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Characterization of a soil heterotrophic nitrifier and its synergistic interactions with Nitrobacter agilis.

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CHARACTERIZATION OF A SOIL HETEROTROPHIC NITRIFIER AND ITS SYNERGISTIC INTERACTIONS WITH *NITROBACTER AGILIS*

A Dissertation Presented

By

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This dissertation is dedicated to my wife Dorothy, to my parents, and to my wife's parents. I dedicate this thesis to them for their constant support, encouragement, and faith in me. This dissertation is also dedicated to my infant daughter, Nancy Alyson, as she represents the hope and future of my life and career.

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ABSTRACT

Characterization of a Soil Heterotrophic Nitrifier

and Its Synergistic Interactions with Nitrobacter Agilis

(September 1980)

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The ecological importance of heterotrophic nitrification has been difficult to assess because of the low levels of nitrification associated with the phenomenon. Local soils (Amherst and Hadley, Massachusetts) were used as inocula in a mineral salts pyruvic-oxime (CH_3-C(=NOH)-COOH) medium. Nitrification of pyruvic-oxime in these enrichments occurred with the production of nitrite and nitrate.

Three isolates, all members of the genus Alcaligenes, proved to be particularly active in the oxidation of pyruvic-oxime to nitrite and nitrate. Quantities as large as 1867 mg nitrite-nitrogen/l and 42 mg nitrate-nitrogen/l were synthesized by the most active of the three isolates (Alcaligenes sp. strain OS1) when cultured in 2.5% pyruvic-oxime mineral salts medium. Growth and nitrification of the Alcaligenes spp. were closely correlated and Alcaligenes sp. strain OS1 demonstrated a remarkable tolerance to hydroxylamine-nitrogen, remaining viable in media containing up to 325 mg hydroxylamine-nitrogen/l.

Alcaligenes sp. strain OS1 was also capable of nitrification in

media at pH 5.4. However, nitrification observed under these conditions was slight and required lengthy periods of time when compared to nitrification by the organism at a neutral pH.

Nitrobacter agilis, American Type Culture Collection #14123, was unable to produce nitrate from pyruvic-oxime. N. agilis was also unable to oxidize nitrite to nitrate when pyruvic-oxime was present in the medium. As little as 5 mg hydroxylamine-nitrogen/l was toxic to N. agilis and prevented the synthesis of nitrate from nitrite when present for one day in the culture medium.

Pyruvic-oxime, however, was oxidized to nitrate when Alcaligenes sp. strain OS1 and N. agilis were cultured jointly. The Alcaligenes sp. oxidized the pyruvic-oxime to nitrite during the first few days of joint culturing followed by the subsequent depletion of nitrite and concomitant production of nitrate in the presence of N. agilis. Thus, the joint culturing of the two organisms resulted in a sequential nitrification pattern not observed when either organism was cultured separately. The ecological significance of such a synergistic system is discussed.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Nitrification is typically regarded as a largely chemoautotrophic process. This classical view, however, does not preclude the role of heterotrophs. Indeed a significant body of literature consistently refers to nitrification which occurs as a result of the metabolic activities of heterotrophs.

Heterotrophic nitrification may have been discounted as an important component of nitrification because of the many intermediates it may generate when compared to autotrophic nitrification. For instance, the oxidation of ammonium ion (NH_4^+) to nitrite (NO_2^-) and of NO_2^- to nitrate (NO_3^-) by the chemoautotrophs Nitrosomonas and Nitrobacter, respectively, results in a more or less clearly defined process when compared to the various products attributed to heterotrophic activity. Also, concentrations of products differ greatly between the two forms of nitrification; the yields of NO_2^- and NO_3^- produced by the chemoautotrophs are immense when compared to the product yields from most heterotrophic oxidations. However, the abundance of different heterotrophic nitrifiers and their occurrence in virtually all environments compels serious consideration when one attempts to assess their importance. In addition, scant attention has been paid to the possible interrelationships between the autotrophs and the heterotrophs. As a result, the processes have been viewed as being quite distinct, if not mutually exclusive, although there is little data to support this conclusion.

The current study was initiated to ascertain if synergistic nitrification could occur between a chemoautotroph and a chemoheterotroph. A major obstacle first had to be overcome, however, if significant work was to be performed: a heterotroph which yielded relatively large concentrations of a nitrification product was needed so that a clearly discernible association between the nitrification processes of both the autotroph and the heterotroph could be studied. Thus, this thesis is concerned both with the nitrification of heterotrophic bacteria which were isolated from local soils and of the subsequent sequential nitrification obtained when the heterotroph and the autotroph (Nitrobacter agilis, ATCC #14123) were cultured jointly.

CHAPTER II

LITERATURE REVIEW

Sources and Products of Heterotrophic Nitrification

Recognition of heterotrophic nitrification. Nitrification due to heterotrophic metabolism was noted as early as 1931. In that year, Cutler and Mukerji (1931) published data which showed that eight bacterial strains isolated from soil oxidized ammonium ion to nitrite. These results were considered highly unusual since ammonium ion oxidation had hitherto been attributed solely to the metabolism of the chemoautotroph Nitrosomonas and other chemolithotrophic ammonium ion oxidizers.

Jensen (1951) noted that three distinct groups of heterotrophs were capable of converting pyruvic-oxime (PO) to NO_2^-. Subsequently, Schmidt (1954) noted that Aspergillus flavus produced NO_3^- from ammonium ion or organic nitrogen (peptone and yeast extract) as well as producing nitrite. The importance of this discovery was twofold: first, it established that fungi could nitrify heterotrophically, and second, that the final stage of classical nitrification, i.e., the production of NO_3^-, could also be accomplished by heterotrophic nitrifiers. Gunner (1963) added further evidence for the heterotrophic nitrification of NH_4^+ to NO_3^- when he reported that Arthrobacter globiformis also effected this conversion. That the ability to nitrify heterotrophically was a ubiquitous trait was shown when 7% of the microbial isolates from twelve different Minnesota soils were observed to be active heterotrophic nitrifiers (Eylar, et al., 1959). Furthermore, these

authors showed that more than 2% of the isolates formed NO_2^- in excess of 0.5 µg NO_2^- -N/ml. With the publication of this landmark study heterotrophic nitrification had to be acknowledged as a widely distributed capacity among the soil microbial populations and not simply as a laboratory artifact. The acceptance of heterotrophic nitrification gradually led to a redefining of the nitrification process to include the heterotrophic contribution. Thus, Alexander, et al., by 1961, proposed a more general definition for nitrification, that is, "as the biological conversion of the nitrogen in organic or inorganic compounds from a reduced to a more oxidized state."

Ammonium ion as a nitrogen source. One of the most commonly studied sources of heterotrophically oxidized nitrogen is ammonium ion. This ion is typically metabolized to NO_2^- by the chemoautotrophic bacteria. Heterotrophic nitrifiers also produce NO_2^- from NH_4^+, but in addition, the heterotrophs also yield a wide variety of other nitrogenous products.

Oxidation of NH_4^+ to NO_2^- has been reported by many investigators (Cutler and Mukerji, 1931; Schmidt, 1954; Fisher, et al., 1956; Marshall and Alexander, 1961; Hirsch, et al., 1961; Marshall and Alexander, 1962; Gunner, 1963; Verstraete and Alexander, 1972a; Verstraete and Alexander, 1972b; Gowda, et al., 1976; Romanovskaya, et al., 1977; Tate, 1977; and Witzel and Overbeck, 1979). Though nitrite is the most commonly observed product of heterotrophic nitrification of NH_4^+, NO_3^- is also frequently produced, reportedly mainly due to fungal activity (Schmidt, 1954; Marshall and Alexander, 1961; Hirsch,

et al., 1961; Marshall and Alexander, 1962; and Remacle, 1977). The concentrations of NO_3^--N produced by the fungi range from 0.16-35 µg NO_3^--N/ml and are generally greater than the concentrations of NO_2^--N observed from both fungal and bacterial nitrification of NH_4^+. Bacteria, however, also metabolize NH_4^+ to NO_3^-, the concentrations of nitrate produced generally being less than those generated by fungal metabolism, in the range of 1.0 - 14.0 µg NO_3^--N/ml (Gunner, 1963; Laurent, 1971; Verstraete and Alexander, 1972a; Verstraete and Alexander, 1972b). The ability to produce the classical products of nitrification, i.e., NO_2^- and NO_3^-, is clearly widely dispersed throughout the microbial community.

One important difference between heterotrophic and autotrophic ammonium ion oxidation is the occurrence of nitrogen oxides other than NO_2^-. Ammonium ion has been noted to yield NH_2OH when oxidized by Arthrobacter spp. (Gunner, 1963; Verstraete and Alexander, 1972a; Verstraete and Alexander 1972b; and Berger, et al., 1979) or when oxidized by Aspergillus niger (Steinberg, 1939) or Penicillium solitum (Bergerova and Zamecnik, 1978). Ammonium ion was also cited as one of the nitrogen sources required for the production of 3-nitropropionic acid, or β-nitroproprionic acid (3-NPA). Among microorganisms, fungi have been identified as responsible for 3-NPA biosynthesis. Both Aspergillus flavus (Marshall and Alexander, 1962) and Penicillium atrovenetum (Raistrick and Stossl, 1958; Shaw and Wang, 1964; and Shaw and McCloskey, 1967) produce 3-NPA and have been studied most in an effort to understand this aspect of the biochemistry of heterotrophic nitrification.

Nitrogen oxides other than 3-NPA are also synthesized from ammonium ion. These oxides include bound hydroxylamines [(bound-NH_2OH) defined by McNary and described by Marshall and Alexander (1962) as "any compound which yields free hydroxylamine upon acid hydrolysis"], hydroxamic acids, primary nitro compounds (Verstraete and Alexander, 1972a; Verstraete and Alexander, 1972b), oximes (R-C=NOH), amine oxides, C-Nitroso compounds (Focht and Verstraete, 1977), and N-Nitroso compounds (Verstraete, 1975). The bound-NH_2OH noted by Verstraete and Alexander (1972a) was identified as 1-nitrosoethanol (1-NE). Amine oxides, N-Nitroso, and C-Nitroso compounds heterotrophically produced include, respectively, pulcherriminic acid, streptozotocin, and ferroverdin, as well as 1-NE (Focht and Verstraete, 1977). Table 1 lists the major products of heterotrophic nitrification of NH_4^+ and gives chemical formulas for some of the lesser known compounds.

NH_2OH as a nitrogen source. Oxidation of NH_2OH and of oximes, which function as constant sources of NH_2OH (Amarger and Alexander, 1968), is effected by heterotrophs. DeGroot and Lichtenstein (1960) noted that extracts of Pseudomonas flourescens catalyzed the formation of two hydroxamic acids when NH_2OH was a substrate. In this study, the first hydroxamic acid produced was β-asparthohydroxamic acid, catalyzed from the reactants L- or D-asparagine and NH_2OH, while the second group of products were α-N-alkyl-β-asparto-hydroxamic acids synthesized from DL-α-N-alkyl derivatives of asparagine and NH_2OH. Similarly, NH_2OH condensation resulted in the formation of bound hydroxylamines when either malic or fumaric acids were added to extracts of E. coli together with NH_2OH (Grossowicz and Lichtenstein, 1961). Resting E. coli

TABLE I

Common heterotrophic nitrification products of ammonium ion.

Compound	Chemical Formula
Free NH_2OH	NH_2OH
Oximes	$R-C(=NOH)-$
Hydroxamic Acids (Mono-,di-, and tri-)	$R-C(=O)-NHOH$
Amine Oxides	$R-N(=R)(-R_2)-O$
N-Nitroso Compounds	$N-NO$
C-Nitroso Compounds	$C-NO$
C-Nitro Compounds	$R-CH(=R)-NO_2$

cells also caused the formation of bound hydroxylamines when incubated with organic acids and NH_2OH. Emery (1963) demonstrated that aspartase may be responsible for the production of hydroxamic acids since partially purified aspartase caused the synthesis of N-hydroxyaspartic acid when its substrates were fumaric acid and NH_2OH. Aspartase may also be responsible for NH_2OH tolerance by B. cadaveris since this microbe grew in the presence of approximately 33 µg NH_2OH/ml, a concentration of NH_2OH considered by Emery to be rather large.

NH_2OH also greatly aids in the synthesis of 3-NPA by Penicillium atrovenetum (Shaw and Wang, 1964). Small additions of NH_2OH, less than 2.0 µmoles/ml, resulted in a dramatic increase in 3-NPA synthesis when P. atrovenetum was grown in an NH_4^+ mineral salts medium. Concentrations of greater than 5.0 µmoles/ml, however, resulted in diminishing both 3-NPA synthesis and the growth of the fungus.

NH_2OH is rapidly metabolized by sewage microbes (Verstraete and Alexander, 1973). Nitrite and nitrate were formed in the sewage, although their concentrations were determined to be much less than the NH_2OH added. Similarly, NH_2OH was also rapidly lost from soil samples when added at a concentration of 10 µg NH_2OH-N/g soil. The production of either NO_2^- or NO_3^-, however, was not observed and it was thus assumed that the NH_2OH-N initially added had either volatilized or was bound to soil components.

A soil microorganism, however, was shown to effect directly the transformation of NH_2OH to NO_2^- (Lees, et al., 1954). Resting cells of Nocardia corallina synthesized a maximum of 0.60 µmol NO_2^- when incubated with an initial concentration of 6 µmol NH_2OH. Other

common heterotrophs were subsequently shown to cause the same transformation of NH_2OH to NO_2^- (Castell and Mapplebeck, 1956). Two Pseudomonas spp., Proteus morganii, Proteus vulgaris, and a Microbacterium sp. were all identified as organisms capable of oxidizing NH_2OH to NO_2^-.

Oximes as nitrogen sources. Oximes also serve as sources of NH_2OH (Amarger and Alexander, 1968). Oximes released NH_2OH when supplied as the carbon-nitrogen source for the organism Tetrahymena pyriformis (Seaman, 1954; Seaman, 1956). This protozoan caused NH_2OH to accumulate in the medium since T. pyriformis contains an enzyme capable of metabolizing pyruvic-oxime to both NH_2OH and pyruvate. T. pyriformis thus differs from many other microorganisms since most other oxime metabolizing microbes either effect very little NH_2OH release from oximes or rapidly convert NH_2OH to NO_2^-.

An important question posed by nitrification researchers is where would oximes occur in the environment. This question has been answered partially by the work of Yamafugi, et al., (1950) and Yamafugi and Akita (1952). Yamafugi, et al., reported the presence of oxime-N in a number of plant tissues, including tobacco, mulberry, spinach, onion, and turnip leaves as well as in turnip stems. Selecting spinach as a model, oxime concentration was noted to reach maximal levels as leaf size approached its maximum. Oxime content of the various plants differed with the plants examined, concentrations ranging from 0.01-0.15 mg oxime-N/kg. Oxime content in animal tissues was generally lower than that determined in plants, with levels varying from 0.0012-0.05 mg

oxime-N/kg. Yet, a wide variety of animal tissues, both fresh and autolysed, were positive for oxime presence. Ox kidney, heart, and liver, in addition to silkworm tissues all contained oxime-N (Yamafugi, et al., 1950). Yamafugi and Akita (1952) studied oxime generation from NH_2OH and concluded that:

> hydroxylamine formed in living tissues rapidly combines with carbonyl groups and that oxime group transfers to other compounds when there exist more suitable acceptors. Oximes of pyruvic, oxaloacetic and α-ketoglutaric acids are thus easily produced in organisms.

It thus seems quite likely that oximes exist as natural components in both plant and animal tissues and would therefore enter the soil when such tissues become subject to degradation.

Many soil microorganisms have the capability of oxidizing oxime-N to NO_2^-. Quastel and Scholefield (1949) observed that perfused soils induced to oxidize NH_4^+ and NO_2^- required a lag time to oxidize PO. Since classical NH_4^+ and NO_2^- oxidation inhibitors (urethane, methionine, and potassium chlorate) caused no inhibition of PO oxidation and since PO oxidizing soils did not oxidize NH_4^+, these authors concluded that heterotrophs were responsible for the PO oxidation observed. Quastel, et al., (1950) reported isolating three soil organisms capable of oxidizing PO to NO_2^-. Two of the isolates were Achromobacter spp. and the third was a Corynebacterium sp.

From soil, Jensen (1951) isolated a Norcardia corallina, an Alcaligenes sp., and an Agrobacterium sp. capable of transforming PO to NO_2^-. When these organisms were cultured with 0.015, 0.020, and 0.030 M PO as a carbon source [approx. 0.15%, 0.20%, and 0.30% (w/v)], NO_2^--N was

produced in concentrations not exceeding 40 µg NO_2^--N/ml. Quastel, et al., (1952) also noted NO_2^--N production from PO oxidation by an Achromobacter (Alcaligenes) sp. isolate. These authors reported that PO served as an excellent source of oxidizable N; 10 µmoles PO resulting in 7.39 µmoles of NO_2^--N produced by the Achromobacter sp.

Doxtader and Alexander (1966) isolated from soil a Fusarium sp. capable of yielding up to 25 mg NO_2^--N/l when grown in a PO medium. Replacement cultures of the Fusarium sp. were more adept at producing NO_2^--N, however, yielding approximately 105 mg/l NO_2^--N. Thus a greater than 90% conversion of PO-N to NO_2^--N was demonstrated.

Similarly large NO_2^--N yields were reported by Obaton, et al, (1968) and Amarger and Alexander (1968). Studying the acetaldoxime metabolism of a Pseudomonas aeruginosa isolated from soil, these authors observed that cultures grown in a glucose-peptone medium converted 40 µmoles acetaldoxime [approximately 0.24% w/v] to 284 µg NO_2^--N/ml. The largest yields of heterotrophic nitrification products reported are of oxime metabolism and may reflect the low concentration of NH_2OH released. Thus, the low NH_2OH concentrations associated with oxime equilibriums may allow microbial metabolism to proceed unimpeded by the toxic effects of larger concentrations of NH_2OH.

Pyruvic-oxime and acetaldoxime are not the only oximes which are metabolized to NO_2^- by soil microbes. Oxaloacetic-oxime (Quastel, et al., 1950), phenylpyruvic acid-oxime, and α-ketoglutaric-oxime are similarly converted to nitrite (Quastel, et al., 1952a). Furil dioxime, arabinose-oxime, and salicylaldoxime are examples of oximes which were not reported metabolized by the soil microflora.

Nitro compounds as nitrogen sources. Nitro compounds, including 3-NPA, undergo oxidation by common heterotrophic microbes. Little (1951) described the oxidation of nitroethane, 1-nitropropane, and 2-nitropropane by Neurospora crassa. Since only aliphatic nitro compounds were metabolized, Little concluded that N. crassa enzymatic activity was limited to this class of compounds. The general mechanism of nitro compound oxidation by N. crassa is represented by the reaction of nitroethane oxidation:

$$CH_3CH_2NO_2 + O_2 + H_2O \leftrightarrow CH_3CHO + HNO_2 + H_2O_2$$ (Little, 1951).

Kido, et al., (1975), working with cultures of Hansenula beijerinckii, Candida utilis, and Penicillium chrysogenum grown in a 0.5% nitroethane medium, described a nitro compound oxidizing enzyme which catalyzed the formation of nitrite and acetone from 2-nitropropane and also of nitrite and acetaldehyde from nitroethane. Pseudomonas aeruginosa metabolizes nitroethane, 2-nitropropane, and 2-nitrobutane in a manner analogous to that of the fungi, with NO_2^- being the nitrogen product (Amarger and Alexander, 1968). Arthrobacter is also capable of oxidizing primary nitro compounds, as both NO_2^- and NO_3^- were synthesized when nitroethane, 2-nitroethanol, and 3-nitropropionate (3-NPA) were used as nitrogen sources (Verstraete and Alexander, 1972c). Corynebacterium simplex produces nitrite when cultured in media containing 0.02% 4,6-dinitro-O-cresol (DNOC) (Sundersen and Jensen, 1956). Once induced to metabolize DNOC, C. simplex also oxidizes related nitro compounds such as p-nitrophenol, 2,4-dinitrophenol, 2,4,6-trinitrophenol (picric acid) and yields NO_2^- as the nitrogen

product. Picric acid, O- and p-nitrophenol, trinitro-resorcinol, and nitrobenzene were oxidized by microbes other than C. simplex (Micromonospora, spp., unidentified proactinomycetes, and a Pseudomonas sp.) and thus led to the conclusion that soil microbes have the capacity to oxidize these types of nitro compounds. The importance of these oxidations becomes evident when one considers that the above mentioned compounds are used as herbicides.

3-NPA, however, differs from the herbicides since it is both a plant and fungal product. Neurospora crassa was as active in converting 3-NPA to NO_2^- as it was in oxidizing nitroethane, the chemical by which N. crassa was induced to metabolize aliphatic nitro compounds (Little, 1951). Indeed, Little hypothesized that since 3-NPA is the natural substrate, perhaps aliphatic nitro compound metabolism in N. crassa relates primarily to 3-NPA metabolism.

Unlike the enzyme of N. crassa, an Aspergillus flavus enzyme capable of yielding NO_2^- from 3-NPA had no apparent activity with the aliphatic nitro compounds tested (Marshall and Alexander, 1962). Thus, A. flavus extracts were without action on nitromethane, nitroethane, 1-nitro-propane, and 2-nitropropane. The disparity between the N. crassa and A. flavus systems is suggestive of the metabolic diversity which exists among the heterotrophic nitrifiers.

Doxtader and Alexander (1966a, 1966b) and Molina and Alexander (1971) observed NO_3^- production from 3-NPA oxidation. Both growing and replacement cultures of A. flavus and replacement cultures of Penicillium atrovenetum were active in the conversion. Growth conditions and physiological state thus are important in defining the nitrification of 3-NPA.

Aromatic nitro compounds are oxidized to NO_2^- and NO_3^- in a fashion similar to aliphatic nitro compound oxidation. Focht and Verstraete (1977) reported that various authors cite such organisms as Aspergillus flavus, Arthrobacter spp., Norcardia sp., Flavobacterium sp., and Pseudomonas sp. as capable of effecting these transformations.

Gaseous nitrogen products. The role of heterotrophic nitrification in the generation of gaseous nitrogen products remains unresolved. Gunner (1963) reported that actively nitrifying Arthrobacter globiformis cultures synthesized gaseous nitrogen products, although the exact nature of the gas or gases was not determined. Though the presence of nitric oxide was indicated, nitric oxide did not account for the total yield of the nitrogen gases and Gunner concluded that other gaseous nitrogen oxides might be produced.

Dissimilar results were recorded by Verstraete and Alexander (1972a). These authors reported that neither nitrous oxide, nitric oxide, nor nitrogen dioxide were detected in the gaseous phase over actively growing and nitrifying cultures of an Arthrobacter sp. Again, the metabolic diversity of heterotrophic nitrifiers is examplified by these studies. The need to monitor gaseous nitrogen products of active heterotrophic nitrifiers is apparent if a better understanding of this phenomenon is to result.

Nitrite as a nitrogen source. Nitrite serves as a source for heterotrophic nitrification in at least two ways. First, it may be metabolized to form toxic nitroso compounds (Ayanaba and Alexander, 1973; Ayanaba, et al., 1973). Nitrite, when present with dimethylamine, was converted

to a nitrosamine by an unidentified bacterium and was also produced by Eschericha coli, Streptococcus epidermis, and Aspergillus oryzae when NO_3^- was substituted for NO_2^-. Ayabana and Alexander reported that soluble enzymes from these organisms catalyzed the formation of dimethylnitrosamine and N-nitrosodiphenylamine. The enzymes from these organisms were also capable of synthesizing N-nitroso compounds from the corresponding dialkylamines.

The second mechanism by which nitrite functions as a heterotrophic nitrogen source is in the synthesis of nitrate (Doxtader and Alexander, 1966b; Molina and Alexander, 1972). As great as 90% of the NO_2^--N present was oxidized to NO_3^--N by Aspergillus flavus extracts. Oxidation of NO_2^- to NO_3^- is not limited to A. flavus, however, as a Penicillium sp. was also noted to effect this reaction (Focht and Verstraete, 1977).

Organic nitrogen and soil extract as nitrogen sources. In addition to the types of organic nitrogen already noted, soil extract and other organic nitrogen sources serve as substrates for heterotrophic nitrification. Much of the earliest work focused on the nitrification of complex media in which either yeast extract (YE), peptone, and amino acids were the nitrogen sources. Aspergillus flavus, A. oryzae, A. wentii, and Penicillium atrovenetum have been noted to synthesize NO_2^- and/or NO_3^- from peptone (Marshall and Alexander, 1962; Schmidt, 1960a; VanGool and Schmidt, 1973; Marshall, 1965; and Gowda, et al., 1976) and from a peptone-YE medium (Schmidt, 1954). In addition, YE served as sole nitrogen source for the heterotrophic nitrifiers A. flavus and A. parasiticus which produced NO_3^- (Shih, et al., 1974).

A wide variety of amino acids function as substrates for heterotrophic nitrification. Hatcher and Schmidt (1971) reported that L-aspartate and L-glutamate were the best sources for nitrification by A. flavus. However, all of the amino acids, with the exception of cysteine, methionine, L-histidine, DL-phenylalanine, and DL-tryptophan, supported some nitrification by A. flavus with bound NH_2OH, NO_2^-, and NO_3^- production occurring. Bergerova and Bernat (1976) reported similar results since their strain of A. flavus nitrified all amino acids except L-cysteine and DL-typtophan.

A. flavus also nitrified amino acids other than the common eighteen amino acids noted. This fungus attacks α-alanine, β-alanine (Alexander, et al., 1960) and α-aminobutyric acid (Doxtader and Alexander, 1966b). Curiously, A. flavus enhances its production of nitrate when either β-alanine or L-aspartate is added to the peptone growth medium (Marshall, 1965; VanGool and Schmidt, 1973). An explanation for this stimulation has not yet been proposed.

Penicillium spp., including P. atrovenetum (Shaw and McCloskey, 1967; Shaw and Wang, 1964) and P. solitum WESTLING (Bergorova and Zamecnik, 1978) also yielded more heterotrophic nitrification products when aspartate was included in the culture medium. However, an unusual fact noted by Bergerova and Zamecnik is that P. solitum WESTLING favors the production of NH_2OH (versus NO_2^-) when cultured on either a L-asparagine or a DL-valine medium. Again, the explanation for such a product preference remains unresolved.

Since much NH_4^+ - N is yielded from the metabolism of YE, peptone, and the amino acids (Schmidt, 1954; Hatcher and Schmidt, 1971), many

authors have attributed the nitrification of such sources as an artifact of NH_4^+ metabolism. Yet reports have noted that NH_4^+ in and of itself was either not preferred to organic-nitrogen sources (Shaw and McCloskey, 1967) or was in no way stimulatory to the production of nitrification products (Ishaque and Cornfield, 1974). Thus, the role of the carbon moiety, with and without its organic nitrogen, appears of great importance in heterotrophic nitrification.

Examples of other organic nitrogen sources for nitrification include urea and oxamide (Ishaque and Cornfield, 1974). Soil microflora also nitrify such urea derivatives as 1,3-dimethylurea, 1,1-dimethylurea, methylurea, phenylurea, and n-butylurea with NO_2^- being produced (Doxtader and Alexander, 1966c). Less well defined sources of organic nitrogen noted to result in heterotrophic nitrification include manure, oat, barley, alfalfa, and wheat straws (Schmidt, 1960b) as well as non-viable cells of the alga Trichodesmium erythraeum (Rajendran and Venugopalan, 1976). It can thus be concluded that a wide variety of organic nitrogen sources, as well as the inorganic nitrogen sources previously described, undergo heterotrophic oxidation.

