CULTURAL PARAMETERS FOR ESTIMATING SIMAZINE EFFECTS ON SOIL MICROBIAL POPULATIONS

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
RAYMOND STEWART SMITH

1967

ABSTRACT

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by Raymond Stewart Smith

Nitrogen sources, growth factors and incubation atmospheres were investigated as cultural parameters which might be useful in dilution plating procedures to detect changes in nitrate reductase activity in soil microbial populations. Criteria were sought which might differentiate between bacterial types in which nitrate reduction is (1) essentially assimilatory and those in which it is (2) essentially or (3) coincidentally dissimilatory.

Simazine, a herbicide known to stimulate nitrate reductase activity in higher plants, was tested for its ability to promote the same adaptive response in 18 strains of known facultative nitrate reducing bacteria. The observed responses of these strains in pure culture were used as the basis for inferences regarding population estimates obtained under parallel cultural conditions for field soils previously treated with simazine.

Simazine introduced into solid media at concentrations of 5 to 50 ppm promoted more rapid development of streak cultures of several nitrate reducing bacteria. This stimulus

to growth appeared to be due to earlier adaptive synthesis of nitrate reductase, since there were no effects of simazine on growth or denitrification when added to broth cultures previously adapted to nitrate.

Field simazine treatments reduced plate counts for major taxonomic groups in the order: bacteria (66%) > actinomycetes (25%) > fungi (15%). The proportion of nitrate reducers in the bacterial population, however, was markedly increased, as evidenced by numbers capable of growth and/or survival in 100 per cent CO_2 or H_2 on media supplying nitrate.

The major proportionate increase in nitrate reducing bacteria occurred among types which differed from representative pure strains of denitrifying bacteria in their ability to grow on nitrate media only after removal from CO_2 into air. It is inferred that these are not actively denitrifying types, but rather types in which nitrate reductase activity, stimulated by simazine, is exploited for assimilitory reduction of nitrate to support growth.

It appeared that the differentiation between assimilatory and dissimilatory nitrate reducers could be clearly made using dilution plating techniques on solid media. The cultural parameters employed with solid media did not provide criteria for the clear distinction between true denitrifiers and coincidental dissimilatory nitrate reducers which is possible with broth media.

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By

Raymond Stewart Smith

A THESIS

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

MASTER OF SCIENCE

Department of Soil Science

1967

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. A. R. Wolcott for his assistance and encouragement throughout this study.

The author wishes to thank all those members of the faculty of Michigan State University whose cooperation has made this study possible.

The author is also grateful to his wife, Cynthia, for her unfailing support, help and encouragement during the course of this work.

The study was supported by Public Health Service research grant CC 00 246, from the National Communicable Disease Center, Atlanta, Georgia.

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INTRODUCTION

Soil microbial responses to pesticides are generally evaluated by monitoring microbial metabolism (CO_2 evolution, oxygen uptake, ammonification, nitrification, cellulose decomposition, etc.), or by estimating numbers of the major taxonomic groups (bacteria, actinomycetes and fungi). These methods obtain no information on the variances within taxonomic or physiological groups. Certain physiologically similar species may find the induced conditions detrimental to survival while others are able to increase in numbers, thus the opposite effects balance and portray a picture of little or no change. Although ideally each microbial specie would be investigated, this is obviously impossible. A compromise approach is to classify the population into increasingly restrictive physiological groups and develop appropriate methods for observing variations at these levels.

Denitrifying bacteria include genera and species which, under anaerobic conditions, reduce nitrate to gases which are lost from the soil. An adaptive mechanism necessary for this dissimilatory reduction is the synthesis of nitrate reductase. Recent studies have shown that simazine, an agricultural herbicide, stimulates nitrate reductase activity in higher plants. Were this response to occur in

the denitrifying bacteria, an enrichment in denitrifying capacity would develop in the soil population.

Enhancement of nitrate reductase activity in the soil microflora need not necessarily increase denitrification losses. Depending upon the balance of responding organisms, an increase in nitrate reductase activity in the soil microbial population could lead to increased losses of nitrogen due to denitrification, or to decreased losses due to reduction to nitrite, ammonium or to amino N in cellular tissues.

Present methods for estimating numbers of denitrifiers in soil populations do not permit distinctions to be made between these different types of nitrate reducing organisms. These methods, involving serial dilutions in broth culture and plus or minus observations on growth or production of gas and alkalinity, must be handled by statistically weak "most probable numbers" techniques.

The main objective of the present study was to investigate cultural parameters which might permit use of dilution plate counts on solid media to detect changes in nitrate reductase activity in soil populations and to differentiate between important types of nitrate reducing organisms.

Effects of nutritional and atmospheric parameters were tested in pure culture with and without simazine. Selected combinations of media and incubation atmospheres were used to estimate population changes in field soil previously treated with simazine.

LITERATURE REVIEW

Denitrification

The loss of gaseous nitrogen from soils may occur under various environmental conditions with several resulting products. Four distinct mechanisms are suggested by Alexander (1):

a) Non-biological losses of ammonia which, according to Willis and Sturgis (109), may be significant only above pH 7.0.

b) Chemical decomposition of nitrite under acid conditions to yield nitrogen oxides. Clark, Beard and Smith (20) suggest reduction of nitrite to nitric oxide as the main mechanism for nitrogen loss in well-aerated acid soils. Wullstein and Gilmour (112) have presented evidence that transition metals may catalyze these reactions.

c) Production of N_2 by the non-enzymatic reaction of nitrous acid with ammonium or amino acids. Bremner (9), Mortland and Wolcott (66), and Stevenson and Swaby (89) consider it quite likely that non-enzymatic reactions of nitrous acid or its equilibrium species with phenolic structures in humus or decomposing biological materials are significant pathways for loss of gaseous nitrogen under field conditions.

d) Enzymatic denitrification, which is a biological process accomplished by facultatively anaerobic bacteria capable of using nitrate or nitrite in place of oxygen as a hydrogen acceptor. This dissimilatory reduction is in contrast with the assimilatory reduction of nitrate or nitrite to the level of ammonium or amino nitrogen, as accomplished by higher plants and many microorganisms to meet their requirements for organic nitrogen.

Considerable research has been directed toward the investigation of the pathways for denitrification. There is fairly general agreement that the reduction sequence includes nitrate, nitrite, nitrous oxide, and molecular nitrogen, in that order. However there is still some disagreement regarding the inclusion of nitrous oxide on the main pathway of denitrification. Allen and Van Niel (4) concluded that nitrous oxide was not the precursor of molecular nitrogen in cultures of Pseudomonas stutzeri. Wijler and Delwiche (108) found nitrous oxide to be the major denitrification product under moist soil conditions. They noted, as did Hauck and Melsted (39), that above pH 7.0 nitrous oxide could be readily reduced to nitrogen, but below pH 6.0 its reduction is strongly inhibited. On the other hand Delwiche (26) found that the relative proportions of N_2O and N_2 were markedly influenced by the concentration of nitrate plus nitrite. Because nitrate and nitrite are preferential hydrogen acceptors N_2O is utilized, and subsequently reduced to N_2, only if the supply

of nitrate and nitrite is limiting. However, most of the published evidence is in agreement with Nommik's (69) conclusion that nitrous oxide is an obligatory precursor of molecular nitrogen.

Several soil environmental factors have a direct influence on denitrification. There is general agreement among several investigators, Valera and Alexander (99), Delwiche (25), and Nommik (69), that microbial denitrification is only of consequence in near-neutral soil habitats (pH 6.0 to 8.0).

Broadbent and Clark (11) stated that the effect of organic matter on denitrification is twofold: since the free energy change in the reduction of nitrate to nitrous oxide or molecular nitrogen is positive, an oxidizable substrate is required which can furnish energy for growth of the denitrifying bacteria and serve as a hydrogen donor for the denitrification process; and secondly, the rate of organic matter decomposition markedly influences the oxygen demand. The first effect is illustrated by McGarity (62) who showed that the addition of glucose markedly increased the rate of denitrification in soil with low content of organic carbon, but the increase was only slight in soils with high native levels of organic carbon.

Denitrification is markedly affected by temperature. The optimum is above 25C. Nommik (69) and Bremner and Shaw (10) reported an optimum as high as 60-65C. Although the reaction is decreased at lower temperatures, Alexander (1) considers it still to be of economic importance.

Moisture level and oxygen availability, which are in an inverse relationship in the soil, are two final environmental factors which influence denitrification. Moisture level has a direct effect on denitrification as reported by Jansson and Clark (40), Nommik (69), and Bremner and Shaw (10). The latter authors observed increased nitrogen losses as a function of moisture content up to 450% water-holding capacity. More recently Mahendrappa and Smith (59) have reported the fastest nitrogen gas production under anaerobic conditions at moisture near saturation, with a reduction in rate at levels above and below this point.

The oxygen supply influences denitrification significantly, for the utilization of a nitrogen oxide as a hydrogen acceptor will occur only when the oxygen supply is insufficient. Most investigators, including Allison and Carter (5), Bremner and Shaw (10), and Alexander (1), conclude that denitrification may continue in anaerobic microhabitats of a well aerated soil, but that the nitrogen loss by this biological mechanism is not significant under these conditions.

Several investigators, Broadbent and Stojanovic (12), Kefauver and Allison (47), Marshall, Dishburger, MacVigar and Hallmark (60), Meiklejohn (63), Treccani (97), and Verhoeven (101), have reported aerobic denitrification, but have not estimated the oxygen levels during their investigation.

In contrast Cady and Bartholomew (17) reported an atmospheric oxygen level less than 7% by volume necessary for appreciable denitrification, while Greenwood (34), and Sherman and MacRae (86) consider denitrification significant only at dissolved oxygen levels below 0.2 ppm in the soil solution.

Differing from the above views, which regard oxygen deficiency as imposing a reliance upon oxidized forms of nitrogen as alternative electron acceptors, Pichinoty (73) and Chang and Morris (18) propose that oxygen inhibits bacterial denitrification by repressing the biosynthesis of nitrate, nitrite, and nitrous oxide reductases. They suggest that oxygen also inhibits the action of these reductases after they are formed.

Denitrifying Bacteria

Many bacterial species have been shown to denitrify, in culture media at least, and large populations of several of the more active bacteria are found in most agricultural soils. Woldendorp (111) has found that non-spore-forming organisms of the genera Pseudomonas, Micrococcus and Spirillum, aerobic spore formers (Bacilli) and a number of other facultative anaerobes can reduce nitrate. The active species are largely limited to the genera Pseudomonas, Achromabacter and Micrococcus. Bacillus strains, though numerous, are rarely important because their abundance is usually only the result of the persistence of the endospores (Alexander, 1). Delwiche

(25) reports more than forty organisms with the ability to denitrify.

From the above summary of denitrifying bacteria, a few physiological similarities are immediately apparent. In order to classify these organisms physiologically and to more fully understand their relationships with plants and other important soil organisms, however, further nutritional and environmental requirements and ecological roles of the denitrifying bacteria must be determined.

The known denitrifying bacteria may be referred to as chemoorganoheterotrophs, that is they depend upon the oxidation of exogenous organic substances for an energy source and may also require an exogenous supply of one or more essential metabolites or growth factors. It seems that all bacteria require a small number of nutrients which may be referred to as universally required foodstuffs (Lamanna and Mallette, 51). These nutrients are water, phosphate, carbon dioxide, and certain mineral salts. The differences among bacteria in the foodstuffs required are rarely due to differences in the need for particular elements. Important differences do appear in the compounds of carbon, nitrogen, and sulfur which they can assimilate.

Ecological investigations of the N_2-releasing bacteria of soil are frequently performed using Giltay's medium, in which nitrate and minerals are supplied, with citrate and asparagine as carbon sources. Valera and Alexander (100) suggest that the energy source selected for denitrification

population estimates is a critical factor. They reported that glycerol and tartrate appeared to yield greater population estimates than the citrate supplied in Giltay's medium. Moreover, the inclusion of yeast extract in the citrate-inorganic salts medium resulted in an eight-fold greater estimate of the numbers of denitrifying bacteria than when Giltay's medium was used. In parallel pure culture studies these authors also concluded that maltose, xylan, mannitol, lactose, xylose, glucose, raffinose and sucrose could serve as carbon sources for most of the denitrifying species examined.

The heterotrophic microflora in soils comprise a very numerous diversity of genera and species, the functional roles of these and their ecological implications are still largely unknown or but little understood. With a view to uncovering some of these functional relationships, efforts have been made to classify soil microorganisms on the basis of nutritional needs. Lochhead and Chase (56) described a classification system based on a determination of growth requirements of soil bacteria first isolated on nonselective plating media. Seven main nutritional groups were recognized, ranging from organisms capable of maximum development in a simple basal medium to types unable to develop with supplements of amino acids, growth factors, or yeast extract, but which require soil extract for growth:

Medium	B	Basal medium	
"	A	"	plus amino acids
"	G	"	plus growth factors (vitamins)
"	AG	"	plus amino acids plus growth factors
"	Y	"	plus yeast extract
"	S	"	plus soil extract
"	YS	"	plus yeast extract plus soil extract

Valera and Alexander (100) studied pure cultures of denitrifying bacteria to ascertain some of their nutritional relationships. They concluded that the N_2-producing microorganisms with which they worked could be divided into four nutritional groups:

a) Bacteria that denitrify in a glucose-inorganic salts medium, a group that included three Pseudomonas aeruginosa strains and Ps. denitrificans.

b) Microorganisms like Achromobacter hartlebii which require ammonium for denitrification.

c) Those apparently needing amino acids for activity, Alcaligenes denitrificans, Ps. stutzeri and Micrococcus denitrificans being included in this category.

d) A fourth group containing Bacillus licheniformis, Denitrobacillus licheniformis, Serratia indica and S. kilensis, none of which denitrified in the test media.

Woldendorp (111), using broth cultures, found that his collection of denitrifying bacteria could be divided into two

groups on the basis of whether they could or could not grow anaerobically in the absence of nitrate. Additional distinctions could be made within each of these groups on the basis of whether or not amino acids or vitamins were required for growth and/or gas production. His observations were, in general, consistent with those of Valera and Alexander (100).

In a comparative survey of the nutrition of the genus Bacillus, Knight and Proom (50) observed the following characteristic nutritional patterns:

1. B. subtilis, B. licheniformis and B. megatherium grew with gluccose as energy source, ammonium as sole nitrogen source and in the absence of added growth factors.

2. B. Cereus and B. brevis grew in the absence of added growth factors but required mixtures of amino acids instead of ammonia only as sources of nitrogen.

3. B. pumilus and B. macerans grew with ammonia only in the presence of biotin and aneurin.

4. B. alvei required amino acids and aneurin; B. circulans and B. coagulans had more complex requirements.

Investigators working with M. denitrificans have observed some physiological characteristics differing from the other known denitrifying bacteria. Kluyver and Verhoeven (48) have observed an adaption in M. denitrificans which permits chemolithotrophic oxidation of hydrogen at the expense of nitrate. Pichinoty (72) reports two nitrate-reductase

enzymes, A and B, in a strain of M. denitrificans. B is a constitutive enzyme without known physiological functions, whereas A is induced by the presence of nitrate but suppressed by oxygen. A third unique characteristic was observed by Mützе (67) who found that exposure to light decreased the oxidative activity of M. denitrificans due to inactivation of cells which do not possess carotenoids to absorb light of injurious shorter wavelengths.

It is apparent from the above studies that denitrifying organisms represent a wide diversity of nutritional requirements with respect to carbon source, energy source, growth factor requirements and the extent to which dissimilatory nitrate reduction is essential for or incidental to growth under anaerobic conditions.

In preliminary work leading to the present study, B. S. Hong[1] investigated the effect of amino acids on the ability of Pseudomonas stutzeri to utilize different carbon sources for growth in vigorously aerated broth cultures. Cells were grown into the exponential phase in nutrient broth. Washed cells were used to inoculate test media to a standardized optical density of .01 or .02 at 600 mμ, and growth was followed turbidimetrically at this wavelength.

Data in Figure 1 show that only acetate could be utilized for maximum growth in the absence of exogenous amino acids. A major deficiency appeared to be for amino acids

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[1]Unpublished data, 1965.

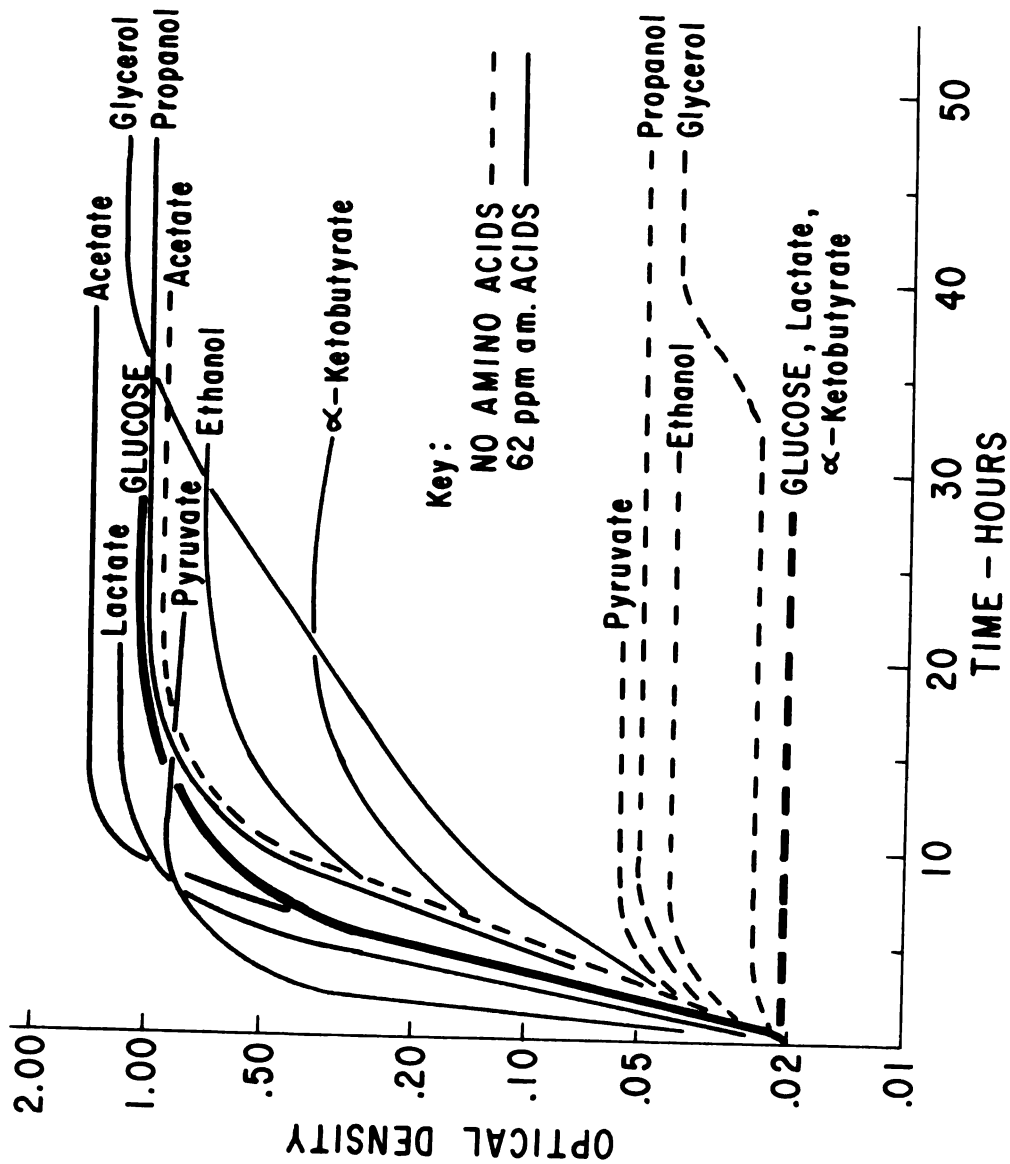

Fig. 1. Effect of amino acids on utilization of glucose and fermentation products by Pseudomonas stutzeri for aerobic growth with N supplied as $(NH_4)_2SO_4$. (B. S. Hong, 1965)

needed in synthesis of enzymes for oxidizing pyruvate to acetic acid. Other deficiencies involved conversion of lactate, alcohols and lipid precursors to acetate or glycolytic intermediates.

