate and others do not.

The fact that nitrous oxide is one of the end products of denitrification makes it extremely probable that this process is the source of this gas in the Earth's atmosphere. It was in 1911 that scientists predicted nitrous oxide as an atmospheric constituent of microbial origin and in 1938 its presence was verified by direct observation. In the early twentieth century there were conflicting ideas about the role of nitrous oxide in denitrification, partly because of inadequate techniques. For example Beijerinck and Minkman (3) and Suzuke (29) maintained that nitrous oxide was always present as the gaseous products of denitrification, whereas Gayon and Dupetit (16) and others claimed that nitrous oxide was entirely absent in some of their experiments. Sacks and Barker (26) rejected entirely nitrous oxide as an intermediate in nitrogen formation while Kluyver and Verhoeven (20) considered that

nitrogen may have a dual origin: partly derived from a hydrogenation of nitrous oxide and partly from direct hydrogenation of the precusors of nitrous oxide.

Recently greater interest in this topic has been stimulated by the hypothesis that nitrous oxide released to the atmosphere leads to the partial distruction of the ozone layer which protects the earth from biologically harmful ultraviolet radiation (6, 7, 8, 23). It has also been recognized that the use of nitrogenous fertilizers and other agricultural practices might increase the atmospheric concentration of nitrous oxide and thereby pose more danger to lives on Earth. Recent calculations by Wang et al. (30) show that if the nitrous oxide in the atmosphere is doubled, it would cause a warming of the planet that could drastically change the climate and thus be harmful to food production. These recent concerns about nitrous oxide and its hazardous effect to man and food production made this ignored product of denitrification a topic of great interest.

At the moment there are many basic questions yet to be answered concerning nitrous oxide production and utilization by denitrifiers. It is known that nitrous oxide is a trace component of the atmosphere and a major sink for nitrous oxide was considered to be photochemical dissociation in the troposphere and stratosphere (2); this has been supported by Schutz et al. (27).

Evidence that soil can also act as a sink for atmospheric nitrous oxide under certain conditions was obtained from studies showing (4) that soil microorganisms have the capacity to remove nitrous oxide from soil atmosphere until the concentration of this gas is much lower than

the concentration in air. This uptake of nitrous oxide by soils was found to be due to microbial reduction stimulated by readily available organic matter.

Certain denitrifiers can grow on nitrous oxide as the sole oxidant (9). Kluyver and Verhoeven (20) concluded that nitrous oxide is an intermediate in denitrification in at least some bacterial species because of the ubiquity of its occurrence. It has also been reported that Pseudomonas stutzeri (1), P. denitrificans (22) and Paracoccus (formerly Micrococcus) denitrificans (25) grew anaerobically using nitrous oxide as an electron acceptor.

Although there exists some literature on nitrous oxide utilization and production by some denitrifiers, there has not been an extensive study of the nitrous oxide utilization and production by a wide variety of isolates from nature. To my knowledge, studies so far carried out have been on one or a few denitrifiers. Because of this, there are still differences in opinion among investigators concerning whether nitrous oxide can be utilized and produced by all denitrifiers and whether it is an obligatory intermediate in denitrification.

As far as ecological interpretations are concerned, it is useful to identify denitrifiers of ecological importance for use in the study of the biochemical and physiological features of the pathway of denitrification. In this regard work with pure cultures is important though care has to be taken when using the results to predict what happens in Nature.

The purpose of this study was to survey the isolates of Gamble (11) for their ability to utilize and produce nitrous oxide. Other studies were undertaken to determine cell growth yields when grown with

nitrous oxide as the terminal electron acceptor and to investigate whether nitrous oxide as the only electron acceptor in MPN tubes could serve as a specific method for enumeration of denitrifiers. The results of these studies may help to elucidate the denitrification pathway. For instance organisms that reduce nitrate to nitrogen but cannot grow on N_2O may help in elucidating the importance of nitrous oxide in respiratory nitrate reduction. Furthermore if nitrous oxide is the only gas produced from nitrate by growing cells of these organisms, then the study of the pathway of denitrification will not be complicated by the production of two gases as often occurs with other denitrifying organisms (20).

MATERIALS AND METHODS

Description of denitrifier strains

A total of 114 isolates confirmed as denitrifiers by Gamble (11) and 10 reference strains of denitrifying bacteria were studied. The reference strains were Pseudomonas fluorescens (ATCC 17822), Pseudomonas perfectomarinus, Hyphomicrobium sp. (WC 24 R, from Peter Hirsch), Pseudomonas denitrificans (ATCC 13867), Paracoccus denitrificans (ATCC 2008), Pseudomonas stutzeri (ATCC 17588), Pseudomonas aureofaciens (ATCC 13985), Pseudomonas mendocino (ATCC 25411), Pseudomonas aeruginosa, Alcaligenes faecalis (ATCC 8750). The origin of these strains is given by Gamble (11). Other strains used as controls to check for O_2 contamination were Pseudomonas strains 388 and 402 obtained from G. E. Becker, University of Iowa and a Pseudomonas strain that grew on NTA. Becker strains 388 and 402 do not grow on N_2O while the NTA consuming strain is an obligate aerobe.

The 114 isolates were confirmed by Gamble to be denitrifers by the production of N_2O and/or N_2 during growth in nitrate broth Gamble (11). A list of these denitrifiers is found in Appendix A. These cultures were isolated by Gamble from soils, fresh water lake sediments and nitrified poultry manure and came from eight countries and a variety of different soils and environments.

Comparison of growth media

The stock cultures used were prepared by T. N. Gamble in sterilized soil in sealed screw cap tubes and had been stored two years in the refrigerator. Aggregrates of soil were aseptically transferred to test tubes containing 10 ml of sterilized nutrient broth (Difco) and tryptic soy broth (Difco). The cultures were incubated aerobically at 30° C. When turbid a loop of the culture was transferred to tubes containing 10 ml of each of the following three test media: (i) 0.8% nutrient broth (Difco, Detroit, MI), (ii) 3% tryptic soy broth (TSB, Difco) and (iii) 3% TSB plus 3.5 mM KNO_3. The first two were incubated aerobically at 30° C and the latter anaerobically in a glove box at room temperature. Growth was scored as visible turbidity after 7 to 14 days.

The above complex media contain grams/litre: Tryptic soy broth- trypticase peptone 17 g, phytone peptone 3 g, NaCl 5 g, dipotassium phosphate 2.5 g and Bacto dextrose 2.5 g; nutrient broth- Bacto-beef extract 3 g and Bacto peptone 5 g.

Preparation of inocula

The soil inoculum was aseptically transferred into 10 ml of sterilized TSB and incubated aerobically at 30° C. After about 3 days growth, 1 ml of this culture was transferred aseptically into another

TSB tube and again incubated aerobically at 30° C until the tubes were inoculum as needed. Every two weeks these "stock cultures" were reinoculated into a fresh medium and grown at 30° C and then stored in the refrigerator until use. This process was repeated as needed through the experimental period.

Experimental culture conditions

Isolates were grown in culture tubes sealed with butyl rubber septa (Hungate tubes, Bellco Glass, Vineland, N.J.). The tubes contained 10 ml of 3% TSB plus either 3.5 mM KNO_3, N_2O or O_2 (air) as the terminal electron acceptor. For anaerobic incubations the air was removed by evacuating and filling with He via needles connected to a manifold which was linked to a vacuum pump and He tank. The flushing cycle was repeated four times with a vacuum of -30 inches Hg achieved for 15 min each cycle. When N_2O was required the desired concentration (generally 0.2 atmosphere) was added by syringe after first removing an equivalent volume of He by syringe. Acetylene was added where indicated at a concentration of 0.1 atmosphere. The tubes were then autoclaved at 121° C and 15 psi for 15 min.

The tubes were inoculated with 1 ml of the refrigerated inoculum. Tubes were incubated inverted to reduce chances of O_2 leakage through the septum, and placed on a rotary shaker operating at 120 rpm. The incubation temperature was 30° C. The incubation period was one week for the nitrate dependent growth and N_2O concentration experiments and until visible growth for the survey experiment. If no growth was visible the tubes were incubated for two weeks before analysis.

Analyses

Turbidity was measured as percent transmission at 660 nm in a Turner Spectrophotometer, Model 330. For growth yields a standard curve of cell dry weight versus optical density (optical density = 2-$\log_{10}$ percent transmission) was used to determine biomass.