Soil extract is also nitrified by various heterotrophs. Gram negative short rods isolated from soil produced significantly less NO_2^- when grown in defined media (either acetate or glucose serving as the carbon source) than when cultured with soil extract as the energy source (Fisher, et al., 1956). Similarly, Tate (1977) noted that an Arthrobacter sp. produced more NO_2^- in an unamended sterile soil than when NH_4^+ was added to the soil. Tate noted, however, that acetate and NH_4^+ resulted in the greatest yield of NO_2^- by the Arthrobacter sp.

Ishaque and Cornfield (1976) noted a similar depression of heterotrophic nitrification when NH_4^+ was added to the soil. Odu and Adeoye (1970) reported that at least three of their bacterial isolates produced neither NO_2^- nor NO_3^- from peptone but did yield these products when cultured in media containing soil organic matter extract. Partial explanations for these results have been proposed by both Odu and Adeoye (1970) and Ishaque and Cornfield (1976). The former authors suggested that pure culture studies are inadequate since microbial metabolic succession does not occur and will thus not reflect the true nature of the soil's organic nitrogen content. The latter authors suggested that the natural organic nitrogen of soils is metabolized to a much greater extent by soil heterotrophs than the defined nitrogen supplied in laboratory studies. Until more exacting data on the nature of heterotrophic nitrification substrates becomes known, it is likely that soil extract may continue to function as a greater source for heterotrophic nitrification than the nitrogen added in defined media studies.

Further metabolism of heterotrophic nitrification products. Heterotrophic nitrifiers also metabolize some of the more oxidized nitrogen products. A primary example, as already noted, is the oxidation of 3-NPA by *Aspergillus flavus* and *Penicillium atrovenetum*, to NO_2^- and/or NO_3^-. An interesting phenomenon is the metabolism of NO_3^- by *P. atrovenetum*. This fungus, when grown in a chemically defined medium with sodium nitrate as the sole nitrogen source, produces 3-NPA as a metabolite (Raistrick and Stossl, 1958). Although 3-NPA yields are better with NH_4^+ as the nitrogen source, significant yields of 3-NPA (approximately 100 mg/l)

were noted with NO_3^- as the nitrogen source.

A more general occurrence is that observed by Cutler and Mukerji (1931). These authors reported that six of eight bacterial isolates cause NO_2^- reduction after the initial phase of nitrification had occurred. The authors suggested that NO_2^- might be metabolized by these bacteria since assimilation of NO_2^- was observed. Accordingly, it is of interest to note that Pseudomonas spp., Alcaligenes spp., Bacillus subtilus, Flavobacterium, and Proteus spp. (Castell and Mapplebeck, 1956) metabolized the NO_2^- produced from NH_2OH. Since all of these microbes are capable of NO_3^-/NO_2^- reduction (Buchanan and Gibbons, 1974) and as Pseudomonas and Alcaligenes spp. are vigorous denitrifiers, perhaps the further metabolism of NO_2^- and NO_3^- reflects the broad metabolic capacities, concerning nitrogen, of these heterotrophic nitrifiers.

Substrates for heterotrophic nitrification may arise from the metabolism of denitrifiers and nitrogen fixers. Saris and Virtanen (1957) reviewed the literature and observed that Blom reported nitrogen gas (N_2) assimilation by Azotobacter cultures which resulted in small concentrations of NH_2OH being produced. Not only did N_2 serve as a source for NH_2OH, but NO_3^- functioned in this role as well. Ammonium ion, however, resulted in the production of no NH_2OH. These same authors also reported that Virtanen and Jurvinen and Virtanen and Hakala observed bound-NH_2OH production by A. vinelandii when either NO_3^-, N_2, or NH_4^+ served as the nitrogen source. Nitrate and N_2 gas, however, resulted in more rapid production of bound-NH_2OH than did NH_4^+. Saris and Virtanen (1957) reported not only bound-NH_2OH synthesis by A. vinelandii, but also the further metabolism of this product such that it was not noted four

days after it had been produced.

Hilali and Molina (1979), working with soil suspensions, noted rapid immobilization of nitrogen when NO_3^- was inoculated into the experimental apparatus. These authors suggested that such immobilized nitrogen might be retained in the microbes and the matrix of the experimental system. Most interesting, however, was their suggestion that the immobilized nitrogen might be in forms such as nitro compounds or bound-NH_2OH. Such molecules, in a soil, could be readily converted to NO_2^- if the appropriate heterotrophic nitrifiers were present. It is therefore possible that heterotrophic nitrifiers may produce their substrates for nitrification under appropriate environmental conditions thus forming an integral component of nitrogen cycling in the soil and other ecosystems.

The Ecology of Heterotrophic Nitrification

The ecology of heterotrophic nitrification is diverse. Nitrifiers from varying habitats have been isolated and a wide phylogenetic range of organisms has been described. Nitrification occurs in widely differing habitats and its occurrence can not always be attributed to either Nitrosomonas and Nitrobacter (Alexander, et al., 1960). Some environments, in particular, would seem more amenable than others to the occurrence of heterotrophic nitrofication (Tate, 1977). An additional facet of heterotrophic nitrification is the enormous metabolic capacity of the many different species involved. Thus, the environments in which heterotrophic nitrification may occur can be as different as are the physiological and environmental stresses which these organisms endure. Perhaps the most striking characteristic of heterotrophic nitrification is the species diversity inherent in this phenomenon and the different habitats from which these organisms have been isolated.

Species diversity. Many organisms are known to heterotrophically nitrify. Current extensive reviews (Verstraete, 1975; Focht and Verstraete, 1977) describe many reports of the various isolates obtained. A partial list of these microbes, along with the types of environments from which they were isolated, is presented in Table 2. The importance of such a compilation lies in the breadth of both the environments and the organisms cited. The diversity and habitats of these nitrifiers is remarkable, and representatives of the plant, animal, and protist kingdoms are included in Table 2.

TABLE 2

An overview of the species and the habitats of heterotrophic nitrification

Species	Isolation Habitat	Reference
Methylobacter ucrainicus		Romanovskaya, et al., 1977
Methane Oxidizing Bacteria		Hutton and Zobell, 1953
Nocardia		Alexander, et al., 1960
Streptomyces		Alexander, et al., 1960
Arthrobacter sp.	Lake	Witzel and Overbeck, 1979
Arthrobacter sp.	Sewage	Verstraete and Alexander, 1972a
Pseudomonas aeruginosa	Soil	Obaton, et al., 1968
Achromobacter sp.	Soil	Quastel, et al., 1952b
Corynebacterium sp.	Soil	Quastel, et al., 1952b
Unidentified Bacteria	Teak Soils	Odu and Adeoye, 1970
Cephalosporium sp.	Soil	Eylar, et al., 1959
Aspergillus flavus	Soil	Eylar, et al., 1959
A. glaucus	Wheat Seed	Eylar, et al., 1959
Penicillium sp.	Soil	Eylar, et al., 1959
Penicillium atrovenetum		Raistrick and Stossl, 1958
Fusarium sp.	Soil	Doxtader and Alexander, 1966c
Neurospora crassa		Little, 1951
Tetrahymena pyriformis		Seaman, 1954
Ankistrodesmus braunii (alga)		Spiller, et al., 1976
Dog		Verstraete, 1975
Man		Verstraete, 1975
Rabbit		Verstraete, 1975
Rat		Verstraete, 1975

The relative activity of the heterotrophic nitrifiers is an important parameter to consider. Eylar, et al., 1959, noted that isolates which were most active came from soils with high nitrification rates. These authors also noted that the most common active fungal nitrifier was A. flavus and that A. flavus generated large yields of nitrate. Considering that 27%, 26%, and 17% of the actinomycetes, bacteria, and fungi, respectively, nitrified when supplied peptone, the importance of the rate and yield of nitrification becomes apparent since only 7% of the isolates produced greater than 0.2 µg product/ml and a sparse 2% yielded greater than 0.5 µg product/ml. Hutton and Zobell (1953) also demonstrated that a large proportion of their isolates (31%) were capable of nitrification and that nitrification paralleled methane oxidation. However, these authors did not describe variation among isolates as did Doxtader and Alexander (1966b) who observed that different strains of A. flavus and A. oryzae yielded from 0 to 215 µg NO_3^--N/ml when cultured with either NH_4^+, 3-NPA, or β-alanine. Variability among heterotrophic nitrifiers is further confused by the results of Schmidt (1960) who reported the loss of nitrifying capacities of A. flavus after repeated laboratory culturing and of the subsequent restoration of heterotrophic nitrification activity by passage through sterile soil. Obviously, both adequate substrates and inducers for heterotrophic nitrification exist in soil and failure to satisfactorily supply such compounds results in the somewhat puzzling data obtained from laboratory studies (Schmidt, 1960b).

The importance of heterotrophic nitrification will be properly assessed when the diversity, metabolic capabilities, and the

activity of the heterotrophic nitrifier population can adequately be determined in the environment. Yet, the potential of heterotrophic nitrifiers may be estimated from knowledge concerning the species involved and their distribution. Shih, et al., (1974) noted that both nitrification and aflatoxigenicity were observed in the same strains of A. flavus and that the broad distribution of this organism may be ecologically important when considering NO_2^-, NO_3^-, and alfatoxin concentrations in water and in various foodstuffs.

The wide distribution and large numbers of heterotrophic nitrifiers has been postulated as being perhaps one means by which these microbes effect nitrification (Eylar, et al., 1959; Alexander, et al., 1960; Fischer, et al., 1956). Thus, the general consensus is that expressed by Odu and Adeoye (1970):

> the production of nitrite or nitrate by heterotrophic organisms may be of ecological importance in environments where the inefficiency of the heterotrophs may be compensated for by their large numbers.

Heterotrophic nitrification yields. The low yield of products of heterotrophic nitrification is one of the primary factors preventing the assessment of the importance of this phenomenon. Many organisms produce minute concentrations of nitrification products, as examplified by the data of Eylar, et al., (1959) in which only 2% of the soil isolates yielded greater than 0.5 µg products/ml .

Focht and Verstraete (1977) summarized the amounts of products yielded by several organisms. Bacteria generally yielded from 0.2-18.0 µg NO_2^--N/ml and 2.0 - 14.1 µg NO_3^--N/ml. The fungi were somewhat more active, producing up to 45.0 µg 3-NPA -N/ml and 75.0 µg

NO_3^--N/ml. Yet, exceptions have been noted. Methylococcus thermophilus produced approximately 150 mg NO_2^--N/l (Romanovskaya, et al., 1977) and Pseudomonas aeruginosa converted acetaldoxime to yield 284 µg NO_2^--N/ml (Amarger and Alexander, 1968). Among the fungi, Fusarium is noteworthy; it produced 190 µg NO_2^--N/ml when pyruvic-oxime served as the substrate (Doxtader and Alexander, 1966c). Oximes are particularly accessible substrates for heterotrophic nitrification and the highest yields recorded occurred when oximes were the nitrogen sources. However, even the 284 µg NO_2^--N/ml synthesized by P. aeruginosa is slight when compared to the yields of Nitrosomonas and Nitrobacter. These microorganisms yield from 2,000 to 4,000 µg N product/ml (Focht and Verstraete, 1977) and thus underline the low yields seemingly inherent in the heterotrophic nitrification process.

One explanation for the low yields of heterotrophic nitrification has been postulated by Verstraete (1975). This author claims that since many heterotrophic nitrification metabolites are toxic or even mutagenic (N-nitroso compounds, C-nitroso compounds, NO_2^- ,and NH_2OH) that perhaps their function is to inhibit or destroy predators, parasites, or competitors. More definitive data, however, will have to be reported before the above hypothesis can be seriously considered. Yet in light of much of the literature cited, such low concentrations may not be serving solely as the endproducts of metabolic pathways.

Nitrification synergisms. The classical nitrification synergism is that which occurs between Nitrosomonas and Nitrobacter and results in NH_4^+ oxidation to NO_3^-. However, the literature does cite studies in which synergisms result in nitrification and are composed of organisms other

than the Nitrosomonas-Nitrobacter combination.

Synergisms may thus occur between the chemoautotrophs and heterotrophs or solely between heterotrophs. The myxobacterium Sorangium symbioticum was observed to enter into a symbiosis with Nitrosomonas (Imsenecki, 1946). Apparently both organisms benefitted since a moist environment was maintained by S. symbioticum while autolysis of some Nitrosomonas cells resulted in a carbon source utilized by the myxobacterium. A similar situation may be postulated for Nitrobacter. Recent studies (Bock, 1976; Steinmuller and Bock, 1976) present data which indicate that Nitrobacter can grow heterotrophically, with pyruvate and acetate as carbon sources and yeast extract and peptone as nitrogen sources. Cells thus cultured experienced no lag time when grown in a medium where NO_2^- was the energy source. Further, a yeast extract-peptone mineral salts medium in which heterotrophic bacteria (Pseudomonas fluorescens, Micrococcus luteus, Bacillus subtilus, Arthrobacter, and Nocardia corallina) had been cultured and were subsequently removed, when added to an autotrophic medium (90% autotrophic: 10% yeast extract-peptone medium), resulted in marked stimulation of NO_2^- oxidation. Therefore, the possibility of either protocooperation or commensalism (as defined by Alexander, 1977) exists in the reported stimulation and growth of Nitrobacter. Also, a synergism between Nitrobacter and a heterotrophic nitrifier which produces NO_2^- may be surmised though as yet unreported. Any such association would result in at least some of the original nitrogen being converted to NO_3^-.

Odu and Adeoye (1970) postulated that sterile soils inoculated with pure cultures of heterotrophs do not adequately allow for the

development of the proper microbial communities which would effect heterotrophic nitrification. Mixed cultures, they argued, would offer a greater chance for the necessary precursors to be synthesized. Cutler and Mukerji (1931) presented data favoring such a hypothesis. A highly active ammonifying bacterium isolated by these authors, when cultured with ammonia oxidizing heterotrophs, caused a great increase in NO_2^- production compared to that produced when the heterotrophic nitrifiers were cultured separately. Obviously, the greater NO_2^- production was thus dependent on the association between the two groups.

More recent evidence also notes a synergistic effect on nitrification when two species of Arthrobacter were cultured simultaneously (Berger, et al., 1979). The organism termed Arthrobacter Q1 produced NH_2OH as its nitrification product. Yet, when this species was cultured with the second Arthrobacter sp., no apparent NH_2OH accumulation occurred. The authors therefore concluded that the second Arthrobacter sp. thus metabolized was not identified. Still a definite nitrogen synergism had occurred and conversion of NH_2OH was effected. Thus nitrogen synergisms are possible not only between autotrophs and heterotrophs but between heterotrophs as well.

Natural occurrences of heterotrophic nitrification. Verstraete and Alexander (1972b; 1973) reported heterotrophic nitrification in samples obtained from various ecosystems. Two of four sewage samples demonstrated nitrification patterns analogous to those observed by an axenic culture of Arthrobacter. That is, when supplied acetate and NH_4^+, the sewage samples produced NH_2OH, a hydroxamic acid, a bound hydroxylamine, a

primary nitro compound, nitrite, and nitrate. The hydroxamic acid and NH_2OH were synthesized earliest among the products, while the bound-NH_2OH, NO_2^-, and NO_3^- were synthesized primarily after 3 days of incubation. Similar nitrification patterns were discerned for river water, lake water, and soil suspension samples. In all cases, the products and their sequence of appearance were similar to that observed with axenic cultures of the Arthrobacter sp.

Other aspects of these studies were the effect of pH on nitrification, the failure of soil samples to nitrify, the occurrence of classical nitrification patterns, and the disappearance of NH_2OH from soil samples. The pH was observed to rise from 7.0 to 9.0 in some samples and in all samples nitrification occurred only at a neutral or alkaline pH. The lack of nitrification in the soil samples is curious since soil suspensions amply nitrified. However, this phenomenon and that of NH_2OH disappearance may be due to binding of nitrogenous substrates to soil colloids.

Classical nitrification patterns were observed in these studies and were an indication of active chemoautotrophic nitrifiers. Yet, many samples exhibited heterotrophic nitrification patterns and the authors' suggestion that Arthrobacter-like heterotrophic nitrification may occur in varied natural ecosystems appears justified.

Heterotrophic nitrification niches. In contrast to heterotrophic nitrification occurring in the common environments listed above, studies have reported specialized niches in which the autotrophic nitrifiers were either absent or present in insufficient numbers to account for the nitrification observed. Nitrification occurring under these

circumstances must be a result, at least in part, of heterotrophic metabolism.

Ishaque and Cornfield (1974; 1976) studied a Bangladesh laterite (pH 4.2) soil in which autotrophic nitrifiers could not be detected yet nitrate accumulated. Gode and Overbech (1972) observed that heterotrophic nitrifying bacteria were much more numerous than autotrophic nitrifiers in the eutrophic PluBsee Lake. They attributed nitrification in this environment to heterotrophic activity. Heterotrophic nitrifiers were present in the mulberry rhizosphere and thus were deemed potential nitrifiers in that ecosystem (Vasantharajan and Bhat, 1968). Similarly, Meiklejohn (1962) observed insufficient numbers of autotrophic nitrifiers to account for the nitrification evident in some Ghana soils. German forest soils were also cited for their lack of autotrophic nitrifiers and for species of actinomycetes and fungi capable of heterotrophic nitrification (Remacle, 1977).

Burning chaparral soils caused the death of Nitrobacter cells (Dunn, et al., 1979). The nitrification occurring in these soils was credited to heterotrophs and found to correspond to fungal growth. Organic mucks also had too few autotrophs to account for the nitrification observed (Tate, 1977). Again, a heterotrophic nitrifier was present (an Arthrobacter sp.) and was thought responsible for at least some of the nitrification noted. In addition, the potent Nitrosomonas inhibitor N- serve diminished but did not eliminate nitrification in the muck, indicating the presence of an active heterotrophic nitrifier population. Gowda, et al., (1976) reported the presence of nitrification in a loam soil treated with 500 p.p.m. benomyl, a known inhibitor of autotrophic

nitrifiers. An Aspergillus carneus isolated from this soil produced NO_2^- from NH_4^+, supporting the hypothesis of functional heterotrophic nitrification in this environment. Also, studies have reported inadequate numbers of Nitrosomonas to account for the Nitrobacter populations present (Ardakani, et al., 1974; Morrill and Dawson, 1967). Thus heterotrophs may be responsible for the NO_2^- in those environments where Nitrosomonas numbers are inadequate to account for the NO_2^- produced.

Perhaps the most unusual ecosystem reported as a heterotrophic nitrification niche is that of the human, guinea pig, and rat intestine (Ralt, et al., 1980). A Pseudomonas aeruginosa isolate was obtained both from human ileal fluid and from caceal fluid of the guinea pig and was capable of converting 90-99% of acetohydroxamic acid nitrogen to NO_2^- when grown in a medium with 0.1-0.2% yeast extract. The organism also could metabolize NH_2OH to NO_2^-.

Thus, many ecosystems are both potential sites for heterotrophic nitrification and contain the organisms which may effect such transformations. The difficulty of identifying those environments in which heterotrophic nitrification occurs is, however, compounded by our limited knowledge of synergistic associations between heterotrophs and the effect of soil particles on this process. Also complicating the problem are the four main differences between heterotrophic and autotrophic nitrification. Heterotrophic nitrification as cited by Witzel and Overbeck (1979) is distinguished by:

(1) A broad spectrum of organisms, N-sources and products;

(2) An organic carbon source is necessary not only for growth of the organism but in addition for nitrification of at least inorganic N-sources;

(3) Heterotrophic nitrifiers are much less active and effective in their nitrification ability than autotrophic ones; and

(4) Heterotrophs mostly accumulate nitrite or nitrate when active growth of the cells has ceased.

Difficulties arising from these differences will have to be resolved if heterotrophic nitrification environments are to be identified definitively.

Simultaneous autotrophic and heterotrophic nitrification. The mulberry rhizosphere has been mentioned as a possible site of heterotrophic nitrification. Heterotrophic nitrifiers, however, do not exclusively populate this environment (Varsantharagan and Bhat, 1968). Instead, a complex association of ammonifying bacteria and both heterotrophic and autotrophic nitrifiers was observed. The authors therefore suggested that the ammonifying flora metabolized any autotrophic nitrification inhibitors (such as methionine) and supplied NH_4^+ to both the heterotrophic and autotrophic nitrifiers.

A study of the Negev Desert soils of Israel revealed patterns of nitrification indicative primarily of heterotrophic nitrification (Etinger-Tulczynska, 1969). At 28°C nitrification of NH_4^+ proceeded as would be expected if autotrophs were effecting the transformation. However, elevated incubation temperatures (38 - 40 C), similar to those observed in the field, caused a decrease in nitrification in those soils ammended with NH_4^+ while untreated soils continued to accumulate NO_3^-. A similar pattern was also observed for NO_2^--oxidation. Since sterilization of the soil resulted in the complete cessation of nitrification, the conversion of soil nitrogen and $NH_4^{\mp}$ to NO_3^- was assumed to be biological in its origin. Etinger-Tulczynska thus concluded that while

both heterotrophic and autotrophic nitrification were operational in these desert soils, most of the nitrification occurred as a result of heterotrophic activity.

Tate (1977) described a Pahokee muck soil in which the indigenous autotrophic nitrifier population was insufficient to account for the NO_3^- observed. After having isolated a nitrifying Arthrobacter sp. from that soil, Tate proposed that heterotrophs were responsible for at least some of the NO_3^- observed. Thus, the occurrence of simultaneous autotrophic and heterotrophic nitrification has been noted in widely varying habitats and may be a ubiquitous phenomenon. If the autotrophic nitrifiers evolved from heterotrophic nitrifiers, as Verstraete (1975) suggests, then the association of the two groups in the same environments and the joint functioning of their respective metabolisms should not be a surprising event.

Physiology of Heterotrophic Nitrification

Many factors affect both the process and the amount of heterotrophic nitrification. However, many heterotrophic nitrifying organisms do not respond similarly to the same stimuli. The more important variables which govern the physiology of heterotrophic nitrification are reviewed in the following section.

Organic carbon effects. Organic carbon is necessary in some form for heterotrophic nitrification. Both the type and the amount needed varies with different organisms. The synthesis of 3-NPA by Aspergillus flavus requires an unknown organic compound derived from appropriate organic

carbon sources (Doxtader and Alexander, 1966a). Penicillium atrovenetum can utilize a number of four carbon dicarboxylic acids (fumarate, malate, succinate, and oxaloacetate) to synthesize 3-NPA and these dicarboxylic acids promoted 3-NPA synthesis when aspartic acid served as the nitrogen source (Shaw and Wang, 1964). Further evidence showed that the carbon skeleton of aspartic acid was necessary for 3-NPA synthesis when the organism was grown with aspartic acid as a carbon source (Birkinshaw and Dryland, 1964; Shaw and McCloskey, 1967). The number 4, 3, and 2 carbons of aspartic acid were directly incorporated into 3-NPA.

Organic carbon (glucose and peptone) was necessary for significant nitrification by Pseudomonas aeruginosa; its omission reduced NO_2^- production from 284 to 3.1 µg/ml (Obaton, et al., 1968). The Arthrobacter sp. of Verstraete and Alexander (1973) exhibited no nitrification when NH_4^+ and propionate, caproate, glycolate, sucrose, and glucose served as carbon-nitrogen sources. However, NH_2OH, NO_2^-, and 1-nitrosoethanol were produced when either succinate or acetate and NH_4^+ were present. Similar results were reported for the Arthrobacter sp. of Witzel and Overbeck (1979). Nitrification by their Arthrobacter sp. either did not occur or was sparse when acetate was supplied below a threshold concentration. These authors observed that either malate, citrate, or ethanol could substitute for acetate as a nitrification carbon source but that pyruvate, glucose, lactose, frustose, lactate, glyoxylate, formate, 2-oxoglutarate, succinate, methanol, glycerol, stearate, and palmitate could not.

Besides being necessary for nitrification, many reports cited organic carbon and organic carbon-nitrogen compounds as being stimulatory.

Verstraete and Alexander (1972c) reported acetate to effect a fourteen fold increase in NH_2OH production when added to resting cells. Similarly, exogenous sources of acetate stimulated 1-nitrosoethanol synthesis by the Arthrobacter sp. Cutler and Mukerji (1933) also reported increased nitrification by their bacterial isolates when exogenous carbohydrates were provided. P. atrovenetum was noted to have maximal production of 3-NPA when auxiliary carbon sources were present. A. flavus increased its oxidation of NO_2^- to NO_3^- when exogenous sources of organic carbon such as 3-chloropropionate and aspartate were present. Finally, organic-nitrogen in the forms of either oxamide, urea, or dried blood resulted in marked increases in the nitrification of Bangladesh laterite soils (Ishaque and Cornfield, 1974; 1976). Thus, a variety of carbon sources and organisms were found to be involved in the stimulation of heterotrophic nitrification.

Organic carbon, however, has also been reported to depress heterotrophic nitrification. Two studies which summarize the inhibition data are those of Witzel and Overbeck (1979) and Jensen (1951). The former authors noted suppression of nitrification when either glucose, tryptone, or yeast extract were present, with 80% inhibition occurring when as little as 1 mmol glucose/1 was added. Jensen noted a similar glucose inhibition and postulated that the inhibition occurred as a result of the stimulation of cell synthesis.

Carbon to nitrogen ratios (C/N ratios). Cultures of Alcaligenes, Agrobacterium, and Nocardia were observed to nitrify in accordance with the amounts of carbon and nitrogen furnished as substrates (Jensen, 1951).

Nitrification of pyruvic-oxime was proportional to the amount of additional pyruvate supplied to these microbes with nitrification occurring at C/N ratios between 7.7 - 15.4. Little NO_2^- was produced by these organisms when the C/N ratio was greater than 15.4. A Streptomyces sp. and a Nocardia petroleophilia were similarly noted to commence nitrification only in those media where there was an excess of ammonium ion (Alexander, et al., 1960).

The fungi, represented by Aspergillus flavus, exhibit the same general response to C/N ratios. Schmidt (1960a) noted nitrification by A. flavus only when the C/N ratio approached 5.9. Hirsch, et al., (1961) confirmed these data and noted significant NO_3^- production by A. flavus when the C/N ratio varied from as low as 1/6 to as high as 5/1.

A somewhat puzzling C/N ratio effect, however, has been cited for an Arthrobacter sp. (Verstraete and Alexander, 1972a). These authors reported that NO_2^- production was proportional to the C/N ratio supplied but that NH_2OH synthesis was not. Why one heterotrophic nitrification product should be governed by the C/N ratio while another is not remains unexplained.

Growth and heterotrophic nitrification. Conflicting data have been reported on the relationship between growth of heterotrophs and nitrification. The spectrum of data ranges from no correlation to strong correlation between the two parameters. Synthesis of 3-NPA and growth were both directly correlated with Penicillium atrovenetum (Shaw and Wang, 1964), and Aspergillus flavus (Doxtader and Alexander, 1966a). Nitrate production by A. flavus increased linearly, however, until seven

days after growth had ceased, at which time its concentration declined. P. atrovenetum produced 3-NPA in the earliest stages of growth with the majority of the final 3-NPA concentrations accumulating before active growth had terminated (Raistrick and Stossl, 1958). Schmidt (1954) reported that NO_3^- was synthesized rapidly during active cellular growth. The bacteria, represented by Nocardia corralina, were observed to oxidize PO to NO_2^- in close accordance with growth (Jensen, 1951).