Several acids of the tricarboxylic acid cycle were also tested (Figure 2). An absolute requirement for supplied amino acids was expressed only for utilization of α-ketoglutarate. In the absence of amino acids, adaptation to utilization of citrate appeared to involve successive stepwise adaptation to utilization of succinate and malate.

In the presence of added amino acids, malate was used directly without lag. An initial rapid utilization of succinate may have occurred via malate, but was apparently terminated by exhaustion of exogenous factors necessary for intermediate steps in the conversion of succinate to malate. In the utilization of citrate, amino acids apparently had no effect on intermediate adaptations to succinate or malate. However, after the adaptation to malate was evidenced by a short period of accelerated growth, subsequent utilization of citrate in the presence of added amino acids was controlled by some mechanism other than the rate of malate utilization.

Utilization of propionate (not a tricarboxylic acid cycle component) apparently involved an adaptive conversion to succinic acid. This adaptation did not occur when no amino acids were added.

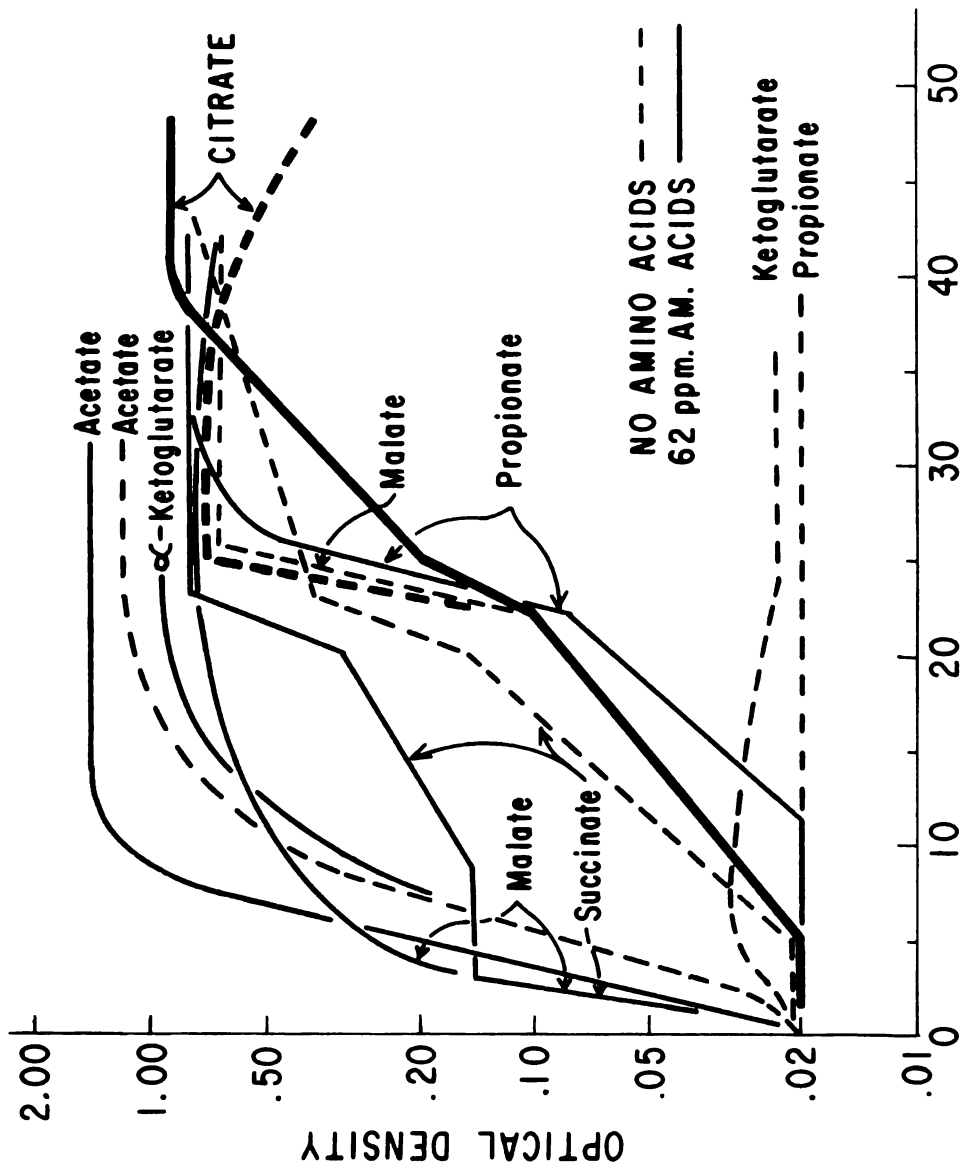

Fig. 2. Effect of amino acids on utilization of tricarboxylic acid cycle components and associated metabolites by Pseudomonas stutzeri for aerobic growth with N supplied as $(NH_4)_2SO_4$. (B. S. Hong, 1965)

In later studies,[2] it was found that the amino acid requirement for glucose utilization was more critical for Ps. stutzeri than for Ps. aeruginosa or Serratia marcescens. None were able to use glucose aerobically or anaerobically with ammonium as the nitrogen source unless amino acids were added to the medium. With transfer from a growth medium supplying a vitamin-free amino acid mixture as sole source of carbon and nitrogen to a glucose-mineral salts medium containing nitrate and as little as 100 ppm of the amino acid mixture, lag times at 30C for S. marcescens, Ps. aeruginosa, and Ps. stutzeri were 0, 5, 10 hours respectively under aerobic and anaerobic conditions. When the amino acids were omitted, the respective lag times were 20, 10 and 30 hours under vigorously aerated conditions and 15, 20 and 40 hours in vaspar-sealed tubes.

The Ps. stutzeri (Woldendorp) strain used in these studies did not demonstrate the absolute requirement for amino acids shown by the ATCC 11607 strain used in studies depicted in Figure 1. It did, nevertheless, express a distinctly longer adaptive lag in glucose utilization than the other two species, even where amino acids were supplied. With all three organisms, lag periods increased as temperature was lowered to 20 and 10C. At 10C, no growth under anaerobic conditions was observed for any organism on any medium over a 2-week period.

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[2] B. S. Hong, Unpublished data, 1966.

Rhizosphere Relationships

In studies of the relationship between the plant rhizosphere and soil microbes, it is almost invariably found that the bacteria in soil respond much more to the presence of plant roots than do actinomycetes, fungi, algae, and protozoa (Katznelson, et al. 44, Clark 19, Starkey 87, Katznelson 42, and Rouatt et al. 81).

Most comparisons between rhizosphere and non-rhizosphere bacteria show a definite selective stimulation by plant roots of gram-negative rods, amino acid requiring organisms, and organisms with increased physiological activity (Clark 19, Katznelson et al. 44, Starkey 87, Rouatt et al. 81, Katznelson 43, Rouatt 79, and Rouatt and Katznelson 80).

The classification of soil and rhizosphere bacteria on the basis of nutritional requirements (Katznelson et al. 45, Lochhead and Chase 56, Lochhead and Thexton 58, Lochhead and Rouatt 57, Wallace and Lochhead 104, Wallace and Lochhead 105 and West and Lochhead 107) has shown that most rhizosphere isolates grow on the basal medium of glucose, nitrate, and salts or require amino acids, while most soil isolates require complex growth factors supplied by soil extract and yeast extract.

Studies on nutritional requirements of rhizosphere bacteria have shown that some organisms can supply essential nutrients for other groups in the rhizosphere (Cook and Lochhead 23, Payne, Roualt and Lochhead 71). Cook and Lochhead

(23) found that many amino acid-requiring bacteria from the rhizosphere synthesized thiamin, biotin, and vitamin B_{12}. This explains why many vitamin-requiring bacteria occur in the rhizosphere (Cook and Lochhead 23, Rovira and Harris 83, and Sulochans 93) in spite of the fact that many root exudates are deficient in the B vitamins (Meshkov 64).

Denitrifying bacteria are an important physiological group which occur in larger numbers in the rhizosphere than in control soil (Katznelson and Rouatt 45, Rouatt 79, Rouatt et al. 81, and Sheng 85).

Starkey (87) reports no evidence that denitrification is rapid in the rhizosphere although it may be more rapid than in soil owing to the greater microbial activity which might provide local areas of anaerobic conditions.

Woldendorp (110, 111), utilizing a permanent grassland at 80 and 96 per cent water-holding capacity, found that the oxygen consumption of living roots was 28 times greater than that of dead roots. Two-thirds of this high consumption was attributable to root respiration and the remainder to the rhizosphere microflora. This high oxygen consumption in the rhizosphere could lower the oxygen level to a point that would favor denitrification. Woldendorp's studies showed that from 15 to 37 per cent of added fertilizer nitrogen was volatilized, the losses from nitrate fertilizer being double those from ammonia. Woldendorp also considers that the release by roots of compounds which serve

as hydrogen donors increases denitrification in the rhizosphere. Among the most efficient hydrogen donors which may be present in root exudates was found to be glutamic acid. It appeared that nitrate reduction was closely linked to oxidative deamination of this amino acid.

Antagonistic Relationships

In the above studies, rhizosphere effects on the microflora have been investigated. It seems also of ecological significance to study effects that rhizosphere microorganisms may have directly on the plant, or indirectly through interactions among microbial species within the rhizosphere population.

Rovira (82) has summarized several investigations which contain evidence that the rhizosphere population can influence the following aspects of plant growth: root morphology, root-to-shoot weight ratio, uptake of calcium and rubidium, uptake of phosphorus and sulphur, mineral content, rate of development and onset of flowering, crop yield, and physiological processes.

A study investigating the effects of pure cultures of denitrifying bacteria on the growth of corn has been reported by Mikhaleva (65). It was found that, when nitrate and peptone were added to the inoculated root medium, growth of corn was strongly inhibited. The author concluded that the inhibition was caused by growth substances and antibiotics secreted by the bacteria, and also by the nitrite

produced in the presence of peptone.

Alexander (2) has described the many interactions among microorganisms in the soil environment. Of particular interest to this review is amensalism which is the microbial production and secretion of antibiotics that kill or stop the growth of other microorganisms. Most investigators have been concerned with the great variety of bacteria, fungi and especially actinomycetes that produce antibiotics which inhibit pathogens of interest to human or animal medicine, or with organisms antagonistic to fungi which are mainly responsible for plant root diseases. Despite this vast literature dealing with antibiosis and synthesis of antibiotics by soil-derived organisms, the ecological function of these antimicrobial agents in soil remains unresolved.

Pseudomonas aeruginosa, which has previously been shown to be an active denitrifying bacterium in the rhizosphere, is well known for its pigment production. Wahba (103) lists four types of diffusible pigments produced by Ps. aeruginosa:

Pigment	Per cent of strains
Blue-green or brownish green	68.2
Light-brown	10.5
Red pyorubrin	3.5
No pigment	17.8

The biological functions of these compounds are not yet clearly understood, although several indications point

to their role in electron transport (De Ley 24, Friedheim 33, and Grossowicz et al. 35).

Pyocyanin, the blue pigment produced by Ps. aeruginosa, has been shown to inhibit certain organisms, especially gram-positive bacteria (Hays et al. 38, Ringen and Drake 78, and Young 113). This pigment is blue only under alkaline conditions and turns red in acid solutions.

Many contributions have been made by numerous investigators toward defining the nutritional requirements for pyocyanin production. The biosynthesis of pyocyanin by Ps. aeruginosa is stimulated by glycerol (Blackwood and Neish 8, and Young 113), Mg^{++} (Grossowicz et al. 35), alanine (Frank and De Moss 31) or several other amino acids and Krebs cycle intermediates (Grossowicz et al. 35).

Liu (54) observed that glucose and phosphate were important in the production of extracellular toxins by Ps. aeruginosa. The most critical factor appeared to be the concentration of phosphate. He also suggested that the products of anaerobic metabolism of glucose are needed in the production of these toxins. Burton et al. (15) found that a synthetic medium consisting of d,l-alanine or glycine at 0.4 per cent concentration, combined with 0.8 per cent l-leucine, 1.0 per cent glycerol and salt mixture was the most suitable of several media for pyocyanin production by five representative strains of Ps. aeruginosa.

Other pigments produced by Pseudomonas species include pyorubrin, which is red in both acid and alkaline

conditions (Wahba, 103; and Ringen and Drake, 78), and Fluorescin. In well aerated conditions, an inverse relationship exists between iron and the production of fluorescin (Lenhoff, 52; and Wasserman, 106).

Lenhoff (52) suggests fluorescin is an alternative product of porphyrin metabolism. In the absence of iron and at high oxygen tensions Ps. fluorescens and Ps. aeruginosa can not synthesize cytochrome C. The investigator suggests that the accumulated metabolic precursors of the porphyrin, therefore, might be metabolized to yield fluorescin.

Composition of Anaerobic Atmospheres

Anaerobic conditions necessary for denitrification exist commonly under field conditions. This inference is supported by the widespread distribution in soil of faculative and obligate anaerobes. To produce such results, the composition of the soil atmosphere must fluctuate between extremes of high and low tensions of oxygen and carbon dioxide. Factors such as restricted drainage, poor soil structure, rainfall, root respiration and microbial activity contribute to fluctuations in partial pressure of these gases in the soil atmosphere.

The rhizosphere, which has been shown to produce the most dense microbial populations and intensified activity, probably experiences greater atmospheric fluctuations than soil a few millimeters distant from the root surface.

There have been numerous investigations concerning the effects on microorganisms of increased carbon dioxide tensions in the presence of oxygen, but only a few studies have considered the significance of carbon dioxide enrichment in relation to soil microbial ecology. Durbin (27) suggested that organisms that can tolerate high carbon dioxide tensions which occur at lower soil depths may find it advantageous because it allows an escape from competition. Burges and Fenton (13) concluded that tolerance to high tensions of carbon dioxide, rather than to low tensions of oxygen, may determine the vertical distribution of soil fungi.

Several investigators have contributed information regarding the effects of carbon dioxide on specific bacteria. Carbon dioxide has been shown to:

1) Be required by Bacillus circulans for initiation of growth of germinated spores, but was not required for the germination of spores (Cook et al., 22).

2) Have caused a modification in microflora of the nitrogen cycle by increasing the aerobic nitrogen fixers and proteolytic bacteria (Cacciari, 16).

3) Inhibit biosynthesis of methionine and vitamin B_{12} by Mycobacterium tuberculosis (Schaefer, 84).

4) Limit the growth of two Pseudomonas species at concentrations greater than 90 per cent carbon dioxide, although at less than 100 per cent carbon dioxide recovery and rapid growth occurred with subsequent incubation in air (Stotzky and Goos, 90).

Stotzky and Goos (90) studied the influence of carbon dioxide on soil microbes by observing the numbers of colonies which developed from soil inocula on dilution plates incubated under various concentrations of carbon dioxide. It was observed that soil organisms are, in general, tolerant to conditions of high tensions of carbon dioxide and low tensions of oxygen. However, greater than 90 per cent carbon dioxide reduced numbers of the major taxonomic groups in the following order: actinomycetes > bacteria > fungi. Most organisms recovered when exposed to air, indicating that organisms were not killed, only growth was inhibited. Essentially no organisms were capable of growing under 100 per cent carbon dioxide.

The inhibition of most microorganisms by high concentrations of carbon dioxide was not caused by a deficiency of oxygen, inasmuch as a higher percentage of organisms were capable of developing under 100 per cent nitrogen than under carbon dioxide tensions greater than 90 per cent.

In a recent study Stotzky and Goos (91) confirmed previous observations (90,92) that the soil microbiota can adapt to conditions of poor aeration. The increase in numbers of microorganisms tolerant to high tensions of carbon dioxide and low tensions of oxygen after conditioning of soil to these conditions were similar to the higher numbers of carbon dioxide tolerant organisms counted in soils stored for three months in plastic bags than from soils analyzed immediately

after sampling in the field. It could not be conclusively established whether conditioning resulted in enrichment of specific groups within the soil population which were already tolerant to these conditions, or whether physiological adaptation occurred.

Simazine Effects

Simazine (2-chloro-4,6-bis(ethylamino)-S-triazine) is a herbicide used for selective weed control on several crops. When used at the recommended field application rate, from 1 to 4 lbs/acre, Alexander (3) and Talbert and Fletchell (94) have reported the persistence of simazine in the soil up to 16 months. Ercegovich (29) has reviewed the literature concerning the mechanisms for disappearance of simazine activity. These include: (a) volatilization, (b) adsorption by the soil colloidal complex, (c) leaching, (d) chemical alteration, (e) photodecomposition, (f) plant removal, and (g) microbial degradation.

Numerous investigators, including Audus (6), Burnside et al. (14), Kaufman et al. (46) and Guillemat (36) have reported fungi and actinomycetes isolated from soil which were capable of degrading simazine and which, in the process, utilized simazine as nearly the sole source of carbon and/or nitrogen. However, Pantos et al. (70) and Farmer et al. (30) included, also, a few bacterial species which were capable of degrading simazine.

It is agreed that the microbial population affects simazine. Conclusions from research into the reverse situation, the effects of simazine on the microbial population, do not receive such general agreement.

Several investigators, including Burnside et al. (14), Eno (28), Guillemat et al. (37), Pantos et al. (70), Steinbrenner et al. (88), Todorović and Grbić (96), and Volk and Eno (102) studied the effects of simazine on the general microbial population. They observed CO_2 production and/or bacterial, fungal and actinomycete numbers. No significant effects were detected when simazine was applied at recommended field rates. However, Ragob and McCallum (74), after treating a soil with ^{14}C labeled simazine, reported a drop in $^{14}CO_2$ produced after 91 hours, indicating simazine inhibition. Similar conclusions were drawn by Klyuchnikov et al. (49), who observed a decrease in bacteria, fungi and actinomycetes with the recommended rate applied to a sandy soil. Nepomiluev et al. (68) suggested that the lower initial microbial population and the greater inhibitory effect of simazine in a sandy soil, compared with a soil which had received high rates of organic matter and fertilizer, were due to the low level of nutrients.

The effect of simazine on the bacteria involved in nitrogen transformations has been a concern to several investigators. No significant effects of simazine on nitrification in soil systems were reported by Burnside et al. (14) for dosages up to 4096 ppm, by Eno (28) up to 16 pounds per

acre, or by Volk and Eno (102) at recommended field rates. On the other hand Tsvelkova (98) obtained data following the application of simazine which indicated a 10-fold increase in nitrification in a soil with pH 5.3 and 2 per cent organic matter.