The composition of gases in the sealed cultures was determined by gas chromatography. The culture was vigorously shaken by hand to ensure equilibration of the gas between the soluble and vapor phase prior to sampling. A sample, usually 0.5 ml, of headspace gas was removed by a 1 ml plastic syringe fitted with a Pressure Lock valve (Precision Sampling Corp., Baton Rouge, LA). The sample was injected into a Carle Model 8515 gas chromatograph (Carle Instruments, Fullerton, CA), equipped with Poropak Q (3 mm x 1.8 m) and Molecular Sieve 5A (3 mm x 1.8 m) columns connected in series by a column switching valve. The detector was a microthermistor. The column temperature was 45° C. The carrier gas was He at a flow rate of 25 ml/min. Peaks were recorded on a chart recorder and were quantified by a computing integrator (Autolab I, Spectra-Physics, Santa Clara, CA). In the survey the integration value in μ volt·sec is recorded to give an indication of the size of each peak since precise quantitation of each component was not necessary.

Growth yield experiments

The reference strains of A. faecalis, P. perfectomarinus, P. stutzeri, and Paracoccus denitrificans were used to determine growth yields on N_2O and O_2 as terminal electron acceptors. Inocula were grown aerobically on 3% TSB and then transferred (4 ml) to side-arm Erlenmeyer flasks (164 ml) which contained 100 ml of 3% TSB. The

flasks were sealed with rubber stoppers pierced by a glass tube capped with a serum stopper for sampling of headspace gas by syringe. The flasks were made anaerobic by evacuation and filling with He as described above. N_2O was added to achieve a gas composition of 0.2 atmosphere in the manner described above. Flasks were incubated at 30° C on a rotary shaker at 150 rpm.

Growth was measured as percent transmission in the side-arm tube at 1 or 2 hour intervals. This value was converted to cell dry weight by means of the standard curve for each organism. At the same time a sample of flask atmosphere was analyzed for N_2O by gas chromatography. The total N_2O content was determined for the vapor plus solution phases using a Bunsen coefficient of 0.67 Smith et al. (28).

The same procedure was used for growth yield experiments with O_2 and nutrient broth as culture components.

Use of N_2O as electron acceptor in MPN tubes

The soils used are described in Table 1. After collection the soils were passed through a 5 mm sieve without drying and were stored in sealed plastic bags at 2° C until used. These soil samples consisted of six subsamples that were freshly collected from the upper horizon of the soil. The same soils were used in other MPN studies of denitrifiers but using different methods so that my results could be directly compared (N. V. Caskey, personal communication).

The first dilution was prepared by blending 10 g of soil in a sterilized Waring blender for 2 min with 90 ml of sterilized distilled water containing 0.85% NaCl. One drop of Tween 80 was added per liter of the distilled water before sterilization. Ten-fold dilutions of the soil samples were prepared. One-tenth milliliter of the appropriate

Table 1. Characteristics of soils used.

Series	Texture	Classification	pH	% Organic matter
Brookston	Loam	Typic argiaquoll	7.6	3.2
Miami	Sandy loam	Typic hapludalf	6.6	2.7
Spinks	Loamy sand	Psammentic hapludalf	6.4	1.5

dilutions of the soil samples were transferred to each of the five Hungate tubes which contained 10 ml of sterilized 3% TSB and 0.5 atm N_2O. The tubes were incubated on a rotary shaker at 30° C and observed daily for turbidity. After 14 days incubation, 0.5 ml of headspace gas was analyzed for disappearance of N_2O by gas chromatography beginning with the tubes showing turbidity at the highest dilution.

RESULTS AND DISCUSSION

Incubation conditions

In Gambles' (11) previous study and from other experiences in the laboratory it was noted that nutrient broth did not always support luxurious and consistent growth of denitrifiers. Therefore, in a preliminary study nutrient broth was compared with tryptic soy broth for support of growth of a variety of denitrifier strains. At the same time the ability of each strain to grow anaerobically on tryptic soy broth and NO_3^- was also examined. The results for each of 123 strains on each medium is recorded in Appendix A. The results are summarized in Table 2. Tryptic soy broth was superior as 98% of the cells grew in this medium while only 75% grew in nutrient broth. Fifteen of the isolates lost their viability as they could not grow aerobically in any of the media while 10 of them could grow aerobically but had lost the ability to grow by denitrification.

To further examine whether TSB was a better medium for growth of the denitrifiers, growth rates of *P. perfectomarinus*, *P. stutzeri* and *A. faecalis* on TSB were compared with those on nutrient broth under anaerobic conditions with 20% N_2O as the electron acceptor. Both rate of growth and N_2O use were much faster in TSB than in nutrient broth

Table 2. Comparison of complex media for supporting growth of denitrifier strains.

Medium	Number that grew	Number that did not grow	Percentage of viable isolates that grew[a]
Nutrient Broth + O_2	84	39	76
Tryptic soy broth + O_2	108	15	98
Tryptic soy broth, 3.5 mM KNO_3, no O_2	98	25	89

[a]Inoculum was pregrown on nutrient broth and tryptic soy broth; 110 of the 123 strains taken from the soil stock culture grew on one or both media.

(Table 3). The improvement in growth rate was 40 to 100% by use of TSB for the strains examined.

Because of the nutritional and physiological differences among denitrifiers, it is not surprising that a single medium is inadequate for their cultivation or enumeration. Tryptic soy broth differs from nutrient broth in that it provides a readily utilizable carbon and energy source (glucose) a plant rather than an animal-derived protein, more total carbon and possibly more growth factors. Whatever the explanation, it would appear that the organic substances in nutrient broth were not adequate to satisfy the nutritional demands of sizeable portion of the denitrifying microflora. Marten (21) has also found that 0.3% Bacto-tryptic soy broth (Difco) solidified with 1.5% agar to be as good as a soil extract based medium for isolation and enumeration of total aerobic bacteria. Thus, a TSB based medium appears adequate for growth of soil denitrifiers in this collection and was the medium of choice for the denitrification study.

Because all denitrifiers and aerobes prefer O_2 over nitrogenous oxides as their electron acceptor, it was necessary to ensure that oxygen contamination could be minimized thus assuring the result was due to nitrogenous oxide dependent growth. Oxygen contamination can result from incomplete air removal during evacuation and flushing, possible air leakage through the septum, introduction of oxygen with needle and inoculum solution, and impurities in the gases, especially N_2O which often contains 0.5 to 1% O_2. The adequacy of the procedure used to minimize the influence of contaminating oxygen is demonstrated in Table 4. The Becker strains (Pseudomonas sp.) are denitrifiers which have lost the ability to reduce N_2O to N_2. They can grow with

Table 3. Comparison of rate of growth of *P. perfectomarinus*, *P. stutzeri* and *A. faecalis* in nutrient broth and tryptic soy broth (TSB) with N_2O as the only terminal electron acceptor.

Strain number	Strains	N_2O utilization (μmol/h)		Generation time (h)		Percent improvement in generation time by use of TSB
		TSB	Nutrient broth	TSB	Nutrient broth	
999	*P. perfectomarinus*	322	223	2.0	4.0	100
998	*P. stutzeri*	337	136	1.2	2.0	67
191	*A. faecalis*	215	154	1.0	1.4	40

Table 4. Validation of Hungate tubes and He flushing regime for assaying N_2O dependent growth.

Strains	Characteristic	Aerobic growth	Growth in tubes flushed with He	Growth in tubes with 80% He and 20% N_2O
Becker #388	lacks N_2O reductase	+[a]	0[a]	0
Becker #402	lacks N_2O reductase	+	0	0
Pseudomonas sp (NTA strain)	obligate aerobe	+	0	0
P. fluorescens	denitrifies to N_2	+	0	+
P. stutzeri	denitrifies to N_2	+	0	+

[a] + = growth shown by turbidity; 0 = no growth

O_2, NO_3^- and NO_2^-. The NTA strain is an obligate aerobe. Since no strains grew under He and the first three did not under He + 20% N_2O, oxygen contamination was judged insignificant. Lack of growth also confirms that the medium does not contain other electron acceptors (eg. NO_3^- or NO_2^-) that could support growth. The medium was adequate for denitrifier growth as shown by growth of P. fluorescens and P. stutzeri with N_2O. The first three organisms were routinely used as controls for oxygen contamination in other experiments.