The Pseudomonas aeruginosa of Obaton, et al., (1968) however, did not directly oxidize acetaldoxime in accordance with growth. Rather, a linear increase in NO_2^- concentrations from approximately 6-160 hours was noted while growth ceased at approximately 72 hours. The Arthrobacter spp. of Verstraete and Alexander (1972a) and Witzel and Overbeck (1979) were noted to nitrify both during and after the logarithmic phase of growth. The Arthrobacter sp. of Verstraete and Alexander produced NH_2OH and a hydroxamic acid during logarithmic growth while a primary nitro-compound, a bound hydroxylamine, NO_2^- and NO_3^- were all synthesized after growth had ceased.

In apparent contrast, the Fusarium sp. of Doxtader and Alexander (1966c) nitrified PO only after active growth had ceased. Aspergillus flavus exhibited a similar nitrification pattern as bound-NH_2OH, a $FeCl_3$ reacting material (presumably a hydroxamic acid), NO_2^-, and NO_3^- all were synthesized as growth entered the stationary phase. VanGool and Schmidt (1973) and Schmidt (1960) both cited similar patterns of NO_3^- production and growth cessation with A. flavus cultures. An actinomycete isolated by Hirsch, et al., responded similarly, with NO_2^-

production becoming apparent only after growth had terminated. Thus, the concept of nitrification correlated with growth is not clearly defined. Indeed, some authors, having noted nitrification occurrences after growth had ceased, and in direct disregard for those studies which show the opposite, have proposed that nitrification by heterotrophs is primarily an autolysis phenomenon (Hirsch, et al., 1961; Marshall and Alexander, 1962).

Energy and heterotrophic nitrification. Since heterotrophic nitrification generally results in low concentrations of products, little or no energy is presumed to become available to the microbes which cause this transformation (Fisher, et al., 1956). Similarly, since heterotrophic nitrification is sometimes not associated with growth, little energy yield is deemed possible from the process (Alexander, et al., 1960; Aleem, 1970).

A different hypothesis, however, was proposed by VanGool and Schmidt (1973). These authors suggested that the function of heterotrophic nitrification may be to provide maintenance energy. They concluded that the low concentrations of nitrification products yielded by A. flavus, in spite of the relatively large concentrations of a nitrifiable substrate, supports such a hypothesis.

In contrast to both of these postulations is the proposal of Romanovskaya, et al., (1977). These authors suggested that the energy needed to initiate oxidation of methane by methane oxidizing bacteria could be furnished by the oxidation of NH_4^+ to NO_2^-, a process which yields -32.54 kilocalories/mole. While there is no substantial evidence

to support this claim, energy yielded from heterotrophic nitrification may play a role, albeit minor, in the energy budget of the heterotrophs concerned.

pH effects. Heterotrophic nitrification is dependent on the pH of the medium. However, there are conflicting reports as to which pH ranges allow for growth and nitrification by heterotrophic organisms.

Most workers agree with the data of Schmidt (1960a). This author reported no nitrification by A. flavus when the pH of the medium was below 6.6. Doxtader and Alexander (1966a) also cited a total lack of nitrification by A. flavus when the initial medium pH was 5.5. Actinomycetes and bacteria responded similarly to pH, with little or no nitrification occurring at pH values lower than 6.0 (Alexander, et al., 1960; Hirsch, et al., 1961).

Verstraete and Alexander (1972a, 1973) noted a peculiar response of their Arthrobacter sp. to the pH of the medium. The initial pH of 7.0 was altered to 9.2 by the growth and nitrification of this organism. While no appreciable changes in pH were noted with either Azotobacter (Saris and Virtanen, 1957) or Achromobacter (Quastel, et al., 1952) spp., neither the Azotobacter nor the Achromobacter would produce substantial quantities of nitrification products below pH 6.0. Accordingly, a pH of 5.5 reduced by 50% the amount of PO oxidized by the Achromobacter sp. when compared to a medium of pH 6.0.

Enzyme fractions of heterotrophic nitrifiers respond similarly. Seaman (1957) reported that an enzyme capable of effecting the hydrolysis of PO to pyruvate and NH_2OH had virtually no activity at pH 5.0 while

optimal rates were noted from pH 6.1 - 7.8. NH_2OH oxidation to NO_2^- by P. aeruginosa extracts occurred maximally in the pH range of 6.8 - 7.0 with activity reported from pH 5.5 - 8.4 (Amarger and Alexander, 1968). A. flavus extracts were cited as producing $NO3^-$ from both 3-NPA and NO_2^- (Molina and Alexander, 1971). However, activity was the greatest at pH 8.0 even though the reactions occurred at pH 6.0 and 7.0.

In contrast to these reports are those that cite nitrification under acidic conditions. Field studies have demonstrated nitrification in soils with pH values as low as 4.0 (Weber and Gainey, 1962). The oxidation of NH_4^+ to NO_2^- was reported in culture media with a pH of 4.8 (Cutler and Mukerji, 1931). A. flavus did not produce either NO_2^-, NO_3^-, or bound-NH_2OH at pH 2.34, yet the fungus did synthesize free NH_2OH at this pH (Marshall and Alexander, 1962). Similarly, A. flavus grown on a yeast extract-sucrose medium yielded both the most NO_3^- and aflatoxin when the pH was 4.60 (Shih, et al., 1974).

Perhaps the strongest evidence for acidic nitrification is that of Becker and Schmidt (1964) who showed that A. flavus synthesis of 3-NPA occurred at a maximal rate when the initial medium pH was 3.5, next at 5.0, and least at 6.5. However, 3-NPA oxidation to NO_3^- was noted at both pH 2.1 and 6.3, yet no 3-NPA oxidation was measured at pH values greater than 6.8. Thus, while most researchers contend that heterotrophic nitrification is a phenomenon of neutral or alkaline pH, the data is not conclusive.

Inhibitors of autotrophic and heterotrophic nitrification. The responses of heterotrophic nitrifiers to autotrophic nitrification inhibitors

exemplify some of the differences and similarities of the processes. Inhibition of autotrophic NH_4^+ oxidation results from exposure of Nitrosomonas to a number of molecules. Ethyl urethane and methionine, two of these autotrophic inhibitors, are, however, without effect on PO oxidation (Quastel and Scholefield, 1949). N-Serve [2-chloro-6-(trichloromethyl) pyridine], perhaps the most potent autotrophic NH_4^+ oxidation inhibitor, was without effect on the oxidation of NH_4^+ by an Arthrobacter sp. (Verstraete and Alexander, 1972a) and was responsible for increasing the heterotrophic nitrifier population (Tate, 1977). Sodium sulphacetamide, another Nitrosomonas inhibitor, drastically diminished NO_2^- accumulation from NH_4^+ while leaving unaffected the synthesis of NO_3^- from soil nitrogen (Etinger-Tulczynska, 1969).

Perhaps the only similarity in the inhibition of autotrophs and heterotrophs is in their response to hydrazine, an inhibitor of NH_2OH oxidation. Amarger and Alexander (1968) reported that both P. aeruginosa and Nitrosomonas europea were approximately equally inhibited (65%) by a 10^{-3} M concentration of hydrazine.

1-allyl-2-thiourea and potassium chlorate were without effect on the Arthrobacter sp. of Verstraete and Alexander (1972a). Nitrourea (Quastel, et al., 1952) and potassium chlorate (Quastel and Scholefield, 1949), other inhibitors of Nitrobacter, were also ineffective as inhibitors of PO oxidation. Etinger-Tulczynska (1969) reported that potassium chlorate was highly effective in limiting NO_3^- production from NH_4^+ while having little effect on NO_3^- accumulation due to soil nitrogen oxidation. This same author noted that chloromycetin, a strong NO_2^-

oxidation inhibitor, depressed NO_3^- formation from soil nitrogen. It is therefore evident that the two nitrification processes must at some point be divergent since the same inhibitors achieve markedly different responses. Apart from hydrazine, the only inhibitors of heterotrophic nitrification cited as effective are sodium azide and sodium cyanide (Seaman, 1957; Quastel and Scholefield, 1949), both of which are vigorous respiratory poisons.

Hydroxylamine and organic-nitrogen toxicity. An important issue in any discussion of the physiology of nitrification is the toxicity of substrates and products of heterotrophic nitrification. Organic nitrogen compounds, such as 4,6-dinitro-O-cresol (DNOC), p-nitrophenol (PNP), 2,4-dinitrophenol (2,4-DNP) and picric acid (2,4,6-trinitrophenol) were reported toxic at concentrations that were slightly above those which permitted growth (Gundersen and Jensen , 1956). DNOC was both bacteriostatic and bactericidal at 0.5% (w/v) while 0.2% PNP was bactericidal. However, 0.05% PNP limits bacterial activity such that PNP is only bacteriostatic. Both 2,4-DNP and picric acid were used as carbon and nitrogen sources at 0.2%, yet growth was extremely slow at these concentrations. However, the majority of the toxicity studies concerning heterotrophic nitrification focus on NH_2OH.

Concentrations of NH_2OH as low as 10 ug/ml are toxic to algae (Berger, et al., 1979). This same bacteriostatic/bactericidal phenomenon of NH_2OH is evident when considering the bacteria. Reports of toxicity at 1-250 mg/l are comron but many bacteria are capable of withstanding 20-30 mg/l NH_2OH (Castell and Mapplebeck, 1956). Among some of the

more NH_2OH resistant microbes are Aerobacter, Eschericia coli, Proteus spp., Serratia marcescens, Alcaligenes spp., Microbacterium spp., and Bacillus spp., all of which grow in media containing 50-100 mg/l NH_2OH (or a maximal equivalent of 42.4 mg NH_2OH-N/l). The fungi, represented by Penicillium, Aspergillus, Trichoderma, Alternaria, Cladosporium, and Oospora spp., were somewhat more resistant to NH_2OH, tolerating 250 mg/1 NH_2OH or 106 mg/1 NH_2OH-N. Jensen (1951) had reported results similar to those of Castell and Mapplebeck noting that greater than 40 mg/1 NH_2OH-N (94 mg/1 NH_2OH) severely restricted the growth of an Alcaligenes sp. and of Nocardia corallina. Bacillus cadaveris behaves similarly, growing in media which contain 33 p.p.m. NH_2OH (Emery, 1963). Shaw and Wang (1964) observed that P. atrovenetum grew in media which contained 28 mg/1 NH_2OH-N. Thus, while the concentration of NH_2OH tolerated varies with the organism, a significant number of heterotrophic nitrifiers remained viable and proliferated in media containing NH_2OH concentrations of 50-100 mg/1.

Oximes present a somewhat different situation. Quastel, et al., (1952), using PO as a model, postulated that the equilibrium between oximes and their corresponding keto acids and NH_2OH ($CH_3 - \overset{O}{\overset{\|}{C}} - COOH + NH_2OH \leftrightarrow CH_3 - \overset{NOH}{\overset{\|}{C}} - COOH + H_2O$) is vastly in favor of the oxime. Thus, NH_2OH from oximes might be present at non-toxic concentrations and therefore undergo metabolism by capable microorganisms. Evidence supporting this hypothesis was presented by Amarger and Alexander (1968) who noted that 1mM NH_2OH (14 p.p.m. NH_2OH-N) inhibited P. aeruginosa growth. However, low concentrations of acetaldoxime (40 μM) were rapidly

metabolized by this P. aeruginosa strain and converted to NO_2^- (Obaton, et al., 1968). Oximes thus serve as valuable research tools since they allow research to be conducted on NH_2OH metabolism while alleviating the toxic effect of NH_2OH concentrations.

Biochemistry of Heterotrophic Nitrification

The biochemistry of heterotrophic nitification is not completely elucidated. As with other parameters of heterotrophic nitrification contradictory data are recorded. Indeed, data obtained by the use of the same microbe are often at odds with previous studies. However, certain concepts have emerged with respect to the biochemistry of this phenomenon and are presented below.

Ammonium ion metabolism. Aleem, et al., (1964) reported the oxidation of NH_4^+ to NH_2OH, NO_2^-, and NO_3^- by extracts of Aspergillus wentii, A. flavus, and P. atrovenetum. Since only small quantities of NH_2OH and NO_2^- were detected during NH_4^+ oxidation to NO_3^-, and since the oxidation products were identical to those of Nitrosomonas and Nitrobacter, these authors proposed that heterotrophic nitrification of NH_4^+ proceeds by a similarly inorganic pathway. However, neither Shaw and McCloskey (1967) nor Shaw and DeAngelo (1969) could substantiate these results using either cells or cell-free extracts of P. atrovenetum. Shaw and McCloskey (1967) noted that NH_4^+ utilization by P. atrovenetum resulted in 3-NPA synthesis. These authors further reported that the amino group of aspartic acid was used preferentially when compared to NH_4^+. Shaw and DeAngelo (1969) observed that repeated attempts using

P. atrovenetum extracts did not result in the oxidation of NH_4^+. Similarly, attempts to demonstrate the subsequent reduction of cytochrome c met with failure. Similar results were also reported for A. flavus (Doxtader and Alexander, 1966b). A. flavus extracts synthesized NO_2^- from 3-NPA but were without action on NH_4^+, NH_2OH, β-alanine, aspartate, and asparagine. Replacement cultures of the fungus differed from the extracts and NO_3^- was produced from 3-NPA. However, the replacement cultures behaved like the extracts by synthesizing no products when supplied with NH_4^+. Verstraete and Alexander (1972c) reported the synthesis of NH_2OH from NH_4^+ by an Arthrobacter sp. However, this organism differs from many others in that NH_2OH is oxidized to 1-nitrosoethanol (1-NE) as well as to NO_2^-. Nitrite, however, was not produced from NH_2OH by extracts of this bacterium. Thus, a strictly inorganic pathway of NH_4^+ oxidation to NO_3^- cannot be surmised from these data.

However, there is no doubt that at least segments of the pathway are inorganic in nature. Verstraete and Alexander (1972d) presented data which showed that molecular oxygen (O_2) was incorporated into the NH_2OH excreted by an Arthrobacter sp. As NH_4^+-N was the only source of nitrogen, the synthesis of NH_2OH was considered to be achieved via an inorganic pathway, although the possibility that an organic molecule might be the substrate was not eliminated.

The stoichiometry of NH_4^+ oxidation remains unresolved. Fisher, et al., (1956) noted close correlations between NH_4^+ disappearance and NO_2^- synthesis. From 63 to 87% of the ammonia lost was accounted for by the subsequent production of NO_2^-. Witzel and Overbeck (1979), however,

reported that NH_4^+ uptake and NO_2^- synthesis were not stoichiometric for their Arthrobacter sp., reflecting a low efficiency of conversion. If, as previously suggested, nitrogen is needed for cell synthesis, then one would not expect stoichiometric conversion of NH_4^+ to any oxidized product. If, however, nitrogen is not incorporated into biomass, then stoichiometric synthesis of products could be expected.

NH_2OH metabolism. Aleem, et al., (1964) reported that extracts of A. wentii oxidized NH_2OH to NO_2^- while concomitantly reducing cytochrome c. Becker and Schmidt (1964), however, noted no conversion of NH_2OH to either NO_2^- or NO_3^- with replacement cultures of A. flavus. Similarly, P. atrovenetum extracts were not observed to synthesize either NO_2^- or NO_3^- when supplied NH_2OH, yet NH_2OH - cytochrome c reductase was noted to be active in such preparations (Shaw and DeAngelo, 1969). Cell-free extracts of an Arthrobacter sp. did metabolize NH_2OH but bound-NH_2OH, NO_2^-, or NO_3^- were not resultant products (Verstraete and Alexander, 1972c). These authors cited extra-cellular proteins as being partially responsible for NO_2^- formation since extra-cellular fractions were capable of synthesizing 7.0 µg NO_2^--N/ml when a total of 29 µg NH_2OH-N/ml was supplied. Thus, the conversion did not result in a stoichrometric synthesis of NO_2^- from NH_2OH. This organism was also unusual in that 1-NE synthesis required that NH_2OH be present.

Hydroxylamine oxidation to NO_2^- has also been reported for bacteria other than Arthrobacter. Alcaligenes, Corynebacterium equii, and Norcardia corallina all effected this conversion (Lees, et al., 1954). Similarly, Pseudomonas aeruginosa caused the oxidation of NH_2OH to NO_2^- (Amarger

and Alexander, 1968). The salient point mentioned by Lees, et al., and Amarger and Alexander was that only at low concentrations of NH_2OH could the organisms effect the conversion. Otherwise, hydroxylamine toxicity developed and the oxidizing metabolism of the organisms was inhibited.

Oxime metabolism. Various reports have cited oxime oxidation to NO_2^- (Lees, et al., 1954; Quastel, et al., 1950; Amarger and Alexander, 1968). However, the biochemical mechanism of this oxidation is yet not fully understood.

Yamafugi and Akita (1952) determined that little free NH_2OH is present in solutions containing dissolved oximes, even if the medium was acidic. Seaman (1954; 1957) reported that Tetrahymena pyriformis has an enzyme capable of splitting PO into pyruvate and free-NH_2OH. The equilibrium of this reaction, however, greatly favors the formation of PO. The enzyme was not specific for PO as α-ketoglutaric oxime was hydrolyzed as well.

Such an enzyme may also be operative in other microbes. Lees, et al., (1954) demonstrated oxidation of either PO or NH_2OH by Alcaligenes, Norcardia corallina, and Corynebacterium equii and suggested that the two substrates were oxidized via the same pathway. Yet the earlier study of Quastel, et al., (1952) cited no NH_2OH oxidation by PO metabolizing cells of Achromobacter and Corynebacterium. These authors suggested that oxidation of PO was a single function process and provisionally named the responsible enzyme pyruvic-oxime oxidase.

Definitive results have, however, been presented more recently.

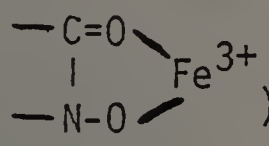
Obaton, et al., (1968) noted two nitrification enzyme systems in P. aeruginosa. The first system oxidizes nitroalkanes while the second nitrifies NH_2OH. Amarger and Alexander (1968) observed that nitromethane was not metabolized by P. aeruginosa extracts and prevented the metabolism of other nitroalkanes. Yet when nitromethane was present, P. aeruginosa extracts formed NO_2^- from acetaldoxime. It was therefore concluded that the low concentrations of NH_2OH resultant from the oxime equilibrium supplied a source of oxidizable nitrogen to the organism. Thus, NH_2OH was furnished in the medium at a constant non-inhibitory concentration until such time as the oxime was exhausted. Additionally, Amarger and Alexander observed no reduction of cytochrome c by P. aeruginosa when extracts were nitrifying NH_2OH. These authors concluded that a dehydrogenation may be responsible for P. aeruginosa nitrification, although the substrate might be a NO_2^- precursor other than NH_2OH.

The degree of similarity between the nitrification sequences of the chemoheterotrophs and the chemoautotrophs is thus still an unanswered question. While many examples of oxime metabolism by heterotrophs are present in the literature, Quastel and Scholefield (1949) observed no nitrification of PO by either Nitrosomonas or Nitrobacter. However, NH_2OH toxicity may be operative in the case of the chemoautotrophs and may thus mask any metabolism similar to that of the heterotrophs.

Hydroxamate metabolism. Hydroxamic acids have at least three functions in microbial metabolism. First, they may be substrates for nitrification, second, they are avid scavengers for metal ions, particularly

iron (Fe), and third, they may act as growth factors (Neilands, 1967).

Verstraete and Alexander (1972c) noted that 1-NE was produced when their Arthrobacter sp. was furnished acetate and NH_2OH. However, aceto-hydroxamic acid was a much better substrate for 1-NE synthesis resulting in approximately a 300% increase in 1-NE production. In addition, hydroxamic acids also function as substrates for NO_2^- synthesis.

Since hydroxamates dissociate protons easily in alkaline media (pKa $\sim$ 9), the attachment of metal ions to form a stable, five-membered ring ) occurs readily (Neilands, 1967). The ferric complex of hydroxamates possibly serves as an Fe transfer molecule since the ferric ion complex is noticeably stable when compared to the ferrous ion complex. Neilands suggested that copious production of hydroxamates affords a cell an efficient means of scavenging for Fe molecules.

Verstraete and Alexander (1972a) substantiated Neilands' work. They found that the concentration of hydroxamic acid(s) produced by their Arthrobacter sp. was inversely proportional to the Fe content of the growth medium. These authors also reported that when Fe was limited, nitrification was generally poor.

1-nitrosoethanol metabolism. 1-nitrosoethanol was reported as a nitrification product of an Arthrobacter sp. (Verstraete and Alexander, 1972c). Exogenous carbon, in the form of acetate, was stimulatory to 1-NE synthesis, yet not essential. NH_2OH, however, was necessary for 1-NE production.

1-NE metabolism represents a paradox in the nitrification sequence

since Arthrobacter sp. extracts are without action on 1-NE, even though they contain a nitrosocleaving enzyme (Verstraete and Alexander, 1972c). 1-NE is resistant to degradation under natural conditions as well, since little 1-NE was metabolized when added to a soil sample (Verstraete and Alexander, 1973).

3-Nitropropionic acid metabolism. Using radioactive carbon compounds, Birch, et al., (1960) established that aspartate was a precursor of 3-NPA. These results were confirmed by Birkinshaw and Dryland (1964) but refuted by Hylin and Matsumoto (1960) and Shaw and Wang (1964). Hylin and Matsumoto noted little 3-NPA syntehsis from aspartate unless an additional source of nitrogen (NH_4^+) was present. However, under these conditions, fumarate or succinate functioned equally well as 3-NPA carbon sources. Shaw and Wang (1964) noted no 3-NPA production from aspartate unless tartarate was present. Shaw and McCloskey (1967), attempting to reconcile these apparent contradictions, suggested that aspartate may be metabolized via several pathways and that such variables as growth medium composition may govern aspartate accessibility for 3-NPA synthesis.

Hylin and Matsumoto (1960) observed that four carbon dicarboxylic acids were the best 3-NPA precursors for P. atrovenetum. These authors also substantiated that β-alanine is not an intermediate in the synthesis of 3-NPA. Formation of 3-NPA was postulated to occur by the incorporation of oxidized nitrogen, such as NH_2OH into one of the four carbon dicarboxylic acids.

Hydroxylamine stimulation of P. atrovenetum 3-NPA synthesis has

been noted (Shaw and Wang, 1964). Additionally, NO_2^- was also observed to increase 3-NPA synthesis by this fungus. NH_2OH was also proposed as a 3-NPA precursor by Birkinshaw and Dryland (1964) when they postulated that the amino group of aspartate was not directly oxidized to the NO_2^- group of 3-NPA. Using radiolabeled pyruvate and acetate, these workers demonstrated that the C-2 ——— C-4 carbons of oxaloacetic acid form 3-NPA with the C-1 of 3-NPA being the C-4 of oxaloacetatic acid.

Shaw and McCloskey (1967) concluded that carbons 4, 3, and 2 of aspartate are directly incorporated into 3-NPA. These authors suggested that the amino group of aspartate is utilized preferentially when compared to NH_4^+. Further research is warranted to determine the nature of the nitrogen incorporated into 3-NPA.

With respect to 3-NPA biosynthesis, Shaw (1967) described an enzyme termed β-nitroacrylic acid reductase which catalyzes the following reaction:

$$\begin{array}{l} CH = CH\text{-}COOH \\ | \\ NO_2 \end{array} + NADPH + H^+ \leftrightarrow \begin{array}{l} CH_2 - CH_2 - COOH \\ | \\ NO_2 \end{array} + NADP^+$$

The enzyme's function was not reversible and its substrate completely inhibited 3-NPA synthesis when aspartate was used as a precursor. Thus, Shaw proposed that the reaction catalyzed by the enzyme was an integral component of 3-NPA synthesis.

3-NPA is not a final metabolite for those fungi which produce it. Both NO_2^- and NO_3^- have been cited as products of 3-NPA metabolism by A. flavus (Doxtader and Alexander, 1966a; Becker and Schmidt, 1964; Marshall and Alexander, 1962; and Molina and Alexander, 1971).

Neurospora crassa is also capable of forming NO_2^- from 3-NPA (Little, 1951). However, since little NO_2^- is produced and mutants of A. flavus capable of oxidizing 3-NPA to NO_3^- cannot oxidize NO_2^- to NO_3^-, it appears that the main pathway of 3-NPA metabolism is one which results in NO_3^- synthesis. Yet, NO_2^- oxidation by a peroxidase has not been discounted (Molina and Alexander, 1971).

Nitrite and nitrate metabolism. The role of NO_2^- in heterotrophic nitrification is unresolved. Nitrite was metabolized to NO_3^- by A. flavus (Becker and Schmidt, 1964; Doxtader and Alexander, 1966b) and was included in the hypothesis of Aleem, et al., (1964) to be one of the inorganic intermediates of NO_3^- production by heterotrophs. However, not all the literature concurs.

Shaw and DeAngelo (1969) failed to observe any NO_2^- oxidation with P. atrovenetum extracts. Extracts of the Arthrobacter sp. of Verstraete and Alexander (1972c) were without action when NO_2^- was supplied. However, NO_2^- and NH_2OH added simultaneously resulted in a decrease of these substrates with neither bound-NH_2OH nor NO_3^- being produced. Hirsch, et al., (1961) reported similar results when they noted no NO_2^- metabolism by Streptomyces strain 259 after the microbe had produced NO_2^- from NH_4^+. Thus, NO_2^- oxidation to NO_3^- does not seem a ubiquitous feature of heterotrophic metabolism.

Nitrate may function as a nitrogen source for heterotrophic nitrification. However, the results of Shaw and McCloskey (1967) show that NH_4^+ is a much better precursor and that any NO_3^- which enters into a heterotrophic nitrification pathway probably is first reduced to

NH_4^+. This was concluded since no $N^{18}O_3^-$ oxygen was detected in any of heterotrophic nitrification products identified.

Influence of iron and other cofactors. Iron influences heterotrophic nitrification. As previously cited, Neilands (1967) noted an inverse relationship between the Fe content of the medium and the quantities of hydroxamic acids syntehsized. The work of Verstraete and Alexander (1972a) confirmed this finding. These authors also noted enhanced yields of NH_2OH, 1-NE, and NO_2^- when Fe was present. The aforementioned study of Aleem, et al., (1964) noted Fe stimulation of NH_4^+ oxidation. In addition, these authors observed that nickel ion (Ni^{2+}) caused an inhibition of heterotrophic nitrification by A. flavus.

Witzel and Overbeck (1979) reported magnesium ion (Mg^{2+}) stimulation of heterotrophic nitrification. Contrary to this report is that of Bergerova and Bernat (1976) who noted only Fe^{2+} stimulation, while also observing Ni^{2+} and Mg^{2+} inhibition. A concentration of 30 mg $FeSO_4 \cdot 7H_2O$/liter caused the greatest increase in nitrification by A. flavus, yet 3 and 120 mg $FeSO_4 \cdot 7H_2O$/liter were also stimulatory (Bergerova and Zamecnik, 1978). Three-hundred mg $FeSO_4 \cdot 7H_2O$/liter caused a marked inhibition in A. flavus nitrification. Finally, copper ions were stimulatory to A. flavus nitrification and a 10^{-3}M concentration of $CuSO_4 \cdot 5H_2O$ resulted in maximum production of NO_2^- from either L-asparagine or DL-valine (Bergerova and Zamecnik, 1978).