In a perfusion study, Farmer et al. (30) found an inhibition of nitrification above 6.0 ppm of simazine. They concluded from pure culture studies that this reaction was stopped by the inhibition by simazine of Nitrobacter and that Nitrosomonas was not inhibited.

Since the solubility of simazine in water is only 5 ppm, Volk and Eno (102) suggest that this is not enough to affect bacterial growth, but that phenomena associated with physical contact of the microbes with simazine particles becomes important in altering growth. Perhaps the simazine enters the organisms by virtue of the fact that it is soluble in components of cell walls or cytoplasmic membranes.

No reports of simazine effects on nitrogen mineralization and denitrification are available. It may be speculated from observations of Nepomiluev et al. (68) that individual microbial species may vary greatly in their susceptibility. These investigators found that non-spore-forming bacteria were more resistant to simazine than fungi and actinomycetes, and much more resistant than spore-forming bacteria.

Hong[3] conducted preliminary tests with simazine to evaluate its effect on growth of three non-spore-forming bacteria which are known denitrifiers: Ps. aeruginosa, Ps. stutzeri and S. marcescens. In vigorously aerated (roll-tube) cultures in glucose-nitrate broth, simazine at concentrations of .005, .5 or 3 ppm had no effect on growth at 30C when amino acids were present (Figure 3). In the absence of growth factors, simazine at 3 ppm reduced the lag period for all three organisms and materially increased the rate of growth of Ps. aeruginosa and S. marcescens.

Tests at lower temperatures were conducted with Ps. aeruginosa only (Figure 4). In the presence or absence of growth factors, lag periods were increased by 3 ppm simazine. At 20C, subsequent growth was more rapid in the presence of simazine. At 10C, the pattern of growth in the presence of amino acids was altered by simazine. In the absence of amino acids, growth at 10C was inhibited by simazine for 180 hours, and very meager development was observed up to 300 hours.

Tests by Hong under anaerobic conditions were inconclusive.

It has been well established that simazine increases the growth and nitrogen content of tolerant plant species (Bartley, 7; Freney, 32; Karnatz, 41; Reis and Gast, 75; and Reis et al. 76). This effect is not due to a lack of weed

[3]B. S. Hong, Unpublished data, 1966.

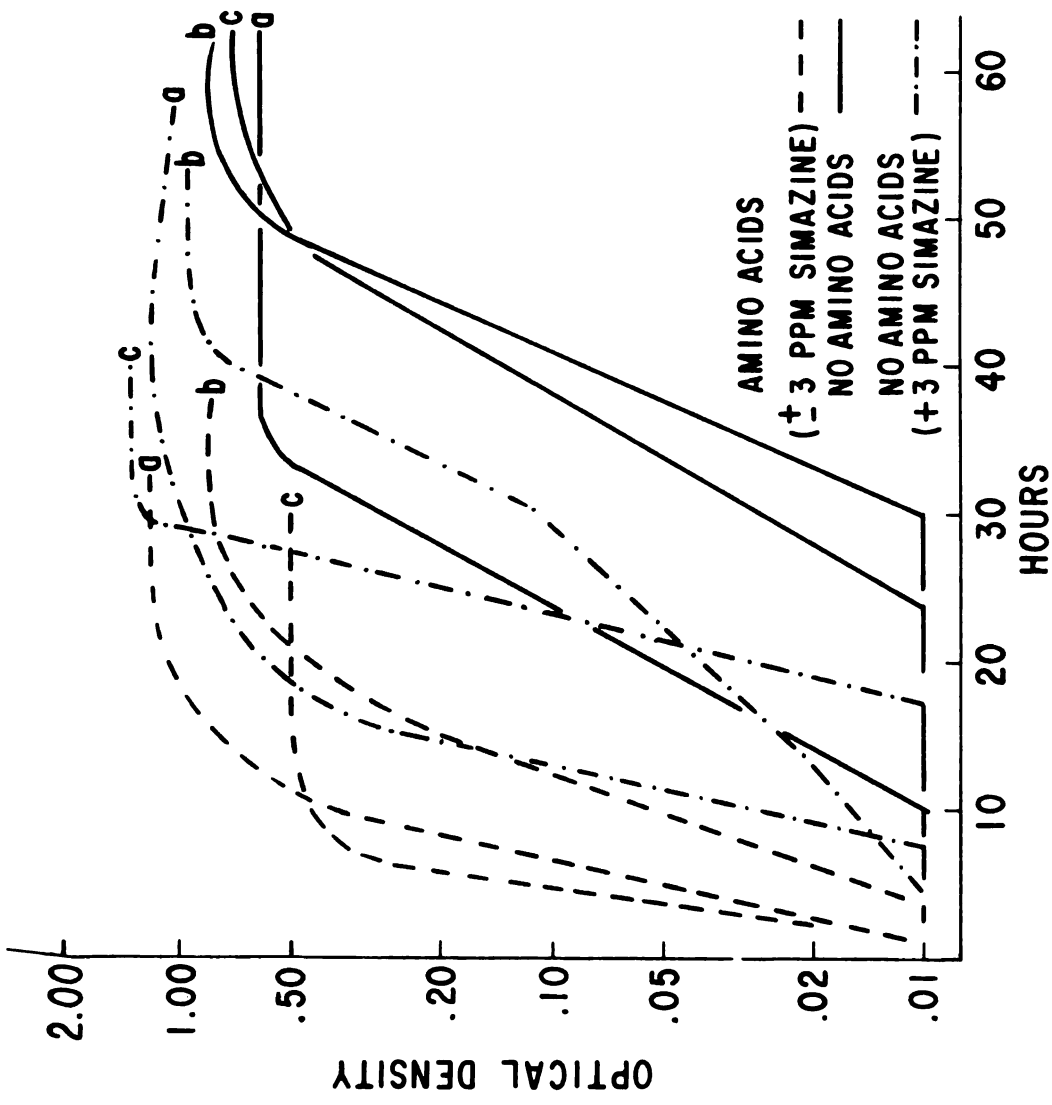

Fig. 3. Interactions of amino acids and simazine on aerobic growth in glucose-nitrate broth at 30C of (a) Pseudomonas aeruginosa, (b) Ps. stutzeri, and (c) Serratia marcescens. (B. S. Hong, 1966)

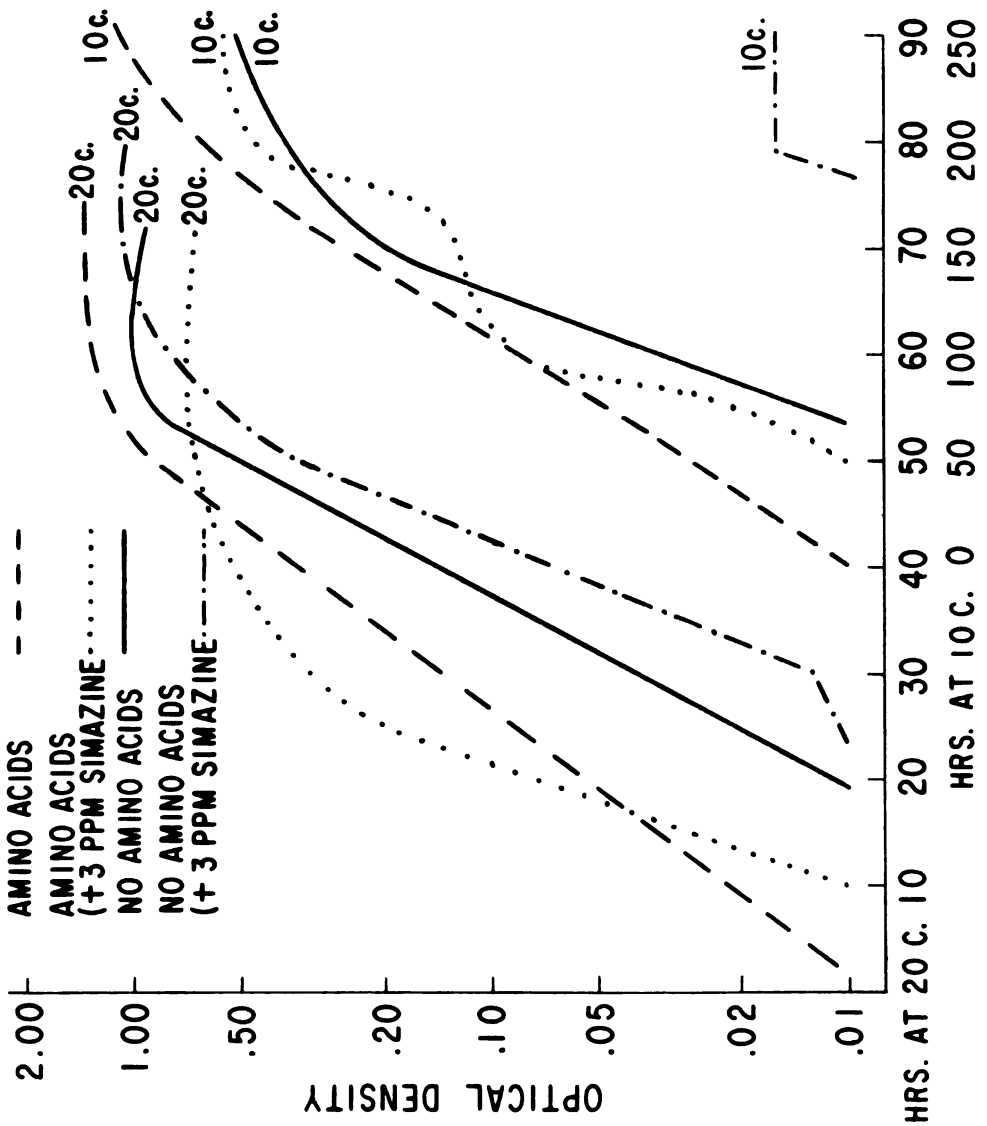

Fig. 4. Interactions of amino acids and simazine on aerobic growth in glucose-nitrate broth of Pseudomonas aeruginosa at 10 and 20C. (B. S. Hong, 1966)

competition (32,75,76), nor to the additional nitrogen available in simazine (75).

In experiments with corn (Zea mays L.), Reis and Tweedy (77) found these responses to simazine occur in plants grown with nitrate, but not in plants grown with ammonium as the source of nitrogen, and are greatest when nitrate and temperature are at sub-optimal levels. They also reported nitrate reductase activity in corn growing on sub-optimal levels of nitrate increases in a linear fashion with simazine concentration. From these observations they presented the hypothesis that simazine enhanced nitrate utilization by increasing nitrate reductase activity.

EXPERIMENTAL METHODS

Nitrate Reducing Bacteria - Pure Culture Studies

In the review of literature it was observed that nitrate-reducing bacteria were found in increased numbers in the soil rhizosphere (45,81,85). One of the causes of this ecological phenomenon has been speculated to be the increased supply of amino acids in the rhizosphere (81), but the nutritional and/or antagonistic interactions producing this increase in denitrifiers have not been described in any detail.

A series of experiments were conducted in petri dishes, using pure cultures of known nitrate-reducing bacteria. The objectives were to find differences in nutritional requirements of representative species which might be used to develop solid media for enumeration and isolation of more restricted physiological types among the total group of nitrate reducers, and to observe interactions between the test species on such media under the influence of various atmospheric conditions. Simazine was included in some instances to determine its effect on the test organisms.

Eighteen species and strains of facultatively anaerobic bacteria with known capacity for reducing nitrate were used (Table 1). Stock cultures were maintained on nutrient agar

Table 1. Nitrate reducing bacteria investigated in pure culture.

Laboratory Number	Organism	Source Identification
15	Achromobacter hartlebii	ATCC[a] 365
9	Bacillus cereus	ATCC 6464
10	Bacillus cereus	ATCC 14579
13	Bacillus circulans	ATCC 4513
7	Bacillus coagulans	Woldendorp[b] 1963 II
5	Bacillus laterosporus	Woldendorp 468B
8	Bacillus licheniformis	Woldendorp P_1
6	Bacillus licheniformis	Woldendorp 430
14	Bacillus licheniformis	ATCC 14580
11	Bacillus macerans	ATCC 843
12	Bacillus macerans	ATCC 8244
16	Micrococcus denitrificans	ATCC 13543
1	Pseudomonas aeruginosa	ATCC 10145
2	Pseudomonas aeruginosa	Woldendorp
18	Pseudomonas denitrificans	ATCC 13867
17	Pseudomonas fluorescens	ATCC 11250
3	Pseudomonas stutzeri	Woldendorp
4	Serratia marcescens	MSU MPH[c]

[a]American Type Culture Collection, Rockville, Maryland.

[b]J. W. Woldendorp, Laboratory of Microbiology, Agricultural University, Wageningen, Netherlands.

[c]Department of Microbiology, Michigan State University.

plus yeast extract, with periodic transfer at 30C followed by storage at 10C after cultures reached log phase.

Eleven experimental media were employed (Table 2). Media 103 through 115 are modifications of media described by Woldendorp (111) and by Valera and Alexander (100). Stock solutions listed in Table 2, when combined in the proportions shown, give the following concentrations of minerals in 1 liter of the final medium: 123 mg Na_2HPO_4, 724 mg KH_2PO_4, 89 mg $MgSO_4 \cdot 7H_2O$, 53 mg $CaCl_2 \cdot 2H_2O$, 380 ug $CuSO_4 \cdot 5H_2O$, 440 ug $ZnSO_4 \cdot 7H_2O$, 310 ug $MnSO_4 \cdot H_2O$, 250 ug H_3BO_4, 5 ug $NaMoO_4 \cdot 2H_2O$ and 1 mg Fe (as FeEDTA) per liter.

The media were prepared by combining the buffer solutions (in proportions to provide media of pH 7.3), mineral salts solutions and agar in a suction flask. These were sterilized by autoclaving at 121C for 15 minutes. The glucose, amino acids, vitamins, iron chelate, and simazine were sterilized by introduction through a Millipore filter. The simazine was added as a concentrated solution in chloroform. The chloroform was removed by aspirator suction in a water bath at 45C. Similar quantities of chloroform were added to and evaporated from control media without simazine.

The media were poured into disposable petri plates, cooled and those plates designated for anaerobic incubation were placed immediately into an H_2 atmosphere for storage until ready for use. Plates for aerobic incubation were stored on the laboratory table under sterilized cloth cover.

Table 2. Experimental media used to supply growth factors

Component	Medium Number			
	103	105	106	107
Stock sol'n. A[a]	510 ml	510 ml	510 ml	510 ml
" " B[b]	90 ml	90 ml	90 ml	90 ml
" " C[c]	180 ml	180 ml	180 ml	180 ml
" " D[d]	10 ml	10 ml	10 ml	10 ml
" " E[e]	10 ml	10 ml	10 ml	10 ml
KNO_3	2.5 g	2.5 g	---	2.5 g
Asparagine	---	---	2.5 g	---
Glucose	2.5 g	2.5 g	2.5 g	2.5 g
Vitamins[f]	---	20 ml	20 ml	---
Amino acids[g]	---	20 ml	20 ml	---
Na thioglycollate	---	---	---	500 mg
Yeast extract	---	---	---	---
Na citrate	---	---	---	---
KH_2PO_4	---	---	---	---
$MgSO_4 \cdot 7H_2O$	---	---	---	---
$CaCl_2 \cdot 6H_2O$	---	---	---	---
$FeCl_3 \cdot 6H_2O$	---	---	---	---
Agar	15 g	15 g	15 g	15 g
H_2O to:	1 liter	1 liter	1 liter	1 liter
pH	7.3	7.3	7.3	7.3

[a]Stock sol'n. A: 1.42 g Na_2HPO_4 per liter (.01 M).

[b]Stock sol'n. B: 1.36 g KH_2PO_4 per liter (.01 M).

[c]Stock sol'n. C: 493 mg $MgSO_4 \cdot 7H_2O$ (.002 M) plus 294 mg $CaCl_2 \cdot 2H_2O$ (.002 M) per liter.

[d]Stock sol'n. D: 38 mg $CuSO_4 \cdot 5H_2O$, 44 mg $ZnSO_4 \cdot 7H_2O$, 31 mg $MnSO_4 \cdot H_2O$, 25 mg H_3BO_3, and 5 mg $Na_2MoO_4 \cdot 2H_2O$ per liter.

[e]Stock sol'n. E: 769 mg Fe EDTA per liter.

and sources of carbon and nitrogen for cultural studies.

Medium Number						
109	110	111	112	113	114	115
510 ml	510 ml	510 ml	510 ml	---	510 ml	510 ml
90 ml	90 ml	90 ml	90 ml	---	90 ml	90 ml
180 ml	180 ml	180 ml	180 ml	---	180 ml	180 ml
10 ml	10 ml	10 ml	10 ml	---	10 ml	10 ml
10 ml	10 ml	10 ml	10 ml	---	10 ml	10 ml
2.5 g	---	1.25 g	1.25 g	1.0 g	2.5 g	2.5 g
---	2.5 g	1.0 g	1.0 g	1.0 g	---	---
2.5 g	2.5 g	2.5 g	2.5 g	---	2.5 g	2.5 g
20 ml	20 ml	20 ml	---	---	20 ml[h]	20 ml[h]
20 ml	20 ml	20 ml	---	---	20 ml	20 ml
500 mg	500 mg	500 mg	500 mg	500 mg	500 mg	---
---	---	---	10 g	---	---	---
---	---	---	---	8.5 g	---	---
---	---	---	---	1.0 g	---	---
---	---	---	---	1.0 g	---	---
---	---	---	---	0.2 g	---	---
---	---	---	---	0.03 g	---	---
15 g	15 g	15 g	15 g	15 g	15 g	15 g
1 liter	1 liter	1 liter	1 liter	1 liter	1 liter	1 liter
7.3	7.3	7.3	7.3	7.3	7.3	7.3

[f]Vitamin stock solution to provide 1 ug biotin, 2 ug vitamin B_{12}, 2 ug folic acid, 100 ug riboflavin, 500 ug thiamine, 500 ug nicotinic acid, 500 ug pyridoxine-HCl, 500 ug ca-pantothenate and 50 mg inositol per liter in the final medium.

[g]Amino acid stock solution to provide 950 mg vitamin-free casamino acids, 50 mg tryptophane and 10 mg cysteine per liter of final medium.

[h]Vitamin stock solution described above without inositol.

Inoculating cultures were prepared by growing in nutrient broth for 12 to 18 hours. To prevent a carry over of nutrients, the cells were spun down, the nutrient broth removed and the cells resuspended in buffer solutions A and B (Table 2) combined to provide a pH of 7.3.

For anaerobic incubations, atmospheres of H_2 and/or CO_2 were used in two cabinet type anaerobic incubators. For H_2 atmosphere incubation the cabinets were evacuated four times to 20 lb vacuum, introducing H_2 each time to 3 lb. This resulted in an H_2 atmosphere containing about 0.5 per cent O_2. Palladium-impregnated asbestos was used as a catalyst to reduce remaining traces of O_2. Small tubes of methylene blue agar, visible through the glass door, were used to indicate the establishment of reducing conditions. Partial or complete replacement of H_2 with CO_2 was effected only after reducing conditions had been established.

Aerobic plates were incubated on the laboratory table covered with a sterilized cloth.

Both aerobic and anaerobic incubations were carried out at 28 to 30C. After various incubation periods, growth was estimated visually on a scale of 0 to 4. Cross-streak inoculations were made and appropriate notations for synergism or antagonism were used.