Initially 20% N_2O was used to minimize any toxic effect that might be due to a highly water soluble, oxidizing gas. However, when N_2O gas concentrations from 10 to 100% were used, cell yield increased in a linear manner (Figure 1). Thus toxicity is not apparent. Furthermore, final cell yield appears limited by the amount of N_2O available. Since the yield is suboptimal at 20% N_2O, higher concentrations of N_2O are recommended for future work.

Distribution of N_2O production and utilization capabilities among denitrifier strains

Eighty-eight strains which grew by denitrification in TSB (Table 2) were examined for their ability to grow on N_2O as their only electron acceptor, as well as for their tendancy to produce N_2O from NO_3^- in the absence and presence of acetylene. The results for each strain are shown in Table 5 with summaries following in Tables 6-8. The viability of each strain under aerobic and denitrifying condition was also noted by observing turbidity in the presence of O_2 and NO_3^-, respectively, as terminal electron acceptors. Eight strains did not grow with O_2 and three more did not grow with NO_3^-. Thus 77 strains were confirmed as active denitrifiers for this survey (Table 6).

Figure 1. Growth of denitrifiers in different N_2O concentration.

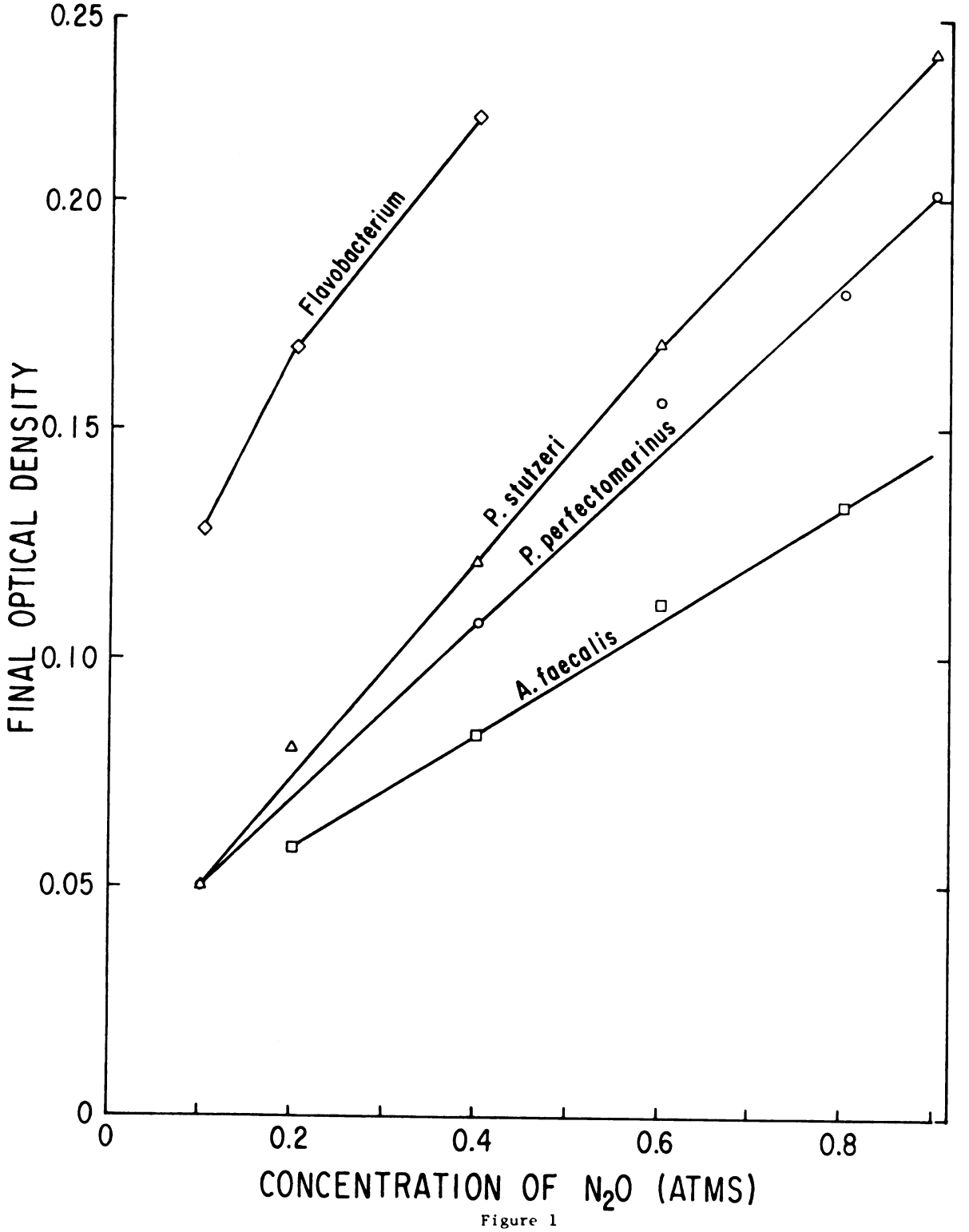
0.25
0.20
0.15
0.10
0.05
0
FINAL OPTICAL DENSITY
Flavobacterium
P. stutzeri
P. perfectomarinus
A. faecalis
0
0.2
0.4
0.6
0.8
CONCENTRATION OF N_2O (ATMS)

Figure 1

Table 5. Utilization and production of N_2O by 79 denitrifier isolates and 9 reference cultures.

Cluster No.	Strain No.	Growth on N_2O as e^- acceptor		Growth on NO_3^- as e^- acceptor (no acetylene)		Growth on NO_3^- as e^- acceptor plus acetylene		Visible growth			Pathway used[a]
		% T	N_2O remaining	N_2O	N_2	N_2O	N_2	N_2O	NO_3^-	Air	
		integrator response in 10^2 μvolt·sec									
	Sterile	100	143	tr	31			0	0	0	
	Becker 388	99	143	25	75			0	+	+	
	Becker 402	99	141	236	61			0	+	+	
	NTA	99	141	0	44			0	0	+	
1D	6	95	156	0	99	4	41	0	+	+	C
1C	12	96	137	169	52	128	45	0	+	+	C
1A	13	91	168	144	49	33	47	0	+	+	C
1C	15	88	tr[b]	tr	256	4	37	++	+	+	A
--	39	95	0	0	197	140	10	+	+	+	A
5	40	92	75	0	367	4	52	++	+	+	A
1A	42	94	102	3	324	171	59	+	+	+	D
5A	43	68	0	0	320	182	52	+++	+	+	A
1A	44	92	120	tr	500	26	6	0	+	+	D
1A	45	94	88	0	574	166	48	+	+	+	A
10	46	95	tr	0	616	37	54	0	+	+	A
1A	49	91	59	tr	341	175	48	++	+	+	A
1A	51	97	145	0	31	--	50	0	0	+	E
1	55	93	tr	1	368	14	38	+++	+	+	A

Table 5. Continued

Cluster No.	Strain No.	Growth on N_2O as e^- acceptor		Growth on NO_3^- as e^- acceptor (no acetylene)		Growth on NO_3^- as e^- acceptor plus acetylene		Visible growth			Pathway used[a]
		% T N_2O remaining		N_2O	N_2	N_2O	N_2	N_2O	NO_3^-	Air	
		----------integrator response in 10^2 μvolt·sec----------									
1	58	92	0	0	687	112	54	++	+	+	A
1A	59	96	13	161	45	169	46	+	+	+	A
1A	61	91	84	tr	314	30	40	+	+	+	A
1A	62	95	109	166	43	178	58	++	+	+	A
1A	63	82	0	0	376	170	48	++	+	+	A
1A	64	89	92	0	427	164	37	+	+	+	A
5	65	90	0	0	462	49	43	+	+	+	A
1A	66	89	13	tr	435	60	52	++	+	+	A
1A	67	85	72	0	507	110	40	+	+	+	A
1A	68	97	130	157	46	nt[b]	nt	0	+	+	C
1A	69	97	177	0	590	179	78	0	+	+	D
1A	70	94	169	182	49	159	48	0	+	+	C
1A	72	95	36	189	50	104	26	+	+	+	A
1A	73	95	75	116	103	162	38	+	+	+	A
1A	74	90	0	0	55	173	47	+++	+	+	A
1A	75	95	118	146	53	176	56	+	+	+	A
1	78	93	0	tr	494	15	62	+	+	+	A
1A	79	95	134	104	119	99	45	+	+	+	A
1D	80	85	65	nt	nt	tr	45	+	+	+	A
1C	81	97	168	nt	nt	nt	nt	0	0	0	E
1A	82	92	97	0	355	17	16	+	+	+	A
1C	83	98	159	0	17	0	16	0	0	0	E
1A	84	95	167	156	45	172	55	0	+	+	C
1B	85	87	0	0	590	39	47	++	+	+	A
1A	89	84	0	0	560	105	43	++	+	+	A