The role of oxygen. The pioneering work of Cutler and Mukerji (1931) noted that aeration stimulated the oxidation of NH_4^+ to NO_2^- by soil bacteria. Subsequent work demonstrated that little NH_2OH was

nitrified when O_2 tensions approached zero (Rajendran and Venugopalan, 1976). Amarger and Alexander (1968) reported similar results and they observed no disappearance of NH_2OH and no NO_2^- production by P. aeruginosa extracts when O_2 was absent. Pyruvic-oxime metabolism also requires O_2, yet in the study of Quastel, et al. (1952) not all the oxime was converted to carbon dioxide, water, and NO_2^-, since only half of the O_2 required for this bioconversion was used. A likely explanation for the discrepancy noted is that some of the carbon of PO was simultaneously converted to biomass while the rest was utilized for energy production.

The first study to show oxygen incorporation into a product of heterotrophic nitrification was that of Kawai, et al., (1965). Using $^{18}O_2$, these authors observed that p-nitrobenzoate contained ^{18}O in both oxygen atoms of the nitro group. The oxygenase which effected $^{18}O_2$ incorporation into the nitro group was deemed a novel enzyme, as was the reaction. Using similar methods, Verstraete and Alexander (1972d) noted that their Arthrobacter sp. incorporated $^{18}O_2$ into NH_2OH. Whether the substrate was NH_4^+ or an organic nitrogen compound was not, however, ascertained.

Coenzymes and heterotrophic nitrification metabolism. Studies such as that of Obaton, et al., (1968) note no NAD^+, $NADP^+$, Mg^{2+}, or manganese stimulation and have reported that heterotrophic nitrification proceeds independently of coenzymes and cofactors. However, other reports have noted coenzyme effects on heterotrophic nitrification.

$NADP^+$ and NAD^+, but not FAD or FMN, enhanced the conversion of

3-NPA to NO_3^- by A. flavus (Molina and Alexander, 1971). The finding that quinacrine reduced both NO_2^- and NO_3^- production and that the inhibition could be reversed by addition of the flavines was, however, observed. Thus, a flavine enzyme was postulated to participate in the reaction sequence which leads to NO_2^- and NO_3^- production from 3-NPA in A. flavus.

Verstraete and Alexander (1972c) noted both stimulation and no effect due to coenzymes. Neither NAD^+ nor $NADP^+$ enhanced NH_2OH oxidation to NO_2^- by an Arthrobacter sp., yet NAD^+ did stimulate 1-NE syntehsis from acetate and NH_2OH. Aleem, et al., (1964) also reported similar coenzyme effects; NH_4^+ oxidation was markedly stimulated by $NADP^+$ but was much less affected by NAD^+.

Enzymology of heterotrophic nitrification. A number of enzymes have been postulated as agents effecting heterotrophic nitrification. Aspartase caused the formation of N-hydroxyaspartic acid when NH_2OH and fumaric acid were furnished as the substrates (Emery, 1963). DeGroot and Lichtenstein (1960) noted that the same enzyme catalyzed a number of different reactions, among them the hydrolysis of D-β aspartohydroxamic acid. VanGool and Schmidt (1973) hypothesized that a shift from cytochrome c terminal respiration to a flavoprotein type of respiration might accompany maturation of A. flavus cultures. Little (1951) noted that a nitrite liberating enzyme from N. crassa was unaffected by high concentrations of azide, cyanide, fluoride, and NH_2OH. Little thus concluded that the enzyme was not a hemoprotein.

Little also noted that the enzyme was inducible and NH_4^+ and alanine

were the nitrogen sources which resulted in the lowest levels of the enzymatic activity per mg dry weight. Induction of NH_2OH oxidizing enzyme(s) from P. aeruginosa occurred only when oxime was present (Amarger and Alexander, 1968). However, Quastel, et al., (1951) reported that no induction period was necessary for PO oxidation.

The location of the enzymes of heterotrophic nitrification appears to vary with the organism. P. aeruginosa had no extracellular enzymes capable of nitrification (Obaton, et al., 1968). Yet, an Arthrobacter sp. was noted to contain both intra- and extracellular nitrification enzymes (Verstraete and Alexander, 1972c).

Pyruvic-oxime has served as the carbon-nitrogen source for a number of nitrification studies. Seaman (1957) reported the presence of an enzyme from T. pyriformis capable of hydrolyzing PO and α-ketoglutaric oxime. Quastel, et al., (1950) described an enzyme they termed pyruvic-oxime oxidase (PO oxidase) which nitrified PO to NO_2^-. The enzyme was inhibited by cyanide, azide, phenyl pyruvic-oxime, D-arabinose-oxime, and α-ketoglutaric-oxime. However, PO oxidase was not totally inhibited by these oximes and the inhibition was of a competitive type. Quastel, et al., (1952) confirmed these results and showed the enzyme to be thermolabile; immersion for one minute into a boiling water bath caused the cessation of all nitrifying activity.

The role of cytochrome c in heterotrophic nitrification remains unresolved. Aleem, et al.,(1964) reported a marked stimulation of NH_4^+ oxidation by A. wentii cell-free extracts when mammalian cytochrome c was present. These authors also reported the presence of a NH_2OH

cytochrome c reductase, which reduced cytochrome c and liberated NO_2^- when NH_2OH functioned as an electron donor. Yet, Shaw and DeAngelo (1969), working with P. atrovenetum, could not duplicate these results even though a cytochrome c reductase was present. Amarger and Alexander (1968) demonstrated that P. aeruginosa did not have a functional NH_2OH cytochrome c reductase. Molina and Alexander (1972) substantiated these findings when they noted that the nitrifying eluates of A. flavus extracts were devoid of cytochromes.

Nitro compounds have also been studied in an attempt to understand the biochemistry of heterotrophic nitrification. Little (1951) described a N. crassa enzyme capable of oxidizing nitroethane. The reaction catalyzed is:

$$CH_3\text{-}CH_2\text{-}NO_2 + O_2 + H_2O \leftrightarrow CH_3\text{-}CHO + HNO_2 + H_2O_2.$$

Interestingly, Little reported that no H_2O_2 accumulated since the enzyme contained catalase activity.

Nitrite-cytochrome c reductase and NO_2^- oxidase were observed in A. wentii extracts (Aleem, et al., 1964). NO_2^- accumulation occurred with A. wentii extracts if NH_2OH was the electron donor and nitrite oxidase was inhibited. Working with A. flavus, Molina and Alexander (1971) reported different results than did Aleem, et al. They noted that A. flavus contained two enzyme systems. The first is a NO_2^--peroxidase and the second is an enzyme system capable of converting 3-NPA to NO_2^- and NO_3^-. Since these authors could demonstrate no NO_3^- reduction, they postulated that NO_3^- produced by A. flavus was due to 3-NPA oxidation.

Since A. flavus extract fractions were distinctly different for catalase and NO_2^- peroxidase activity, it was concluded that these reactions were functions of separate enzymes in A. flavus (Molina and Alexander, 1972). Yet these authors reported that catalase, peroxidase, and H_2O_2 enhanced the transformation of NH_2OH and NO_2^-, with NO_3^- being the ultimate product. The authors postulated that NO_2^- oxidation by A. flavus may proceed by a dehydrogenation sequence even though it would not be cytochrome linked as in the chemoautotrophs. Alternately, either catalase or peroxidase may function in NO_2^- oxidation as well.

Nitrite and nitrate reductase are not postulated as contributing significantly to heterotrophic nitrification. This conclusion was reached since P. atrovenetum contained these enzymes only when grown in a NO_3^- medium (Shaw and DeAngelo, 1969). Yet, the organism produced 3-NPA, NO_2^-, and NO_3^- when grown in NH_4^+ medium. These authors also noted that P. atrovenetum extracts capable of the greatest 3-NPA oxidation were those which also contained the highest β-nitroacrylate reductase activity. This observation lends support to the hypothesis that β-nitroacrylate is an integral part of 3-NPA metabolism.

Numerous researchers have proposed either catalase or peroxidase as enzymes active in heterotrophic nitrification (Alexander, et al., 1960; Hirsch, et al., 1961; Molina and Alexander, 1962; Verstraete and Alexander, 1972c). A. flavus extracts had both catalase and peroxidase activity (Molina and Alexander, 1972). Yet, as no clear distinction between A. flavus' catalase, peroxidase, and NO_2^- oxidizing system could be made, neither catalase nor peroxidase could be confirmed as necessary

for nitrification. Difficulties such as those encountered by Molina and Alexander have prevented conclusions with respect to catalase or peroxidase activity in heterotrophic nitrification. However, speculation on the role that these enzymes play persists since many heterotrophic nitrifiers, including P. aeruginosa (Amarger and Alexander, 1968), A. flavus (Hirsch, et al., 1961; VanGool and Schmidt, 1973), and Arthrobacter (Verstraete and Alexander, 1972c) contain these enzymes.

A final note on the enzymes of heterotrophic nitrification concerns the function of oxygenase. Kawai, et al., (1965) demonstrated that O_2 incorporation into p-nitrobenzoate was caused by a novel oxygenase and that both O_2 atoms of p-nitrobenzoate were incorporated from molecular O_2. Similarly, Verstraete and Alexander (1972d) noted molecular O_2 inclusion into heterotrophically synthesized NH_2OH. Oxygenases may be further involved in heterotrophic nitrification since many key reactions and enzymes await elucidation.

Biochemical pathways of heterotrophic nitrification. Evidence supporting both an inorganic and an organic pathway of heterotrophic nitrification is in the literature. Verstraete and Alexander (1972d) showed that O_2 incorporation into NH_4^+ resulted in NH_2OH synthesis. Similarly, Amarger and Alexander (1968) demonstrated that P. aeruginosa does not oxidize acetaldoxime through an organic pathway but rather by oxidizing NH_2OH. Aleem, et al., (1964) stated that A. wentii, A. flavus, and P. atrovenetum oxidized NH_4^+, NH_2OH, and NO_2^- to NO_3^- in a manner analogous to the postulated inorganic oxidations of the chemoautotrophs. Yet, a clear

distinction between solely inorganic or organic pathways cannot be established, since Arthrobacter yields NH_2OH, hydroxamic acids, 1-NE, NO_2^-, and NO_3^- when supplied with NH_4^+ and acetate as substrates (Verstraete and Alexander, 1972c). Clearly, both types of pathways are operative with this organism.

Concerning fungal nitrification, a number of studies have directly contradicted the results of Aleem, et al., (1964). Radio-labeled ^{14}C-aspartic acid resulted in ^{14}C-3-NPA when used as a substrate for P. atrovenetum (Birch, et al., 1960). It has been proposed that ammonium ion was transaminated to an amino acid or another organic compound before giving rise to 3-NPA (Shaw and Wang, 1964). 3-NPA was observed not to be converted to NO_2^-, but rather directly oxidized to NO_3^- by A. flavus (Becker and Schmidt, 1964). Working with the same microbe, Marshall (1965) proposed that aspartate is converted to β-alanine and 3-NPA before being metabolized to NO_2^- and NO_3^-.

Doxtader and Alexander (1966a; 1966b) demonstrated only 3-NPA synthesis by growing cultures of A. flavus supplied with NH_4^+ as a nitrogen source. Further, these authors noted no production of any nitrogenous products when replacement cultures were supplied with NH_4^+. However, these same cultures syntehsized NO_3^- when 3-NPA was furnished.

Shaw and DeAngelo (1969) found no involvement of inorganic compounds in 3-NPA syntehsis by P. atrovenetum. Aspartic acid was concluded to be the source of oxidizable nitrogen for this microbe. Hatcher and Schmidt (1971) confirmed these results and asserted that

there was no relationship between NH_4^+ and the synthesis of bound-NH_2OH, NO_2^-, and NO_3^-. Similar solely organic reactions were noted by Kawaii, et al., (1965) and Little (1951), who also described electron transfer in the nitroethane oxidation reaction by N. crassa.

It is therefore clear that heterotrophic nitrification does not proceed strictly by an inorganic or an organic pathway, and the biochemical pathways described by Focht and Verstraete (1977) appear at the moment to best account for the conflicting data existent in the literature. The scheme proposed by these authors is:

N Oxidation State	-3	-1	+1	+3	+5
Inorganic Pathway	NH_4^+	$\rightarrow$ NH_2OH	$\rightarrow$ [HNO]	$\rightarrow$ NO_2^-	$\rightarrow$ NO_3^-
Organic Pathway	$R\text{-}NH_2$	$\rightarrow$ $R\text{-}NHOH$	$\rightarrow$ $R\text{-}NO$	$\rightarrow$ $R\text{-}NO_2$	

R = NOH

Future study will hopefully elucidate the specific details of this general pathway scheme.

The significance of heterotrophic nitrification. Since NO_3^- is the principal form of nitrogen utilized by photosynthetic organisms (Schmidt, 1954), any process which ultimately results in NO_3^- synthesis is of vast importance to both ecology and agriculture. The organisms bringing about heterotrophic nitrification are ubiquitous (Eylar, et al., 1959) and thus the phenomenon may be of great significance even though the rate of product yield is low (Verstraete and Alexander, 1973). Further, since no induction period is necessary for certain aspects of the process to occur (Quastel, et al., 1952) heterotrophic

nitrification may be an important supplement to autotrophic nitrification when one considers nitrogen cycling in various ecosystems.

C H A P T E R III

MATERIALS AND METHODS

Enrichments: media and procedures. The enrichment media for the initial selection of heterotrophic nitrifiers varied according to the particular carbon and nitrogen source utilized. However, the mineral salts component of the medium remained constant throughout this study and was a modified version of the medium (VM) listed by Verstraete and Alexander (1972a). The modified mineral salts component consisted of:

KH_2PO_4	8.2 g
NaOH	1.6 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$CaSO_4 \cdot 2H_2O$	5.0 mg
$CuSO_4 \cdot 5H_2O$	5.0 mg
$FeCl_3 \cdot 6H_2O$	5.0 mg
$ZnSO_4 \cdot H_2O$	5.0 mg
Distilled H_2O	1 liter

pH adjusted to 7.0.

All chemicals used in this study were reagent grade or better. The respective quantities of the mineral salts medium (usually 100 ml) were dispensed to appropriate erlenmeyer flasks equipped with cotton-cheese cloth stoppers. The flasks were sterilized by autoclaving at 15 pound per square foot pressure for 20 minutes. The $MgSO_4 \cdot 7H_2O$ component, as well as the carbon and nitrogen sources, were filter sterilized using a 0.20 μm GA-8 Gelman membrane filter (Gelman, Ann Arbor, Michigan) and

added aseptically. Filter sterilizing of these chemicals avoided precipitate formation (with $MgSO_4$) and chemical reactions of the carbon and nitrogen sources.

The various carbon-nitrogen sources used as heterotrophic nitrification enrichments included acetohydroxamic acid (AHA) (Aldrich, Milwaukee, Wisconsin), 1-aminoethanol (Eastman Kodak Co., Rochester, N.Y.), acetamide (Baker Chemical Co., Phillipsburg, N.J.), ammonium acetate ($NH_4O_2CCH_3$)(Fisher, Medford, Massachusetts), ammonium sulfate [$(NH_4)_2SO_4$] (Fisher), dextrose (Fisher), hydroxylamine-hydrochloride ($NH_2OH \cdot HCl$) (Fisher), sodium citrate (Fisher), sodium nitrite (Mallinckrodt, St. Louis, Missouri), sodium acetate (Baker Chemical Co., Phillipsburg, New Jersey), sodium succinate (Fisher), trypticase soy broth (TSB) and agar (TSA) (B.B.L., Cockeysville, Maryland), M-Standard Methods agar (PCA) (M-trypticase glucose yeast broth) (B.B.L.), yeast extract (B.B.L.) and the amino acids β-alanine, L-asparagine (Eastman-Kodak), L-alanine (Nutritional Biochemicals Corp., Cleveland, Ohio), DL-asparagine, (Mallinckrodt), L-leucine, and L-aspartate (Fisher). Since pyruvic-oxime (PO) was not commercially available, it was synthesized as described by Quastel, et al., (1952).

In view of the indications in the literature that heterotrophic nitrification was favored by a C/N ratio of 10/1 or less (see the C/N ratio section of the literature review), carbon and nitrogen sources used as enrichments were added in quantities sufficient to establish a C/N ratio of 5.0/1 or less.

In addition, certain carbon and nitrogen sources were added

together so that 2 or more sources of either carbon or nitrogen were usually present. Thus glucose and citrate were the carbon sources for enrichment and $(NH_4)_2SO_4$ was the nitrogen source. A medium in which NH_2OH and NO_2^- served as the nitrogen sources and glucose as the carbon source was also tested. Yeast extract, with rare exceptions as noted, was employed solely as a vitamin and cofactor source and was added at a concentration of 50 mg per liter.

Sterilized soil (1 g/100 ml) was added to some enrichments to determine if soil components might be necessary for nitrification. Sterilized soil was used as a supplement primarily after isolates obtained from enrichments had been purified and were being tested for their nitrification capacities. Soil extract, prepared as described by Tate (1977), was employed in some enrichment and isolate nitrification studies.

Isolation procedures. Since there exists no method to selectively favor the growth of heterotrophic nitrifiers, enrichment media were inoculated with local soils (2g/1) and periodically assayed (7, 14, and 21 days) for either NO_2^- or NO_3^- production. The local soils used as inocula included a Hadley lawn and corn field and Amherst field and residential yard soils.

Enrichment cultures were placed on a rotary shaker (118 oscillations/min.) at 27C in the dark. When nitrification became apparent, i.e., a positive nitrite test was obtained (none of the enrichments were NO_3^- positive), 1.0 ml of the enrichment was transferred to 100 ml of fresh sterile medium. The process was repeated and then 0.1 ml of

the enrichment was plated on the same medium solidified by the addition of 1.5% agar (Fisher). Well isolated colonies were chosen and transferred to fresh plates. The transfer of organisms to a fresh plate was repeated and inocula from isolated colonies were streaked on fresh plates of the agar enrichment and of PCA and TSA plates. The PCA and TSA plates were checked for isolate purity and any isolate giving rise to more than one colony type was reinoculated onto the enrichment agar and reisolated until pure cultures were obtained.

The nitrification activity of the isolates was confirmed by adding one drop each of the NO_2^- reagents to the inoculated enrichment plates and observing the pink to red color indicative of NO_2^-. Isolates which did not yield a positive NO_2^- test were discarded. The general procedure utilized in this study to isolate heterotrophic nitrifiers was developed from similar methods described by Doxtader and Alexander (1966c) and Quastel, et al., (1952b).

Isolates which gave indication of the ability to nitrify were subsequently tested further for their nitrification capacities. The enrichment agar plates were used to inoculate fresh, sterile, enrichment medium. These cultures were grown in liquid medium as noted above and were used to determine the various nitrification products synthesized. Spot plates were used for the preliminary determination of NH_2OH and NO_2^- production, by means of the chemical tests noted below, since the number of isolates (100 examined) was large. Finally, only those microbes which consistently synthesized satisfactory amounts of nitrification products (>5 µg product/ml) were maintained as stock cultures.

Characterization of isolates. The identification of actively nitrifying bacteria isolated in this study was based on the biochemical and morphological tests listed in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). The tests performed included catalase (3% H_2O_2), oxidase (Difco, 1972), SIM (Sulfide, Indole, Motility) (Difco, 1972), triple sugar iron (Difco, 1972), indole (Difco, 1972), oxygen sensitivity and O_2 requirement for metabolism (Hugh-Leifson) (Holding and Collee, 1971) arginine dihydrolase (Holding and Collee, 1971), fluorescence and phenazine production (King, et al., 1954), 2-ketogluconate (Holding and Collee, 1971), and 3-ketolactose (Holding and Collee, 1971). Utilization of the following compounds was performed according to the methods of either Holding and Collee (1971) or Stanier, et al., (1966):

acetate	glucose	L-alanine
pyruvate	rhamnose	L-arginine
succinate	starch	L-hisitidine
benzoate	mannitol	aspartate
citrate	fructose	asparagine
butyrate	xylose	acetamide
propionate	ribose	casein
glycerol	ribitol	plate gelatin.
phenol	cellobiose	
ethanol	sucrose	
	maltose	
	arabinose	

The capacity of isolates to metabolize nutrient gelatin was tested as described in the Difco Manual. The litmus milk test (Difco, 1953) was performed as described by Holding and Collee (1971).

Nitrogen use by isolates was also determined. Nitrate utilization was assayed as described by Stanier, et al., (1966) except that sodium acetate was substituted for lactate as a carbon source. Denitrification analysis was performed as described by Stanier, et al., (1966). Nitrogen fixation analysis was performed using Burke's medium (Gunner, 1977), the method described by Stanier, et al., (1966), and the acetylene reduction test (Postgate, 1972).

Isolate metabolism of the macromolecules cellulose and chitin was determined by the methods of Tansey (1971) and Daoust (Ph.D. Thesis, University of Massachusetts, 1978), respectively. Growth in media at various pH values was determined as described by Stanier, et al., (1966) as was the determination of the ability of an isolate to grow at various temperatures. Temperature preference was determined by observing isolate growth on the PO mineral salts-agar medium. Chemolithotrophic growth with hydrogen gas was determined as described by Stanier, et al., (1966).

Stains were employed to characterize isolates. The Acid-fast, Gram, and Negative stains utilized were described by Pelczar and Chair (1972). Hiss' capsule stain and the intracellular lipid stain used were described by Norris and Swain (1971). The flagella stain method of Clark (1976) and Negative stain (phosphotungstic acid) electron microscopy (Lickfield, 1976) were used to determine both the number and position of

flagella.

DNA-guanine plus cytosine analysis. DNA was obtained from the Alcaligenes sp. using the modified technique of Marmur (1961) described by Kowalski (Ph.D. Thesis, University of Massachusetts, 1975). Escherichia coli B served as the standard. The thermal denaturation temperature (Tm) of DNA was determined by the technique of Mandel and Marmur (1968) using the equation (for DNA dissolved in 0.1X Saline-Sodium citrate solution):

$$\% \; G + C = (Tm - 53.9)\; 2.44 \text{ where}$$

$$Tm = \frac{\text{final O.D.} - \text{initial O.D.}}{2}$$

or the midpoint temperature of the thermal denaturation curve.

Growth and nitrification determination. Pure cultures of active nitrifiers were inoculated into PO-mineral salts medium and growth and nitrification recorded. Growth was measured by correlating absorbance at 660 nm with dry weights (Mallette, 1969). Aliquots of both control and experimental flasks were removed aseptically and were assayed for the various products monitored. Control and experimental flasks were then immediately incubated on a rotary shaker in the dark, unless otherwise stated.

Acidic nitrification studies. The mineral salts medium of Verstraete and Alexander (1972a) used in the growth curve procedures was unsatisfactory for the monitoring of acidic nitrification since it was buffered at pH 7.0. Thus, two alternate media, maleate buffer and citrate-phosphate buffer (Gomori, 1955) were employed. Both buffers had an

initial pH of 5.20 - 5.50. One ml/l of trace minerals solution (Schmidt, et al., 1973), 1 ml $MgSO_4 \cdot 7H_2O$ solution/100 ml (added aseptically) (Schmidt, et al., 1973), 0.01% yeast extract, 0.5g KCl/l, 80mg KH_2PO_4/l, 5mg $FeCl_3 \cdot 6H_2O$/l, 5mg $CaSO_4 \cdot 2H_2O$/l, and 0.2% PO were added to the maleate buffer to supply necessary mineral salts and a carbon-nitrogen source. The citrate-phosphate buffer was amended similarly except that yeast extract was not present, and all flasks were incubated in the dark on a rotary shaker (118 oscillations/min) at 27C unless otherwise noted. Aliquots were removed aseptically so that pH, growth, NO_2^-, NO_3^-, bound-NH_2OH, and free-NH_2OH measurements could be determined.

Phosphate buffered resting cell experiments. The method used to assess the Alcaligenes sp. resting cell metabolism was derived from that described by Lees, et al. (1954).

The various carbon sources in a pH 7.0 phosphate buffer (Gomori, 1955), were prepared to determine if NH_2OH in combination with these energy sources would be oxidized by Alcaligenes sp. resting cells. A parallel study of NH_2OH oxidation in the absence of a carbon source was also conducted. Alcaligenes sp. cells, grown for forty-two hours in 0.5% PO-VM, were aseptically harvested by centrifugation (Sorvall super-speed RC-2 centrifuge, 10,000 x g for 15 minutes), washed twice in the sterile phosphate buffer (the approximate volume of the cells taken from the growth vessel). Dry weights were then determined by correlating growth to absorbance. Five ml of the resuspended cells were subsequently transferred to 12.5, 25, or 50 ml Erlenmeyer flasks (each

concentration of a substrate was present in flasks of equal volume, although the results show that this was not a factor). When appropriate to the experimental design, control samples were at this time boiled for 5 minutes in a 100C waterbath. After cooling, 1 ml of either sterile NH_2OH (filter sterilized), sterile PO, sterile Na acetate, sterile Na acetate and sterile NH_2OH, sterile Na pyruvate, or sterile Na pyruvate and sterile NH_2OH, in sufficient quantities to achieve the desired final concentrations, were added aseptically to each flask. The Alcaligenes sp. resting cells were next incubated for 2 hours on a rotary shaker (118 oscillations/min.) at 27C in the dark. Cultures were then centrifuged at 10,000 x g for 5 minutes and aliquots of the clear supernatant were used to determine NO_2^--N concentrations.

Nitrobacter agilis growth and maintenance. Nitrobacter agilis, ATCC 14123, was purchased from the American Type Culture Collection (Rockville, Maryland). Growth and purity were maintained as described by Schmidt, et al., (1973) except that purity checks were made by plating 0.1 ml of the N. agilis cultures onto trypticase - soy agar and M-Standard Methods agar.

Nitrobacter agilis metabolism testing. The ability of Nitrobacter agilis to metabolize PO, or to metabolize NO_2^- in the presence of PO, and its tolerance of NH_2OH were tested during the course of this study. In all experiments, trace mineral - VM [Verstraete and Alexander (1972a) mineral salts medium plus 1 ml $MgSO_4 \cdot 7H_2O$ solution/100 ml, and 1 ml trace mineral salts (Schmidt, et al., 1973)], was used as the mineral salts medium.

To test the ability of N. agilis to metabolize PO, 0.25 ml of a pure stationary phase N. agilis culture was inoculated into 100 ml of sterile trace mineral - VM containing 0.5% PO. Control flasks were identical except they contained no N. agilis cells. Viability of the N. agilis cells was checked by inoculating 0.25 ml of the N. agilis culture into 100 ml of Schmidt, et al., (1973) Nitrobacter medium containing 0.14% $NaNO_2$.

The metabolism of NO_2^- by N. agilis when PO was present was investigated by inoculating 0.5 ml of a pure stationary phase N. agilis culture into 100 ml of sterile trace-mineral VM containing 0.5% PO and 0.14% $NaNO_2$. Control flasks received no N. agilis inoculum. N. agilis viability was monitored by inoculating 0.5 ml of N. agilis into 100 ml of sterile trace mineral - VM containing only 0.14% $NaNO_2$.