Black and white Polaroid photographs proved to be useful as a permanent visual record of growth and interaction responses. Attempts to record pigmentation contrasts on various types of color film were not successful.

Bartholomew and Mittwer's "Cold" method spore staining procedure (21) was used to identify spores.

Microbial Counts From Simazine Treated Plots

An experiment was designed to investigate effects of simazine on soil microbial populations. Soil samples were taken in the fall of 1966 from an orchard pesticide experiment on Hillsdale fine sandy loam.[4] Duplicate samples were taken of untreated soil and soil which had received 4 lb simazine each year in 1964, 1965, and 1966. In 1966, 2 lb. amitrole-T was combined with the simazine. Each sample was composited from three field randomized plots, four core samples in each plot. Samples were sifted through a 6 mm sieve, placed in plastic bags and stored in a cold room at 40C for about a week before estimates of microbial numbers could be made.

An estimate of the total population of fungi, actinomycetes, and bacteria in untreated and treated soil was obtained by use of soil dilution plate counts. The actinomycete population was estimated after 10 days growth on chitin agar (53), the fungal population after 7 days on Martin's Rose Bengal agar (61), and aerobic bacteria after 8 days on soil extract agar (55). Anaerobic bacteria were estimated

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[4]Courtesy Dr. S. K. Ries, Department of Horticulture, M.S.U. The experiment was located on the University Farm, East Lansing.

on media 103, 105, and 106 (Table 2) incubated in 100% H_2 or 90% CO_2- 10% H_2 for 14 days. Bacteria suppressed by the H_2 and CO_2, but still viable, were estimated by counting the additional colonies which had appeared 3 days after removal from anaerobic conditions. All incubations were at 28 to 30C.

Denitrification In Liquid Broth

In an effort to establish nitrate-reducing and denitrifying capacities of the eighteen pure culture bacteria (Table 1) in the presence and absence of simazine, a study was undertaken using liquid media in Durham fermentation tubes.

Media 103, 105, 106, and 113 were employed (Table 2). In preparing media 103, 105, and 106 the ingredients were combined to form two separate solutions. The first solution containing buffer solutions, mineral salts, and nitrate or asparagine was sterilized by autoclaving at 121C for 15 minutes. One-half of each medium contained bromthymol blue indicator. Five ml of this first solution was dispensed into Durham tubes (outer tube, 16 x 125 mm: inner vial, 10 x 75 mm, inserted upside down) by means of a sterile automatic syringe.

The second solution, containing glucose, vitamins, amino acids, and iron chelate, was sterilized through a millipore filter into a suction flask maintained at 45C in a water bath. Sufficient simazine, dissolved in chloroform, was added to the appropriate quantity of each medium through

the Millipore filter to achieve the desired final medium concentration. Vacuum at 45C was employed to remove the chloroform before this second solution was added to the Durham tubes, using a sterile automatic syringe.

Medium 113 (Timonin's medium, 95) was prepared and dispensed similarly, with appropriate variations.

All tubes were put into anaerobic chambers and reducing conditions obtained by evacuating and filling the chambers with H_2. During the process the gas trapped in the inner vial was evacuated and the vial filled with the respective medium.

Each bacterial culture was prepared for inoculation by growing for 18 hours in nitrate broth. To minimize carryover of nutrients, 0.10 ml of each turbid culture was pipetted aseptically into 10 ml of a sterile solution formed by combining solution's A and B (Table 2) in a ratio of 7:3. The resulting pH was 7.3. This suspension was mixed and 0.10 ml inoculated into each Durham tube. The tubes were placed in anaerobic incubators. To disperse the inoculum through the medium, and also to provide anaerobic conditions, the anaerobic chambers were evacuated and filled with N_2. The cultures were allowed to incubate for 10 days at 28 C in this N_2 atmosphere.

For each organism, duplicate tubes were incubated at each factorial combination of four media with and without bromthymol blue, with and without simazine (5 ppm).

During incubation, the tubes with the indicator, initially light blue because of the inclusion of bromthymol blue, changed to an intense blue as the solution became more alkaline, an indication of denitrification, or to a yellow or green as the solution became more acid. This reaction change was recorded as "alkaline" if the color was intense blue, or "acid" if yellow or green, or "-" if unchanged.

A second indication of denitrification was the production of gas which was collected in the inner vial. Gas production was estimated visually on a scale from - to $^{+++}$.

A third observation was the test for microbial reduction of nitrate to nitrite. Approximately 1.0 ml of sulfanilic acid solution and a 1.0 ml of dimethyl-alpha-naphthylamine was added to each of the tubes which did not contain bromthymol blue. The development of a red or maroon color indicated the presence of nitrite. A negative test (no development of color) could be interpreted to mean, either that nitrate was not reduced, or that nitrate reduction had occurred but the reaction had gone beyond the nitrite stage to ammonia or gaseous nitrogen. Therefore, it was necessary to test for nitrate in those tubes giving a negative reaction for nitrite. This was done by the addition of a very small amount of powdered zinc. Zinc reduces nitrate to nitrite, and the color characteristic of a positive nitrite test developed if the nitrate had not been completely reduced.

RESULTS

Cultural Studies on Solid Media

Pure culture studies were undertaken with eighteen species and strains of nitrate-reducing bacteria. Observations of their behavior on different media and under various atmospheric conditions were made in an effort to establish a differential counting procedure, using solid media. The test organisms selected represented different physiological types based on studies by Woldendorp (111) and Valera and Alexander (100). The chemically defined media of Woldendorp were used with slight modifications (see Table 2). Buffer concentration and the concentration of Ca and Mg were reduced to avoid precipitation of phosphate and iron. The vitamin component was augmented by addition of inositol and vitamin B_{12} as in media described by Lochhead and Chase (56).

Plates were streaked by loop inoculation with washed cell suspensions from log phase cultures grown in nutrient broth.

A major difficulty in preliminary anaerobic studies was the complete elimination of traces of oxygen at the beginning of incubation. Traces of oxygen can permit initiation of growth and adaptation to anaerobic conditions in

the case of some facultative anaerobes (Valera and Alexander, 100). To prevent this the plates were chemically reduced by storage in H_2 prior to streaking and precautions were taken to minimize the length of time that media were exposed to air during inoculation.

Responses to inositol and thioglycollate

The major difference in media used in this study and the comparable media used by Woldendorp (111) was the addition of vitamin B_{12} and inositol to the list of vitamins supplied as growth factors. A preliminary study was designed to investigate effects of inositol on growth of representative test organisms when added at the rate of 50 mg per liter as in media described by Lochhead and Chase (56). The possibility was also investigated that sodium thioglycollate might contribute to clearer differentiation of anaerobic responses by minimizing oxidation during the time that plates were necessarily exposed to air while being streaked.

Aerobic growth responses of four bacterial strains are presented in Table 3. All organisms made better growth with asparagine as source of nitrogen (medium 110) than nitrate (medium 109). With nitrate as nitrogen source, three of the four organisms made more rapid growth when inositol was left out of the complement of growth factors (medium 114). The further elimination of thioglycollate in medium 115 produced no further changes in growth.

Table 3. Aerobic growth[a] on agar media[b] at 28C.

Organism	Medium 109		Medium 110		Medium 114		Medium 115	
	1 day	4 days	1 day	4 days	1 day	4 days	1 day	4 days
2. Ps. aeruginosa Woldendorp	2	3	3	5	2	3	2	3
7. B. coagulans 1963 II(W)	0	3	½	4	½	3	½	3
14. B. licheniformis ATCC 14580	0	1	2	4	1	3	1	3
16. M. denitrificans ATCC 13543	0	2	½	4	½	3	½	3

[a]Growth estimated visually on a scale of 0 to 5.

[b]Medium 109 - (glucose + nitrate + amino acids + vitamins including inositol + sodium thioglycollate).

Medium 110 - (glucose + asparagine + amino acids + vitamins including inositol + sodium.thioglycollate).

Medium 114 - 109 without inositol.

Medium 115 - 109 without inositol or sodium thioglycollate.

Observations of growth under three different anaerobic atmospheres are presented in Table 4. Of the four organisms, only Ps. aeruginosa was unable to grow in the absence of nitrate (medium 110) in a 100 per cent H_2 atmosphere. All made very good growth on this medium in atmospheres containing high partial pressures of CO_2, although growth of M. denitrificans was depressed when 10 per cent H_2 was included to assure catalytic reduction (on palladium) of traces of oxygen in the CO_2.

In the three media supplying N as nitrate, the inositol in medium 109 strongly suppressed growth of M. denitrificans in the 100 per cent H_2 atmosphere, and to some extent growth of the two Bacillus species also. This effect was not expressed in the two CO_2 atmospheres. Elimination of thioglycollate in medium 115 promoted a release of growth of M. denitrificans in 100 per cent CO_2 and some reduction in growth of Ps. aeruginosa in this atmosphere.

During the course of this experiment, it was observed that reducing conditions could be established in methylene blue agar within one hour after replacement of air with H_2 in the presence of the palladium catalyst. The time required to streak a group of plates for incubation was no more than one and one-half hours. Thus, the total exposure of experimental inocula to aerobic conditions was something less than two and one-half hours. There appeared to be no advantage to use of thioglycollate, so it was eliminated from media used in later studies.

Table 4. Anaerobic growth[a] on agar media[b] at 28C.

Organism	Medium 109	Medium 110	Medium 114	Medium 115
		100% H_2[c]		
2. Ps. aeruginosa Woldendorp	2	0	2	2
7. B. coagulans	2	2	3	3
14. B. lichiniformis ATCC 14580	2	2	3	3
16. M. denitrificans ATCC 13543	½	1	3	3
		100% CO_2[c]		
2. Ps. aeruginosa Woldendorp	2	3	2	1
7. B. coagulans 1963 II(W)	3	4	3	3
14. B. lichiniformis ATCC 14580	3	4	3	3
16. M. denitrificans ATCC 13543	3	4	2	4
		90% CO_2 - 10% H_2[d]		
2. Ps. aeruginosa Woldendorp	1	3	1	1
7. B. coagulans 1963 II(W)	3	4	3	3
14. B. lichiniformis ATCC 14580	3	4	3	3
16. M. denitrificans ATCC 13543	3	2	3	3

[a]Growth estimated visually on a scale of 0 to 4.

[b]Medium 109 - (glucose + nitrate + amino acids + vitamins including inositol + sodium thioglycollate).

Medium 110 - (glucose + asparagine + amino acids + vitamins including inositol + sodium thioglycollate).

Medium 114 - 109 without inositol.

Medium 115 - 109 without inositol or sodium thioglycollate.

[c]15 days growth.

[d]11 days growth.

In spite of the evidence in Tables 3 and 4 that inositol influenced growth behavior of some species unfavorably under certain atmospheric conditions, it was retained in later media as being a growth factor likely to exist in soils. Later experience suggests that the concentration may have been so high as to be unnecessarily restrictive, particularly for estimation of aerobic elements in soil populations.

Interactions of nutrition, atmospheric composition and simazine on growth

To study growth, pigmentation and organism interactions, the selected test organisms were streaked on agar[5] media 103, 105, and 106 (see Table 2) and incubated in aerobic, H_2, or CO_2 atmospheres. Following the anaerobic incubations, plates from H_2 and CO_2 were incubated under aerobic conditions to allow recovery of the test organisms.

Because Ps. aeruginosa [organism 1] had been observed in earlier studies to obtain good growth under adverse nutritional conditions and also had shown strong antagonism to several other organisms, it was streaked vertically on the plates as a standard test organism. Three organisms were streaked at right angles to Ps. aeruginosa on each plate to allow each to express possible interactions with this organism.

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[5]DIFCO Bacto Agar was used.

Growth responses for 18 organisms tested are tabulated in Table 5. Growth of Achromobacter hartlebii [organism 15] was erratic on all test media. Unique cultural requirements of this organism were not satisfactorily resolved.

Under aerobic incubation, most organisms grew best on medium 106 with vitamins, amino acids, and asparagine as the nitrogen source (Table 5).

Aerobic growth of all organisms was restricted on glucose-nitrate without growth factors (medium 103). Ps. aeruginosa [organisms 1 and 2], M. denitrificans [16] and Ps. fluorescens [17] made distinctly better aerobic growth than the others on this medium but did respond to the growth factors in medium 105. Marked responses to growth factors were expressed by S. marcescens [4], several Bacillus strains [5, 6,8,9,10,14] and by Ps. denitrificans [18]. Ps. stutzeri [3] and the slower growing B. macerans [11,12] and B. circulans [13] were less responsive to growth factors when confined to nitrate as source of nitrogen.

However, growth patterns and requirements changed when grown under anaerobic atmospheric conditions. Without oxygen, other compounds or elements must act as electron acceptors to enable growth. With the organisms studied, nitrate effectively promoted growth under strongly reductive anaerobic conditions. This can be observed in Table 5 where medium 105 with nitrate, vitamins, and amino acids provided the best medium for growth in an H_2 atmosphere.

Table 5. Growth[a] of denitrifying bacteria on solid media[b] in various atmospheres.

Organism[c]	Aerobic			H_2			H_2 + aerobic			CO_2[d]		
	103	105	106	103	105	106	103	105	106	103	105	106
1	3	4	5	2	2	1	2	3	5	2	3	5
2	3	4	5	2	2	½	2	2	5	2	4	5
3	1	2	4	1	2	0	1	2	1	0	½	0
4	1	3	5	2	3	1	2	3	3	1	2	3
5	1	3	3	2	3	1	2	3	1	1	2	4
6	1	4	5	3	4	2	3	4	4	1	4	4
7	2	3	4	4	5	2	4	6	3	2	4	5
8	1	4	5	2	4	2	2	4	4	1	4	4
9	1	4	5	2	3	1	2	4	3	1	3	5
10	1	4	5	2	5	2	2	6	5	½	2	5
11	½	1	2	2	3	1	2	3	1	2	2	3
12	½	1	2	2	3	3	3	3	3	2	2	3
13	½	1	3	2	3	2	3	4	2	1	2	3
14	1	4	5	3	4	2	3	4	4	1	4	4
15	--	--	--	--	--	--	--	--	--	--	--	--
16	3	4	5	2	3	1	2	3	3	0	½	1
17	3	4	5	0	0	0	0	0	2	½	½	0
18	1	4	5	1	2	1	1	2	2	2	3	5

[a]Growth at 28C estimated visually on a scale of 0 to 6.

[b]Medium 103 - (glucose-nitrate).
Medium 105 - (glucose + nitrate + amino acids + vitamins including inositol).
Medium 106 - (glucose + asparagine + amino acids ± vitamins including inositol).

[c]See Table 1.

[d]No additional growth in CO_2 + aerobic.

Medium 106 with asparagine, vitamins, and amino acids produced growth with most organisms in H_2, though less than in air, and with organisms 8,10,12,13, and 18 growth was equal to that in the basic nitrate medium without growth factors. With the probable exception of organisms 1,2,3,16 and 18, all of the strains tested are known to have ready access to non-respiratory (fermentative) pathways of metabolism. However, it must be recognized, also, that the sparse growth observed visually and designated in the table by values of 1 or ½ may have occurred at the expense of electron acceptors which accumulated, exogenously or endogenously, by chemical or enzymatic oxidations during the 1½ hours that the plates were exposed to air while being inoculated. The fact that respiratory pathways are used preferentially for growth by at least half of these organisms is demonstrated by their rapid growth on medium 106 when plates were removed from H_2 into air for three days.

B. coagulans [organism 7] produced moderate to heavy growth on both nitrate media under H_2. The three B. licheniformis strains [6,8,14] and one of the B. cereus strains [10] also grew well using nitrate in the presence of growth factors. These and all other organisms listed, except the first three and the last three, were able to utilize nitrate more effectively in H_2 than in air when growth factors were not supplied.

With H_2 incubation, Ps. fluorescens [17] grew only on the asparagine medium and then only after exposure to air.

This indicates that the H_2 killed the inoculum on nitrate media and residually inhibited or delayed growth with asparagine. A number of other organisms may have been similarly inhibited residually by H_2 on one or both nitrate media, since they failed to make additional growth during the subsequent three day exposure to aerobic conditions. By contrast, only organisms 5,11,12, and 13 failed to make additional growth on the asparagine medium when exposed to air. These were all bacilli (B. laterosporus, B. macerans and B. circulans).

With CO_2 incubation, all organisms were either residually inhibited by exposure to the 100 per cent CO_2 atmosphere, or growth made in CO_2 exhausted the medium of some component essential for growth. At any rate, no additional growth occurred during subsequent aerobic incubation, so only observations made at the end of the incubation in CO_2 are recorded in Table 5.

During incubation in CO_2 most organisms produced their greatest growth with asparagine as the nitrogen source. The CO_2 atmosphere greatly inhibited organisms 3,16, and 17 on all media. For a number of others, CO_2 was a less favorable atmosphere than H_2 for growth on either nitrate medium. For most organisms, CO_2 was more favorable than H_2 for growth when asparagine was the nitrogen source. Except for organisms 3,16, and 17, growth in CO_2 was amazingly similar to growth under aerobic conditions on all three media.

Stotzky and Goos (91) found that more than 90% CO_2 reduced the number of organisms developing on soil extract dilution plates, but most organisms recovered when the plates were subsequently incubated in air. From this observation and the present study it may be inferred that, if any of these test organisms (except numbers 3,16, and 17) were present in the soil utilized by Stotzky and Goos, they would have fully developed on their media while in CO_2 incubation. The differential responses to nitrogen source and growth factors observed here under different atmospheres provide additional criteria for isolation and enumeration of more restricted physiological groups within the soil population.

Simazine effects on the growth of the pure culture test organisms were noted after 2 and 12 days' incubation under H_2 and 90% H_2 + 10% CO_2 atmospheric conditions on solid media 107,109, and 110. Simazine was supplied at 0 and 50 ppm in the media.

After 2 days' growth under H_2 incubation several organisms responded with a slight increase in growth on both nitrate and asparagine media treated with simazine. However, after 12 days' incubation only a few Bacillus species still indicated a better growth response on simazine media. With the inclusion of 10% CO_2 in the H_2 atmosphere, this growth response to simazine was limited to a very few Bacillus species on the nitrate media after 2 days' growth and was diminished after 12 days' incubation. Thus it appears that

simazine may have decreased the lag phase for some of the test organisms, especially under 100% H_2 atmospheric conditions, with a very slight increase in total growth on the nitrate media.

This apparent tendency for simazine to promote more rapid development on solid media is consistent with the reduced lag times and more rapid growth observed with Ps. aeruginosa, Ps. stutzeri and S. marcescens by Hong in broth cultures at 30C when amino acids were left out of a medium containing glucose, nitrate, vitamins and minerals (see Figure 3). It should be noted that this adaptive stimulus was reversed at lower temperatures and that simazine was strongly inhibitory at 10C (cf Figure 4).

Interactions of nutrition, atmospheric composition and simazine on antagonism by Pseudomonas aeruginosa

The physical and chemical characteristics of soil, both natural and man-induced, determine the nature of the environment in which microorganisms are found. These varying environmental characteristics in turn affect the composition of the microbial population both quantitatively and qualitatively by producing numerous ecological niches. These niches are essentially situations of opportunity for exploitation by individual species whose evolutionary and physiological adaptations give them a unique advantage in that particular environment.

In this study only a few of the stimuli to which components of the soil microflora respond will be considered, including nutritional factors, microbial interactions, atmospheric conditions and the effect of the herbicide, simazine.