Table 5. Continued

Cluster No.	Strain No.	Growth on N_2O as e^- acceptor	Growth on NO_3^- as e^- acceptor (no acetylene)		Growth on NO_3^- as e^- acceptor plus acetylene		Visible growth			Pathway used[a]
		% T N_2O remaining	N_2O	N_2	N_2O	N_2	N_2O	NO_3^-	Air	
		integrator response in 10^2 μvolt·sec								
--	97	97	138	0	30	nt	nt	0	0	0	E
--	98	76	tr	0	417	3	38	+++	+	+	A
5B	104	99	161	0	29	tr	56	0	0	0	E
1A	105	83	0	0	564	75	62	+++	+	+	A
2	107	87	tr	tr	240	8	41	+++	+	+	A
--	108	86	1	0	376	125	45	+	+	+	A
1C	110	93	126	0	649	169	41	+	+	+	D
1A	111	82	0	1	383	107	45	+	+	+	A
3	1181	97	166	3	20	nt	nt	0	+	+	C
3	129	98	158	4	39	nt	nt	0		+	C
3	135	98	135	0	30	tr	44	0	0	+	E
3	141	98	154	0	62	0	39	0	0	+	E
2	143	83	0	0	318	15	48	+++	+	+	A
2	149	99	147	0	22	nt	nt	0	0	+	E
6	151	95	143	0	318	0	44	0	+	+	D
--	154	89	tr	nt	nt	nt	nt	++	+	+	A
8	155	89	0	0	410	132	41	+++	+	+	A
4	156	94	113			1	43	0	+	+	C
2	163	90	0	0	357	4	40	+++	+	+	A
4	167	94	124	151	60	184	53	0	+	+	C
10	175	99	172	tr	31	0	37	0	0	0	E
10	177	91	0	0	509	86	4	+	+	+	A
--	179	86	118	tr	34	nt	nt	+	+	+	A
1B	183	95	126	42	199	9	40	+	+	+	A
1B	185	95	130	146	88	9	40	0	+	+	C

Table 5. Continued

Cluster No.	Strain No.	Growth on N_2O as e^- acceptor	Growth on NO_3^- as e^- acceptor (no acetylene)		Growth on NO_3^- as e^- acceptor plus acetylene		Visible growth			Pathway used[a]
		% T N_2O remaining	N_2O	N_2	N_2O	N_2	N_2O	NO_3^-	Air	
		integrator response in 10^2 μvolt·sec								
2	188	87	0	0	562	19	38	+++	+	+	A
--	189	98	72	0	444	46	45	0	+	+	A
1	190	85	0	0	363	112	49	+++	+	+	A
5C	191	81	0	0	281	6	43	+++	+	+	A
--	193	80	56	0	109	25	40	+	+	+	A
--	195	94	0	0	386	6	38	+++	+	+	A
1	196	88	0	2	433	160	44	++	+	+	A
--	202	95	39	0	244	39	5	+	+	+	A
--	204	96	152	1	125	1	105	+	+	+	B
1A	205	89	0	2	401	177	56	+	+	+	A
2	206	89	57	63	131	182	47	+	+	+	A
9	224	74	30	tr	255	nt	nt	++	+	+	A
9	231[2]	95	0	0	497	2	32	+	+	+	A
7	232	97	160	0	33	0	62	0	0	0	E
2	234	86	0	0	241	6	56	++	+	+	A
P. denitrificans	991	90	0	tr	317	100	50	++	+	+	A
P. aureofaciens	992	94	184	155	32	57	37	0	+	+	C
P. mendocino	993	99	137	nt	nt	8	14	0	0	0	E
P. fluorescens	995	90	0	0	617	nt	nt	+++	+	+	A
P. aeruginosa	996	85	0	2	318	nt	nt	+++	+	+	A
Paracoccus	997	87	0	nt	nt	nt	nt	++	+	+	A
P. stutzeri	998	84		tr	339	138	34	+++	+	+	A
P. perfectomarinus	999	89	tr	0	301	tr	1	+++	+	+	A
Hyphomicrobium		24	14	tr	456	133	95	+++	+++	+	A

[a] See text for description of pathway

[b] tr, trace detected; nt, no test performed

Table 6. Number of denitrifier strains showing the indicated denitrification pattern.

Description of denitrification pathway used by isolates	Number of denitrifiers[a]
1. Produce and utilize N_2O (A)	64
2. No N_2O production and utilization but reduction of NO_3^- to N_2 (B)	1
3. Produce N_2O but do not utilize it (C)	12
4. Reduce $NO_3^- \rightarrow N_2$ but do not grow on N_2O (D)	5
5. Produce N_2O in the presence of acetylene	66
6. Lost ability to denitrify and/or not viable (E)	11
7. Able to use NO_3^- as a terminal e-acceptor [A+B+C]	77

[a]Total number of organisms studied was 88

Confirmation of ability of the denitrifiers to utilize N_2O was based on the following:-

1. Partial or total disappearance of N_2O when grown on N_2O.
2. Increase in N_2 when grown on NO_3^-.
3. Visual turbidity when grown on N_2O.
4. Accumulation of N_2O from NO_3^- in the presence but not in the absence of 0.1 atmosphere of acetylene.
5. Partial or total disappearance of N_2O from NO_3^- in the absence of acetylene and a subsequent increase in N_2.
6. Increase in CO_2 production when grown with N_2O.

Results in Table 5 show some variability in extent of N_2O reduction and growth. This was partially due to my collection of data after the appearance of turbidity, but not necessarily at the same stage of growth. Nonetheless, all of the above criteria could easily be distinguished and gave a consistent interpretation for 69 of the 77 strains. For the remaining eight strains it was turbidity that was not clearly discernable. In these cases the tubes were scored as positive for N_2O use if the concentration of N_2O had diminished significantly. In these cases the limited growth also limited N_2O reduction. Despite the first impression of variability of data, the number of clear-cut conclusions was high.

Sixty-four or 83% of the 77 strains reduced N_2O (Table 6), while 13 strains produced but could not use N_2O. One strain (No. 204) exhibited reduction of NO_3^- to N_2 but did not produce or use N_2O suggesting that N_2O may not be a freely diffusable intermediate in this case. Five strains (Nos. 42, 44, 69, 110, 151) reduced NO_3^- to N_2 but did not have the capacity to grow on N_2O. This is apparently because

these strains lack the capacity for phosphorylation associated with the N_2O reduction. The fact that most strains which reduce N_2O can also grow on N_2O suggests that the capacity for N_2O reduction and phosphorylation are generally linked.

One of the following hypothetical schemes can be assigned to each of the strains from the data in Table 5.

A. $NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$; N_2O $\downarrow\uparrow$ N_2O ATP

B. $NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$; N_2O $\downarrow\uparrow$ N_2O ATP?

C. $NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \not\longrightarrow N_2$; N_2O $\downarrow\uparrow$ N_2O ?

D. $NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$; N_2O $\downarrow\uparrow$ N_2O ? ATP

E. $NO_3^- \not\longrightarrow NO_2^- \xrightarrow{?} NO \xrightarrow{?} N_2O \xrightarrow{?} N_2$; N_2O $\uparrow$ N_2O

Pathway A shows that N_2O is a freely diffusable intermediate while B represents no production and utilization of extracellular N_2O by denitrifiers. C represents production but no utilization of N_2O. Here the end product of denitrification is N_2O. D represents reduction of NO_3^- and perhaps N_2O to N_2 but no growth occurs on N_2O since ATP is not generated. E represents those cells which have lost their ability

to grow. All strains unable to grow on NO_3^- were also unable to grow on N_2O. The numbers of denitrifiers fitting the above schemes are summarized in Table 6.