The tolerance of N. agilis to NH_2OH was tested by inoculating 0.5 ml of a pure stationary phase N. agilis culture into 100 ml of sterile trace mineral-VM containing approximately 5.3 mg NH_2OH-N/l. Control flasks were treated similarly except no NH_2OH was present. After 1 day the contents of the flasks were filtered aseptically through a 0.20 µm membrane filter (Gelman, Ann Arbor, Michigan) thus trapping the N. agilis cells on the filters. The filters were aseptically removed and placed into 100 ml of sterile 0.14% $NaNO_2$ mineral salts medium (Schmidt, et al., 1973). At this time, 3 uninoculated flasks were added as controls. Measurements for determining NO_2^-, NO_3^-, and free-NH_2OH were made throughout the experiment.

Synergistic nitrification experiments. The occurrence of synergistic

nitrification was assayed by jointly culturing N. agilis and the Alcaligenes sp. Sterile pyruvic-oxime was added to 100 ml of sterile trace mineral - VM and 1 ml of an Alcaligenes sp. culture (grown in 0.5% PO-VM and washed and resuspended in sterile trace mineral - VM), was added. Depending on the experimental design, 0.5 ml of a pure, stationary phase N. agilis culture was either aseptically added immediately (i.e., at T = 0 days) or after 7 days of incubation. Uninoculated flasks and flasks with only Alcaligenes sp. present served as controls.

Analytical Methods

Nitrogen compounds. Ammonia was measured by the phenate procedure detailed in Standard Method for the Examination of Water and Wastewater (Rand, et al., 1976). Free hydroxylamine was assayed by a modification of the method of Magee and Burriss (1954). Samples received 1 ml of 0.5% aqueous sulfamic acid solution per 1 ml of sample, as described by Verstraete and Alexander (1972a), thereby eliminating nitrite interference. Samples were then developed to test for free hydroxylamine as described by Magee and Burris.

Bound hydroxylamine concentrations, a method of determining PO concentrations, were measured by the method of Verstraete and Alexander (1972a). The N-(1-naphythyl)-ethylenediamine dihydrochloride-sulfanilamide method described by Rand, et al., (1976) was used to measure nitrite. Nitrate concentrations were assessed by the chromotropic acid method described by Rand, et al., (1976).

Spot plate analyses for free-NH_2OH and NO_2^- were used to determine

qualitatively if either enrichment cultures or fresh isolates were capable of nitrification. Some of the enrichment and screening culture experiments necessitated the use of activated charcoal (Fisher, Medford, Massachusetts) to remove colors imparted to medium supernatants. After treatment with activated charcoal, analyses were performed as described above. At no time did any of the Alcaligenes sp. or synergistic nitrification experiments require such treatment. Removal of cells by centrifugation resulted in a clear, colorless supernatant which was used to determine products not associated with growth.

Additional analyses. The lanthanum nitrate method (Dawes, et al., 1971) was employed to determine acetate concentrations. Viability of Alcaligenes sp. cells subjected to large concentrations of PO and thus NH_2OH was monitored as follows: 0.1 ml of Alcaligenes sp. cells cultured in PO-VM was distributed onto plates of 0.3% PO agar, trypticase soy agar, and M-Standard Methods agar. The plates were incubated for a maximum of 7 days and were scored either positive (ample growth present) or negative.

The determination of pH was made using either pH papers (Micro Essential Laboratory, Brooklyn, New York) or by using either the Fisher Accumet pH meter (Fisher, Medford, Massachusetts) or a Beckman Zeromatic pH meter. When pH meters were utilized, standard buffers were used to calibrate the meters. Both the IEC Model HT (International Equipment Company, Boston, Massachusetts) and the Sorvall Superspeed RC-2 centrifuge (Dupont) were used either to remove cells from the growth medium

(for nitrogen compound analyses) or for the harvesting and washing of cells.

Statistical analyses. All data reported are means of three replicate samples. The Student's t-test was used to determine significant differences when two means were compared. An analysis of variance followed by Duncan's Multiple Range Test was used to determine significant differences when three or more means were compared.

C H A P T E R IV

RESULTS

Enrichments and Isolation of Heterotrophic Nitrifiers

Acetohydroxamic acid enrichments and isolates. Initial attempts to enrich for heterotrophic nitrifiers employed mineral salts VM supplemented separately with 0.2% acetamide, 0.2% 1-aminoethanol, and 0.6% sodium acetate with 0.2% $(NH_4)_2SO_4$. Two grams per liter of soils from either a local pasture or forest (Amherst, Massachusetts) were added to the enrichment media. After five days of incubation, only those enrichments containing AHA showed any nitrification, i.e., the production of NO_2^--N. Both the field and forest soil inocula were rated as positive since greater than 300 µg NO_2^--N/l was observed while uninoculated control media (both liquid medium and AHA agar plates) failed to demonstrate the presence of NO_2^--N. The AHA-VM enrichments were continued (sterilized soil was added as a supplement in one flask) and 10^{-3}, 10^{-4}, and 10^{-5} dilutions of the enrichments were made onto 0.2% AHA-0.1% YE, PCA, TSA, and nutrient agar plates in an attempt to isolate the responsible organism(s).

While the AHA enrichments were continuing, samples of local soils (Amherst, Massachusetts) were also used to inoculate into 0.3% sodium citrate, 0.3% dextrose, and 0.2% $(NH_4)_2SO_4$-VM. This medium allowed ample growth of many bacteria and fungi. However, none of the enrichment flasks produced NO_2^- or NO_3^- and the glucose-citrate-$(NH_4)_2SO_4$ enrichment was thus discarded.

Both fungal and bacterial isolates were obtained from the AHA, PCA, TSA, and nutrient agar plates inoculated with the dilutions of the AHA-VM enrichment. The isolates were checked for purity on the above-mentioned agar plates and were then monitored for their ability to nitrify in either TSB, glucose-yeast extract-sterile soil-VM (0.3%, 0.15%, and 1g/100 ml, respectively), glucose-yeast extract-VM (0.3% and 0.15%, respectively) and AHA-yeast extract-VM (0.2% and 0.1%, respectively). Of the 71 isolates thus tested, 12 produced NH_2OH-N, (<5 p.p.m.) 4 yielded NO_2^--N (ca. 100 p.p.b.), and none synthesized NO_3^--N.

Inoculating 9 of the more active isolates into media composed of 0.3% sodium acetate, 0.3% dextrose, and 0.3% $(NH_4)_2SO_4$, both with and without sterilized soil, resulted in similarly low yields of products, with only one bacterium producing slight concentrations of NH_2OH-N. The entire set of AHA isolates was tested for nitrification twice when using TSB and 0.3% sodium acetate, 0.3% $(NH_4)_2SO_4$ (with and without sterilized soil) media. Activated charcoal was employed in the analyses since some of the isolates secreted colored substances into the growth medium. Only negligible concentrations of NH_2OH-N, NO_2^--N, and NO_3^--N were produced by 6 of the isolates.

Several AHA isolates were next tested for their capacities to nitrify 0.2% ammonium acetate, 0.15% dextrose, and 0.1% yeast extract, both with and without 1g sterilized soil/100 ml. Again, negligible amounts of NH_2OH-N, NO_2^--N, and NO_3^--N were produced. Only those cultures which contained sterilized soil had NO_3^--N, but this finding

was of little consequence since the sterilized soil accounted for the NO_3^--N present. That is, the sterilized soil contained approximately 0.3 mg NO_3^--N/l and was thus responsible for the low NO_3^--N concentrations observed, 0.3 - 0.6 mg/l.

The amino acids L-asparate, DL-asparagine, and β-alanine were also tested as substrates for nitrification. The amino acids were added to VM at concentrations of 0.1% and a small addition (0.01%) of dextrose was added to serve as an energy source in order that induction to amino acid metabolism might occur. The AHA bacterial isolate used in this study did not cause any nitrification of the amino acids noted. Indeed, growth was either sparse or absent.

Since repeated culturing on laboratory media is, in some situations, known to reduce the capacity of heterotrophs to nitrify, fresh acetamide enrichments were inoculated with local field soils (Amherst, Massachusetts) and also with an AHA isolated bacterium. This attempt to find a heterotrophic nitrifier, however, proved futile as neither NH_2OH, NO_2^-, nor NO_3^- was synthesized. These negative results, which at best yielded less than 0.065 mg NO_2^--N/l by the AHA isolates, led to the initiation of a new procedure to isolate heterotrophic nitrifiers.

Succinate-NO_2^- enrichments and isolations. Enrichment media were prepared in which glucose and NH_2OH and NO_2^- (300 µg dextrose/l, 100 µg NH_2OH-HCl/l, and 100 µg $NaNO_2$/l), sodium succinate and NH_2OH (300 µg sodium succinate/l, 100 µg $NH_2OH \cdot HCl$/l) and sodium succinate and NO_2^- (300 µg sodium succinate/l and 100 µg $NaNO_2$/l) served as the carbon and nitrogen sources respectively. The concentrations of NH_2OH and NO_2^-

were maintained at low levels in the media because of the known toxicity of these compounds. Correspondingly, the concentrations of glucose and succinate were small in order that a carbon to nitrogen ratio of less than 10 to 1 was maintained. Local soils (Amherst and Hadley, Massachusetts) were used to inoculate the enrichment media. While none of the enrichment media demonstrated any activity greater than the controls in the oxidation of NH_2OH (i.e., significant production of NO_2^-), six of the enrichments did show production of NO_3^--N. Aliquots from those enrichment media which yielded NO_3^--N were re-inoculated into fresh medium and monitored for NO_3^- synthesis. Only the succinate-NO_2^- enrichments demonstrated consistent NO_3^- synthesis; inoculated with soils from the banks of the Mill River (Amherst, Massachusetts), a yard (Amherst, Massachusetts), and a corn field (Hadley, Massachusetts), these enrichments all produced more than 4.5 mg NO_3^--N/l. These enrichments were, therefore, diluted and plated onto agar-solidified succinate-NO_2^--yeast extract (50 mg/l) medium.

Ten bacteria were isolated from the succinate-NO_2^- plates. The bacteria were grown in L-leucine-L-asparagine-yeast extract (0.15%, 0.15%, and 50 mg/l, respectively) - VM and were inoculated into the same medium as well as sodium succinate - $(NH_4)_2SO_4$ VM (0.3% sodium succinate and 0.1% $(NH_4)_2SO_4$) in order that the nitrifying activities of the isolates could be observed in greater detail.

After seven days incubation, 9 of the 10 bacteria had produced at least 0.40 mg NO_3^--N/l from the amino acid medium. Similarly, 8 of the 10 isolates had synthesized more than 0.45 mg NO_3^--N/l from the

succinate-NH_4^+ medium.

This general pattern was repeated on the 12th and 21st days with all ten bacterial isolates producing from 0.35 - 1.2 and 0.65 - 1.8 mg NO_3^--N/l from the amino acid and the succinate -NH_4^+ medium, respectively, by the 21st day.

One of the ten isolates, L104, was tested to determine if NH_4^+ and soil extract stimulated L-leucine-L-asparagine nitrification. Soil extract was added at the rate of 100 ml/l medium containing 0.1% L-leucine, 0.1% L-asparagine, and 0.05% $(NH_4)_2SO_4$. Soil extract did not increase NO_3^- production by the bacterium. Indeed, NO_3^- synthesis was less than when the bacterium was cultured in L-leucine-L-asparagine-VM without soil extract. At this point in the study, however, attention was shifted from the succinate-NO_2^- isolates to those isolates demonstrating much more nitrifying activity with pyruvic-oxime as their substrate.

Pyruvic-oxime enrichments and isolates. Verstraete mineral salts medium containing 0.375% pyruvic-oxime was used as an enrichment medium. Four local soils, a corn field and a lawn (Hadley, Massachusetts) and a river bank and a yard soil (Amherst, Massachusetts), were used as inocula and were added at the rate of 1g/100 ml of medium. All the soil enrichments were strongly NO_2^- positive in spot plate tests and 1.0 - 6.6 mg NO_3^--N/l were present by the 7th day of the enrichment. After 40 days of incubation, NO_2^--N was still present in large concentrations and from 1.7 - 10 mg NO_3^--N/l was evident. The pyruvic-oxime enrichments were therefore transferred to fresh PO enrichment

media and again monitored for nitrite and nitrate synthesis. One week of incubation proved sufficient to achieve results similar to those of the primary enrichment and 14 days of incubation resulted in large NO_2^--N concentrations with 10.6 - 17.2 mg NO_3^--N/l being produced. Aliquots of the second set of enrichments were removed, diluted, and inoculated into 0.2% PO-VM agar plates. Isolates thus obtained were transferred to 0.2% PO-VM and were checked for purity by growth on the enriched media TSA and PCA. In this manner, isolate purity was confirmed.

Five bacteria isolated from the corn field environment, designated as OS1-OS5, were tested for their ability to nitrify either 0.2% PO-VM, succinate-NH_4^+-VM (0.3% sodium succinate and 0.15% $(NH_4)_2SO_4$), and L-asparagine-L-monosodium glutamate-NH_4^+-VM (0.2% L-asparagine, 0.2% 2-monosodium glutamate, and 0.035% $(NH_4)_2SO_4$). Two of the bacteria were able to metabolize PO, producing 142 and 47 mg NO_2^--N/l and 9.7 and 3.8 mg NO_3^--N/l by the 21st day. Bacterium OS1 nitrified the amino acids slightly while OS3 was without action on either the amino acids or the succinate-NH_4^+-VM. Interestingly, OS4 and OS5, which nitrified neither PO-VM nor the amino acids, did yield 0.8 - 0.9 mg NO_3^--N/l when cultured in the succinate-NH_4^+-VM.

This experiment was then repeated, with the modification that L-alanine was substituted for L-monosodium glutamate, using bacteria isolated from the other PO-VM enrichments. After 21 days of culturing only 2 bacteria were observed to be active PO nitrifiers, yielding up to 145 mg NO_2^--N and 11.3 mg NO_3^--N/l. These organisms, termed MR1 and

GS1 did not nitrify in the succinate-NH_4^+-VM and GS1 yielded only a small quantity, 1.1 mg/l, of NO_3^--N, when grown in the amino acid-VM. Thus, the four active PO nitrifiers, OS1, OS3, GS1, and MR1, were subsequently maintained on PO-VM, TSA, and PCA plates.

Since PO had functioned as an easily nitrified substrate for the bacteria monitored, the 10 bacteria isolated from the succinate-NO_2^- enrichments were cultured in 0.3% PO-VM to determine if they were capable of PO nitrification. All of these isolates were initially capable of PO nitrification yielding NO_2^- and NO_3^- by the 21st day of culturing. Of these bacteria, only one was capable of producing quantities at a level similar to those produced by OS1, OS3, GS1, and MR1. This bacterium, designated L3, yielded 135 mg NO_2^--N and 8.9 mg NO_3^--N/l when cultured in PO-VM for 21 days. The other succinate NO_2^- isolates yielded only 1.5 - 3.3 mg NO_2^--N/l and negligible amounts of NO_3^--N.

It was therefore decided that subsequent studies would be performed with OS1, OS3, GS1, MR1, and L3. However, culturing of MR1 and L3 on enriched media resulted in a decrease in their production of NO_2^- and NO_3^- from PO, i.e., a reduction from 150 mg NO_2^--N/l to 5 mg NO_2^--N/l and of 10-30 mg NO_3^--N/l to less than 0.3 mg NO_3^--N/l. It was conjectured that this repression might be due to the culturing of MR1 and L3 on the enriched media TSA and PCA. An attempt was therefore made to induce these microbes to nitrify PO by culturing them repeatedly on 0.3% PO-VM agar plates. While the organisms did grow, 16 successive passages of MR1 and L# on the PO-VM plates did not return to them their

previous nitrification capabilities. These organisms were thus discarded in favor of OS1, OS3, and GS1 which had been maintained on 0.2% PO-VM plates and were highly active in nitrifying PO. Transfers of OS1, OS3, and GS1 on PO-VM plates were and are continuing and these stock cultures were the source of cells for the various morphological and biochemical tests described in the appendix. The OS1, OS3, and GS1 stock cultures have remained active and will be so maintained in order to avoid the repression of PO oxidizing activity.

Nitrification of pyruvic-oxime by an Alcaligenes sp. After having isolated and established that OS1, OS3, and GS1 could nitrify PO, their nitrification capacities were further characterized and a preliminary attempt was made to establish their taxonomic position. As shown in the appendix, all three microbes are members of the genus Alcaligenes. However, species identification was not possible since various characteristics of each isolate deviated from the species descriptions listed in Bergey's Manual of Determinative Microbiology (18th ed.). That the organisms originally designated OS1 and GS1 are the same microbe was deduced from the morphological and biochemical data shown in the appendix, while OS3 is either a subspecies of this bacterium or a closely related species.

The Alcaligenes sp. known as GS1 was also capable of oxidizing 0.25% PO-VM to NO_2^- and NO_3^- (Table 3) and produced maxima of 95.3 mg NO_2^--N/l and 9.5 mg NO_3^--N/l. Growth of this organism in the PO-VM was closely correlated with nitrification, reaching a maximum value of 19.3 mg/ml and then declining to 8.80 mg/ml by the end of the experiment (14 days).

TABLE 3

Nitrification of 0.25% PO-VM by Alcaligenes sp. strain GS1.

Time (days)	Growth (mg/ml)		pH		NH_4^+-N (Spot Test)		NO_2^--N (mg/ml)		NO_3^--N (mg/ml)	
	[1] Con.	[2] Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0.00	<.193	<.193	7.33	7.29	-	-	.022	.084^{+}	<.30	<.30
0.12	<.193	<.193	7.40	7.32	-	-	.025	.283^{+}	<.30	<.30
0.29	<.193	.775^{++}	7.42	7.32	-	-	.042	1.10^{++}	<.30	<.30
0.50	<.193	1.80^{++}	7.53	7.38	-	-	.064	3.70^{++}	<.30	<.30
1.00	<.193	11.2^{++}	7.40	7.32	-	-	.015	82.5^{++}	<.30	3.9^{+}
2.00	<.193	19.3^{++}	7.38	7.37	-	-	.048	94.7^{++}	<.30	5.6
5.00	<.193	14.4^{++}	7.43	7.47	-	-	.100	86.3^{++}	<.30	5.3
7.00	<.193	13.7^{++}	7.36	7.44	-	-	.071	91.7^{++}	<.30	4.0
14.00	<.193	8.80^{++}	7.41	7.45	-	-	.054	95.3^{++}	<.30	9.5^{++}

[1] Uninoculated control

[2] Alcaligenes sp. strain GS1

$^{+}$ Significantly different from control ($p \leq .05$)

$^{++}$ Significantly different from control ($p \leq .01$)

Ammonium ion was not present in either the control or the Alcaligenes sp. medium and the pH of the cultures did not differ from one another. Since PO in solution is in equilibrium with pyruvate, it was postulated that acetate might be a product of PO metabolism. However, at no time was acetate present in either the control or Alcaligenes sp. cultures.

The Alcaligenes sp. termed OS3 also nitrified PO. However, this microbe differed from the Alcaligenes spp. OS1 and GS1 in that 50 mg yeast extract/l and static conditions stimulated its oxidation of PO (Table 4). As with Alcaligenes sp. strain GS1, nitrification and growth were closely associated; 7.87 mg/ml was the maximum cell density obtained. Concentrations of up to 47.2 mg NO_2^--N/l and 1.3 mg NO_3^--N/l were produced by Alcaligenes sp. strain OS3. Similar to the data of Table 3, neither NH_4^+ nor acetate was synthesized at any time during this experiment and no significant pH differences between the control and the Alcaligenes sp. were noted.

The capacity of Alcaligenes sp. strain OS1 to nitrify when cultured in 0.2% PO-VM and 0.3% PO-50 mg/l yeast extract-VM is reflected in the data shown in Tables 5 and 6. The results obtained were similar to those for the Alcaligenes sp. strains OS3 and GS1. The nitrification of PO by strain OS1 was also closely correlated to its growth. NO_2^--N and NO_3^--N production rose steadily with the growth of the bacterium and reached maximal values of 197 and 13.2 mg/l, respectively, after 14 days. The pH of the medium was not affected by PO metabolism and neither NH_4^+-N nor acetate was produced in any of the culture flasks. Bound-NH_2OH-N, a measure of PO concentration, decreased rapidly with

TABLE 4

Nitrification of 0.2% PO-0.005% Yeast Extract-VM by Alcaligenes sp. strain OS3.

Time (days)	Growth (mg/ml)		pH		NH_4^+-N (Spot Test)		NO_2^--N (mg/ml)		NO_3^--N (mg/ml)	
	[1] Con.	[2] Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0.00	<.095	<.095	7.01	7.05	-	-	<.007	<.007	<.30	<.30
0.15	<.095	<.095	7.10	7.11	-	-	.054	.120^{++}	<.30	<.30
0.29	<.095	<.095	7.07	7.12	-	-	.046	.360^{++}	<.30	<.30
0.44	<.095	1.40^{++}	7.11	7.14	-	-	.102	1.28^{++}	<.30	<.30
1.00	<.095	2.83^{++}	7.14	7.18	-	-	.056	9.83^{+}	<.30	.48^{++}
2.00	<.095	4.90^{++}	7.13	7.14	-	-	.060	19.2^{+}	<.30	1.3
5.00	<.095	7.35^{++}	7.11	7.07	-	-	.093	42.2^{++}	<.30	.95^{+}
7.00	<.095	7.87^{++}	7.19	7.20	-	-	.129	39.7^{++}	<.30	.44
14.00	<.095	3.65^{+}	7.20	7.20	-	-	.133	47.2^{++}	<.30	1.2^{++}

[1] Uninoculated control

[2] Alcaligenes sp. strain OS3

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$Significantly different from control ($p \leq .01$)

TABLE 5

Nitrification of 0.2% PO-VM by *Alcaligenes* sp. strain OS1.

Time (days)	Growth (mg/ml)		pH		Bound-NH_2OH-N (mg/ml)		NO_2^--N (mg/ml)		NO_3^--N (mg/ml)	
	[1]Con.	[2]*Alcal.*	Con.	*Alcal.*	Con.	*Alcal.*	Con.	*Alcal.*	Con.	*Alcal.*
0.00	<.560	<.560	7.10	7.10	159	154	.052	.069	<.30	<.30
0.19	<.560	<.560	7.10	7.10	138	136	.037	.192^{+}	<.30	<.30
1.00	<.560	1.50^{++}	7.18	7.20	161	119^{++}	.067	11.0^{++}	<.30	.44
3.00	<.560	4.80^{++}	7.17	7.19	119	<.050^{++}	.096	70.0^{++}	<.30	6.8^{++}
7.00	<.560	4.00^{++}	7.11	7.09	143	<.050^{++}	.159	69.2^{++}	<.30	11.3^{++}
14.00	<.560	2.63	7.13	7.10	131	<.050^{++}	.680	63.8^{++}	<.30	3.0

[1]Uninoculated control

[2]*Alcaligenes* sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$ Significantly different from control ($p \leq .01$)

TABLE 6

Nitrification of 0.3% PO-50 mg Yeast Extract/l-VM by Alcaligenes sp. strain OS1.

Time (days)	Growth (mg/ml)		pH		NH_4^+-N (Spot Test)		NO_2^--N (mg/ml)		NO_3^--N (mg/ml)	
	Con.[1]	Alcal.[2]	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0.00	<.560	<.560	7.20	7.18	-	-	.011	.173^{++}	<.30	<.30
0.17	<.560	<.560	7.20	7.18	-	-	.013	1.23^{++}	<.30	<.30
0.33	<.560	.587	7.23	7.17	-	-	.022	3.40^{++}	<.30	<.30
0.50	<.560	1.60^{++}	7.19	7.30^{+}	-	-	.023	10.8^{++}	<.30	<.30
1.00	<.560	16.6^{++}	7.20	7.18	-	-	.024	161^{++}	<.30	3.5
2.00	<.560	13.2^{++}	7.19	7.22	-	-	.047	175^{++}	<.30	17^{++}
4.00	<.560	11.5^{++}	7.19	7.30	-	-	.057	184^{++}	<.30	3.2^{++}
7.00	<.560	10.1^{++}	7.18	7.24	-	-	.104	196^{++}	<.30	10.1^{++}
14.00	<.560	8.70^{++}	7.06	7.18	-	-	.185	197^{++}	<.30	13.2^{++}

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$Significantly different from control ($p \leq .01$)

the proliferation of strain OS1 and was below the limit of resolution by the third day of the experiment, as shown in Table 5.

Since Alcaligenes sp. strain OS1 demonstrated active nitrification of PO, it was selected as the strain to further characterize the metabolism of PO in this study. Alcaligenes sp. strain OS1 did not require yeast extract to nitrify PO. Nitrification of 0.3% PO-VM by this microbe proceeded well when the 50 mg yeast extract/l was omitted from the medium (Table 7). Henceforth, yeast extract was excluded from PO-VM in which the Alcaligenes sp. strain OS1 was cultured. This omission did not alter NO_2^- or NO_3^- synthesis, diminish PO metabolism, nor cause an accumulation of either acetate or NH_4^+.

Characterization of PO nitrification by Alcaligenes sp. strain OS1.

Since the 197 mg NO_2^--N/l (Table 6) produced by strain OS1 was a relatively large yield, initial PO-VM concentrations were increased in order to assess the maximum NO_2^--N synthesizing capacity of the organism. The Alcaligenes sp. was cultured in 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5% PO-VM. By noting the nitrification patterns of strain OS1 at these PO concentrations, a profile of its nitrification capacities was established. Since neither acetate nor NH_4^+ had been produced by any of the isolates, and since the pH of the medium had not varied as a consequence of PO metabolism by the Alcaligenes spp., these parameters were not measured in the following experiments.

The Alcaligenes sp. nitrified readily in 1.0% PO-VM (Table 8). Bound-NH_2OH-N concentrations decreased with the growth of the bacterium and with the consequent synthesis of NO_2^- and NO_3^-. Large concentrations

TABLE 7

Nitrification of 0.3% PO-VM by *Alcaligenes* sp. strain OS1.

Time (days)	Growth (mg/ml)		pH		NO_2^--N (mg/ml)		NO_3^--N (mg/ml)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
1	<.560	7.50	7.20	7.10	<.007	52.5	<.30	6.9
7	<.560	7.50	7.20	7.10	.047	70.0	<.30	3.2
19	<.560	1.30	7.30	7.30	.082	67.5	<.30	2.3

[1]Uninoculated control

[2]*Alcaligenes* sp. strain OS1

TABLE 8

Nitrification of 1.0% PO-VM by Alcaligenes sp. strain OS1.

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0.00	<.560	<.560	891	882	.021	.123^{+}	<.30	<.30
0.25	<.560	<.560	723	761	.017	.158	<.30	<.30
1.00	<.560	1.60	849	612	.041	7.48	<.30	<.30
3.00	<.560	37.2	840	44.8^{++}	.077	272^{++}	<.30	17
7.00	<.560	24.5^{++}	926	0.630^{++}	.110	675^{++}	<.30	21^{++}
14.0	<.560	20.7^{++}	831	0.050^{++}	.250	733^{++}	<.30	12^{++}

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$Significantly different from control ($p \leq .01$)

of NO_2^--N and NO_3^--N were produced by the Alcaligenes sp., up to 733 and 21 mg/l, respectively.

In Tables 9 and 10 are shown the nitrification of the Alcaligenes sp. in 1.5% PO. Again, the metabolism of PO (bound NH_2OH-N) corresponds with growth and nitrification. NO_2^--N and NO_3^--N concentrations rose to 1023 and 22 mg/l, respectively.