In natural soil environments, a number of relationships exist between individual microbial species. Members of the microflora may rely upon others for certain growth substances, but at the same time they may exert detrimental influences on still other components. Beneficial, harmful and neutral associations can be demonstrated in isolated systems. Their significance in complex natural soil systems is much more difficult to evaluate. Nevertheless, the numerous studies that have been made support the general ecological principle that the composition of the microflora of any soil habitat is governed by the biological equilibria created by the associations and interactions of all individuals found in the population.

It is well realized that the results of <u>in vitro</u> studies with pure bacterial cultures on chemically defined media cannot be directly assumed to apply to natural situations. However, some indication of possible interactions between bacterial species may be observed and general tendencies inferred for well defined nutritional and atmospheric conditions.

Although man is an animal, the environmental stimuli which derive from his technological inputs are both quantitatively and qualitatively different than those of any other

animal. His use of pesticides is a unique example of qualitatively new and different stimuli being introduced into natural environments. The pesticide technologies in vogue are based upon their most obvious effects on target species. The potential significance to man of their effects on non-target species and on systems of species important to him has been a serious concern to researchers for a much longer period of time than it has to the general public.

With reference to the soil microbial population, effects of pesticides on taxonomic groups have frequently been negligible when tested at 10 to 100 fold normal rates of application. It appears likely that specific effects on species or physiological groups of bacteria, fungi or actinomycetes may be masked by compensating changes in other groups or species. If such specific effects are expressed, it will be necessary to look for them in these more restricted groups. At some point in such research, it will be necessary to look for effects on individual species and interactions between them.

The pure culture studies reported here were undertaken to look for differential effects of simazine on representative denitrifying species. Different nutritional and atmospheric conditions were imposed with a view to defining parameters for differentiating physiological types within the group for later population and isolation studies in field experiments.

In preliminary studies by Sheng (85), it was observed that several nitrate reducing bacteria in the collection exerted antagonistic effects on others. Both Ps. aeruginosa strains were particularly active in this regard, but the ATCC strain was active against a larger number of other strains, including the Woldendorp strain of Ps. aeruginosa.

It appeared useful to set up pure culture studies in such a way that effects of simazine on individual species and on this interaction with Ps. aeruginosa ATCC 10145 could be observed at the same time.

Accordingly, organisms were cross-streaked against Ps. aeruginosa [organism 1] so that interactions with this standard organism might be observed. As can be seen in Table 6 growth inhibition of several organisms by organism 1 occurred under aerobic incubation. The complete nitrate medium 105 provided conditions most suitable for a wide spectrum of growth inhibition, since only B. cereus [10], Ps. fluorescens [17], and Ps. denitrificans [18] failed to be inhibited. However, the most extensive inhibition occurred with the three B. licheniformis strains [6,8, and 14] and B. coagulans [7], as shown in Figures 5 and 6, on the basal nitrate medium 103.

Other instances of growth inhibition occurred under CO_2 incubation with Ps. aeruginosa [2] on all test media (see Figure 7), and B. coagulans [7] on media 103 and 106 (see Figure 8).

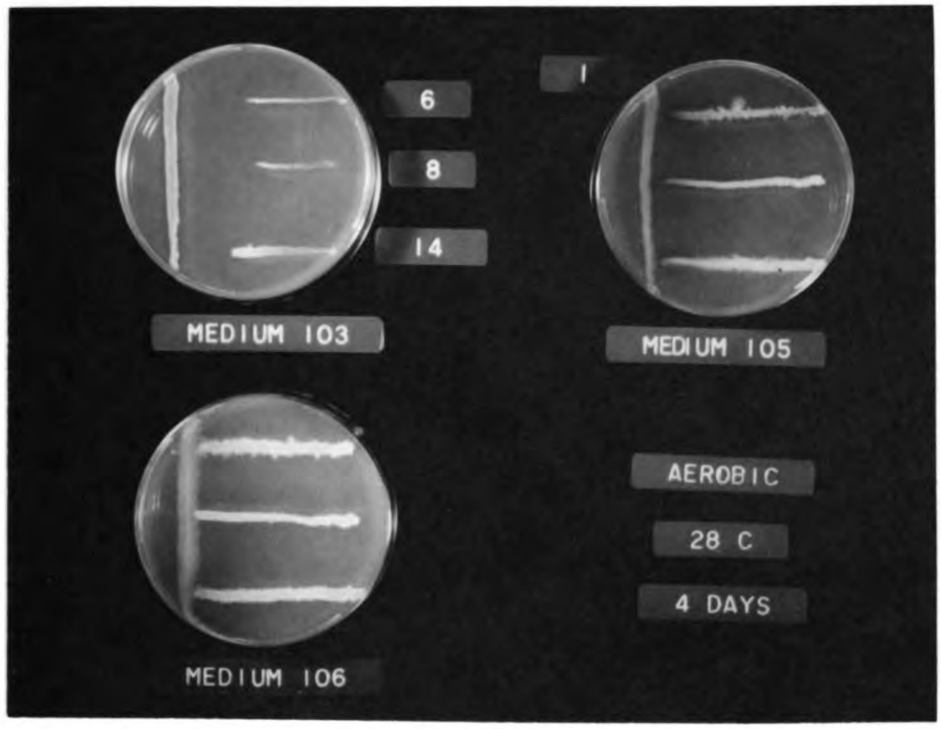

Fig. 5. Aerobic growth and interactions of Ps. aeruginosa (strain 1) and B. licheniformis (strains 6, 8, and 14).

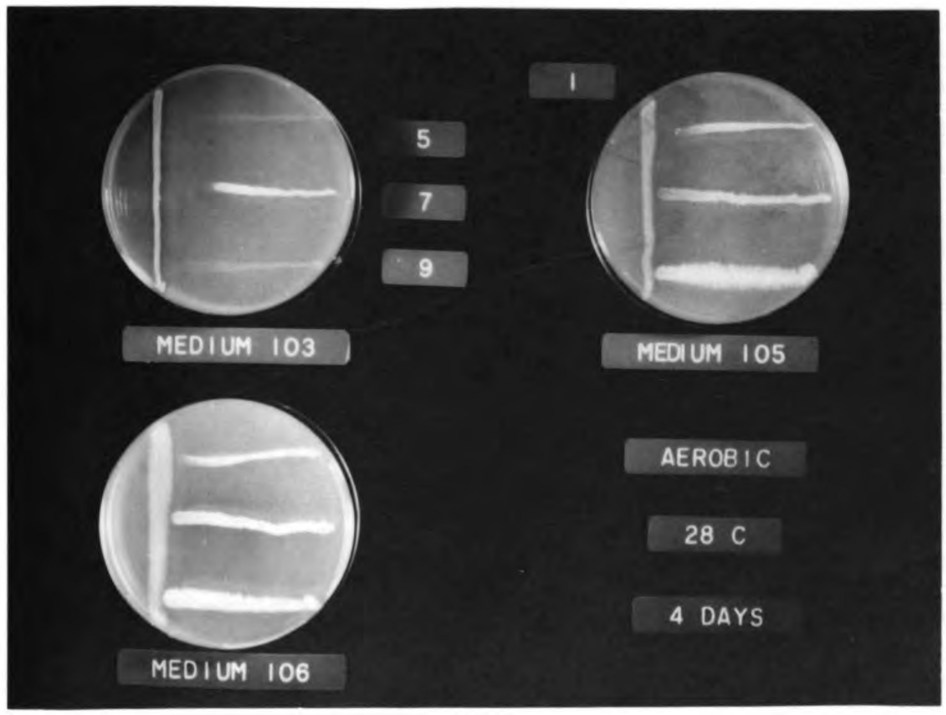

Fig. 6. Aerobic growth and interactions of Ps. aeruginosa (strain 1) and B. laterosporus [5], B. coagulans [7] and B. cereus [9].

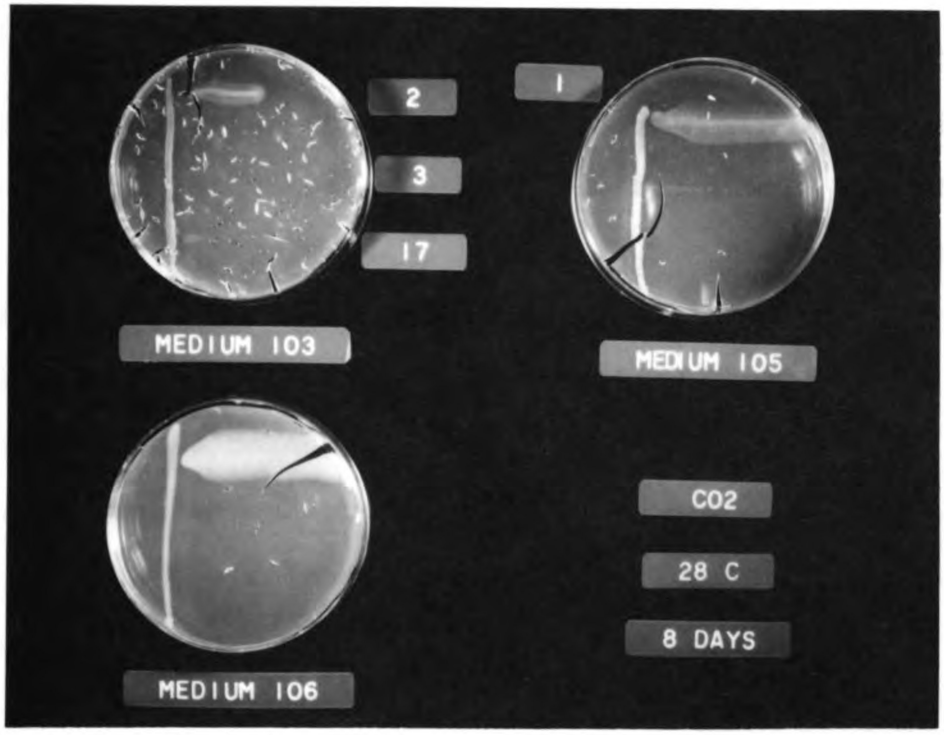

Fig. 7. Growth and interactions in CO_2 of Ps. aeruginosa (strains 1 and 2), Ps. stutzeri [3] and Ps. fluorescens [17].

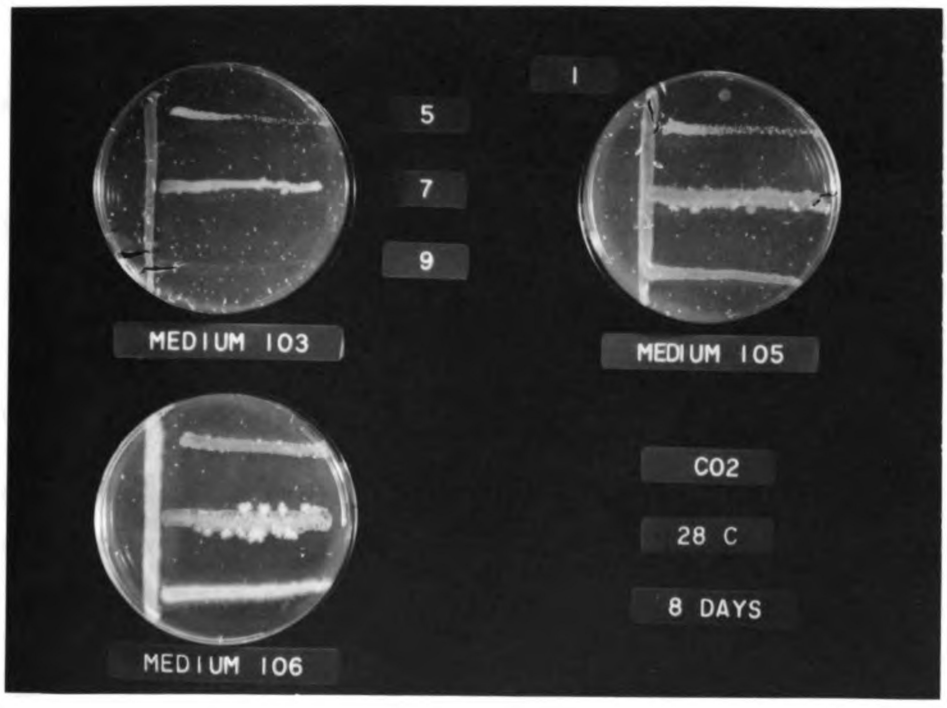

Fig. 8. Growth and interactions in CO_2 of Ps. aeruginosa (strain 1) and B. laterosporus [5], B. coagulans [7] and B. cereus [9].

In contrast to the strong inhibition of B. licheniformis [6,8, and 14] under aerobic incubation, the ATCC strain of Ps. aeruginosa [1] markedly stimulated growth of these B. licheniformis strains under H_2 on media 103 and 105 (see Figure 9). Similar stimulation to growth of several organisms was observed during growth in H_2 and in CO_2. In most cases, this commensalistic effect disappeared or, at least, was seldom enhanced and never reversed during subsequent growth in air (see Table 6). In CO_2, the stimulation was most pronounced on Medium 105.

A comparison of Figures 9 and 10 illustrates the marked release of growth which occurred with many organisms on medium 106 (no nitrate) when removed from H_2 into air (see Table 5).

Test organisms were also cross-streaked against Ps. aeruginosa [1] on solid media with 0, 5, 25, and 50 ppm simazine. Variations occurred in the intensity of antagonism by Ps. aeruginosa against the test organisms at the different simazine levels.

On the nitrate media under aerobic conditions, the antagonistic action of organism 1 against Ps. stutzeri [3], S. marcescens [4], B. laterosporus [5], and B. licheniformis [8] was increased when simazine was applied, but its antagonistic effect against B. coagulans [7] under these same conditions was decreased. Under H_2 conditions on nitrate media, the antagonistic effect against B. laterosporus [5] increased

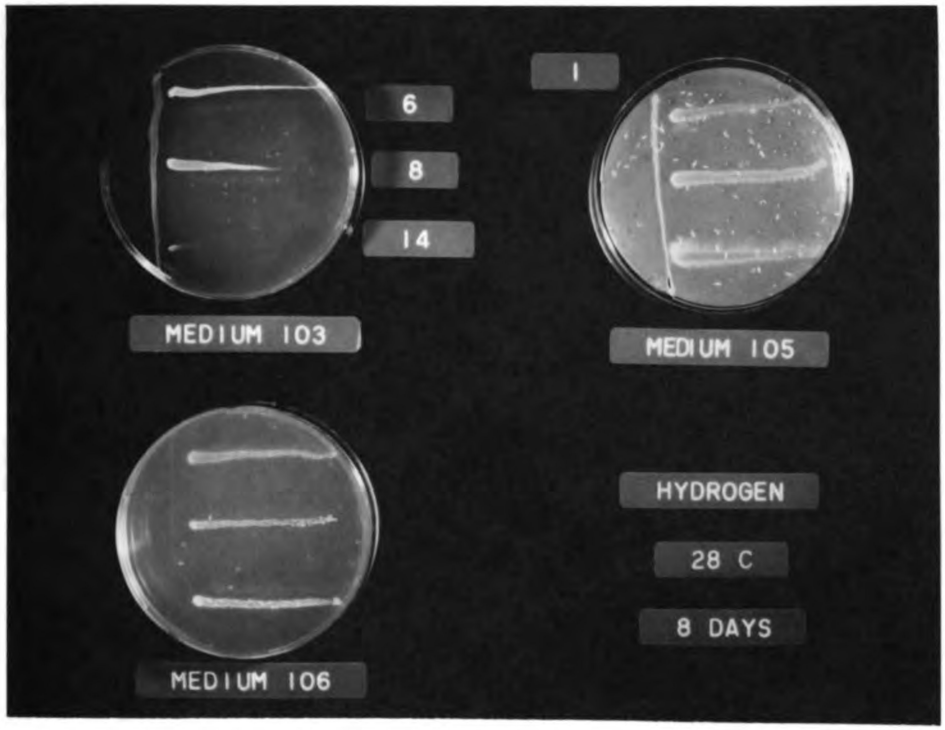

Fig. 9. Growth and interactions of Ps. aeruginosa (strain 1) and B. licheniformis (strains 6, 8, and 14) in a hydrogen atmosphere.

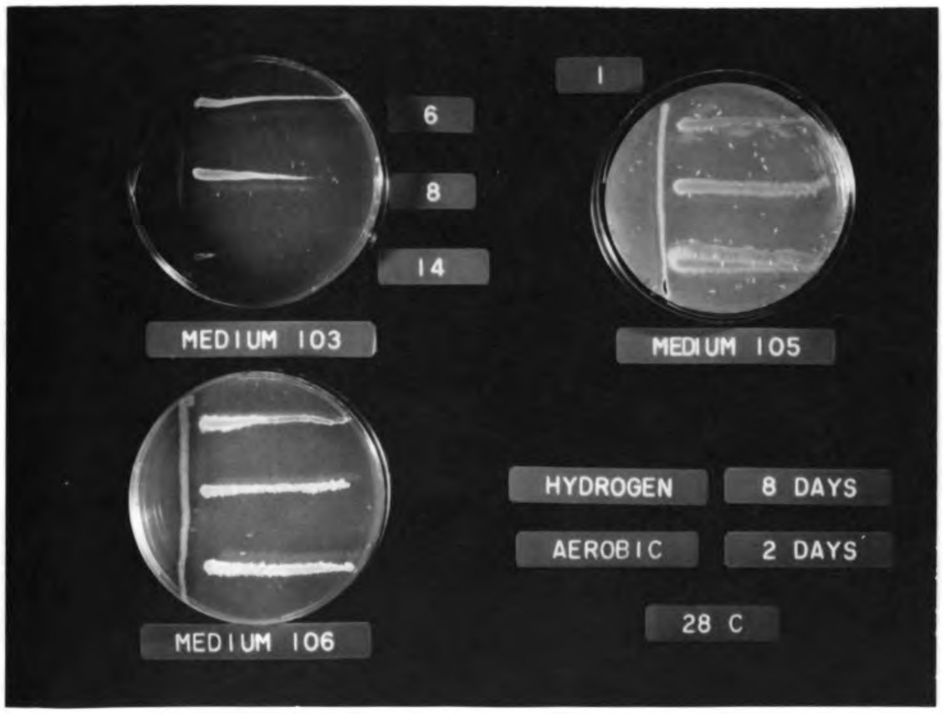

Fig. 10. Growth and interactions of Ps. aeruginosa (strain 1) and B. licheniformis (strains 6, 8, and 14) after 2 days in air following 8 days in hydrogen.

Table 6. Growth inhibition and stimulation[a] of various denitrifying bacteria by Pseudomonas aeruginosa (ATCC 10145) under various atmospheres on cross-streaked agar plates.

	Aerobic			H_2			H_2 + aerobic						CO_2						CO_2 + aerobic		
	Antagonism[b]			Stimulation			Antagonism			Stimulation			Antagonism			Stimulation			Stimulation		
Organism	103	105	106	103	105	106	103	105	106	103	105	106	103	105	106	103	105	106	103	105	106
2	1	2	2					2					3	3	3						
3	3	2																			
4		2																			
5		2	1																		
6	4	2		3	1							1				1					
7	4	2	1										1		2						
8	4	2		3	1											1					
9		2																			
10				1	2					1	2						2			3	
11		2	1			2						2				1	2				
12		2	1		1					1	2					2	2				
13		2		1	2					1	2	1				1	2	1			
14	4	2		2	1											1					
16	2	1															2				
17																					
18																1	1				

[a]Estimated visually on a scale of 1 to 4, representing suppressive or stimulatory responses observed 5 to 30 mm distant from limits of growth of ATCC 10145.