Table 7 shows the number and percentage of strains in major taxonomic clusters capable of N_2O utilization while Table 8 indicates the percentage of major species identified by conventional means that utilize N_2O. Unfortunately many of the groupings had too few strains to draw a conclusion on correlation of N_2O reduction capacity with phenotype. For most groups 2/3 or more of the strains reduced N_2O. The most noteable exception is P. aeruginosa. None of the strains clustered as this species grew on N_2O (Table 7). The one strain classified as P. aeruginosa that did grow on N_2O (Table 8) was a reference strain originally obtained from W. J. Payne (Gamble, 12). The absence of growth on N_2O by P. aeruginosa is supportive of the same observation noted by W. P. Payne and J. L. Ingraham (personal communications to J. M. Tiedje). All strains of P. stutzeri studied utilized N_2O as a terminal e-acceptor. No strain of Pseudomonas sp. type 2 utilized N_2O.

P. fluorescens, biotype II was the most commonly encountered denitrifier in the world-wide survey conducted by Gamble et al. (12). A high proportion of these strains reduced N_2O (Table 7) which suggests that the capacity for N_2O reduction exists in most soils.

For several strains the presence of acetylene did not cause the dramatic increase in N_2O expected. It is not clear if this is due to ineffective inhibition by acetylene of some strains or whether the sampling was premature. In the absence of acetylene N_2O produced by reduction of NO_3^- persisted in some of the tubes for only short periods.

Table 7. Distribution of the capacity to grow on N_2O among the similarity clusters of denitrifier strains found by Gamble *et al.* (12).

Cluster number	Probable identity of cluster	Total number of denitrifiers studied	Total number of N_2O utilizers	Denitrifiers that can utilize N_2O (%)[a]
1	*P. fluorescens*	39	33	85
2	*Pseudomonas* sp.	6	6	100
3	*P. aeruginosa*	2	0	0
4	*P. aeruginosa*	2	0	0
5	*Pseudomonas* sp. and *Alcaligenes* sp.	4	4	100
6	*Pseudomonas* ?	1	0	0
7	*Pseudomonas* sp.	1	1	100
8	*Pseudomonas* sp.	2	2	100
9	*Flavobacterium* sp.	2	2	100
10	Ungrouped isolates	10	10	100
11	Reference cultures	8	6	75
	Total	77	64	83

[a] Percentage based on viable isolates.

Table 8. Major species that utilized N_2O.

Species[a]	Number of N_2O utilizers	Number studied[b]	Percentage of N_2O utilizers in number studied[b]
1. *P. fluorescens* II	20	25	80
2. *A. faecalis*	3	4	75
3. *Pseudomonas* sp. type 2	0	5	0
4. *Pseudomonas* sp. type 4	1	1	100
5. *P. aureofaciens*	2	3	67
6. *Pseudomonas* sp. type 5	2	3	67
7. *P. aeruginosa*	1	3	33
8. *Flavobacterium* sp.	3	2	67
9. *P. stutzeri*	4	4	100
10. *P. fluorescens* (?)	4	4	100
Total	40	54	

[a] Tentative identification given by Gamble (11).

[b] Percentage based on viable isolates.

This observation is in harmony with previous studies by Blackmer and Bremner (5) where they found that microorganisms accumulated N_2O for a short time and subsequently reduced it to N_2. N_2O also accumulated temporarily and then was converted to N_2 in both soil and microbial culture experiments (10, 24).

This survey shows that 83% of these isolates produce and use N_2O. N_2O was the end product of denitrification for 17% of the isolates.

In concluding it is likely that soil can be a sink as well as a source of atmospheric N_2O because of the high numbers of denitrifiers that can utilize and produce N_2O. The percentage of N_2O users may have even been higher if these cultures were freshly isolated, since some of them may have lost the ability to synthesize N_2O reductase during their period in the laboratory. This argument is supported by literature records which suggested that at least for fresh isolates from soil, essentially all reduce N_2O to N_2 (24). Also Garcia (13) working with soil showed a high correlation between denitrification rates measured by Warburg and N_2O reduction. Gamble (12) also noted that a large percentage of his fresh isolates which originally denitrified no longer produced N_2 gas after subculturing.

Growth yields

Koike and Hattori (18) have reported that nitrate respiration is about 40% less efficient than aerobic respiration (4.5 vs. 7.5 g cells/mole glutamate with NO_3^- vs. O_2, respectively). Though I did not determine growth yield for O_2 as the electron acceptor, the aerobic generation time was less than with N_2O, 0.5 vs. 1.0 hour on O_2 vs. N_2O, respectively, by A. faecalis in TSB and 0.8 vs. 2.0 for P. perfectomarinus

in nutrient broth. This was a 50-60% reduction in growth rate due to N_2O. The above authors found a greater reduction in growth rate by denitrification, 1/5 to 1/7 of that with O_2.

Koike and Hattori (19) also reported that P. denitrificans showed identical cell yields per electron transferred when NO_3^-, NO_2^- and N_2O were electron acceptors. Their data are summarized in the lower half of Table 9 to facilitate comparison with my data. Plots used to obtain my data are in Figure 2. Their data does not include maintenance energy which becomes more significant as growth rate decreases. The A. faecalis strain, which grew very quickly, had a yield similar to their values. The other strains had lower growth yields. The two with the lowest yield also had the slowest growth rate. Thus a large maintenance energy cost may be at least partially responsible for the lower yields.

Use of N_2O as an electron acceptor in MPN tubes

There is no reported attempt to use N_2O as the terminal electron acceptor in the enumeration of denitrifiers by the MPN procedure. This approach has the advantage that only denitrifiers can reduce N_2O to N_2 under these growth conditions. Thus this method would be specific for denitrifiers, a feature not found in currently used methods. Other methods measure disappearance of NO_3^- and NO_2^-. Problems are false positives due to dissimilatory nitrate reducers and the sometimes slow reduction of NO_2^- possibly due to its toxic effect. A potential problem of the N_2O reduction approach was the uncertainity as to how many denitrifiers could grow on N_2O. This concern has been alleviated by the finding that 4/5 of the denitrifiers surveyed could reduce N_2O. This error would be encompassed by the statistical error inherent in the 5-tube MPN method. Use of TSB with N_2O would retain the nutritional

Table 9. Growth yields of several denitrifier strains when grown in batch culture on N_2O as electron acceptor.

Strain	Electron acceptor	Carbon source	Growth yield (g cells/mol acceptor)	Yield per electron transferred (g cells/e^-)	Generation time (h)
A. faecalis	N_2O	TSB	11.2	5.6	1.0
P. stutzeri	N_2O	TSB	8.2	4.1	1.2
Paracoccus denitrificans	N_2O	TSB	6.1	3.0	2.2
P. perfectomarinus	N_2O	TSB	4.4	2.2	2.0
P. denitrificans (from Koike & Hattori, 18)[a]	NO_3^-	Glutamate	28.6	5.7 (3.6)	
	NO_2^-	Glutamate	16.9	5.6	
	N_2O	Glutamate	8.8	4.4	

[a] Maintenance energy not included; data from my study was done in batch culture and could not be corrected for maintenance. Value in parenthesis was also done in batch culture (Koike and Hattori, 17) and is included for comparative purposes.

Figure 2. Growth yields of four denitrifier strains with N_2O as terminal electron acceptor.