The oxidation of 2.0 and 2.5% PO-VM by the Alcaligenes sp. gave results confirming the observations of the previous experiments (Tables 11 and 12). Growth increased rapidly as nitrification proceeded and PO was depleted. Significantly high concentrations of nitrification products were synthesized, with maximal values reaching 1867 mg NO_2^--N/l and 42 mg NO_3^--N/l. Further study, however, showed that 2.5% PO-VM was the concentration of substrate which yielded the greatest concentration of nitrification products. In Figure 1 is shown the production of NO_2^- and NO_3^- by the Alcaligenes sp. when cultured in 2.5% PO-VM.

As shown in Table 13 the concentration at which inhibition of nitrification by Alcaligenes sp. occurred was 3.0%. Growth, NO_2^- and NO_3^- production, and PO metabolism were inhibited at 3.0% PO though patterns of nitrification similar to those previously noted did occur. The concentrations of NO_2^--N and NO_3^--N synthesized did rise as growth increased and both bound- and free-NH_2OH-N levels decreased as PO was oxidized. Thus, 3.0% PO caused a reduction in nitrification by the Alcaligenes sp., but did not prevent the microbe from exhibiting a typical pattern of PO utilization.

The results of culturing the Alcaligenes sp. in 3.5% PO-VM

TABLE 9

Nitrification of 1.5% PO-VM by *Alcaligenes* sp. strain OS1.

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]*Alcal.*	Con.	*Alcal.*	Con.	*Alcal.*	Con.	*Alcal.*
0.00	<.560	<.560	1101	1027	.048	.233^{++}	<.30	<.30
0.25	<.560	<.560	1237	1325	.042	.700^{++}	<.30	<.30
1.00	<.560	4.87^{++}	1400	1204	.052	23.0^{++}	<.30	<.30
3.00	<.560	48.8^{++}	1237	72.8^{++}	.072	668^{++}	<.30	4.3^{+}
7.00	<.560	29.8^{++}	1097	.050^{++}	.093	850^{++}	<.30	7.5^{+}
14.0	<.560	22.7^{++}	1255	.050^{++}	.173	850^{++}	<.30	1.4^{+}

[1]Uninoculated control

[2]*Alcaligenes* sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$Significantly different from control ($p \leq .01$)

TABLE 10

Nitrification of 1.5% PO-VM by *Alcaligenes* sp. strain OS1.

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0.00	<.560	<.560	1260	1591	.187	.313^{++}	<.30	<.30
0.25	<.560	<.560	1379	1493	.179	.783^{++}	<.30	<.30
1.00	<.560	3.60^{+}	1598	1533	.204	14.1^{++}	<.30	.30
3.00	<.560	48.4^{++}	1412	124.2^{++}	.206	683^{++}	<.30	11^{+}
7.00	<.560	36.3^{++}	1417	.050^{++}	.233	1023^{++}	<.30	19^{++}
14.0	<.560	23.7^{++}	1528	.050^{++}	.257	983^{++}	<.30	22^{++}

[1]Uninoculated control

[2]*Alcaligenes* sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$Significantly different from control ($p \leq .01$)

TABLE 11

Nitrification of 2.0% PO-VM by Alcaligenes sp. strain OS1.

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	<.560	<.560	1731	1692	.029	.075^{++}	<.30	<.30
1	<.560	.773	1680	1680	.048	3.20	<.30	<.30
3	<.560	33.6^{+}	1715	602^{++}	.025	306	<.30	2.6
7	<.560	51.6^{++}	1400	61.2^{++}	.397	1242^{++}	<.30	20^{+}
14	<.560	51.6^{++}	1878	.050^{++}	.086	1517^{++}	<.30	42^{+}

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$Significantly different from control ($p \leq .01$)

TABLE 12

Nitrification of 2.5% PO-VM by *Alcaligenes* sp. strain OS1.

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]*Alcal.*	Con.	*Alcal.*	Con.	*Alcal.*	Con.	*Alcal.*
0	<.560	<.560	2023	1965	.036	.147^{++}	<.30	<.30
1	<.560	<.560	1946	1953	.053	2.50^{++}	<.30	<.30
3	<.560	5.07	1888	1843	.079	71.7^{+}	<.30	.43
7	<.560	58.0^{++}	1657	380.0^{++}	.096	1198^{++}	<.30	17^{++}
14	<.560	22.3^{++}	1969	3.27^{++}	.115	1867^{++}	<.30	40^{++}

[1]Uninoculated control

[2]*Alcaligenes* sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$Significantly different from control ($p \leq .01$)

Figure 1. Nitrification of 2.5% PO-VM by Alcaligenes sp. strain OS1.

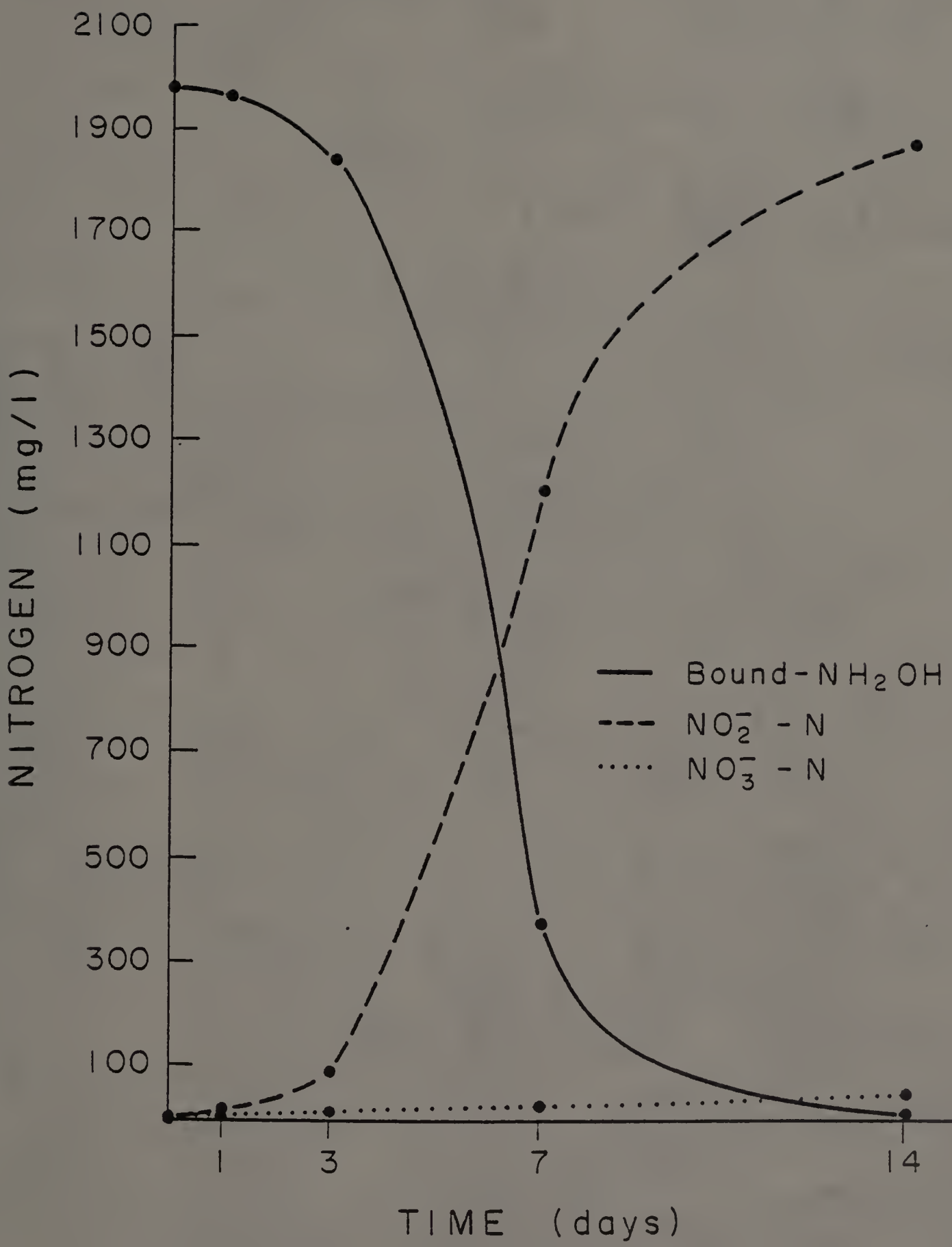

2100
1900
1700
1500
1300
1100
900
700
500
300
100
NITROGEN (mg/l)
Bound-NH2OH
NO2- - N
NO3- - N
1
3
7
14
TIME (days)

TABLE 13

Nitrification of 3.0% PO-VM by *Alcaligenes* sp. strain OS1.

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	<.560	<.560	3232	2917	152	147	.010	.190^{++}	<.30	<.30
1	<.560	.740	3197	2917	170	155	.029	5.30	<.30	<.30
3	<.560	12.7	3640	2905	210	168	.052	184	<.30	2.0
7	<.560	6.13	3080	1937^{+}	504	288^{+}	.062	705^{+}	<.30	18
15	<.560	10.1^{+}	3127	57.4^{++}	155	30.7^{++}	.075	675^{++}	<.30	16^{+}
18	<.560	9.33^{+}	3640	56.0^{++}	214	33.8^{++}	.107	957^{+}	<.30	24^{+}
25	<.560	9.63^{+}	4013	31.3^{++}	282	24.0^{++}	.135	967^{+}	<.30	18^{++}

[1]Uninoculated control

[2]*Alcaligenes* sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$Significantly different from control ($p \leq .01$)

(Table 14) support the data of Table 13. Inhibition of PO nitrification was clearly evident since only 608 mg NO_2^--N/l and 9.9 mg NO_3^--N/l were synthesized. Concomitant with the low yields of NO_2^- and NO_3^- was the apparent inability of the Alcaligenes sp. to completely oxidize both PO and NH_2OH. Thus, after 28 days of culturing, PO and NH_2OH were still present in the growth medium. Incomplete metabolism of PO is also reflected in the low growth yield of the microbe with 4.00 mg dry weight/ml the maximum obtained.

Complete cessation of PO metabolism by the Alcaligenes sp. occurred when the culture medium was either 4.0 or 4.5% PO-VM (Tables 15 and 16). The Alcaligenes sp. failed to grow and neither NO_2^- nor NO_3^- was synthesized. Free-NH_2OH was not metabolized as evidenced by the comparable NH_2OH-N concentrations of the control and Alcaligenes sp. cultures throughout the experiments. The viability of the Alcaligenes sp., when subjected to large concentrations of PO and thus NH_2OH, was monitored by plating aliquots of the 4.0% PO-VM culture medium onto plates of 0.3% PO-VM, PCA, and TSA (Table 17). As the data indicate, the Alcaligenes sp. tolerated 3700 mg PO-N/l and 325 mg NH_2OH-N/l, even though the organism was unable to metabolize any PO at this concentration. The Alcaligenes sp. is thus able to withstand large quantities of both PO and NH_2OH.

In Figure 2 is illustrated the maximum production of NO_2^- and NO_3^- at various concentrations of PO by the Alcaligenes sp. Synthesis of NO_2^--N rose linearly with initial concentrations of PO from 0.2 - 2.5%. Above 2.5% PO, a marked decrease in the capability

TABLE 14

Nitrification of 3.5% PO-VM by Alcaligenes sp. strain OS1.

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	< .560	< .560	3593	3640	150	150	.014	.126^{++}	<.30	<.30
1	< .560	< .560	3768	3920	198	203	.027	1.97^{++}	<.30	<.30
3	< .560	< .560	3173	3232	267	388	.037	14.2	<.30	<.30
7	< .560	.573	4095	4042	235	344	.032	21.2	<.30	<.30
14	< .560	4.00^{+}	3617	477.7^{++}	250	230	.088	582^{+}	<.30	7.4^{++}
21	< .560	2.67^{+}	3199	55.8^{++}	147	36.8^{++}	.233	508	<.30	2.8
28	< .560	3.53^{++}	3745	51.6^{++}	174	39.5^{++}	.250	608	<.30	9.9^{++}

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{++}$Significantly different from control ($p \leq .01$)

TABLE 15

Inhibition of nitrification by Alcaligenes sp. strain OS1 in 4.0% PO-VM.

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	<.560	<.560	3360	3407	181	189	.032	.046	<.30	<.30
1	<.560	<.560	3827	4025	195	190	.044	.167$^+$	<.30	<.30
3	<.560	<.560	3593	3722	333	357	.056	.333$^+$	<.30	<.30
7	<.560	<.560	4433	4352	334	325	.140	.520	<.30	<.30
14	<.560	<.560	3593	4247	251	389	.187	.777	<.30	<.30

[1] Uninoculated control

[2] Alcaligenes sp. strain OS1

[+] Significantly different from control ($p \leq .05$)

TABLE 16

Inhibition of nitrification by *Alcaligenes* sp. strain OS1 in 4.5% PO-VM

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	<.560	<.560	3430	3290	256	227	.027	.072[+]	<.30	<.30
1	<.560	<.560	3500	3243	141	128	.030	.410[+]	<.30	<.30
3	<.560	<.560	3955	4256	179	179	.042	.500[+]	<.30	<.30
7	<.560	<.560	3967	3558	411	290	.100	.700[+]	<.30	<.30
14	<.560	<.560	3500	3080	146	118	.185	.783[+]	<.30	<.30

[1]Uninoculated control

[2]*Alcaligenes* sp. strain OS1

[+]Significantly different from control ($p \leq .05$)

TABLE 17

Viability of Alcaligenes sp. strain OS1 in the presence of 4.0% PO-VM.*

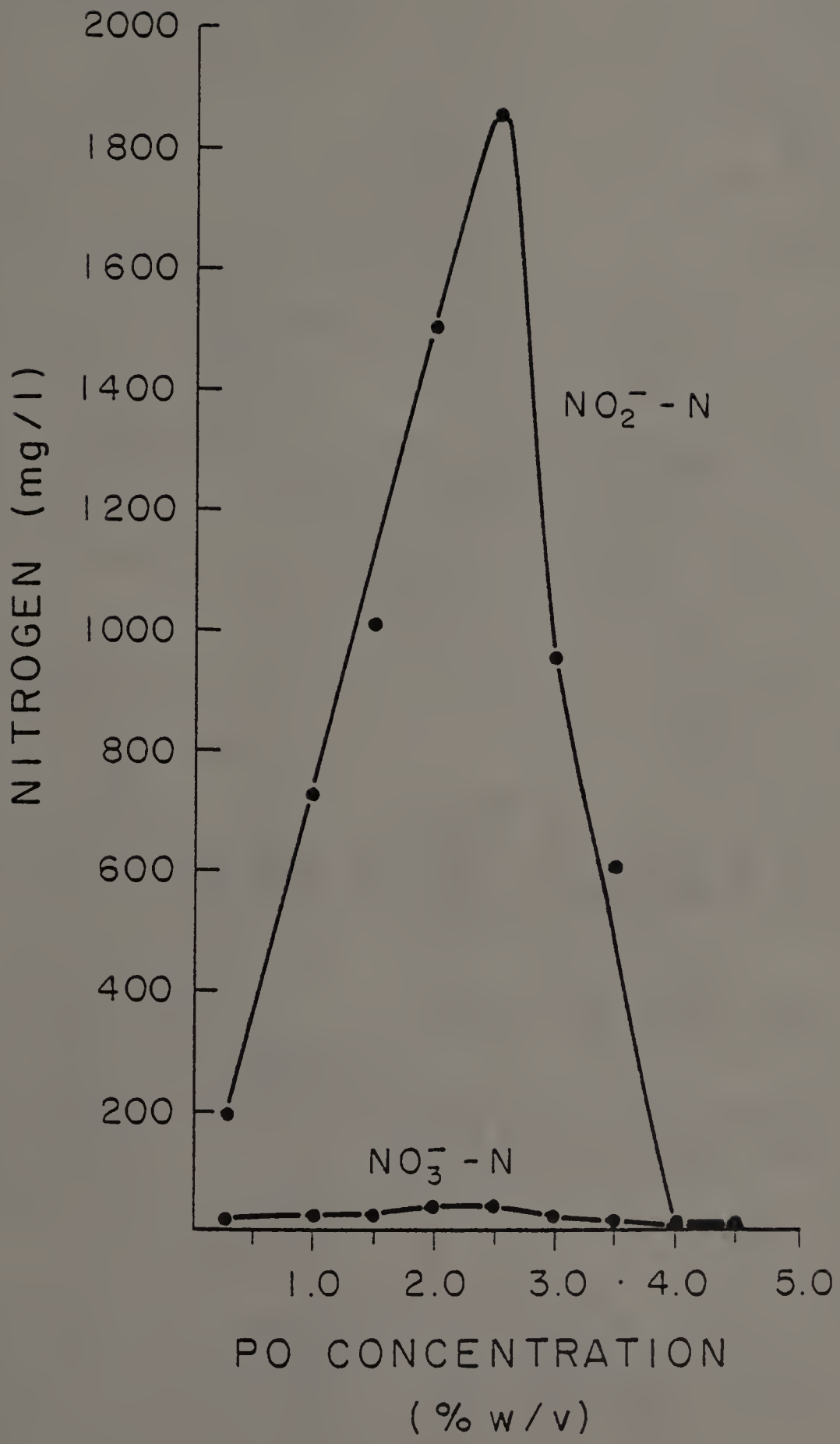
Time (days)	Aliquot 1			Aliquot 2			Aliquot 3		
	PO	PCA	TSA	PO	PCA	TSA	PO	PCA	TSA
0	+	+	+	+	+	+	+	+	+
1	+	+	+	-	-	+	-	-	-
3	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+

*A 10^{-1} ml aliquot from each growth vessel was used to inoculate agar plates of 0.3% PO, M-Endo Standard Methods Agar (PCA), and Trypticase Soy Agar.

+Positive (Abundant Growth)

-Negative (No Growth)

Figure 2. NO_2^- and NO_3^- production by Alcaligenes sp. strain OS1 at various concentrations of PO.


2000
1800
1600
1400
1200
1000
800
600
400
200
NITROGEN (mg/l)
NO_2^- - N
NO_3^- - N
1.0
2.0
3.0
4.0
5.0
PO CONCENTRATION
(% w/v)

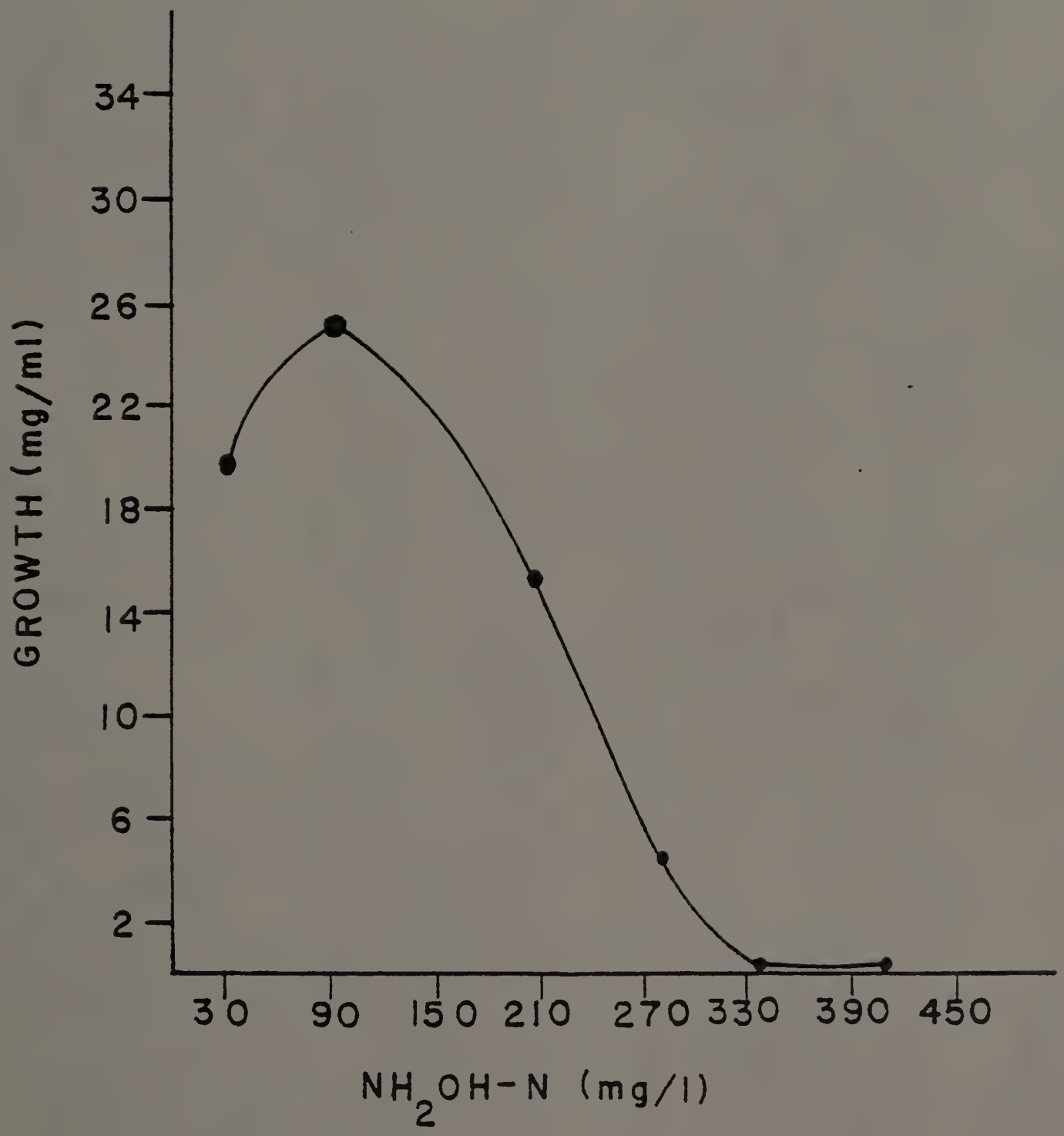
of the Alcaligenes sp. to produce NO_2^- is evident with no NO_2^- being produced at either 4.0 or 4.5% PO. Nitrate production, while distinctly less than NO_2^--N synthesized, was subject to the same general pattern. Concentrations of NO_3^--N were maximal at 2.0 - 2.5% PO (42 mg NO_3^--N/l) and declined thereafter to negligible values at 4.0 and 4.5% PO.

A plot of growth vs. NH_2OH-N concentrations (Figure 3) shows patterns similar to those of PO concentrations and nitrification. Growth was maximal at the lower concentrations of NH_2OH-N, while concentrations greater than 250 mg NH_2OH-N/l resulted in a pronounced decrease in the growth (and subsequent PO metabolism) of the Alcaligenes sp. Although the growth of Alcaligenes sp. did not occur at 325 mg NH_2OH-N/l, the organism remained viable (Table 17).

Oxidation of NH_2OH by the Alcaligenes sp. Pyruvic-oxime dissociates in solution yielding pyruvic acid and NH_2OH. In the previous experiments vigorous oxidation of PO by the Alcaligenes sp. and the concomitant growth and nitrification associated with PO metabolism was noted. However, the question as to whether or not NH_2OH could be oxidized by the Alcaligenes sp. remained to be answered. Therefore, the following set of experiments were performed to determine if NH_2OH was metabolized by the Alcaligenes sp. In addition, stimulation of NH_2OH oxidation by microbial metabolism of exogenous carbon was also investigated.

Resting cells of the Alcaligenes sp. were prepared as described in the Materials and Methods section. Boiled cells of the Alcaligenes sp. were used as controls and secondary controls were maintained by

Figure 3. Growth of Alcaligenes sp. strain OS1 at various concentrations of NH_2OH in equilibrium with PO.


GROWTH (mg/ml)
34
30
26
22
18
14
10
6
2
30
90
150
210
270
330
390
450
NH2OH-N (mg/l)

monitoring NO_2^--N production in flasks when no NH_2OH, either with or without exogenous carbon, was present.

Resting cells of the Alcaligenes sp. were found to be capable of oxidizing NH_2OH (Table 18). Significantly greater amounts of NO_2^- were synthesized by the resting cells in all cases when NH_2OH was present. The data also show, however, that under these circumstances, the efficiency of NH_2OH oxidation was low. That is, an average of only 4.6% of the NH_2OH initially present was converted to NO_2^- by the resting cells during the two hours of incubation.

The data in Table 19 show the marked stimulation of NO_2^--N synthesis when PO functioned as the nitrogen source. The resting cells of the Alcaligenes sp. were quite active in metabolizing PO. These cells synthesized copious quantities of NO_2^--N, converting an average of 52% of the initial PO-N to NO_2^--N by the end of the two hour incubation period.

The addition of acetate carbon caused a slight stimulation of NH_2OH oxidation (Table 20) when compared to those cultures which received only NH_2OH (Table 18). An average of 6.3% of the NH_2OH-N initially present was transformed to NO_2^--N. The low efficiency of NH_2OH conversion to NO_2^- may perhaps be explained on the basis of induction. Although the Alcaligenes sp. does metabolize acetate (see Appendix), it is possible that the two hour incubation period was insufficient to allow the induction of the enzymes that would metabolize acetate. Additionally, since the resting cell experiments were conducted in phosphate buffer, cofactors necessary for acetate metabolism may have been absent.

TABLE 18

Oxidation of NH_2OH-N by resting cells of Alcaligenes sp. strain OS1.*

Initial NH_2OH-N Concentration (mg/l)	NO_2^--N Synthesized (mg/l)	
	[1]Control	[2]Alcaligenes sp.
0.00	< .007	<.007
2.85	< .007	.146++
3.00	.083	.154+
5.70	< .007	.246++
6.00	.083	.265+
12.0	< .007	.483++

*The cell density was approximately 17.2 mg/ml.

[1]Control cultures were Alcaligenes sp. strain OS1 resting cells boiled for 5 minutes in a 100C water bath.

[2]Alcaligenes sp. strain OS1 resting cells.

+Significantly different from control ($p \leq .05$)

++Significantly different from control ($p \leq .01$)

TABLE 19

Oxidation of Pyruvic-Oxime by resting cells of Alcaligenes sp. strain OS1.*

Initial PO-N Concentration (mg/l)	NO_2^--N Synthesized (mg/l)	
	[1]Control	[2]Alcaligenes sp.
0	<.007	<.007
3	<.007	1.55[++]
6	<.007	2.91[++]
12	<.007	6.75[++]

*The cell density was approximately 17.2 mg/ml.

[1]Control cultures were Alcaligenes sp. strain OS1 resting cells boiled for 5 minutes in a 100C water bath.

[2]Alcaligenes sp. strain OS1 resting cells.

[++]Significantly different from control ($p \leq .01$)

TABLE 20

Oxidation of NH_2OH-N by resting cells of Alcaligenes sp. strain OS1 in the presence of sodium acetate.*

Initial NH_2OH-N Concentrations (mg/l)	Initial Acetate-C Concentrations (mg/l)	NO_2^--N Synthesized (mg/l)	
		[1]Control	[2]Alcal.
0.00	12.0	<.007	<.007
2.85	6.0	.071	.249++
5.70	12.0	<.007	.312++
12.0	12.0	<.007	.560++

*The cell density was approximately 16.7 mg/ml.

[1]Control cultures were Alcaligenes sp. strain OS1 resting cells boiled for 5 minutes in a 100C water bath.

[2]Alcaligenes sp. strain OS1 resting cells.

++Significantly different from control ($p \leq .005$).

Sodium pyruvate allowed the transformation of NH_2OH to NO_2^- to proceed much quicker than did either sodium acetate or NH_2OH alone (Table 21). Resting cells of the Alcaligenes sp. converted an average of 32.5% of the initial NH_2OH-N present to NO_2^--N when pyruvate was present. Only PO permitted a more rapid conversion to NO_2^- of NH_2OH.