[b]Medium 103 - (glucose + nitrate).
Medium 105 - (glucose + nitrate + amino acids + vitamins including inositol).
Medium 106 - (glucose + asparagine + amino acids + vitamins including inositol).

in the presence of simazine, but decreased against B. macerans [11 and 12]. In general the influence of simazine on microbial interactions was not distinct and requires more investigation.

Interactions of nutrition and atmospheric composition on pigment production by Pseudomonas aeruginosa

Ps. aeruginosa is well known for its production of several pigments, as well as colorless compounds, with antibiotic properties. Several investigators have shown that pyocyanin, a blue pigment, inhibits certain organisms, especially gram-positive bacteria. Others have defined nutritional requirements for pyocyanin production. These include magnesium, alanine or several other amino acids, and Krebs cycle intermediates.

In the antagonism studies described in the previous section, pigment production by Ps. aeruginosa was found to be dependent upon the nitrogen source, and the amount of pigment was influenced by the atmosphere of incubation. With nitrate as the nitrogen source, as in medium 103 without vitamins and amino acids and in medium 105 with vitamins and amino acids, a blue-green pigment was produced by one strain of Ps. aeruginosa only (ATCC 10145), and then only during aerobic incubation following H_2. No pigment was produced during or after exposure to CO_2 on either nitrate medium. Under completely aerobic conditions, the blue pigment was produced only on medium 105. However, with asparagine as nitrogen

source in medium 106 with vitamins and amino acids, both ATCC 10145 and the Woldendorp strain of Ps. aeruginosa [organisms 1 and 2 respectively] produced a yellow-green pigment in air. The intensity of the pigment increased in the following order: aerobic < aerobic succeeding CO_2 < aerobic succeeding H_2.

It was observed that even under the most favorable conditions for production of yellow-green pigment by ATCC 10145, the pigment did not appear when this organism was growing near several of the other test organisms.

To observe this phenomenon, ATCC 10145 was cross-streaked against each of the remaining test organisms on medium 106 and incubated for 8 days in H_2, followed by 4 days in air. As a control, Ps. denitrificans [18], which allowed intense development of yellow-green pigment by ATCC 10145, was streaked against this Ps. aeruginosa strain on each plate together with one other organism.

As noted in Table 7, S. marcescens [4] and several bacillus organisms, including B. laterosporus [5], B. coagulans [7], B. cereus [9 and 10], B. macerans [11 and 12], and B. circulans [13] prevented visible formation of pigment.

Growth of ATCC 10145 was not affected, but the typical yellow-green pigment did not appear in a diffusion zone extending 5 to 20 mm beyond the limit of growth of the organisms producing the effect. No attempt was made to determine whether production of the pigment was inhibited or whether

Table 7. Inhibition by various denitrifying bacteria of pigmentation in *Pseudomonas aeruginosa* (ATCC 10145) on solid media[a] after exposure to an H_2 atmosphere.[b]

	Organism	Pigment inhibition
2.	*Ps. aeruginosa* Woldendorp	no
3.	*Ps. stutzeri* "	"
4.	*S. marcescens* MSU-MPH	yes
5.	*B. laterosporus* 468 B(W)	"
6.	*B. licheniformis* 430 (W)	no
7.	*B. coagulans* 1963 II(W)	yes
8.	*B. licheniformis* P_1 (W)	no
9.	*B. cereus* ATCC 6464	yes
10.	*B. cereus* ATCC 14579	"
11.	*B. macerans* ATCC 843	"
12.	*B. macerans* ATCC 8244	"
13.	*B. circulans* ATCC 4513	"
14.	*B. licheniformis* ATCC 14580	no
15.	*A. hartlebii* ATCC 365	"
16.	*M. denitrificans* ATCC 13543	"
17.	*Ps. fluorescens* ATCC 11250	"
18.	*Ps. denitrificans* ATCC 13867	"

[a]Medium 106 - (glucose + asparagine + growth factors - see Table 2).

[b]Pigment inhibition after growth for 8 days in H_2 plus 4 days in air.

the pigment was actually produced but not in its chromatic form because of potentiometric or ionic effects.

The remaining test organisms had no observable effect on pigment production by the ATCC strain of Ps. aeruginosa.

There was no apparent relation between pigmentation phenomena and the antagonistic or stimulatory responses associated with proximity to ATCC 10145. Thus, the most extensive inhibition occurred on media 103 in aerobic conditions where Ps. aeruginosa [1] failed to produce a visible pigment. Also organisms 6, 12, and 13, which were inhibited under aerobic conditions where pigments were produced by Ps. aeruginosa ATCC 10145, were stimulated by this organism under H_2 and subsequent aerobic incubation where pigments were also produced. Ps. aeruginosa strains are known to produce several antimicrobial products, some of which are pigments, some which are not [24]. It is entirely probable that different antibiotics with different spectra of activity, are produced under different nutritional or environmental conditions. On the other hand, pyocyanin has been implicated in electron transfer [24,33,35]. It is possible that exogenous redox systems involving pyocyanin may be poised favorably for a given organism under anaerobic conditions but unfavorably under aerobic conditions.

These results serve to illustrate the complexity of interactions which may occur in natural soil habitats where

an extremely heterogenous mixed inoculum is always present to respond to environmental and nutritional stimuli imposed by weather or the activities of plants, animals or man.

Population Studies with Soils Treated in the Field with Simazine Plus Amitrole-T

Stotzky and Goos (90) demonstrated that segments of soil microbial populations could be characterized on solid media by their ability to tolerate high CO_2 tensions and/or low O_2 tensions. High CO_2 tensions were more restrictive than low concentrations of O_2. Tolerance to high CO_2 was most prevalent among fungi and least characteristic of actinomycete species in the microflora of soils which had not been conditioned previously in atmospheres high in CO_2 and low in O_2. With such prior conditioning, however, the increase in numbers of CO_2-tolerant bacteria and actinomycetes was much greater than the increase in tolerant fungi. It was not established whether these increases were due to enrichment of specific groups which were already tolerant or whether physiological adaptation had occurred.

The denitrifying microflora in soils are, characteristically, facultative anaerobes. For some denitrifying species, the adaptation to anaerobic conditions involves a shift to non-respiratory (fermentative) pathways of metabolism for which nitrate is not essential, although nitrate may be reduced coincidentally if it is present. For others nitrate is essential, in the absence of oxygen, as a terminal

electron acceptor in respiration. It is generally agreed that the latter type of physiological adaptation is characteristic of the most active denitrifying species (Delwiche, 25; Alexander, 1; Woldendorp, 111; Verhoeven, 101).

The term, denitrification, refers specifically to the dissimilatory reduction of nitrate to gaseous products (principally N_2 and N_2O) which are lost from the soil. Adaptive mechanisms which lead to these losses include the synthesis of the non-constitutive enzyme, nitrate reductase. Studies of Ries and Tweedy (77) have shown that simazine stimulates nitrate reductase activity in higher plants. Were this effect to be expressed on a major component in the microflora of the soil or rhizosphere, it could alter patterns of nitrogen transformation and transport external to the plant root in ways which might materially influence the nutrition of the plant.

The pure culture studies of Hong, in broth media, which were cited in the Literature Review, and the studies on solid media described in earlier sections of this report are consistent in supporting the view that simazine does, in fact, promote the adaptation to utilization of nitrate by a number of bacterial species that have been implicated in denitrification losses of nitrogen from soils. There is reason to suspect that exposure of a soil population to simazine will, over time, produce changes consistent with an enrichment in nitrate reducing capacity.

A major objective of the present study was to investigate cultural parameters which might be employed to detect such population changes by dilution plating on solid media, rather than by the tedious, statistically equivocal, most probable numbers technique which involves serial dilution in broth media.

Accordingly, the orchard experiment described earlier (p. 34) was selected as a source of soil samples. Dilution platings were made on solid media to estimate numbers of the major taxonomic groups (bacteria, actinomycetes and fungi) and numbers of bacteria which might respond uniquely to nutritional and atmospheric variables employed in cultural studies described in previous sections.

Dilution platings were made of each duplicate field sample which had been taken to represent treated and untreated soil--a total of four soil samples. For each soil sample and each medium, five plates were poured for each of four dilutions. Colonies were counted on 3 to 5 plates of the significant dilution (55).

Colony counts, factored for dilution, were converted to logarithms for analysis of variance. Table 8 shows that the probabilities for no difference between mean logarithms for control and treated soils ranged from 5 to greater than 50 per cent. This generally low level of significance for treatment differences was due to unexplained field variation, rather than to excessive variability in counting and plating procedures in the laboratory. Standard deviations for

Table 8. Microbial population estimates and statistical probabilities for differences between controls and soils with previous history of field treatment with simazine plus amitrole-T.

Incubation atmosphere	Medium[a]	Mean log numbers per g dry soil: Control	Mean log numbers per g dry soil: Simazine plus amitrole-T	S.D.	t	P
Air	118	5.4371	5.3651	0.123	0.585	50
"	117	6.1211	6.0025	0.090	1.318	50
"	116	7.7030	7.2265	0.023	20.717	5
"	111	7.0835	6.4159	0.139	4.803	20
CO_2	103	5.7240	5.5749	0.055	2.711	30
" plus air[b]	"	6.5287	6.3749	0.144	1.349	50
CO_2	105	5.7649	5.6310	0.138	0.970	>50
" plus air	"	6.2817	6.2188	0.148	0.425	>50
CO_2	106	5.7536	5.4524	0.085	3.544	20
" plus air	"	6.2199	5.5465	0.420	1.603	40
H_2	103	5.6913	5.4610	0.131	1.758	40
" plus air	"	5.6645	5.2794	0.071	5.424	20
H_2	105	5.7222	5.5847	1.009	0.136	>50
" plus air	"	5.5259	5.1499	0.014	26.857	5
H_2	106	5.5166	5.4834	0.028	1.186	50
" plus air	"	5.7076	5.6185	0.238	0.374	>50

[a]Medium 116, soil extract agar (55); medium 117, chitin agar (53); medium 118, rose bengal agar (61); media 103, 105 and 106--see Table 2.

[b]Plates exposed 3 days in air after 8 days in CO_2 or H_2. Only new colonies which developed in air were counted.

dilution plate counts within soil samples ranged between 2 and 5 percent of the mean, whereas standard deviations for field variation not associated with replication or treatment ranged up to 19 per cent of the mean. For future studies, it appears essential to increase field replication in sampling.

In spite of the low order of statistical significance for most comparisons, certain relationships between the different combinations of media and incubation conditions should be considered seriously for further study.

Geometric mean numbers are presented in Table 9. From the last column of this table, it can be seen that the general effect of chemical treatment was to reduce numbers in the major taxonomic groups and in most cultural sub-groups capable of growth on media 103, 105 and 106. Fungi were reduced 15 per cent, actinomycetes by 25 per cent, and bacteria on soil extract by 66 per cent. This reduction in bacteria was significant at the 5 per cent level of probability.

The completely synthetic medium 111 was much more restrictive for bacteria under aerobic conditions than the soil extract medium 116. However, 111 is the appropriate complete medium for comparing effects of nitrogen source and growth factors in 103, 105 and 106 (see Table 2).

Total bacteria capable of growth on medium 111 were almost 80 per cent less in treated than in control soil. The distribution of cultural sub-groups within this bacterial population was drasticly different in treated and untreated soil. This can be seen more clearly in Figure 11.

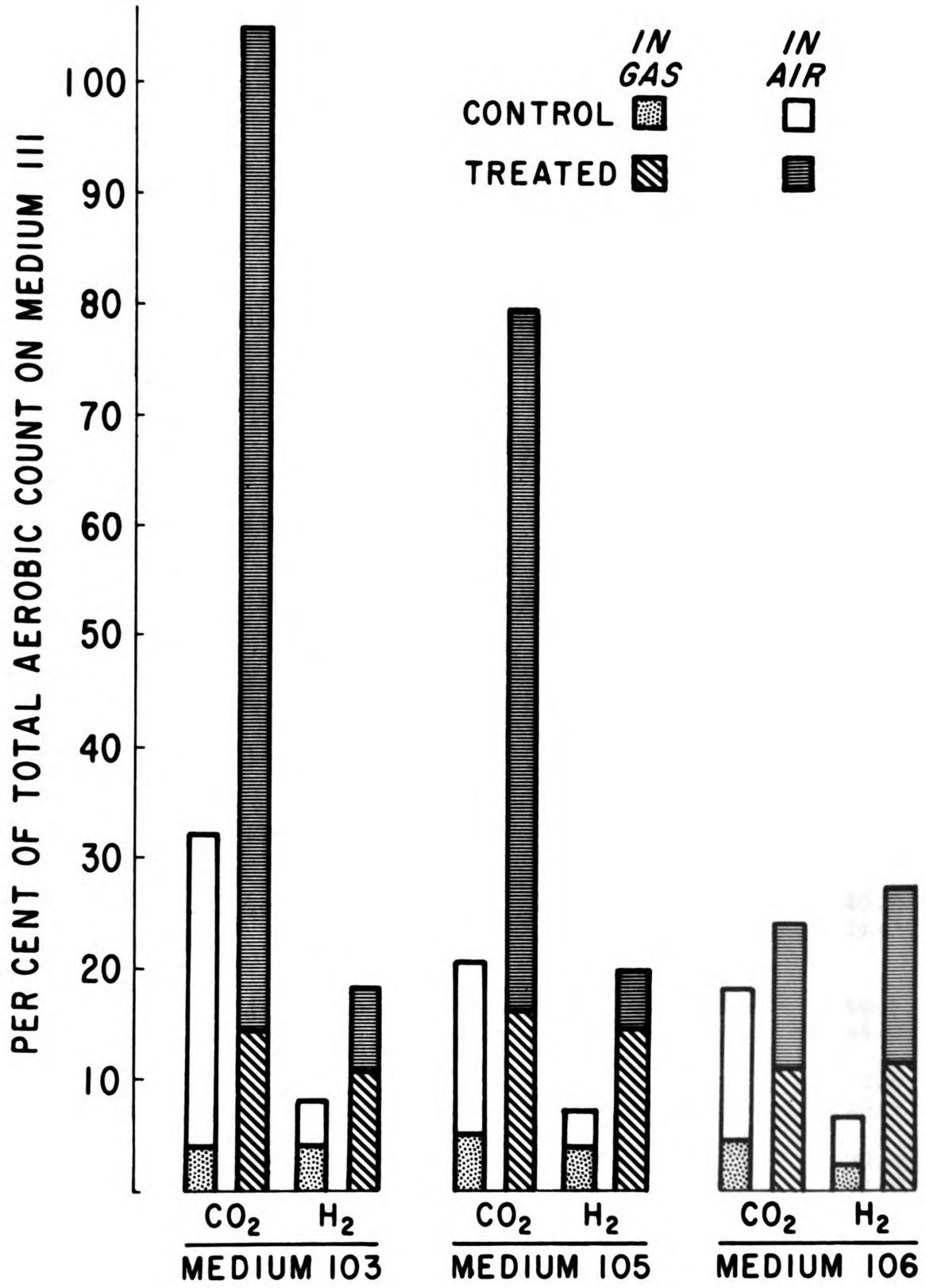

Fig. 11. Effects of field treatment with simazine plus amitrole-T on the distribution of cultural groups in the bacterial population defined by aerobic plate counts on medium 111.

Table 9. Geometric mean numbers and relative numbers of microbial groups in control soils and soils with previous history of field treatment with simazine plus amitrole-T.

Incubation atmosphere	Medium[a]	Microbial group	Geometric mean numbers per g dry soil: Control	Geometric mean numbers per g dry soil: Simazine plus amitrole-T	Treated soil as % of control
			x10^4	x10^4	
Air	118	Fungi	2.7	2.3	84.8
"	117	Actinomycetes	13.2	10.0	76.1
"	116	Bacteria	504.7	168.5	33.4
"	111	Bacteria	121.2	26.1	21.5
CO_2	103	"	5.3	3.8	71.0
"$_2$ plus air[b]	"	"	33.8	23.7	70.4
CO_2	105	"	5.8	4.3	73.4
"$_2$ plus air	"	"	19.1	16.6	86.5
CO_2	106	"	5.7	2.8	49.9
"$_2$ plus air	"	"	16.6	3.5	21.3
H_2	103	"	4.9	2.9	58.8
"$_2$ plus air	"	"	4.6	1.9	41.3
H_2	105	"	5.3	3.8	72.9
"$_2$ plus air	"	"	3.6	1.4	42.1
H_2	106	"	3.3	3.0	92.6
"$_2$ plus air	"	"	5.1	4.2	81.5

[a]Medium 116, soil extract agar (55); medium 117, chitin agar (53); medium 118, rose bengal agar (61); media 103, 105, and 106--see Table 2.

[b]Plates exposed 3 days in air after 8 days in CO_2 or H_2. Only new colonies which developed in air were counted.

In Figure 11, the assumption is made that organisms which developed on Media 103,105 and 106 would have also developed aerobically on medium 111 which contained the growth factors and both nitrogen sources which were systematically eliminated to achieve the more restrictive nutritional conditions represented by the other three media. This assumption is open to criticism, but it provides the basis for a useful first approximation.

In the control soil, only 20 to 35 per cent of the bacterial population defined by aerobic growth on medium 111 was able to grow and/or survive in CO_2 when nitrate was the only source of nitrogen (in media 103 and 105). In treated soil, it appeared that this population consisted almost entirely of CO_2-tolerant types. When asparagine was substituted for nitrate as a nitrogen source (medium 106), differences in CO_2 tolerance between control and treated soils were negligible, considering the weak basis for statistical inference. However, it should be pointed out that the proportion of bacteria capable of growth in CO_2 (bottom bar segments in Figure 11) was consistently greater in treated soils on all three media.

It is recognized that a 100 per cent CO_2 atmosphere is an unrealistic environment. It is possible that the commercial source of CO_2 used may have carried ppm traces of oxygen (this was not checked). Nevertheless, enhanced CO_2 concentrations are certainly present in the vicinity of decomposing biological detritus (plant, animal or microbial) and in the rhizosphere. As Stotzky and Goos (90) have

pointed out, tolerance to CO_2 is a demonstrable ecological factor which has received inadequate attention in soil microbiology. The relationships between CO_2 tolerance and treatment of soils with simazine and/or amitrole-T which appear in Figure 11 need to be taken seriously in terms of future research.

A very pertinent consideration in this regard is the fact that the major increase in CO_2 tolerant bacteria in treated soil appeared to involve aerobic types which could only use nitrate for rapid growth after removal from CO_2 into air. It may be postulated that these were organisms in which dissimilatory nitrate reduction may have supported a maintenance level of respiration to permit them to survive but not to grow and multiply in the absence of air. When air was supplied, rapid assimilatory nitrate reduction, supporting rapid growth, was possible because of an enhanced nitrate reductase activity which appeared to be rather clearly related to chemical treatment. Unfortunately, two chemicals were involved, simazine and amitrole-T. Further studies to investigate this relationship with both chemicals are needed, using experimental designs which will provide a more reliable basis for statistical inference.