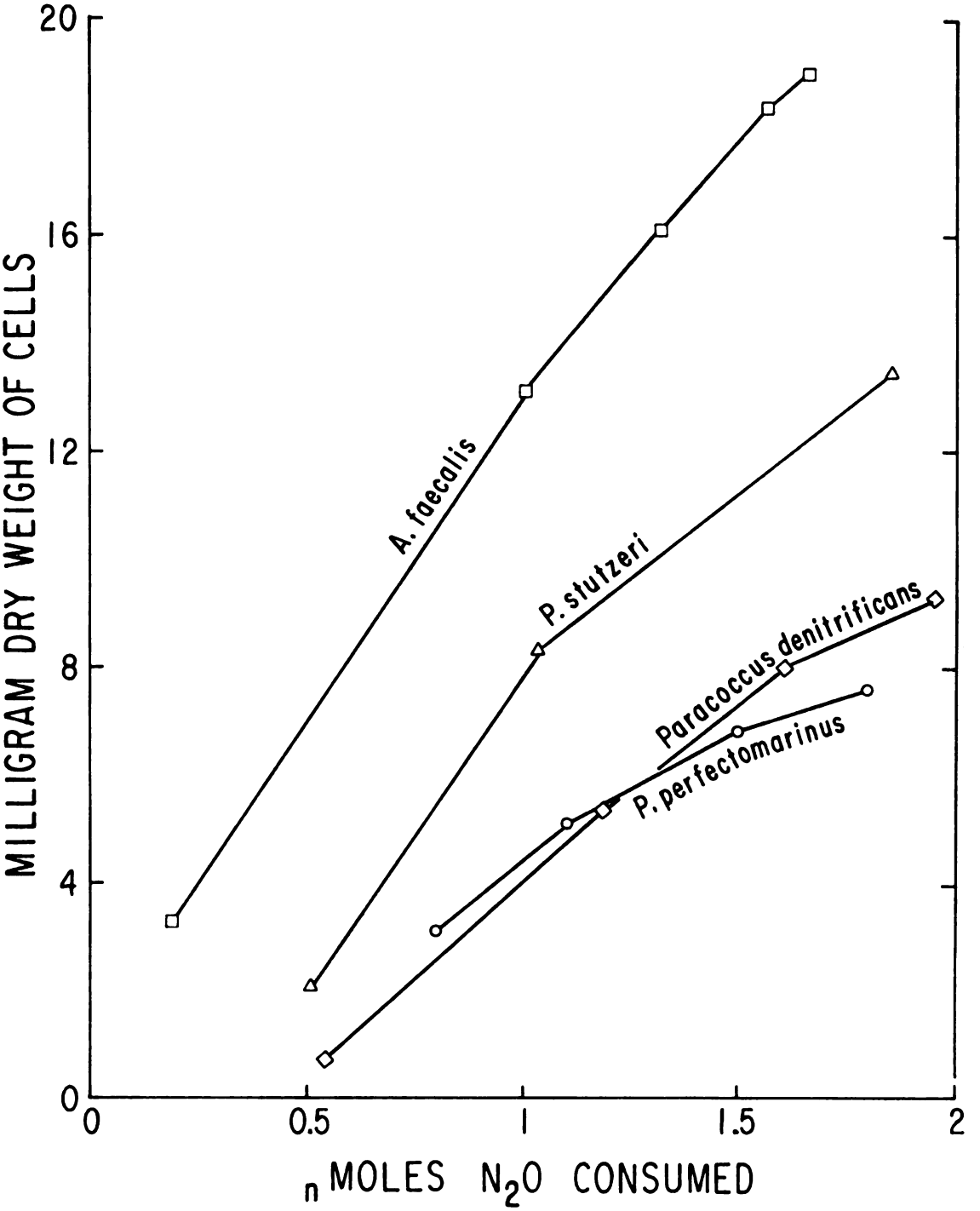
MILLIGRAM DRY WEIGHT OF CELLS
20
16
12
8
4
0
0
0.5
1
1.5
2
n MOLES N2O CONSUMED
A. faecalis
P. stutzeri
Paracoccus denitrificans
P. perfectomarinus

Figure 2

advantages of TSB, decrease in vitro competition due to simultaneous growth of NO_3^- reducers, and simplify the requirement for a positive test for denitrifiers by simply determining partial or total disappearance of N_2O from culture tubes. Therefore the use of the MPN procedure to enumerate denitrifiers capable of N_2O utilization was tested with samples of three soils.

The results were unexpected. No disappearance of N_2O was observed in any tube, even at dilutions of 10^{-3} and 10^{-4}/g. Other methods had shown at least 10^6 organisms/g in these soils. The cause of the lack of N_2O use could be due to absence of enough metabolizable carbon after faster growing aerobes and fermenters had used up the original substrate. It could also be due to the inability of cells to synthesize N_2O reductase under these conditions. To discover the reason for this behavior I added fresh filter sterilized TSB + NO_3^- (concentrated) to half of the tubes and TSB only to the other half. The additional carbon should have overcome any energy limitation and the NO_3^- could serve as an inducer. However, no N_2O disappearance was again observed after one week. In some tubes with added NO_3^- a larger N_2O peak was noted indicating that organisms capable of reduction of NO_3^- to N_2O were present and active. In the survey (Table 6), pure cultures grown in the same medium commonly reduced N_2O to N_2. The difference between the MPN and pure culture results is puzzling.

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LITERATURE CITED

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CHAPTER II

EFFECT OF SOIL pH ON DENITRIFICATION

INTRODUCTION

Many bacteria are quite tolerant of acidity and are able to grow and develop over a wide range of H^+ activity while others are restricted to either acid or alkaline conditions. Although it is generally assumed that denitrification is favored in neutral to alkaline habitats, few studies have been performed with the active species and with soils. Though there exists a considerable literature on denitrification, many reports on the physiological and ecological characteristics of denitrifying microbes are contradictory. There are differences of opinion among investigators concerning the chemical and/or biological processes which lead to the production of nitrogen oxides. The size and activity of the denitrifying flora in different ecological circumstances are key factors in determining the rate of loss of nitrogen from soils but there is little known of the environmental factors regulating the abundance or activity of these microorganisms.

The effect of acidity on denitrification may be exerted in a number of distinctly different ways and the sparse denitrifying population in an acid environment may be a reflection of an influence upon growth rather than an effect upon the denitrifying mechanism itself.

Two ways of establishing the significance of pH to microbial denitrification are by a determination of the effect of the H^+ activity on the size of the denitrifying population in natural circumstances and by a characterization of specific organisms with regard to their capacity

to liberate dinitrogen at various pH levels. Studies with pure cultures will help to support the ecological investigations of the influence of acidity. However, since the conditions designed in the laboratory have to be quite different from those existing in the microenvironments within the soil, the application of these results is difficult. The comparison of the activity of denitrifying microorganisms in a liquid growth medium and after inoculation into sterilized soil is one approach to determine whether various H^+ concentrations significantly affect the denitrifying potential of certain microbes.

Recent work has provided indirect evidence that significant gaseous loss of fertilizer nitrogen can occur through chemodenitrification, i.e. by chemical decomposition of nitrite formed by nitrification of ammonium yielding fertilizers in soils (1, 2, 5, 10, 23). Most workers have assumed that the rate and extent of nitrite decomposition in soils increase with a decrease in soil pH because solution studies have shown that decomposition of nitrite is promoted by acidity. Studies on denitrification products show that acid conditions are more favorable for formation of N_2O and NO than neutral and alkaline conditions which favor N_2 formation (9, 24, 26).

Nevertheless, there are differences of opinion among investigators concerning the nature and importance of chemical and/or biological processes which lead to the production of the nitrogen oxides.

The aim of this project was to determine the effect of H^+ activity on biological denitrification and the extent to which N_2O is produced by enzymatic or chemical reactions.

The objectives were therefore, (1) to determine the rate and products of denitrification by soil samples differing in pH; and (2) to

determine the effect of pH adjustment on denitrification rate and products of the soil samples.

SUMMARY OF PREVIOUS INVESTIGATIONS

Earlier investigations have shown that denitrification is favored by a relatively low hydrogen ion concentration. Broadbent (6) reported that denitrification is favored below pH 7 where as other investigators (14, 4) concluded that nitrogen loss was considerably suppressed under acidic conditions. In another report (16), it is concluded that no correlation between pH and denitrification parameters could be found. It is generally assumed that denitrification is favored in a neutral to alkaline ecological system and that denitrifying populations in otherwise optimal environmental conditions fail to release gaseous nitrogen at high H^+ activities (18).

Dawson and Murphy (12) have shown that denitrification rates give parabolic curves as a function of pH with a peak at 7.0. The rates at pH 6.0 and pH 8.0 were approximately halved. However, Wiljer and Delwiche (24) and Bremner and Shaw (4) have shown that the rate of denitrification increases linearly from pH 4, levels off between pH 7 and 8, then declines, though not ceasing until at pH 9.5. Neutral to slightly alkaline pH ranges not only effect faster rates of denitrification but also the complete reduction to N_2.

Bollag, et al. (3) concluded that formation of nitric oxide in acid soils was largely chemical since sterilized soils evolved as much nitric oxide as controls upon addition of nitrite. Reuss and Smith (19) found that small amounts of N_2 and N_2O are formed by decomposition

of nitrite in acid soils. They also indicated that the amount of nitrite formed increased with a decrease in soil pH and that soil sterilization has little effect on the amount of N_2 or N_2O formed by treatment of acidic soils with nitrite.