The data of Tables 18-21 collectively confirmed that NH_2OH is readily oxidized to NO_2^- by cells of the Alcaligenes sp. which were grown in PO-VM. Additionally, an exogenous carbon source, either acetate or pyruvate, stimulated the conversion of NH_2OH-N to NO_2^--N. The presence of exogenous pyruvate caused a 7 fold increase of NO_2^--N synthesis over the NO_2^--N produced by resting cells incubated with NH_2OH only.

Nitrification of PO in acidic media by the Alcaligenes sp. As mentioned previously, the three isolates used in this study were collected from local soils. Since the pH of soils in Hadley, Massachusetts is approximately 4.5 - 5.5, investigations were initiated to determine if the Alcaligenes sp. was capable of nitrifying PO when cultured in acidic media. The first set of acidic nitrification experiments were performed with maleate buffer (pH 5.4 - 5.5) amended with mineral salts, KH_2PO_4(40 mg/l), and pyruvic-oxime.

In Table 22 are the results of culturing the Alcaligenes sp. in the maleate buffer medium at pH 5.5. Nitrification of PO by the bacterium did not occur. Little if any NO_2^--N was synthesized and no NO_3^- was produced. Similarly, neither bound- nor free-NH_2OH-N was metabolized and growth was not evident.

TABLE 21

Oxidation of NH_2OH-N by resting cells of Alcaligenes sp. strain OS1[2] in the presence of sodium pyruvate.*

Initial NH_2OH-N Concentration (mg/l)	Initial Pyruvate-C Concentration (mg/l)	NO_2^--N Synthesized (mg/l)	
		1 Control	2 Alcaligenes
0.0	12	<.007	<.007
6.0	6.0	.031	2.08+
12.0	12	.089	3.57+

*The cell density was approximately 15.5 mg/ml.

[1]Control cultures were Alcaligenes sp. strain OS1 cells boiled for 5 minutes in a 100C water bath.

[2]Alcaligenes sp. strain OS1 resting cells.

+Significantly different from control ($p \leq .005$)

TABLE 22

Nitrification by Alcaligenes sp. strain OS1 of 0.2% PO at pH 5.5.

Time (days)	pH		Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	5.53	5.53	<.560	<.560	149	133	<.05	<.05	<.007	.015	<.30	<.30
1	5.57	5.56	<.560	<.560	126	128	4.1	3.9	.021	.177[+]	<.30	<.30
3	5.57	5.57	<.560	<.560	121	120	8.0	7.4	.033	.185[+]	<.30	<.30
7	5.55	5.57	<.560	<.560	143	133	6.0	5.5	.057	.222[+]	<.30	<.30
14	5.54	5.51	<.560	<.560	143	133	8.6	7.7	.042	.179	<.30	<.30

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

[+]Significantly different from control ($p \leq .025$)

The data of Table 23 confirm the above results. The addition of 0.01% yeast extract appears to have caused a slight production, 1.25 mg/l, of NO_2^--N. However, nitrification was minimal with minute quantities of NO_2^--N and no NO_3^--N synthesized. Growth of the Alcaligenes sp. was not evident and the metabolism of either bound- or free-NH_2OH-N was absent. It therefore appeared that no PO nitrification by the Alcaligenes sp. occurred under these conditions.

However, since 0.01% yeast extract had caused a slight stimulation of PO nitrification at pH 5.5, an attempt was made to determine if pH 5.4 PO-VM supplemented with 0.1% yeast extract would be nitrified by the Alcaligenes sp. (Table 24). The results were quite similar to the previous experiment. Only 1.68 mg NO_2^--N/l was synthesized by the Alcaligenes sp. and no NO_3^--N was produced. Again, growth was absent and oxidation of either bound- or free-NH_2OH did not occur.

These results suggested that the maleate buffer might be inhibiting the metabolism of the Alcaligenes sp. One indication of such an inhibition was the absence of growth when yeast extract was provided as a carbon-nitrogen source at pH 5.5. The Alcaligenes sp. was capable of abundant growth with yeast extract as a substrate at a pH of 4.5 (see appendix). Thus, a citrate-phosphate buffer with a medium pH of 5.4 substituted for the maleate buffered medium.

From Table 25 will be seen that acidic nitrification by the Alcaligenes sp. did occur, in a limited fashion, in the 0.2% PO-citrate-phosphate buffered medium. Statistically significant quantities of both NO_2^--N and NO_3^--N (4.42 and 1.1 mg/l, respectively) were

TABLE 23

Nitrification by *Alcaligenes* sp. strain OS1 of 0.2% PO at pH 5.5.*

Time (days)	pH		Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1] Con.	[2] Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	5.49	5.48	<.560	<.560	184	187	5.4	5.1	.012	.093+	<.30	<.30
1	5.45	5.44	<.560	<.560	217	212	6.7	7.0	.009	1.25+	<.30	<.30
3	5.41	5.40	<.560	<.560	229	205	9.0	8.5	.015	1.04+	<.30	<.30
7	5.43	5.42	<.560	<.560	208	219	8.5	7.7	<.007	.367+	<.30	<.30
14	5.43	5.42	<.560	<.560	219	220	13	12	<.007	.115+	<.30	<.30

*0.01% yeast extract was added to the culture medium.

[1]Uninoculated control.

[2]*Alcaligenes* sp. strain OS1.

+Significantly different from control ($p \leq .025$)

TABLE 24

Nitrification by Alcaligenes sp. strain OS1 of 0.2% PO-0.1% yeast extract at pH 5.4

Time (days)	pH		Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	5.43	5.42	<.560	<.560	222	222	5.7	5.7	.022	.045[+]	<.30	<.30
1	5.43	5.43	<.560	<.560	212	226	11	8.2[+]	.039	.520[+]	<.30	<.30
4	5.43	5.43	<.560	<.560	212	229	8.0	7.8	<.007	.400[+]	<.30	<.30
7	5.46	5.47	<.560	<.560	175	185	9.8	9.1	.016	.253[+]	<.30	<.30
14	5.46	5.47	<.560	<.560	229	219	11	10	<.007	1.68	<.30	<.30

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

[+]Significantly different from control ($p \leq .05$)

TABLE 25

Nitrification by Alcaligenes sp. strain OS1 of 0.2% PO-Citrate-Phosphate medium at pH 5.4.

Time (days)	pH		Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	5.42	5.42	<.560	<.560	205	212	4.2	4.3	<.007	<.007	<.30	<.30
1	5.44	5.43	<.560	<.560	194	208	4.5	4.6	.017	.047	<.30	<.30
4	5.45	5.45	<.560	<.560	205	213	5.8	6.2	.019	.022	<.30	<.30
7	5.45	5.45	<.560	<.560	210	222	15	15	.009	.066	<.30	<.30
9	5.43	5.44	<.560	<.560	199	203	8.8	9.0	.010	1.87	<.30	.35
11	5.42	5.43	<.560	<.560	220	219	12	13	.015	3.83[+]	<.30	.40
14	5.39	5.40	<.560	<.560	215	201	18	18	.015	4.42[+]	<.30	<.30
21	5.36	5.41	<.560	<.560	212	191	13	9.9	<.007	1.90[+]	<.30	1.1[+]

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

[+]Significantly different from control ($p \leq .05$)

produced. Bound-NH_2OH-N concentrations, while not statistically relevant, decreased during the 14th and 21st days of the experiment. However, growth of the Alcaligenes sp. was not observed.

The absence of observable growth and only slight nitrification were noted when this experiment was repeated at pH 5.2 (Table 26). Again, NO_2^--N and NO_3^--N were synthesized (maximum concentrations of 6.90 and 1.6 mg/l, respectively), and bound-NH_2OH (PO) concentrations decreased significantly by the 28th day of the experiment. During the final 14 days of the experiment, the cultures were incubated statically. This change in culturing was associated with the highest yields of NO_2^--N and NO_3^--N of the experiment. Subsequently, all cultures were statically incubated.

The results of a static culture experiment are shown in Table 27. Nitrification occurred when the Alcaligenes sp. was statically incubated in the medium at pH 5.4. However, the microbe altered the pH of the medium (7 and 14 day measurements) and was thus able to better utilize PO as a carbon-nitrogen source. Indeed, two of the three Alcaligenes sp. growth vessels contained neither bound- nor free- NH_2OH-N nor NO_3^- and had less than 0.040 mg NO_2^--N/l by the 14th day. Thus, the organism had not only oxidized the PO, but had probably also utilized this NO_2^--N and NO_3^--N for growth. That is, excess nitrogen was probably converted to cell nitrogen when the bacterium had become induced to use citrate as a carbon source. Since the Alcaligenes sp. does use citrate as a carbon source (see appendix), the rise in pH to 6.78 by the 14th day and the concomitant decrease of NO_2^--N from 8.8 ml/l (7th day) to less

TABLE 26

Nitrification by Alcaligenes sp. strain OS1 of 0.2% PO-Citrate Phosphate medium at pH 5.2.*

Time (days)	pH		Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	5.19	5.18	<.560	<.560	208	201	6.1	5.6	<.007	.032^{+}	<.30	<.30
3	5.19	5.17	<.560	<.560	201	180	9.0	7.4	<.007	1.91^{++}	<.30	<.30
7	5.17	5.19	<.560	<.560	191	180	15	13	.011	1.62^{++}	<.30	.36
14	5.19	5.21	<.560	<.560	217	205	9.8	8.0	<.007	.373	<.30	.39
21	5.19	5.21	<.560	<.560	184	160	11	9.1	<.007	1.92	<.30	.33
28	5.20	5.24	<.560	<.560	205	150^{++}	12	10^{+}	<.007	6.90^{+}	<.30	1.6

*All cultures were statically cultured during the final 14 days of incubation.

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

$^{+}$Significantly different from control ($p \leq .05$)

$^{+}$Significantly different from control ($p \leq .01$)

TABLE 27

Nitrification by statically grown Alcaligenes sp. strain OS1 of 0.2% PO-Citrate-Phosphate medium at pH 5.4

Time (days)	pH		Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	[1]Con.	[2]Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	5.44	5.46	<.560	<.560	189	171	6.9	6.2	.030	.052^{+}	<.30	<.30
3	5.49	5.45	<.560	<.560	177	170	8.2	7.4	.022	1.67	<.30	<.30
7	5.57	5.72^{++}	<.560	1.90	184	124^{+}	7.5	7.0	.021	8.88^{++}	<.30	.31
14	5.52	6.78	<.560	26.0	180	21.0^{++}	8.8	1.5^{+}	.017	1.26	<.30	.57

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

$^{+}$Significantly different from control ($p. \leq .05$)

$^{++}$Significantly different from control ($p. \leq .025$)

than 0.040 mg NO_2^--N/l in two of the growth flasks indicated that at least some of the growth of the Alcaligenes sp. was due to the utilization of citrate and NO_2^- as carbon and nitrogen sources, respectively.

The data of Tables 25 - 27 indicate that the Alcaligenes sp. does nitrify PO at a pH of approximately 5.4. However, acidic nitrification of PO by the bacterium is both sparse and requires a lengthy period when compared to the nitrification of PO under neutral conditions.

Ammonium ion oxidation by the Alcaligenes sp. Since the Alcaligenes sp. was capable of nitrifying NH_2OH, the ability of the microbe to oxidize NH_4^+ was examined. Preliminary results had shown no production of NO_2^- or NO_3^- when the organism was cultured with NH_4^+ as the nitrogen source. Sodium pyruvate and $(NH_4)_2SO_4$ were provided as the carbon and nitrogen sources. A 0.6% pyruvate-carbon-0.2% NH_4^+-N-VM was used as the growth medium so that a low carbon to nitrogen ratio of 3 to 1 was maintained. The results are listed in Table 28.

Though growth of the Alcaligenes sp. was abundant, no nitrification was observed and neither free-NH_2OH, NO_2^-, nor NO_3^- was synthesized. Slight amounts of bound-NH_2OH were noted. However, since both the control and the Alcaligenes sp. culture flasks had traces of bound-NH_2OH present, it is doubtful that the Alcaligenes sp. was synthesizing bound-NH_2OH. Therefore, it may be concluded that while the Alcaligenes sp. readily oxidizes PO and NH_2OH, the organism does not nitrify ammonium ion.

TABLE 28

Nitrification by Alcaligenes sp. strain OS1 of 0.6% Pyruvate-C-0.2% NH_4^+-N-VM.

Time (days)	Growth (mg/ml)		Bound-NH_2OH-N (mg/l)		Free-NH_2OH-N (mg/l)		NO_2^--N (mg/l)		NO_3^--N (mg/l)	
	1 Con.	2 Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.	Con.	Alcal.
0	<.560	<.560	<.05	<.05	<.05	<.05	<.007	<.007	<.30	<.30
1	<.560	.590	<.05	<.05	<.05	<.05	<.007	<.007	<.30	<.30
3	<.560	5.03+	1.1	1.0	<.05	<.05	<.007	<.007	<.30	<.30
7	<.560	49.6+	1.1	1.3	<.05	<.05	<.007	<.007	<.30	<.30
14	<.560	35.2+	1.4	1.2	<.05	<.05	<.007	<.007	<.30	<.30

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1

+Significantly different from control ($p \leq .05$)

Metabolism of PO, NO_2^- and PO, and NH_2OH by N. agilis, ATCC #14123.

The Alcaligenes sp., isolated from the Hadley, Massachusetts soils, produced abundant amounts of NO_2^--N from PO. It was therefore postulated that the NO_2^- synthesized by the Alcaligenes sp. might function as an energy source for the chemolithotroph Nitrobacter. However, experiments assessing the responses of N. agilis to pyruvic-oxime, NO_2^- and pyruvic-oxime concurrently, and NH_2OH were needed in order to define any synergistic interactions between the Alcaligenes sp. and N. agilis.

Metabolism of PO by N. agilis. Six (2 groups of 3) N. agilis (ATCC 14123) cultures were inoculated into 0.5% PO-VM. Two additional cultures of the organism were inoculated into 0.14% $NaNO_2$ Schmidt, Molina, and Chiang (1973) medium to monitor the viability of the microbe. The results of the experiment are reported in Table 29.

While the N. agilis cultures inoculated into $NaNO_2$ medium oxidized NO_2^- to NO_3^-, those N. agilis cells cultured in 0.5% PO-VM failed to produce NO_3^--N. Furthermore, visual inspection of the $NaNO_2$ medium noted a slight turbidity associated with the growth of pure cultures of N. agilis. At no time during this experiment was any such turbidity observed with either the N. agilis PO-VM or the uninoculated control cultures. The slight concentration of NO_3^--N (1.20 mg/l) noted in the 0.5% PO-VM cultures of N. agilis was due to NO_3^- added to the medium with the N. agilis inoculum. The experiment demonstrated that N. agilis was not capable of metabolizing PO to NO_3^--N in the VM mineral salts medium.

TABLE 29

Metabolism of 0.5% PO-VM by Nitrobacter agilis.

Time (days)	NO_3^--N (mg/l)			
	Uninoculated Control	N. agilis in PO-VM Group 1	N. agilis in PO-VM Group 2	N. agilis in $NaNO_2^-$ medium[a]
0	<.300	1.07	0.92	2.90
7	<.300	1.20	1.02	29.5
14	<.300	1.18	0.88	125
21	<.300	1.23	1.07	132
28	<.300	1.20	1.15	120

[a]0.14% (w/v) $NaNO_2$ Schmidt, Molina, and Chiang (1973) medium with no pyruvic-oxime.

Metabolism of NO_2^- by N. agilis in the presence of 0.5% PO. Since pure cultures of N. agilis were incapable of metabolizing PO, the ability of N. agilis to metabolize NO_2^- if PO was present was tested. Two sets of pure N. agilis cultures were inoculated into trace mineral VM containing 0.5% PO and 0.14% NaNO. In addition, a pure culture of N. agilis was inoculated into 0.14% $NaNO_2$ medium of Schmidt, Molina, and Chiang (1973) to serve as a viability check.

Results of this experiment are recorded in Table 30. NO_2^- was not oxidized and NO_3^- was not synthesized by the N. agilis cells cultured in the $NaNO_2$-PO medium, even though N. agilis cells cultured normally were viable. It was concluded, therefore, that either PO, or the NH_2OH in equilibrium with it, was bacteriostatic or bactericidal to N. agilis cells. An experiment was thus devised to determine the toxicity of NH_2OH to N. agilis.

Effect of NH_2OH on the Metabolism of N. agilis. Two sets of N. agilis cultures were incubated for 1 day in trace mineral VM initially containing 5 mg NH_2OH-N/1. A set of N. agilis cultures treated similarly, except that the trace mineral VM contained no NH_2OH, served as the control. After 1 day of incubation, each culture was aseptically collected on a 0.20 μm membrane filter and transferred to sterile 0.14% $NaNO_2$ medium (Schmidt, Molina, and Chiang, 1973). Three uninoculated flasks of this medium were added at this time to function as a secondary control. In Table 31 are shown the results of this experiment.

TABLE 30

Nitrification by Nitrobacter agilis of 0.14% $NaNO_2$ in the presence of 0.5% PO.[ab]

Time (days)	Bound-NH_2OH-N (mg/l)			Free-NH_2OH-N (mg/l)			NO_2^--N (mg/l)			NO_3^--N (mg/l)		
	[1]Unin. Con.	[2]N. ag. Group 1	[3]N. ag. Group 2	Unin. Con.	N. ag. Group 1	N. ag. Group 2	Unin. Con.	N. ag. Group 1	N. ag. Group 2	Unin. Con.	N. ag. Group 1	N. ag. Group 2
0	681	639	677	28	33	32	233	250	217	3.0	5.1	5.7
7	635	630	588	22	26	24	229	225	212	18	25	29
14	572	595	560	24	26	24	250	275	225	4.5	18	20
19	679	688	787	30	29	29	208	192	200	2.7	21	21
28	611	611	611	37	34	32	233	217	250	2.7	31	28

[a]N. agilis cells were viable as an identical culture grown without PO in the medium yielded 160 mg NO_3^--N/l and only 0.007 mg NO_2^--N/l by the 14th day.

[b]The slight increase in NO_3^--N levels of the N. agilis cultures represents NO_3^- transferred with the N. agilis inocula.

[1]Uninoculated control

[2]N. agilis group 1 [3]N. agilis group 2

TABLE 31

Nitrification of 0.14% $NaNO_2$ by N. agilis after incubation in 5 mg NH_2OH-N/l for 1 day.

Time (days)	Free-NH_2OH-N (mg/l)				NO_2^--N (mg/l)				NO_3^--N (mg/l)			
	[1]Unin. Con.	[2]N. ag. Con.	[3]N. ag. Group 1	[4]N. ag. Group 2	Unin. Con.	N. ag. Con.	N. ag. Group 1	N. ag. Group 2	Unin. Con.	N. ag. Con.	N. ag. Group 1	N. ag. Group 2
0	-	<.05	5.4	5.3	-	<.007	.232	.300	-	2.40	2.43	2.80
1	-	<.05	<.05	<.05	-	.042	.607	.590	-	3.82	2.53	2.57
1[a]	<.05	<.05	<.05	<.05	358	303	315	307	4.57	3.93	4.57	4.40
8	<.05	<.05	<.05	<.05	292	281	290	290	35.7	40.7	35.8	38.5
15	<.05	<.05	<.05	<.05	310	.044[+]	290	295	11.0	312[+]	16.6	22.2
22	<.05	<.05	<.05	<.05	308	.027[+]	323	301	4.78	322[+]	15.5	22.3

[1]Uninoculated control

[2]N. agilis control (not incubated with 5 mg NH_2OH-N/l)

[3]N. agilis group 1

[4]N. agilis group 2

[a]First day measurements after the transferring of the sterile membrane filters into sterile 0.14% $NaNO_2$ Schmidt, Molina, and Chiang (1973) medium.

[+]Significantly different from the uninoculated control, N. agilis group 1, and N. agilis group 2 ($p \leq .01$)

N. agilis cultures subjected to 5 mg NH_2OH-N/l for 1 day did not metabolize NO_2^- to NO_3^-. These cultures did not differ at any time from the uninoculated controls. In sharp contrast, the *N. agilis* cultures collected on the membrane filter, but not incubated with NH_2OH, nitrified normally. Only 0.027 mg NO_2^--N/l remained in these cultures after 22 days compared with approximately 310 mg NO_2^--N/l which was present in the uninoculated control, *N. agilis* group 1, and *N. agilis* group 2 cultures. Further, the synthesis of NO_3^--N did not occur in these three groups while the *N. agilis* controls yielded 322 mg NO_3^--N/l by the 22nd day. It is thus evident that the initial 5 mg NH_2OH-N/l was toxic to *N. agilis* and prevented the bacterium from converting NO_2^- to NO_3^- when incubated in a standard medium intended for that purpose.

Synergistic nitrification between the *Alcaligenes* sp. and *N. agilis*.

In light of the potential for the toxicity of NH_2OH to *N. agilis*, a preliminary experiment to establish nitrification synergy between the autotroph and the *Alcaligenes* sp. was conducted. The *Alcaligenes* sp. was cultured for 7 days in 1.0% PO medium and was therefore able to convert the NH_2OH present to NO_2^-. During the seventh day, the *N. agilis* cultures were added and nitrification of NO_2^- by the *Alcaligenes* sp. was observed. The results are shown in Table 32.

The *Alcaligenes* sp. cultured separately exhibited a pattern typical of the organism's capacity for PO nitrification. Both bound- and free-NH_2OH were oxidized by the *Alcaligenes* sp. cultures

TABLE 32

Synergistic nitrification by Alcaligenes sp. strain OS1 and Nitrobacter agilis.[ab]

Time (days)	Bound-NH_2OH-N (mg/l)				Free-NH_2OH-N (mg/l)			
	[1]Uninoc. Control	[2]Alcal. alone	[3]Alcal.-N Group 1	[4]Alcal.-N Group 1	Uninoc. Control	Alcal. alone	Alcal.-N Group 1	Alcal.-N Group 2
0	845	588	831	896	96	74	67	66
1	1237	810	1178	1178	104	90	69	74
3	1447	528$^+$	152$^+$	196$^+$	77	18$^+$	18$^+$	27$^+$
7	1377	< .050$^+$	< .050$^+$	< .050$^+$	51	< .05$^+$	< .05$^+$	< .05$^+$
14	1493	< .050$^+$	< .050$^+$	< .050$^+$	74	< .05$^+$	< .05$^+$	< .05$^+$
21	723	< .050$^+$	< .050$^+$	< .050$^+$	141	< .05$^+$	< .05$^+$	< .05$^+$
28	1377	< .050$^+$	< .050$^+$	< .050$^+$	112	< .05$^+$	< .05$^+$	< .05$^+$
35	1309	< .050$^+$	< .050$^+$	< .050$^+$	70	< .05$^+$	< .05$^+$	< .05$^+$

[a] 1.0% PO added as the sole carbon-nitrogen source.

[b] N. agilis added after the seventh day measurements had been completed.

[1] Uninoculated control

TABLE 32 CONTINUED

Time (days)	NO_2^--N (mg/l)				NO_3^--N (mg/l)			
	Uninoc. Control	Alcal. alone	Alcal.-N. Group 1	Alcal.-N. Group 2	Uninoc. Control	Alcal. alone	Alcal.-N. Group 1	Alcal.-N. Group 2
0	.144	.131	.132	.132	<.300	<.300	<.300	<.300
1	.150	30.0	19.2	13.6	<.300	<.300	<.300	<.300
3	.167	342^{+}	553^{+}	492^{+}	<.300	5.10^{+}	8.40^{+}	6.90^{+}
7	.308	392^{+}	742^{+}	658^{+}	<.300	7.80^{+}	8.13^{+}	11.3^{+}
14	.343	425^{+}	665^{+}	642^{+}	<.300	44.3^{+}	72.8$^{+\#}$	38.0^{+}
21	.264	408^{+}	165^{+}	172^{+}	<.300	7.00	262$^{@*}$	63.2$^{+\#}$
28	.375	433^{+}	.147$^{\#}$	10.1*	<.300	<.300	877$^{@*}$	470
35	.350	500^{+}	.192$^{\#}$	.210$^{\#}$	<.300	17.8	850$^{+\#}$	967$^{+\#}$

[2] *Alcaligenes* sp. strain OS1 only

[3] *Alcaligenes* sp. strain OS1 and *N. agilis* group 1.

[4] *Alcaligenes* sp. strain OS1 and *N. agilis* group 2.

*Significantly different from *Alcaligenes* sp. strain OS1 only ($p \leq .05$)

#Significantly different from *Alcaligenes* sp. strain OS1 only ($p \leq .01$)

$^{@}$Significantly different from uninoculated control ($p \leq .05$)

$^{+}$Significantly different from uninoculated control ($p \leq .01$)

and neither of these compounds were present in the culture medium by the seventh day of incubation. Concomitantly NO_2^--N concentrations rose to approximately 500 mg/l and remained at this level throughout the experiment. NO_3^--N concentrations rose to a maximum of 44.3 mg/l and thereafter decreased, probably as a result of denitrification by the Alcaligenes sp.

The mixed Alcaligenes sp.-N. agilis cultures showed similar patterns of NO_2^- and NO_3^- production for the first 14 days of the study. However, from the fourteenth to the thirty-fifth days NO_3^--N concentrations increased from approximately 55 mg/l to 900 mg/l by the end of the experiment. Since the uninoculated controls maintained constant concentrations of free- and bound-NH_2OH, NO_2^-, and NO_3^-, it can only be concluded that the mixed cultures were responsible for the conversion of PO nitrogen to NO_3^-.

Culturing the Alcaligenes sp. and N. agilis together from the onset of the experiment resulted in a similar synergistic nitrification pattern (Table 33). The uninoculated controls showed constant levels of bound- and free-NH_2OH, NO_2^-, and NO_3^-. Cultures with the Alcaligenes sp. only again exhibited typical patterns of nitrification for that organism. Jointly cultured Alcaligenes sp. and N. agilis cells, however, converted PO nitrogen to NO_3^-. Concentrations of only 0.10 mg NO_2^--N/l remained after 21 days of simultaneous culturing and NO_2^--N levels of 248 mg NO_3^--N/l were produced. Therefore, even though NH_2OH prevents the metabolism of pure cultures of N. agilis (Table 31), joint culturing of the bacteria, even in the

TABLE 33

Synergistic nitrification by Alcaligenes sp. strain OS1 and Nitrobacter agilis inoculated simultaneously[a]

Time (days)	Bound-NH_2OH-N (mg/l)				Free-NH_2OH-N (mg/l)			
	[1]Uninoc. Control	[2]Alcal. alone	[3]Alcal.-N. Group 1	[4]Alcal.-N. Group 2	Uninoc. Control	Alcal. alone	Alcal.-N. Group 1	Alcal.-N. Group 2
0	633	577	730	595	20	17	14	13
1	700	341$^+$	362$^+$	373$^+$	23	15$^+$	17$^+$	17$^+$
3	467	<.050$^+$	<.050$^+$	<.050$^+$	29	<.05$^+$	<.05$^+$	<.05$^+$
7	653	<.050$^+$	<.050$^+$	<.050$^+$	21	<.05$^+$	<.05$^+$	<.05$^+$
14	656	<.050$^+$	<.050$^+$	<.050$^+$	39	<.05$^+$	<.05$^+$	<.05$^+$
21	665	<.050$^+$	<.050$^+$	<.050$^+$	23	<.05$^+$	<.05$^+$	<.05$^+$

[a]0.5% PO added as the sole carbon-nitrogen source.