A 100 per cent H_2 atmosphere is less realistic than one containing only CO_2. It was used in this study to assure a strongly reducing environment. It is of interest that the proportion of the total population which was able to grow in

this atmosphere on all three media was greater in treated than in control soils, as it was with CO_2. The ability to grow in one or both atmospheres was shown by a number of the denitrifying species and strains that were used in the pure culture studies described in earlier sections of this report. Similar studies need to be carried through to characterization of isolates to determine the extent to which nutritional and atmospheric variables can be combined to permit estimation of denitrifying populations by dilution plating techniques.

An incidental observation of significance for future investigations was the appearance on a number of plates of medium 103 of a fungus which was isolated and identified as a specie or strain of Trichoderma viride. The fungus was found only on plates inoculated from the soils treated with simazine and amitrole-T. It was found capable of vigorous growth on a mineral medium with simazine as sole source of carbon and nitrogen. Later studies on medium 103 showed that, while spore germination, growth and sporulation occurred rapidly in air, only spore germination and limited growth occurred in a CO_2 atmosphere. In an H_2 atmosphere, germination of spores and growth of mycelium were inhibited. On removal from CO_2, a ring of spores formed quickly along the periphery of growth made previously in CO_2, and sporulating mycelium developed rapidly to cover the remainder of the plate. After removal from H_2, spore germination occurred

promptly and growth and sporulation proceeded rapidly to cover the plate. Under some conditions, not clearly defined, a concentric pattern of alternating white vegetative hyphae and green sporulating mycelium would appear.

Effects of Nutritional Parameters and Simazine on Growth and Denitrification in Broth Media

The growth studies of Hong in broth cultures (cited in the Literature Review) indicated that simazine acted both to reduce the lag period for adaptation to nitrate and to increase the rate of growth after adaptation. Observations on development of streak cultures on solid media were consistent with this interpretation.

Inocula used by Hong were grown in a broth medium in which amino acids were the sole source of both carbon and nitrogen. Inocula for the studies on solid media were grown on nutrient broth (peptone plus beef extract) amended with yeast extract. In both groups of studies, simazine effects were observed in test media in which glucose (or citrate in medium 113) were supplied as carbon sources. The observed effects of simazine on lag periods and rates of growth could have been due to effects on adaptive enzyme changes involved in glucose metabolism as well as the inferred stimulus to development of nitrate reductase activity (Woldendorp, 111). These relationships and their significance for actual losses of nitrogen by denitrification need more critical study.

As a first step in this direction, an experiment was designed to determine whether simazine effected growth or denitrification in the presence of glucose by organisms in which nitrate reductase activity had been induced by prior cultivation in the absence of glucose but in the presence of nitrate. Accordingly, cultures were adapted by serial transfer in nitrate broth (Difco: peptone, beef extract, KNO_2). Eighteen-hour cultures in this growth medium were used to inoculate test media dispensed in Durham fermentation tubes.

Test media included 103, 105, 106, and a standard medium for denitrifiers (medium 113, after Timonin, 95). Bromthymol blue was added to a separate series of all media to observe changes in reaction (Valera and Alexander, 100). Simazine, at the rate of 5 ppm, was added to a second complete series of all media, with and without bromthymol blue.

Duplicate tubes of each composition were inoculated and incubated at 28C in an atmosphere consisting of 90 per cent N_2 plus 10 per cent H_2. Observations made after 10 days are recorded in Tables 10 to 13.

With reference to the main objective of this experiment, the most significant observation is that 5 ppm simazine had no effect on response of any of these adapted cultures.

Several incidental observations of value for future work should be pointed out.

A serious error in procedure was the failure to distinguish in the growth notation in column 2 between positive

Table 10. Cultural responses of denitrifying bacteria in medium 103 broth[a] with and without simazine.[b]

Organism[c]	Without Bromthymol Blue				With Bromthymol Blue	
	Growth	Nitrate Reduction	Nitrite Production	Gas Production	Gas Production	Change in reaction
1	+	+	-	+++	+++	Alkaline
2	+	+	-	+++	+++	Alkaline
3	+	+	-	+++	+++	Alkaline
4	+	+	+++	-	-	Alkaline
5	+	+	+++	-	-	Acid
6	+	+	+++	-	-	Acid
7	+	+	+++	-	-	Acid
8	+	+	+++	-	-	Acid
9	+	+	+++	-	-	Acid
10	+	+	+++	-	-	Acid
11	+	+	+++	-	-	Acid
12	+	+	+++	-	-	Acid
13	+	-	-	-	-	Acid
14	+	+	+++	-	-	Acid
15	+	-	-	-	-	-
16	+	+	-	+++	+++	Alkaline
17	+	+	+++	-	-	-
18	+	+	-	+++	+++	Alkaline

[a]Glucose + nitrate + minerals. (See Table 2.)

[b]Responses observed were the same in the presence or absence of 5 ppm simazine.

[c]See Table 1.

Table 11. Cultural responses of denitrifying bacteria in medium 105 broth[a] with and without simazine.[b]

Organism[c]	Without Bromthymol Blue				With Bromthymol Blue	
	Growth	Nitrate Reduction	Nitrite Production	Gas Production	Gas Production	Change in reaction
1	+	+	+	+++	+++	Alkaline
2	+	+	+	+++	+++	Alkaline
3	+	+	+	+++	+++	Alkaline
4	+	+	+++	+	+	Alkaline
5	+	+	+++	-	-	-
6	+	+	+++	-	-	-
7	+	+	+++	+	-	Acid
8	+	+	+++	-	-	Acid
9	+	+	+++	+	-	-
10	+	+	+++	+	-	-
11	+	+	+++	-	-	Acid
12	+	+	+++	+	-	Acid
13	+	-	-	-	-	-
14	+	+	+++	-	-	Acid
15	+	-	-	-	-	-
16	+	+	+	+++	+++	Alkaline
17	+	+	+++	-	-	Alkaline
18	+	+	+	+++	+++	Alkaline

[a] Glucose + nitrate + growth factors + minerals. (See Table 2.)

[b] Responses observed were the same in the presence or absence of 5 ppm simazine.

[c] See Table 1.

Table 12. Cultural responses of denitrifying bacteria in medium 106 broth[a] with and without simazine.[b]

Organism[c]	Without Bromthymol Blue				With Bromthymol Blue	
	Growth	Nitrate Reduction	Nitrite Production	Gas Production	Gas Production	Change in reaction
1	+	-	-	-	-	-
2	+	-	-	-	-	-
3	+	-	-	-	-	-
4	+	-	-	+	+	Acid
5	+	-	-	-	-	-
6	+	-	-	-	-	-
7	+	-	-	-	-	-
8	+	-	-	-	-	-
9	+	-	-	-	-	-
10	+	-	-	-	-	-
11	+	-	-	++	-	Acid
12	+	-	-	+++	+	Acid[d]
13	+	-	-	-	-	-
14	+	-	-	-	-	-
15	-	-	-	-	-	-
16	+	-	-	-	-	-
17	+	-	-	-	-	-
18	+	-	-	-	-	-

[a]Glucose + asparagine + growth factors + minerals. (See table 2.)

[b]Responses observed were the same in the presence or absence of 5 ppm simazine.

[c]See Table 1 .

[d]With organism 12, medium later became alkaline.

Table 13. Cultural responses of denitrifying bacteria in medium 113 broth[a] with and without simazine.[b]

Organism[c]	Without Bromthymol Blue				With Bromthymol Blue	
	Growth	Nitrate Reduction	Nitrite Production	Gas Production	Gas Production	Change in reaction
1	+	+	-	+	+	Alkaline
2	+	+	-	+++	+++	Alkaline
3	+	+	-	+++	+++	Alkaline
4	+	+	+++	-	-	-
5	+	+	++	-	-	-
6	+	+	+++	-	-	-
7	+	+	+++	-	-	-
8	+	+	+++	-	-	-
9	+	+	+++	-	-	-
10	+	+	+++	-	-	-
11	-	-	-	-	-	-
12	-	-	-	-	-	-
13	-	-	-	-	-	-
14	+	+	+++	-	-	-
15	-	-	-	-	-	-
16	+	+	-	-	++	Alkaline
17	+	-	-	-	-	-
18	+	+	-	+++	+++	Alkaline

[a]Timonin medium (95): citrate + nitrate + asparagine + minerals.

[b]Responses observed were the same in the presence or absence of 5 ppm simazine.

[c]See Table 1 .

growth and questionable growth evidenced by only slight turbidity (Tables 10 to 13). A positive growth notation should be questioned in these tables if there is no other evidence of activity in the culture. Thus, it is unlikely that B. circulans [13] developed significantly in any medium except 103, where its activity was evident because of the increase in acidity apparent with bromthymol blue. A. hartlebii [15] was as problematic in its growth in these broth cultures as it was on solid media.

Ps. aeruginosa [1 and 2] and Ps. denitrificans [18] behaved clearly as reported by others. They were unable to grow (slight turbidity) in the absence of nitrate on medium 106. In the other three media, they grew vigorously, reduced all available nitrate and produced gas and a strongly alkaline reaction with bromthymol blue. The production of both gas and alkalinity are necessary as evidence of denitrification (100).

M. denitrificans [16] grew and denitrified vigorously on glucose, both in the absence of amino acids and vitamins (medium 103) and in their presence (medium 105). It behaved in the same way for Woldendorp in vaspar-sealed tubes (111). Valera and Alexander (100), however, found this organism unable to grow on glucose in a purified N_2 atmosphere unless amino acids were also supplied. Its ability to do so in the present study can be accounted for by the presence of 10 per cent H_2 in the N_2 atmosphere. M. denitrificans is faculatively capable of chemolithotrophic growth, utilizing H_2 as an

energy source in the presence of nitrate (48).

The extent to which the 10 per cent H_2 in the incubation atmosphere may have influenced the behavior of other organisms in the test is a matter for further investigation. A number of responses were inconsistent with those reported by other researchers.

For example, Ps. stutzeri [3] has been found incapable of using glucose or a number of other carbohydrates unless amino acids are also supplied. In the present study, organism 3 grew and denitrified as vigorously on medium 103 as did Ps. aeruginosa [1 and 2] or Ps. denitrificans [18]. Hong (see p. 12) had observed that this strain behaved differently than the ATCC strain of Ps. stutzeri with which he began his investigations. Its identity is obviously questionable, although in numerous cultural test it has complied with other criteria for Ps. stutzeri. The possibility that H_2 enrichment of the atmosphere may influence behavior of this organism needs to be investigated.

A number of Bacillus species are reported incapable of anaerobic growth on glucose and nitrate in the absence of amino acids or growth factors. Here, on medium 103, they were found capable of sparse growth, reducing nitrate to nitrite in the process and producing acids. There was some evidence of gas production by several of these strains when amino acids and vitamins were supplied in medium 105, but none produced alkalinity as confirming evidence for denitrification on any medium. Woldendorp had reported gas production

as evidence of denitrification for several of these bacilli when amino acids or amino acids plus vitamins were supplied with glucose and nitrate (111). Valera and Alexander did not find the confirming increase in alkalinity in similar media for B. licheniformis, the only Bacillus species in their tests. None of these investigators checked specifically for reduction of nitrate or production of nitrite.

S. marcescens [4] was not studied by any of these researchers. It is of interest that it gave evidence of denitrification on medium 105 (production of gas and alkalinity) and presumptive evidence of denitrification on medium 103 (production of alkalinity without gas). On citrate without growth factors (medium 113) it produced neither gas or alkalinity. A similar observation may be made in the case of Ps. fluorescens [17], which produced alkalinity (but no gas) only on medium 105.

Woldendorp (111) has proposed that glucose may be a more discriminating energy source for differentiating between true denitrifiers (such as Ps. aeruginosa) and those which produce N_2 or N_2O coincidentally to fermentation (such as the Bacillus species) than are glycolytic intermediates and by-products or Krebs cycle intermediates. His suggestion is based on his own observation and those of others that B. licheniformis is unable to denitrify on glucose as energy source, whereas Verhoeven (101) had classified it as a true denitrifier on the basis of its behavior in a medium supplying

glycerol as energy source. All of the Bacillus species in Woldendorp's tests were able to use glycerol for growth and produced gas anaerobically.

Because of its availability to a large proportion of the soil microflora, glycerol is probably the energy source of choice for estimating total numbers of denitrifying organisms, as is indicated by studies of Valera and Alexander (100). The usefulness of glucose for discriminating between true and coincidental denitrifiers within the total group of denitrifiers is strikingly illustrated by the results which are presented for media 103 and 105 in Tables 10 and 11. Equally clear definition on solid media will be more difficult to obtain because the criterion of gas production will be less readily observed. Production of alkalinity appears to provide a basis for differentiation which could be exploited on solid media.

Woldendorp found that the oxidative deamination of glutamic acid was closely linked with nitrate reductase activity in Ps. aeruginosa but not B. licheniformis. The possibility needs to be investigated that glutamic acid may be a more specific and discriminatory energy source than glucose for differentiating between true and coincidental denitrifiers.

DISCUSSION

The present investigations were undertaken to define nutritional and atmospheric parameters which would permit use of dilution plate counts on solid media to detect changes in nitrate reductase activity in soil populations and to differentiate between important types of nitrate reducing organisms.

The population changes observed in simazine treated field soils suggest that dissimilatory nitrate reducers can be distinguished from organisms which only reduce nitrate assimilatively for growth on the basis of the ability of the former to grow in a CO_2-enriched atmosphere on solid media supplying nitrate and the ability of the latter to grow on the same media only after removal from CO_2 into air.

On the other hand, the parameters tested do not provide any basis for differentiating on solid media between organisms, such as Ps. aeruginosa, in which anaerobic growth involves respiratory pathways dependent upon nitrate for terminal electron transfer, and the Bacillus types which derive their energy under anaerobic conditions from fermentation reactions which may or may not be accompanied by reduction of nitrate to gaseous products. In broth culture, the two criteria of gas production and increased alkalinity do clearly differentiate between these "true" and "coincidental"

denitrifiers when glucose is used as the energy source. However, even in broth culture, a given organism may fail to denitrify on glucose but do so when some intermediate or by-product of glycolysis, such as glycerol, is present in the medium. The nature of growth factors supplied can further complicate the picture.

It is apparent that no specific energy source has yet been identified for making the distinction between these two types of dissimilatory nitrate reduction, in either broth or solid media, in a way that clearly relates to soil conditions and the types of substrates which may be expected to predominate in specific ecological situations such as the rhizosphere. Woldendorp (111) suggests that glutamic acid may be uniquely involved as a hydrogen donor in reduction of nitrate to gaseous products. This amino acid is found in root exudates. Its possible usefulness for making a meaningful distinction between types of nitrate reducers should be investigated, both in broth and solid media.

A serious difficulty with agar media is the fact that strictly defined nutritional conditions cannot be achieved because of impurities in even highly purified agar. In the present study, growth ratings of ½ to 1 on streak cultures may have been due in part to metabolites present as impurities in the agar. These impurities become an even more serious factor when highly restrictive agar media are used for counting purposes. Due allowance should be made for the contribution of the agar to colony counts in interpreting numerical

estimates on solid media.

There are distinct advantages in the use of solid media for the purposes entertained in this study. Atmospheric composition in the immediate environment of organisms developing in thin layers of agar can be more precisely controlled than in tubes of broth. The possibility of observing the effect of changing the atmosphere cannot be entertained in broth culture. The release of growth after removing cultures from CO_2 into air in the present study provided the unique criterion for distinguishing nitrate reduction from those which reduce nitrate assimilatively for growth. With careful standardization of suspending and dilution procedures, the statistical reliability of plate counts is much greater than of the most probable numbers treatment of observations obtained from serial dilution.

The relationships reported here between field treatment and the distribution of cultural sub-types in the bacterial population need to be verified by further studies. For one thing, the fact that amitrole-T was included with the simazine treatment in the third year makes it impossible to ascribe the observed effects specifically to either compound. More field replication is needed, also to give more acceptably low probabilities for error.

However, there was clear evidence that simazine stimulated more rapid adaptation to use of nitrate in pure cultures of bacteria. The increase in the proportion of nitrate utilizing bacteria in field populations exposed to

simazine is consistent with this *in vitro* behavior. The fact that the major proportionate increase was in types that appeared to be not active denitrifiers suggest that simazine may act in the field to conserve nitrate nitrogen by promoting its temporary immobilization in microbial tissues. If this action can be verified, it may provide another useful application for this chemical.

SUMMARY

Cultural parameters were investigated which might be useful in detecting changes in nitrate reductase activity in soil bacterial populations. A major concern was to develop solid media which could be used for isolation and enumeration of nitrate reducing bacteria by dilution plating rather than by serial dilution in broth. Equally important was the search for criteria which could be used to differentiate between three types of organisms: those in which nitrate reduction is (1) essentially assimilatory and those in which it is (2) essentially or (3) coincidentally dissimilatory.

Three parameters were considered: (1) nitrogen sources, (2) growth factors, and (3) incubation atmospheres. Nitrate and asparagine were compared as nitrogen sources. Media with no growth factors were compared with media providing both vitamins and amino acids. Aerobic responses were compared with anaerobic responses in CO_2, H_2 or N_2, alone and in various combinations. Glucose was used as the carbon source, except as comparison was made with a standard medium for denitrifiers in which the energy source was citrate.

Simazine, a herbicide known to stimulate nitrate reductase activity in higher plants, was tested for its ability to promote the same adaptive response in 18 strains of known

faculative nitrate reducing bacteria. The observed responses of these pure strains to simazine in the various cultural situations which were imposed were used as the basis for inferences regarding population estimates obtained under parallel cultural conditions for field soils previously treated with simazine.

Several distinct relationships were observed which support the validity of the objectives undertaken and point to useful directions for further research:

1. Simazine introduced into solid media at concentrations of 5 to 50 ppm promoted more rapid development of streak cultures of several nitrate utilizing bacteria. These observations supported earlier studies, under this project, in broth culture.

2. This stimulus to growth appeared to be due to earlier adaptive synthesis of nitrate reductase, since there were no effects on growth or denitrification when simazine was added to broth cultures previously adapted to nitrate.

3. Previous annual applications of simazine over a three-year period in the field reduced plate counts (relative to untreated controls) for major taxonomic groups in the order: bacteria (66%) > actinomycetes (25%) > fungi (15%).

4. The field simazine treatments increased, strikingly, the proportion of nitrate reducers in the bacterial population. This was evidenced by increased numbers of colonies capable of growth and/or survival in 100 per cent CO_2 or H_2 atmospheres on media supplying nitrate.

5. The major proportionate increase in nitrate reducing bacteria, however, occurred among types which differed from representative pure strains of denitrifying bacteria in their ability to grow on nitrate media only after removal from CO_2 into air. It is inferred that these are types in which nitrate reductase activity, stimulated by simazine, is exploited for assimilatory reduction of nitrate to support growth.

6. Field treatment with simazine produced negligible proportionate changes in bacterial types capable of growth and/or survival in CO_2 or H_2 on a medium which did not contain nitrate.

7. It appeared that a clear distinction could be made between dissimilatory and assimilatory nitrate reducers on solid media supplying nitrate on the basis of the ability of the former to grow in CO_2 and the ability of the latter to grow only after removal from CO_2 into air. The cultural parameters employed with solid media did not provide criteria for distinguishing between true denitrifiers and coincidental dissimilatory nitrate reducers as clearly as is possible in broth media.

8. Specific areas are identified for further research to define cultural parameters more specificly in relation to unique physiological processes. The need for greater replication in field studies to provide a broader base for statistical inference is indicated.