Denitrification and chemical nitrite decomposition seem to be the two predominant processes in volatilization of nitrogen, but it is not clear which one of the two mechanisms is of greater practical importance. Some investigators hold the biological reaction of denitrification most responsible for nitrogen losses from the soil (18); whereas, other studies tend to emphasize more the chemical volatilization (17,7). There is little doubt that both processes are influenced by factors such as pH, organic matter and others. Bremner and Shaw (4) demonstrated that the type of organic matter, pH, temperature and the aeration are among the chief variables governing the rate and magnitude of nitrogen loss.

Valera, et al. (22), found that regardless of seasonal changes the number of denitrifiers was found to be positively correlated with pH, the coefficient of correlation (r) ranging from 0.66 to 0.97. They also found that the size of both the denitrifier population and the total bacterial population was positively correlated with soil pH but that the denitrifying bacteria were more sensitive to acid environments than the bacterial microflora as a whole. On the other hand, in an investigation of Australian soils, Jensen (15) found no relationship between H^+ activity and microbial number although he did note a positive correlation with organic matter content.

In this study I have investigated phase II denitrification rates in Nigerian and Michigan soils which vary from strongly acid to neutral.

The purpose was to determine whether biological denitrification occurs under acid conditions and whether denitrifying populations of difference acid tolerance might have developed in the various habitats. Phase II denitrification rates have been defined by Smith and Tiedje (21) as reflecting the amount of denitrifying enzymes that can be produced by the population of denitrifying organisms present in the natural soil. Thus the rates reported here are not rates expected in nature but reflect the potential of the indigenous population.

MATERIALS AND METHODS

Soils

The collection of the samples involved taking six subsamples of fresh surface soil (0-15 cm deep, including litter layer) from an approximately 10 m^2 homogenous area. The six subsamples were made into one composite sample. Approximately 0.5 kg of the composite sample was enclosed in a plastic bag sealed without drying and stored at 2° C. Nigerian samples were immediately shipped by air to the laboratory; all carried a non-sterilization entry permit.

Five Nigerian soils ranging from acid to neutral pH were obtained for the study. Their major characteristics are summarized in Table 10. Samples 1, 4 and 5 were collected from a forestry reserve that had not been cultivated for over 50 years while sites 2 and 3 have experienced slight cultivation. No evidence of addition of any form of nitrogen fertilizer was indicated at any site. Samples were supplied with information on the crop grown, soil type, previous crops, approximate location, i.e., distance and direction from nearest geographical location, whether site was cultivated or not, drainage, mean rainfall and other useful information.

Table 10. Characteristics of Nigerian soils used in study.

Nigerian soil	pH[a]	Drainage	NO_3-N	NO_2-N
			------ ppm N ------	
N1	3.8	Somewhat well drained	50.0	0.05
N2	4.4	Well drained	44.0	0.11
N3	6.3	Well drained	51.0	0.11
N4	6.7	Well drained	7.0	10.06
N5	4.5	Poorly drained	21.5	0.08

[a] Soil pH measured with a glass electrode pH meter (Beckman Model 4500 Digital pH meter using a 1:1 soil water suspension).

Acid soil samples were also obtained from experimental plots on the Michigan State University farm (courtesy of Dr. A. R. Wolcott, Dept. Crop and Soil Sciences). These plots have been receiving different carriers of nitrogen fertilizer since 1959 (8). One heavy textured soil was obtained with the help of Dr. Christenson from the Saginaw experimental farm. The characteristics of the Michigan soils are summarized in Table 11. The soils are coded N, Nigerian; W, Wolcott and SAG, Saginaw.

Assay of denitrification

Moist soil taken from the stored samples was passed through a 5 mm sieve and 50 g was placed in 125 ml Erlenmeyer flasks. Thirty milliliters of water were added to make a slurry and the flask was sealed with a rubber stopper. The acetylene inhibition method was used to measure the rate of denitrification Smith et al., (21). No additional NO_3^- was added. The soil was made anaerobic by evacuating and filling the flask three times with He. Acetylene was added by syringe to achieve a concentration of 0.1 atm after withdrawing an equal volume of He. The flasks were then incubated at 30° C on a rotary shaker operating at 250 rpm. All treatments were replicated three times.

The headspace gas was sampled periodically by syringe to determine N_2O (and CO_2) concentrations by a microthermistor detector after separation by gas chromatography as previously described (Chapter I). Quantitation of N_2O was by a standard curve and included corrections for N_2O solubility.

Autoclaving and propylene oxide were investigated as methods to achieve sterile controls. The autoclave treatment was 30 min at 121° C, 15 psi, three times with intervals of at least 8 h between. The

Table 11. Characteristics of Michigan soils used in study.

Plot	pH	Treatment	NO_3^--N[a]	NO_2^--N[a]
			------- ppm-N -------	
W1	5.8	$Ca(NO_3)_2$	17.5	0.03
W2	5.0	$(NH_4)_2SO_4$	11.0	0.04
W4	5.9	Control	21.5	0.03
W8	6.1	$NaNO_3$	14.0	0.03
W18-1[b]	7.0	$Ca(NO_3)$	18.5	0.05
W15-1	6.7	$(NH_4)_2SO_4$	4.5	0.04
W20-1	6.8	Control	-	-
W13-1	7.0	$NaNO_3$	19.0	0.03
SAG	7.2	-	9.0	1.41

[a] NO_3^--N and NO_2^--N determined by standard Technician Auto Analyzer II procedure.

[b] Analogous to above treatments but recently limed.

propylene oxide treatment was 2 ml of propylene oxide dispersed over the 25 g of soil, sealed and let set for 2 days after which the flasks were opened in a hood to let remaining propylene oxide diffuse away. The effect of sterilization treatments on pH was determined by measuring soil pH in a 1:1 water slurry before and after treatment.

RESULTS AND DISCUSSION

Evaluation of sterilization methods

The effect of the sterilization treatment on pH is shown in Table 12. The pH change due to autoclaving soil was insignificant (0.04 pH units) but pH was significantly changed by propylene oxide (increase of 0.8 pH units). This had also been noted by Skipper *et al.* (20). Both methods seemed to effectively sterilize the soil as measured by lack of CO_2 production. Neither treatment stimulated N_2O production.

Because of the potential significance of acid catalyzed chemodenitrification to this study, the propylene oxide method was rejected since the original pH could not be maintained in a sterilized control. Thus autoclaved soil was used as the control for chemodenitrification in the following studies.

Denitrification rates in soils of different pH.

The soil pH did not change substantially (<0.01 pH unit) during the short anaerobic incubation period (Tables 13 and 14). Apparently the soils had adequate buffering capacities to maintain their original pH despite the consumption of NO_3^-. In all cases the slight pH change was upward, probably reflecting the loss of the anion.

The denitrification rates of the Nigerian soils are summarized in Table 4. The soil of highest and lowest pH showed no denitrification.

Table 12. Effect of autoclaving and addition of propylene oxide on pH of Saginaw soil.

Treatment	pH			ΔCO_2	ΔN_2O
	Initial	Final	ΔpH	(10^3 x IV.)[a]	
1. Non sterile soil	7.62	7.66	0.04	3.7	2.9
2. Propylene oxide[b]	7.62	8.42	0.8	0.3	0
3. Autoclaved soil	7.62	7.61	0.01	0.4	0

[a] Integration value; these treatments were incubated for 10 hours and in the presence of 0.1 atm of acetylene.

[b] 2 ml added/25 g soil in flask.

Table 13. Rate of denitrification in Nigerian soils of various pHs.

Soil	Initial pH	Final[a] pH	Rate of denitrification[a] (nmol $N_2O \cdot g\ soil^{-1} \cdot h^{-1}$)
N1	3.8	3.88	0[b]
N2	4.4	4.45	8
N5	4.5	4.55	6
N3	6.3	6.34	5
N4	6.67	6.71	0

[a] Incubation was for 13 hours in the presence of 0.1 atm acetylene and no O_2.

[b] A slight increase in N_2O was noted for the first hour only.

Table 14. Rate of denitrification in Michigan soils; soils are paired to compare unlimed and lime treatment.

Soils	Initial pH	Final pH	Rate of denitrification (nmol $N_2O \cdot g\ soil^{-1} \cdot h^{-1}$)	r^2
W2	5.0		2	0.99
W15-1[b]	6.73		11	1.00
W1	5.8		∿1	
W18-1	7.01	7.05	15	
W4	5.95		4	0.99
W20-1	6.8		7	0.85
W8	6.1	5.6	∿1	
W13-1	7.00	7.04	15	
SAG	7.2	7.26	55	

[a] Incubation was for 13 hours in the presence of 0.1 atm acetylene and no O_2.

[b] Limed soil; the preceding soil is identical except unlimed.

Most interesting, however, was the substantial denitrification rate of the two acid soils, i.e. pH 4.4 and 4.5. Autoclaved soils showed no N_2O production. Biological reduction of NO_3^- to NO_2^- could be followed by chemical decomposition of HONO. However, the product is primarily NO and not N_2O, which was measured in this study. Thus, these results are interpreted to mean that acid-tolerant denitrification did occur in these low pH Nigerian soils.

Similar studies were conducted with Michigan soils which had been decreasing in pH since 1959 due to regular additions of different N fertilizer salts (termed "carriers"). In this case the pH's were not as low (5.0 to 6.1) as the Nigerian soils. However, the denitrification rate was very low in all soils. The same soils which had been limed showed much higher denitrification rates. Thus, for the Michigan soils, it appears that acid tolerant denitrifier populations did not develop in the comparatively short period of acid conditions. The Saginaw soil, which is a much heavier textured soil, showed much higher denitrification rates than the other soils. This is expected since a denitrifier population as well as derepression of denitrifying enzymes would be expected in this more O_2 limited habitat.

Considering the results of pH influence on denitrification one does get the impression that there are denitrifier populations which vary in their sensitivity to acidity. The data are consistent with adaption or selection of acid tolerant communities in soils which have been acid for long periods of time.

Future work

This study is only preliminary; substantial additional work is needed before a comprehensive picture of pH influence on denitrification

can be established. Ideas for future experimental work are itemized below:

1. Isolation of acid tolerant denitrifiers.
2. Determination of the ratio of acid tolerant to total denitrifiers in the soil.
3. Alteration of the pH by addition of a base or an acid and determine the rate of denitrification of the soils under adjusted pH's.
4. Use of selected pure cultures to support the ecological investigations on the influence of acidity.
5. Use of autoclaving to sterilize soil and then carrying out more studies on possible chemodenitrification at low pH.

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APPENDIX

APPENDIX A. Capability of denitrifier isolates to grow aerobically on three different media.

Isolate number[a]	Isolate name[a]	Nutrient broth, O_2	TSB, NO_3^- no O_2	TSB, O_2
4	A. faecalis	+[b]	0[b]	0[b]
6	P. type 2	+	0	+
12	P. type 2	0	+	+
13	P. fluorescens I	+	+	+
15	P. fluorescens (?)	+	+	+
17	A. faecalis	0	0	0
18	A. faecalis	0	+	+
20	A. faecalis	0	0	+
21	A. faecalis	0	+	+
28	A. faecalis	0	0	+
30	A. faecalis	0	0	+
31	A. faecalis	0	+	+
36	Unknown type 3	+	+	+
39	Unknown type 3	+	+	+
40	A. faecalis	+	+	+
41	A. faecalis	0	0	0
42	P. fluorescens II	+	+	+
43	A. faecalis	+	+	+
44	P. fluorescens II	+	+	+
45	P. fluorescens II	+	+	+
46	Flavobacterium sp.	+	+	+
47	P. fluorescens II	+	+	+
49	P. fluorescens II	+	+	+
51	P. type 2	+	0	+
52	P. fluorescens II	+	+	+
53	P. fluorescens II	+	+	+
54	P. type 2	+	+	+
55	P. type 4	+	+	+
56	P. type 2	0	0	+
58	P. fluorescens II	+	+	+
59	P. aureofaciens II	+	+	+
61	P. fluorescens II	+	+	+
62	P. aureofaciens II	+	+	+
63	P. fluorescens II	+	+	+
64	P. fluorescens II	+	+	+
65	A. faecalis	+	+	+
66	P. fluorescens II	+	+	+
67	P. fluorescens II	+	+	+
68	P. fluorescens II	+	+	+
69	P. fluorescens II	+	+	+
70	P. fluorescens II	+	+	+
71	P. type 5	+	0	0
72	P. fluorescens II	+	+	+
73	P. fluorescens II	+	+	+

Isolate number[a]	Isolate name[a]	Nutrient broth, O_2	TSB, NO_3^- no O_2	TSB, O_2
74	P. type 5	+	+	+
75	*P. fluorescens* II	+	+	+
78	*P. fluorescens* II	+	+	+
79	*P. fluorescens* II	+	+	+
80	*P. fluorescens* II	+	+	+
81	P. type 2	0	0	0
82	P. type 5	+	+	+
83	P. type 2	0	0	0
84	P. type 6	+	+	+
85	P. type 7	+	+	+
87	P. type 8	0	+	+
89	*P. fluorescens* II	+	+	+
90	*A. faecalis*	0	0	0
97	P. type 9	0	0	0
98	*P. fluorescens* II	+	+	+
99	*A. faecalis*	0	0	0
101	*A. faecalis*	+	+	+
102	*A. faecalis*	0	0	+
103	P. type 10	0	0	+
104	*A. faecalis*	0	0	0
105	*P. fluorescens* II	+	+	+
106	*A. faecalis*	+	0	+
107	P. type 11(not 2)	+	+	+
108	Unknown type 3	+	+	+
110	P. type 12	+	+	+
111	*P. fluorescens* (?)	+	+	+
115	Unknown type 15	0	0	0
1181	P. type 16	+	+	+
126	P. type 18	+	+	+
129	P. type 18	+	+	+
133	P. type 18	+	0	+
141	P. type 16	0	0	+
143	P. type 11	+	+	+
144	*A. faecalis*	+	+	+
148	*A. faecalis*	0	+	+
149	P. type 11	+	+	+
151	P. type 19	+	+	+
153	P. type 19	0	+	+
154	*A. eutrophus*	+	+	+
155	P. type 20	+	+	+
156	*P. aeruginosa*	+	+	+
163	P. type 11	+	+	+
164	*P. aeruginosa*	0	+	+
167	*P. aeruginosa*	+	+	+
171	*A. faecalis*	+	+	+
172	P. type 16	0	+	+
174	P. type 16	0	0	0
175	*Flavobacterium* sp.	0	0	0

Isolate number[a]	Isolate name[a]	Nutrient broth, O_2	TSB, NO_3^- no O_2	TSB, O_2
177	Flavobacterium sp.	0	+	+
179	P. type 19	0	+	+
183	P. fluorescens IV	+	+	+
185	P. fluorescens IV	+	+	+
188	P. type 11	+	+	+
189	Unknown type 21	+	+	+
190	P. fluorescens (?)	+	+	+
191	A. faecalis	+	+	+
192	Bacillus sp.	0	+	+
193	Bacillus sp.	0	+	+
195	P. stutzeri	+	+	+
196	P. fluorescens (?)	+	+	+
199	Unknown type 22	0	0	0
202	P. type 23	0	+	+
204	Unknown type 24	0	+	+
205	P. fluorescens II	+	+	+
206	P. fluorescens IV	+	+	+
221	P. stutzeri	+	+	+
224	P. stutzeri	+	+	+
2312	P. stutzeri	0	+	+
232	P. type 25	+	0	+
234	P. type 11	0	+	+
991	P. denitrificans ATCC 13867	+	+	+
992	P. aureofaciens ATCC 13985	+	+	+
993	P. mendocino ATCC 25411	0	0	0
994	A. faecalis ATCC 8750	+	+	+
995	P. fluorescens ATCC 17822	+	+	+
996	P. aeruginosa	+	+	+
997	Pa. denitrificans ATCC 2008	+	+	+
998	P. stutzeri ATCC 17588	+	+	+
999	P. perfectomarinus	+	+	+
Pseudomonas sp. (Becker strain 388)		+	+	+
Pseudomonas sp. (Becker strain 402)		+	+	+
Pseudomonas sp. (NTA strain)		+	0	+

[a] Isolate numbers are tentative identifications and were given by Gamble (12).

[b] Growth indicated by turbidity (+) or lack of it (0) after incubation at 30° C for 14 days.