BIBLIOGRAPHY

1. Alexander, M. 1961. Introduction to soil microbiology. John Wiley and Sons, Inc., New York. 472 p.

2. Alexander, M. 1964. Biochemical ecology of soil microorganisms. Ann. Rev. of Microbiol. 18:217-252.

3. Alexander, M. 1965. Persistence and biological reactions of pesticides in soils. Soil Sci. Soc. Am Proc. 29:1-7.

4. Allen, M. B., and Van Niel, C. B. 1952. Experiments on bacterial denitrification. Journ. Bacteriol. 64: 397-412.

5. Allison, F. E., and Carter, J. N. 1960. Investigations on denitrification in well-aerated soils. Soil Sci. 90:173-177.

6. Audus, L. J. 1964. Herbicide behavior in the soil. p. 163-203. In L. J. Audus (ed.). The physiology and biochemistry of herbicides. Academic Press, London and New York.

7. Bartley, C. E. 1957. Simazine and related triazines as herbicides. Agr. Chem. Vol. 12, No. 5:34-36.

8. Blackwood, A. C., and Neish, A. C. 1957. Pyocyanine formation from labelled substrates by Pseudomonas aeruginosa. Can. Jour. Microbiol. 3:165-169.

9. Bremner, J. M. 1957. Studies on soil humic acids: II, Observations on the estimation of free amino groups. Reactions of humic acid and lignin preparations with nitrous acid. Jour. Agr. Sci. 48:352-353.

10. Bremner, J. M., and Shaw, K. 1958. Denitrification in soil. II. Factors affecting denitrification. Jour. Agr. Sci. 51:40-52.

11. Broadbent, F. E., and Clark, F. E. 1965. Denitrification. p. 344-359. In W. V. Bartholomew and F. E. Clark (eds.). Soil Nitrogen. American Society of Agronomy, Inc., Madison.

12. Broadbent, F. E., and Stojanovic, B. J. 1952. The effect of partial pressure of oxygen on some soil nitrogen transformations. Soil Sci. Soc. Am. Proc. 16: 359-363.

13. Burges, A., and Fenton, E. 1953. The effect of CO_2 on the growth of certain soil fungi. Trans. Brit. Mycol. Soc. 36:104-108.

14. Burnside, O. C., Schmidt, E. L., and Behrens, R. 1961. Dissipation of simazine from the soil. Weeds 9:477-484.

15. Burton, M. O., Eagles, B. A., and Campbell, J. J. R. 1948. The mineral requirement for pyocyanin production. Can. Jour. Res., C, 26:15-22.

16. Cacciari, I. 1966. Modifications in the biological activity of soils treated with CO_2. Agrochimica 10: 142-148.

17. Cady, F. B., and Bartholomew, W. V. 1961. Influence of low pO_2 on denitrification processes and products. Soil Sci. Soc. Am. Proc. 25:362-365.

18. Chang, J. N., and Morris, J. G. 1962. Studies on the utilization of nitrate by Micrococcus denitrificationes. Jour. Gen. Microbiol. 29:301-310.

19. Clark, F. E. 1949. Soil microorganisms and plant roots, p. 241-282. In A. G. Norman (ed.). Advances in agronomy, 1. Academic Press, New York.

20. Clark, F. E., Beard, W. E., and Smith, D. H. 1960. Dissimilar nitrifying capacities of soils in relation to losses of applied nitrogen. Soil Sci. Soc. Am. Proc. 24:50-54.

21. Conn, H. J., Bartholomew, J. W., and Jennison, M. W. 1957. Staining methods, p. 21. In Manual of Microbiological Methods. McGraw-Hill Book Company, Inc., New York, Toronto and London.

22. Cook, D. W., Brown, L. R., and Tischer, R. G. 1964. A carbon dioxide requirement for the initiation of growth of germinated spores of two thermophilic Bacilli. Developments Indust. Microbiol. 5:326-333.

23. Cook, F. D., and Lochhead, A. G. 1959. Growth factor relationships of soil microorganisms as affected by proximity to the plant root. Can. Jour. Microbiol. 5:323-334.

24. De Ley, J. 1964. Pseudomonas and related genera. Ann. Rev. Microbiol. 18:17-46.

25. Delwiche, C. C. 1956. Denitrification, p. 233-256. In W. D. McElroy and B. Glass (ed.) Inorganic nitrogen metabolism. Johns Hopkins Press, Baltimore.

26. Delwiche, C. C. 1959. Production and utilization of nitrous oxide by Pseudomonas denitrificans. Jour. Bact. 77:55-59.

27. Durbin , R. D. 1955. Straight-line function of growth microorganisms at toxic levels of CO_2. Science 121: 734-735.

28. Eno, C. F. 1958. Nitrification in Florida soils. Fla. Agr. Exp. Sta. Ann. Rpt. Project 771. 160 p.

29. Ercegovich, C. D. What happens to the triazines in soil. Geigy Agr. Chem. Ardsley, New York.

30. Farmer, F. H., Benoit, R. E., and Chappell, W. E. 1965. Simazine, its effect on nitrification and its decomposition by soil microorganisms. Northeastern Weed Conf. 350-352.

31. Frank, L. H., and DeMoss, R. D. 1959. On the biosynthesis of pyocyanine. Jour. Bacteriol 77:776-782.

32. Freney, J. R. 1965. Increased growth and uptake of nutrients by corn plants treated with low levels of simazine. Australian Jour. Agri. Res. 16:257-263.

33. Friedheim, E. 1931. Pyocyanin, an accessory respiratory enzyme. Jour. Exptl. Med. 54:207-221.

34. Greenwood, D. J. 1962. Nitrification and nitrate dissimilation in soil. II. Effect of oxygen concentration. Plant and Soil 17:378-391.

35. Grossowicz, N., Hayat, P., and Halpern, Y. S. 1957. Pyocyanine biosynthesis by Pseudomonas aeruginosa. Jour. Gen. Microbiol. 16:576-583.

36. Guillemat, J. 1960. Interactions entre la simazine et la mycoflore de sol. Acad. Sci., Compt. Rend. 250:1343-1344.

37. Guillemat, J., Charpentier, M., Tardieux, P., and Pochon, J. 1960. Interactions between a chloro-aminotriazine herbicide and the fungal and bacterial microflora of the soil. Ann. Agron. Paris 11:261-294.

38. Hays, E. E., and Wells, I. C. 1945. Antibiotic substances produced by Pseudomonas aeruginosa. Jour. Biol. Chem. 159:725-750.

39. Hauck, R. D., and Melsted, S. W. 1956. Some aspects of the problem of evaluating denitrification in soils. Soil Sci. Soc. Am. Proc. 20:361-364.

40. Jansson, S. L., and Clark, F. E. 1952. Losses of nitrogen during decomposition of plant material in the presence of inorganic nitrogen. Soil Sci. Soc. Am. Proc. 16:330-334.

41. Karnatz, H. 1964. Weitere Versuchsergebnisse bei der Bekampfung von Ungrasern in Obstanlagen. Mitt. Obstbauversuchsringes Alten Landes 19:109-117.

42. Katznelson, H. 1960a. Observations on the rhizosphere effect, p. 192-201. In D. Parkinson and J. S. Waid (eds.) The ecology of soil fungi. Liverpool Univ. Press, Liverpool.

43. Katznelson, H. 1960b. Recent studies on the rhizosphere phenomenon. Int. Congr. Soil Sci., Trans. 7th (Madison, Wis.) II:537-542.

44. Katznelson, H., Lochhead, A. G., and Timonin, M. I. 1948 Soil microorganisms and the rhizosphere. Bot. Rev. 14:543-587.

45. Katznelson, H., and Rouatt, J. W. 1957. Studies on the incidence of certain physiological groups of bacteria in the rhizosphere. Can. Jour. Microbiol. 3:265-269.

46. Kaufman, D. D., Kearney, P. C., and Sheets, T. J. 1965. Microbial degradation of simazine. Jour. Agri. Food Chem. 13:238-241.

47. Kefauver, M., and Allison, F. E. 1957. Nitrite reduction by Bacterium denitrificans in relation to oxidation-reduction potential and oxygen tension. Jour. Bacteriol. 73:8-14.

48. Kluyver, A. J., and Verhoeven, W. 1954. Studies on true dissimilatory nitrate reduction. IV. An adaption in Micrococcus denitrificans. Antonie van Leeuwenhoek 20:337-358.

49. Klyuchnikov, L., Yu., Petrova, A. N., and Polesko, Yu. A. 1964. Effectof simazine and atrazine on microflora of sandy soil. Mikrobiologiya 33:992-996.

50. Knight, B. C., and Proom, H. 1950. A comparative survey of the nutrition and physiology of mesophilic species in the genus Bacillus. Jour. Gen. Microbiol. 4:508-538.

51. Lamanna, C., and Mallette, M. F. 1965. Basic bacteriology, its biological and chemical background. Williams and Wilkins Co., Baltimore. 1001 p.

52. Lenhoff, H. 1963. An inverse relationship in the effects of oxygen and iron on the production of fluorescin and cytochrome by Pseudomonas fluorescens. Nature 199:601-602.

53. Lingappa, Y., and Lockwood, J. L. 1962. Chitin media for selective isolation and culture of actinomycetes. Phytopath. 52(4):317-323.

54. Liu, P. V. 1964. Factors that influence toxigenicity of Pseudomonas aeruginosa. Jour. Bacteriol. 88:1421-1427.

55. Lochhead, A. G. 1940. Qualitative studies of soil microorganisms: III. Influence of plant growth on the character of bacterial flora. Can. Jour. Res., C, 18:42-53.

56. Lochhead, A. G., and Chase, F. E. 1943. Qualitative studies of soil microorganisms. V. Nutritional requirements of the predominant bacterial flora. Soil Sci. 55:185-195.

57. Lochhead, A. G., and Rouatt, J. W. 1955. The rhizosphere effect on the nutritional groups of soil bacteria. Soil Sci. Soc. Am. Proc. 19:48-49.

58. Lochhead, A. G., and Thexton, R. H. 1947. Qualitative studies of soil microorganisms. VII. The rhizosphere effect in relation to the amino acid nutrition of bacteria. Can. Jour. Res., C, 25:20-26.

59. Mahendrappa, M. K., and Smith, R. L. 1967. Some effects of moisture on denitrification in acid and alkaline soils. Soil Sci. Soc. Am. Proc. 31:212-215.

60. Marshall, R. O., Dishburger, H. J., MacVicar, R., and Hallmark, G. D. 1953. Studies on the effect of aeration on nitrate reduction by Pseudomonas species using N^{15}. Jour. Bacteriol. 66:254-258.

61. Martin, J. P. 1950. Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. Soil Sci. 69:215-233.

62. McGarity, J. W. 1961. Denitrification studies on some South Australian soils. Plant and Soil 14:1-21.

63. Meiklejohn, J. 1940. Aerobic denitrification. Ann. Appl. Biol. 27:558-573.

64. Meshkov, N. V. 1959. The effect of thiamin and biotin on the development of some soil microbes. Mikrobiologiya 28:894-899.

65. Mikhaleva, V. V. 1965. Effect of denitrifying bacteria on plant growth. Agrobiologiya No. 4:587-593.

66. Mortland, M. M., and Wolcott, A. R. 1965. Sorption of inorganic nitrogen compounds by soil materials, p. 150-197. In W. V. Bartholomew and F. E. Clark (ed.) Soil nitrogen. Amer. Soc. Agron. Inc., Madison, Wisconsin.

67. Mützе, B. 1963. The effect of light on Micrococcus denitrificans. Arch. Mikrobiol. 46:402-408.

68. Nepomiluev, V. F., Grechin, I. P., and Kuzyakina, T. I. 1966. Effect of simazine on microflora and microbiological processes in dernopodzolic soil tilled by various methods. Izv. timiryazev. sel-khoz. Akad No. 2:128-136.

69. Nommik, H. 1956. Investigations on denitrification in soil. Acta Agri. Scand. VI:195-228.

70. Pantos, G., Gyurko, P., and Takacs, T. 1964. The effect of weedkillers used in practice on some species of the microflora and microfauna in soil and on certain mycorrhizal fungi, and the biological decomposition of herbicides. Acta Agron. Acad. Sci. Hungary 13:21-60.

71. Payne, T. M. B., Rouatt, J. W., and Lochhead, A. G. 1957. The relationship between soil bacteria with simple nutritional requirements and those requiring amino acids. Can. Jour. Microbiol. 3:73-80.

72. Pichinoty, F. 1964. The nitrate reductases of a denitrifying bacterium. Biochim. Biophys. Acta 89:378-381.

73. Pichinoty, F. 1965. The inhibition by oxygen of bacterial denitrification. Suppl. Annls. Inst. Pasteur, Paris 109, No. 3:248-255.

74. Ragab, M. T. H., and McCollum, J. P. 1961. Degradation of C^{14}-labeled simazine by plants and soil microorganisms. Weeds 9:72-84.

75. Ries, S. K., and Gast, A. 1965. The effect of simazine on nitrogenous components of corn. Weeds 13: 272-274.

76. Ries, S. K., Larsen, R. P., and Kenworthy, A. L. 1963. The apparent influence of simazine on nitrogen nutrition of peach and apple trees. Weeds 11:270-273.

77. Ries, S. K., and Tweedy, J. A. 1967. Effect of simazine on nitrate reductase activity in corn. Plant Physiol. 42:280-282.

78. Ringen, L. M., and Drake, C. H. 1952. A study of the incidence of Pseudomonas aeruginosa from various natural sources. Jour. Bact. 64:841-845.

79. Rouatt, J. W. 1959. Initiation of the rhizosphere effect. Can. Jour. Microbiol. 5:67-71.

80. Rouatt, J. W., and Katznelson, H. 1957. The comparative growth of bacterial isolates from rhizosphere and non-rhizosphere soils. Can. Jour. Microbiol. 3: 271-275.

81. Rouatt, J. W., Katznelson, H., and Payne, T. M. B. 1960. Statistical evaluation of the rhizosphere effect. Soil Sci. Soc. Am. Proc. 24:271-273.

82. Rovira, A. D. 1965. Interactions between plant roots and soil microorganisms. Ann. Rev. Microbiol. 19: 241-266.

83. Rovira, A. D., and Harris, J. R. 1961. Plant root excretions in relation to the rhizosphere effect. V. The exudation of B-group vitamins. Plant and Soil 14:199-214.

84. Schaefer, W. B. 1957. Studies on the inhibiting effect of CO_2 on the growth of two mutant strains of Mycobacterium tuberculosis. Jour. Bact. 73:52-55.

85. Sheng, M. W. 1966. Microbiological studies on the physiological disease of rice in Taiwan. M.S. Thesis. Mich. State Univ. 122 p.

86. Sherman, V. B. D., and Mac Rae, I. C. 1957. The influence of oxygen availability on the degree of nitrate reduction by Pseudomonas denitrificans. Can. Jour. Microbiol. 3:505-530.

87. Starkey, R. L. 1958. Interrelationships between microorganisms and plant roots in the rhizosphere. Bact. Rev. 22:154-172.

88. Steinbrenner, K., Naglitsch, F., and Schlicht, I. 1960. The effect of the weedkillers simazine and W 6658 on the microorganisms and fauna in soil. Albrecht-Thaer.-Arch. 4:611-631.

89. Stevenson, F. J., and Swaby, R. J. 1964. Nitrosation of soil organic matter: I. Nature of gases evolved during nitrous acid treatment of lignins and humic substances. Soil Sci. 28:773-778.

90. Stotzky, G., and Goos, R. D. 1965. Effect of high CO_2 and low O_2 tensions on the soil microbiota. Can. Jour. Microbiol., 11:853-868.

91. Stotzky, G., and Goos, R. D. 1966. Adaptation of the soil microbiota to high CO_2 and low O_2 tensions. Can. Jour. Microbial. 12:849-861.

92. Stotzky, G., Goos, R. D., and Timonin, M. I. 1962. Microbial changes occurring in soil as a result of storage. Plant and Soil 16:1-19.

93. Sulochana, C. B. 1962. Cotton roots and vitamin-requiring and amino acid-requiring bacteria. Plant and Soil 16:335-346.

94. Talbert, R. E., and Fletchell, O. H. 1964. Inactivation of simazine and atrazine in the field. Weeds 12:33-38.

95. Timonin, M. I. 1946. Microflora of the rhizosphere in relation to the manganese deficiency disease of oats. Soil Sci. Soc. Am. Proc. 11:284-292.

96. Todorović, M., and Grbić, V. 1965. Effect of simazine and atrazine on the soil microflora in vineyards without summer cultivation. Zemlj. Biljka 14:37-50.

97. Treccani, V. 1961. Investigations on the denitrifying power of soil, and a quantitative method of its estimation. Ann. Microbiol. 11:15-18.

98. Tsvetkova, S. D. 1966. The action of simazine and atrazine on agrochemical properties of soil. Vest. Sel-khoz. Nauki, Mosk. 11, No. 2:125-127.

99. Valera, C. L., and Alexander, M. 1959. The significance of pH in bacterial denitrification. Bact. Proc. 25.

100. Valera, C. L., and Alexander, M. 1961. Nutrition and physiology of denitrifying bacteria. Plant and Soil 15:268-280.

101. Verhoeven, W. 1952. Aerobic sporeforming nitrate reducing bacteria. Thesis, Delft.

102. Volk, G. M., and Eno, C. F. 1962. The effect of simazine and atrazine on certain of the soil microflora and their metabolic processes. Florida soils and Crop Sci. Soc. 22:49-56.

103. Wahba, A. H. 1965. Pyrorubrin-producing Pseudomonas aeruginosa. Appl. Microbiol. 13:291-292.

104. Wallace, R. H., and Lochhead, A. G. 1949. Qualitative studies of soil microorganisms. VIII. Influence of various crop plants on the nutritional groups of soil bacteria. Soil Sci. 67:63-69.

105. Wallace, R. H. and Lochhead, A. G. 1950. Qualitative studies of soil microorganisms. IX. Amino acid requirements of rhizosphere bacteria. Can. Jour. Res., C, 28:1-6.

106. Wasserman, A. E. 1965. Absorption and fluorescence of water soluble pigments produced by four species of Pseudomonas. Appl. Microbiol. 13:175-180.

107. West, P. M., and Lochhead, A. G. 1940. Qualitative studies of soil microorganisms. IV. The rhizosphere in relation to the nutritive requirements of soil bacteria. Can. Jour. Res., C, 18:129-135.

108. Wijler, J., and Delwiche, C. C. 1954. Investigations on the denitrifying process in soil. Plant and Soil 5:155-169.

109. Willis, W. H., and Sturgis, M. B. 1944. Loss of nitrogen from flooded soil as affected by changes in temperature and reaction. Soil Sci. Soc. Am. Proc. 9:106-113.

110. Woldendorp, J. W. 1962. The quantitative influence of the rhizosphere on denitrification. Plant and Soil 17:267-270.

111. Woldendorp, J. W. 1963. The influence of living plants on denitrification. Medeelingen van de Landbouwhogeschool te Wageningen. Nederland. 63(13):1-100.

112. Wullstein, L. H., and Gilmour, C. M. 1964. Non-enzymatic gaseous loss of nitrite from clay and soil systems. Soil Sci. 97:428-430.

113. Young, G. 1947. Pigment Production and antibiotic activity in cultures of Pseudomonas aeruginosa. Jour. Bact. 54:109-117.