[1]Uninoculated control

[2]Alcaligenes sp. strain OS1 only

[3]Alcaligenes sp. strain OS1 and N. agilis group 1

[4]Alcaligenes sp. strain OS1 and N. agilis group 2

TABLE 33 CONTINUED

Time (days)	NO_2^--N (mg/l)				NO_3^--N (mg/l)			
	Uninoc. Control	Alcal. alone	Alcal.-N. Group 1	Alcal.-N. Group 2	Uninoc. Control	Alcal. alone	Alcal.-N. Group 1	Alcal.-N. Group 2
0	.037	.200	.100	.083	<.300	<.300	<.300	<.300
1	.072	16.3^{+}	10.1^{+}	6.33^{+}	<.300	.883*	1.60$^{+\#}$	1.37^{+*}
3	.048	156^{+}	157^{+}	151^{+}	<.300	35.3^{+}	39.0^{+}	46.7$^{+@}$
7	.052	165^{+}	167^{+}	173^{+}	<.300	47.3^{+}	79.5$^{+\#}$	54.2^{+}
14	.067	162^{+}	.233$^{+\#}$	.193$^{+\#}$	<.300	3.67	238$^{+\#}$	250$^{+\#}$
21	.067	170^{+}	.131$^{*\#}$	.102$^{\#}$	<.300	4.67*	248$^{+\#}$	233$^{+\#}$

*Significantly different from uninoculated control ($p \leq .05$)

$^{+}$Significantly different from uninocluated control ($p \leq .01$)

$^{@}$Significantly different from Alcaligenes sp. strain OS1 alone ($p \leq .05$)

$^{\#}$Significantly different from Alcaligenes sp. strain OS1 alone ($p \leq .01$)

presence of 17 mg NH_2OH-N/l, resulted in the oxidation of PO to NO_2^- and of NO_2^- to NO_3^-.

CHAPTER V

DISCUSSION

Synthesis of oximes, hydroxylamine, and bound hydroxylamines has been noted in various microorganisms, plants, and animals. Gunner (1963) observed synthesis of NH_2OH from NH_4^+ by Arthrobacter globiformis. Similarly, Arthrobacter spp. produced NH_2OH when grown in media containing NH_4^+ salts (Verstraete and Alexander, 1972a; Berger, et al., 1979). Aspergillus niger also produced NH_2OH from NH_4^+ (Steinberg, 1939). However, NH_2OH is not exclusively synthesized from NH_4^+; Seaman (1957) reported that extracts of Tetrahymena pyriformis yielded NH_2OH from PO.

Bound hydroxylamines have been produced by various microbes, many of which are common soil and water inhabitants. Azotobacter chroococcum yielded bound hydroxylamine when cultured with either NO_3^-, molecular N_2, urea, or NH_4^+ as nitrogen sources (Saris and Virtanen, 1957). E. coli (Grossowicz and Lichtenstein, 1961) and Arthrobacter (Verstraete and Alexander, 1972b) also have syntehsized bound hydroxylamines. Aspergillus flavus is another ubiquitous soil microbe capable of synthesizing bound hydroxylamine (Marshall and Alexander, 1962).

Additionally, the production of hydroxamic acids by various bacteria and fungi has been noted by numerous researchers (Verstraete, 1975). Neilands (1967) lists over thirty hydroxamic acids which are "biologically active" in that they function as growth factors, antibiotics, and as regulators of cell division.

One report suggests the production of bound hydroxylamine and nitro compounds by an organism known to oxidize another bound hydroxylamine (acetaldoxime), that is, P. aeruginosa (Hilali and Molina, 1979). When denitrifying, cultures of P. aeruginosa immobilize some of the nitrogen and thus probably synthesize bound hydroxylamines. Nitrogen thus immobilized would be accessible since both bound hydroxylamines and nitro compounds are readily converted to nitrite.

Reports in the literature also note the formation of oximes. Virtanen and Laine (1939) and Virtanen, et al., (1949) found quantities of oxime when Azotobacter was grown with NO_3^-, NO_2^-, molecular N_2, and NH_4^+. Virtanen and Laine (1939) reported oxaloacetic-oxime as a product of pea plant root nodules. The microorganisms A. flavus (Alexander, et al., 1960) and Streptomyces achromogenes (Wiley, et al., 1965) also produce oximes.

Yamafugi, et al. (1950), observed the presence of oxime in tobacco leaves, mulberry leaves, spinach leaves, turnip leaves and stems, onion leaves, ox liver, ox heart, ox kidney, and in the silkworm. Yamafugi and Akita (1952) thus concluded that oximes are synthesized by many organisms.

Quastel and Scholefield (1949) theorized that oxime nitrogen may be a significant component of nitrification. These authors noted that unlike other organic nitrogen compounds, pyruvic-oxime required little or no lag time to be oxidized. This ready transformation of PO to NO_2^- thus suggests a soil microbial population already induced for PO oxidation. Quastel, et. al (1950), substantiated this hypothesis when

they isolated three common soil heterotrophs (2 Achromobacter (Alcaligenes) sp. and 1 Corynebacterium sp.) which readily oxidized PO.

Jensen (1951) further supported the oxime nitrification hypothesis when he stated that:

> Since hydroxylamine can be definitely formed by denitrification and other processes of nitrate and nitrite reduction, the occasional presence of oximes under natural conditions is quite likely. In such cases the oxime-decomposing bacteria might bring about a renitrification of nitrogen previously reduced from the nitrate or nitrite stage. A heterotrophic nitrification of oximes not resulting from nitrate reduction might take place in the rhizosphere of leguminous plants if nitrogenous compounds are excreted from the nodules.

Thus there exists a number of environments in which oxime nitrogen has been either observed directly or postulated as very likely to be present.

The Alcaligenes sp. of this study resembles the soil microflora noted by Quastel and Scholefield (1949). Both the initial enrichment and pure cultures containing the Alcaligenes spp. showed that little or no lag time was necessary for PO oxidation. In addition, the Alcaligenes spp. were isolated from soil and organisms of the genus Alcaligenes are known to be common soil heterotrophs.

Alcaligenes sp. strain OS1 did, however, differ from previously studied microbes in a number of ways. The 1867 mg NO_2^--N/l produced by strain OS1 when grown in 2.5% PO-VM is, to the author's knowledge, the largest concentration of NO_2^--N synthesized by a microbe with the exception of the chemoautotroph Nitrosomonas. The Pseudomonas

aeruginosa of Obaton, et al., (1968) synthesized up to 284 μg NO_2^--N/ml when supplied acetaldoxime, but the organism failed to use the oxime as a carbon-nitrogen source. Substantial amounts of NO_2^--N were produced by P. aeruginosa only when the medium was supplemented with 0.1% glucose and 0.4% peptone. Replacement cultures of a Fusarium sp. isolated by Doxtader and Alexander (1966c) yielded 190 mg NO_2^--N/l when incubated with PO. Growth of the fungus with PO serving as a carbon-nitrogen source, however, was not reported; the culture conditions listed peptone and beef extract as the main carbon-nitrogen sources with only 2.8 mg PO/l present in the medium.

The Alcaligenes sp. of this study differed from the P. aeruginosa and Fusarium sp. isolates in that it required no organic supplements to grow in media containing PO. The only heterotrophic microbes cited as being similar to the Alcaligenes spp. in needing no organic carbon supplements and yet yielding large amounts of NO_2^- were the methane oxidizing bacteria of Romanovskaya, et al., (1977). Methylomonas, Methlobacter, and Methylococcus species were reported by these authors to nitrify significant amounts of NH_4^+, producing from 150-180 mg NO_2^--N/l.

An abundant supply of organic carbon was not necessary for nitrification by resting cells. The Alcaligenes sp. converted NH_2OH to NO_2^- when exogenous carbon was either absent or present as pyruvate or acetate. Significant amounts of NH_2OH and NO_2^- were synthesized from NH_4^+ and NH_2OH, respectively, by the Arthrobacter sp. of Verstraete and Alexander (1972c) only when a ratio of 3 to 1 or less

of acetate-carbon to either NH_4^+-N or NH_2OH-N was present. The Alcaligenes sp. and the Arthrobacter sp. were alike, however, in that exogenous carbon did stimulate nitrification. Both microbes yielded more product when acetate was present. Similarly, the Alcaligenes sp. also yielded more NO_2^--N when pyruvate was present with NH_2OH than when it was absent.

The Alcaligenes spp. of this study are also the first heterotrophic microorganisms to be authentically identified as producing NO_3^- from an oxime. The reviews of Verstraete (1975) and Focht and Verstraete (1977) erroneously reported that both the P. aeruginosa of Obaton, et al., (1968) and the Fusarium sp. of Doxtader and Alexander (1966c) synthesized NO_3^- when cultured with acetaldoxime and pyruvic-oxime, respectively. However, neither original paper reports NO_3^- production from the oximes and lists NO_2^- as being the only nitrification product. Indeed, both of the references state specifically in their respective summary and abstracts that NO_3^- is not a product.

The Alcaligenes sp. is somewhat unusual in that nitrification was closely associated with growth. Jensen (1951) reported that PO nitrification by Nocardia corallina was very closely correlated with the growth of the organism. Similarly, Berger, et al., (1979) noted that only actively growing Arthrobacter cells produced NH_2OH. Raistrick and Stossl (1958) also correlated nitrification with growth as their Penicillium atrovenetum strain produced large amounts of 3-NPA before the end of active growth. However, many authors have noted patterns which did not indicate a positive correlation between growth

and nitrification.

The oxime nitrification studies of Obaton, et al., (1968) and Doxtader and Alexander (1966c) stated that nitrification occurred after active growth had ceased. While A. flavus was noted to produce 3-NPA during active growth, NO_2^- and NO_3^- formation occurred only after the cessation of growth (Doxtader and Alexander, 1966a). The reports of Marshall and Alexander (1962), VanGool and Schmidt (1973), and Schmidt (1954; 1960) state that nitrification by A. flavus occurs either during the later stages of active growth or in the stationary phase of growth. Bacteria such as Streptomyces (Hirsch, et al., 1961) and Arthrobacter (Verstraete and Alexander, 1972a; Witzel and Overbeck, 1979) also nitrify primarily after growth has stopped. Thus, the strong correlation between the nitrification of PO and growth of the Alcaligenes sp. of this study represents the exception, rather than the rule, in this transformation.

Alcaligenes sp. strain OS1 also exhibited a remarkable tolerance to NH_2OH. The organism remained viable in media containing 325 mg NH_2OH-N/l and grew in media with 275 mg NH_2OH-N/l. These concentrations of NH_2OH are quite large when compared to the data of other investigators.

Azotobacter, one of the microbes most susceptible to NH_2OH, tolerates less than 0.44 mg NH_2OH-N/l (Novak and Wilson, 1948). The P. aeruginosa strain capable of oxidizing acetaldoxime would not grow in media containing greater than 14 mg NH_2OH-N/l (Amarger and Alexander, 1968). Penicillium atrovenetum, cited as being remarkably

tolerant to NH_2OH, tolerated only 28 mg NH_2OH-N/l (Shaw and Wang, 1964). Similarly, other microorganisms noted for their resistance to NH_2OH tolerated concentrations much less than those noted for the Alcaligenes sp.

Bacillus cadaveris grew in the presence of 33 mg NH_2OH-N/l (Emery, 1963) and an organism similar to E. coli grew in media containing 44 mg NH_2OH-N/l (Grossowicz and Lichtenstein, 1961). Castell and Mapplebeck (1956) tested a number of microbes and noted that Aerobacter (Klebsiella) aerogenes, Aerobacter (Enterobacter) cloacae, E. coli, Proteus vulgaris, Serratia marcescans, Alcaligenes spp., Micrococcus spp., Bacillus spp., and yellow halophiles were able to grow when concentrations of up to 44 mg NH_2OH-N/l were present. Yet, fungi such as Aspergillus, Alternaria, and Penicillium were the most NH_2OH resistant, growing in media containing as much as 106 mg NH_2OH-N/l. In view of these data, the resistance of the Alcaligenes sp. to NH_2OH (275-325 mg NH_2OH-N/l) is noteworthy.

Perhaps one mechanism displayed by the Alcaligenes sp. permitting resistance to NH_2OH is its ability to oxidize NH_2OH to NO_2^-, thereby relieving itself of a potentially toxic compound. Lees, et al. (1954) demonstrated that resting cells of Nocardia corallina oxidized PO and NH_2OH to NO_2^-. This organism was very similar to the Alcaligenes sp. in its conversion of these substrates to NO_2^-. The N. corallina strain incubated with 6.0 µmol. of NH_2OH and 6.0 µmol. of PO converted 7.5 and 35% of these substrates, respectively, to NO_2^-. The Alcaligenes sp. converted 4.4 and 48% of 6.0 mg NH_2OH-N/l and 6.0 mg PO-N/l,

respectively, to NO_2^-.

Thus, the respiratory capacity of the Alcaligenes sp. may, in part, be responsible for the organism's tolerance to NH_2OH. Jensen (1951) noted that free hydroxylamine was highly toxic to cultures of N. corallina and an Alcaligenes sp. He also noted that NO_2^--N present in the culture medium represented unutilized nitrogen and was thus a waste product. Considering the toxicity of NH_2OH, perhaps its oxidation to NO_2^- by the respiratory enzymes of the Alcaligenes sp. of this study not only created a less toxic substance but also altered the equilibrium between PO, pyruvate, and NH_2OH such that more pyruvate was available as both a carbon and energy source. The result of such a change would be the production of NO_2^- accompanied by simultaneous growth. It is of interest, therefore, that such a pattern was observed when the Alcaligenes was cultured in PO-VM.

The Alcaligenes sp. resembled other oxime nitrifiers not only in the demonstration of a strong correlation between growth and nitrification but also in its inability to oxidize NH_4^+. Quastel and Scholefield (1949) noted that soils enriched with oxime nitrifiers were unable to nitrify NH_4^+. Jensen (1951) reported that neither an Alcaligenes sp., an Agrobacterium sp., nor Nocardia corallina isolated from oxime enrichments could produce NO_2^- from either $(NH_4)_2SO_4$, asparagine, or alanine. It therefore appears that NH_4^+ oxidation is not a phenomenon commonly displayed by oxime nitrifiers.

Pyruvic-oxime nitrification in acidic media by the Alcaligenes sp. was generally slow and ineffective when compared to nitrification

at pH 7.0. Ishaque and Cornfield (1974) noted that a Bangladesh laterite soil (pH 4.2), which had no autotrophic nitrifiers, did, nevertheless, nitrify and accumulate NO_3^-. However, the rate of nitrification was quite slow and the period needed for the conversion was thus lengthy. The authors attributed these deviations from the norm to the heterotrophic nitrifying microflora of the laterite soil.

Heterotrophic nitrification at a low pH has been observed by other researchers as well. Becker and Schmidt (1964) noted nitrification by A. flavus from a pH of 3.5 - 6.5. This same microbe oxidized NH_4^+ to NO_3^- and simultaneously produced aflatoxin when the culture medium had a pH of 4.65 - 5.90 (Shih, et al., 1974). Similarly, Cutler and Mukerji (1931) noted that six of their bacterial isolates synthesized NO_2^- from NH_4^+ when the pH of the medium varied from 4.8 to 7.3.

The studies of Rice and Pancholy (1972; 1973; 1974) noted nitrification in the ecosystems of a tall grass prairie, a post-oak blackjack forest, and a mature oak-pine forest. These authors noted that in the mature oak-pine forest, autotrophic nitrifiers were in insufficient numbers to account for the NO_3^- produced and were, in some cases, absent altogether. Additionally, large concentrations of tannins were present in the mature ecosystems and the pH varied from 5.42 - 6.06. That heterotrophic nitrification contributes to nitrification in the mature oak-pine forest seems apparent since both the pH and the tannins of this environment were inhibitors of Nitrosomonas and Nitrobacter. Yet, the importance of the autotrophs to nitrification

was evident as the immature ecosystems contained greater numbers of these bacteria and had a much greater rate of nitrification than did the mature forests. If organisms such as the Alcaligenes sp. of this study contribute to nitrification in mature environments, the lengthy time required for the process to occur becomes evident. That is, since nitrification by the Alcaligenes sp. was slow and yields were low, then one would expect the low rate of NO_3^- synthesis observed in the mature oak-pine forest. Such an effect has been observed in the laboratory when the heterotrophic nitrification of Aspergillus flavus was studied (Doxtader and Alexander, 1966b; Schmidt, 1960a).

Static culturing of the Alcaligenes sp. caused an increase of both nitrification and growth when the organism was cultured in acidic media. Eylar, et al. (1959) and Doxtader and Alexander (1966b) noted that a number of their isolates responded in this manner. However, Becker and Schmidt (1964) observed that their culture of A. flavus, in acidic media, nitrified best when cultured on rotary shaker. Thus, as Doxtader and Alexander (1966b) suggest, oxidation of nitrogenous substrates varies not only with the microbial strain but with agitation and aeration as well.

Acidic nitrification by the Alcaligenes sp. was stimulated when yeast extract was present. Ralt, et al., (1980) noted active nitrification by P. aeruginosa when 0.1 - 0.2% yeast was present in the culture medium. However, since growth of the microbe was not stimulated,

the nature of yeast extract stimulation of the Alcaligenes sp. remains undefined. Yet, growth need not be evident for nitrification to occur. Verstraete and Alexander (1973) amended sewage, river water, and lake water with acetate and NH_4^+. Nitrification patterns, similar to those produced by Verstraete and Alexander's Arthrobacter sp. isolate, occurred in these samples but active growth and proliferation of a heterotrophic nitrifier was not observed.

The low rate of heterotrophic nitrification of Alcaligenes sp. strain OS1 and the fact that the most active cultures were statically cultured may reflect a need to alter the pH of the medium by the organism if nitrification in an acidic environment is to occur. Remacle (1977) suggested that micro-niches with a neutral or a basic pH could be created by soil microbes and serve as a site for heterotrophic nitrification. In the acidic PO-VM, the Alcaligenes sp. nitrified well only when it was statically cultured and when it altered the pH of the buffered medium. Perhaps, static culturing of the Alcaligenes sp. allowed the organism to alter the pH of the "micro-niche" such that nitrification could occur.

The production of NO_2^- from NH_2OH in the citrate-phosphate medium by the Alcaligenes sp. probably resulted from the oxidation of PO. However, NO_2^--N yields did not reach the levels observed in PO-VM experiments. This could reflect the assimilation of the NO_2^--N for growth in the citrate-phosphate medium. As the Alcaligenes sp. became induced to utilize citrate, NO_2^--N was probably used as the nitrogen source. In this manner both citrate and NO_2^- were removed from the medium.

(and the pH correspondingly increased from 5.4 to 6.7) while NO_2^--N levels decreased to minimal concentrations.

An organism may enter into a number of synergistic associations with other organisms. Among these are the associations known as protocooperation and commensalism. Alexander (1977) defines these terms as:

> Protocooperation: an association of mutual benefit to the two species but without the cooperation being obligatory

and

> Commensalism: in which only one species derives benefit while the other is unaffected.

Since the association between the Alcaligenes sp. of this study and N. agilis is not obligatory for their growth and metabolism, the synergistic nitrification observed between these microbes would most appropriately be classified as either protocooperation or commensalism.

The toxic metabolite, NO_2^-, produced by the Alcaligenes sp., serves as an oxidizable substrate for N. agilis. The conversion of NO_2^- to NO_3^- by N. agilis may constitute protocooperation since both the Alcaligenes sp. and N. agilis would derive benefit from the association. Conversely, if large concentrations of NO_2^--N are not inhibitory to the Alcaligenes sp., then the association would be termed commensalism since only N. agilis would be deriving benefit. However, the Alcaligenes sp. may be deriving benefit from the association even if NO_2^--N is not inhibiting the organism. Since Nitrobacter is known to secrete organic matter (Schmidt, Molina, and

Chiang, 1973), the association of the two microbes could therefore be supplying the Alcaligenes sp. with additional organic substrates.

Conversion of PO nitrogen to NO_3^- occurred readily when the Alcaligenes sp. and N. agilis were either simultaneously or sequentially cultured after several days of growth by the Alcaligenes sp. The nitrite produced by the Alcaligenes sp. was rapidly transformed to nitrate by N. agilis and in this manner was analogous to the classical synergism between Nitrosomonas and Nitrobacter. That the synergism between the Alcaligenes sp. and N. agilis was quite active should not be surprising in light of the data of Steinmuller and Bock (1976). These authors observed that the culturing of N. agilis with filtrates of media in which various heterotrophic bacteria (E. coli, Serratia, Pseudomonas, Arthrobacter, Nocardia, etc.) had grown resulted in a marked stimulation of nitrification. It is thus also possible that the joint culturing of the Alcaligenes sp. and N. agilis may have aided the metabolism of NO_2^- by N. agilis by providing various growth stimuli.

This study is the first, to the author's knowledge, to report a nitrification synergism between a heterotroph and one of the chemoautotrophic nitrifiers. However, nitrification synergisms between heterotrophs have been both theorized and observed.

Odu and Adeoye (1970) suggested that nitrification synergisms between soil heterotrophs were responsible for some of the NO_3^- produced in a Nigerian Teak soil. Indeed, they hypothesized that pure culture heterotrophic nitrification studies are insufficient since the

proper microbial interactions which cause nitrification are absent.

Cutler and Mukerji (1931) observed that the heterotrophic nitrification of five of their isolates was stimulated when an ammonifying bacterium was cultured with the isolates. In all cases, association of the heterotrophic nitrifiers with the ammonifying bacterium resulted in increased production of nitrite. Berger, et al. (1979) noted synergistic nitrification when their two species of Arthrobacter cultured simultaneously produced nitrite while separate culturing yielded only hydroxylamine.

The phenomena of autotrophic and heterotrophic nitrification should not be perceived as being mutually exclusive. The data of this investigation show that the soil heterotroph Alcaligenes and N. agilis are capable of entering into a nitrification synergism. The data of Tate (1977) demonstrate that both autotrophic and heterotrophic nitrification can occur simultaneously. The muck soils of Tate's study contained only 0.1% of the autotrophic population necessary to account for the nitrate observed. Tate noted an abundance of heterotrophic nitrifiers and thus suggested that the two processes were functioning together in the muck soils.

As noted above, PO dissociates to form pyruvic acid and NH_2OH. It is thus possible that pyruvic acid may be an inhibitor

of Nitrobacter agilis. However, this seems unlikely. Quastel and Scholefield (1949) reported that pyruvate did not affect nitrite oxidation by nitrifying organisms. Indeed, they concluded that organic matter, by itself, does not alter the oxidation of nitrite by these microbes.

Bock (1976) observed that Nitrobacter agilis was not only unaffected by pyruvate but also utilized pyruvate as a carbon and energy source after a suitable induction period had passed. Thus, N. agilis was capable of chemoorganotrophic growth when pyruvic acid, as well as acetate and formate, were supplied as carbon and energy sources. One must therefore conclude that the inhibition of the metabolism of N. agilis noted in this investigation must be attributed to either pyruvic-oxime per se or to the hydroxylamine resulting from it. The possibility also exists that both pyruvic-oxime and hydroxylamine are either bacteriostatic or bactericidal to N. agilis.

The absence of sufficient numbers of Nitrosomonas cells to account for the population of Nitrobacter in soils has been noted. Focht and Verstraete (1977) stated that approximately three times as much nitrogenous substrate is necessary to produce equivalent numbers of Nitrobacter when compared to Nitrosomonas. That is, the free energy change in NH_4^+ oxidation to NO_2^- is -65 kcal/mole and only -20 kcal/mole for NO_2^- oxidation to NO_3^-. Thus, three times as

many Nitrosomonas cells would be produced as Nitrobacter cells when the metabolism of one mole of NH_4^+ to NO_3^- had occurred. Yet, Morrill and Dawson (1967) reported equal numbers of Nitrosomonas spp. and Nitrobacter spp. in soil. Ardakani, et al., (1974) observed soil populations of Nitrobacter as high as 10^6 cells/cm^3 and an average Nitrobacter population of 10^5 cells/cm^3. These authors also reported that in the same soils Nitrosomonas populations were maintained at a constant level of approximately 10^4 organisms/cm^3. Thus, in the soils studied by Morrill and Dawson and Ardakani et al., Nitrosomonas populations were clearly insufficient to have supplied enough substrate, i.e., NO_2^-, such that the population of Nitrobacter noted would have arisen. One must therefore surmise that the nitrite was produced by populations other than Nitrosomonas present in the soil.

The current study demonstrates that a mechanism does exist for nitrite generation other than by the oxidation of ammonium ion by Nitrosomonas. The literature records many organisms capable of producing hydroxylamines, bound hydroxylamines, and oximes. The conversion of such compounds to nitrite was demonstrated by the Alcaligenes spp. described in this study. Further, synergistic nitrification of these compounds to nitrate was observed when Alcaligenes sp. strain OS1 and N. agilis were jointly cultured. It is therefore possible that such associations could be of significance in soil and other environments. The numbers of Nitrobacter cells in such ecosystems would reflect the production of nitrite and ultimately nitrate synthesized by such heterotrophic-autotrophic nitrification

synergisms.

The toxicity of NH_2OH to N. agilis was clearly demonstrated when the organism was cultured for one day in media containing 5 mg NH_2OH-N/l. Yet, when N. agilis was jointly cultured with the Alcaligenes sp. nitrite was converted to nitrate by N. agilis even though 17 mg NH_2OH-N/l was present in the medium for at least one day. Thus, the resistance to NH_2OH by the Alcaligenes sp. strain OS1 was extended to N. agilis when the organisms were simultaneously cultured, and this phenomenon allowed nitrite produced by the Alcaligenes sp. to be converted to nitrate by N. agilis.

Further study appears warranted to determine if, and to what extent, synergistic nitrification between heterotrophs and autotrophs occurs in various environments. Accordingly, these investigations would assess both the possibility that chemoautotrophs draw on a pool of nitrogen oxides generated by a wide variety of heterotrophic nitrifiers and the possible extension of toxic compound tolerance to the chemoautotrophs by heterotrophs capable of oxidizing toxic organic or inorganic metabolites.

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APPENDIX

The biochemical and morphological criteria employed to characterize the PO nitrifying isolates OS1, GS1, and OS3 are listed in the attached Appendix as well as other descriptive data. In addition to exhibiting traits associated with the genus Alcaligenes, the isolates also demonstrated various physical and biochemical characteristics not listed in the description of this genus. Data conforming to the description of the genus Alcaligenes are presented in Tables 34-36.

The responses of the isolates to various physiological tests are presented in Table 34. These results show that the isolates have a strictly respiratory metabolism and do not ferment. As such these bacteria are strict aerobes with the exception that NO_2^- or NO_3^- may substitute for oxygen as alternate electron acceptors.

The nitrogenous nutritional requirements of these microbes are simple (Table 36) with either NH_4^+ or NO_3^- able to serve as the sole nitrogen source. The isolates do not fix molecular nitrogen.

All of the isolates are oxidase positive (Table 34) and do not hydrolyze either cellulose, chitin, or agar (Table 36). The organisms grow optimally between 20-37C and grow well at pH 7.0 (Table 34). The G + C content of OS1 is 66.1 moles % (Table 34) and all three bacteria were of terrestrial origin. Thus, the characteristics of the isolates OS1, GS1, and OS3 are clearly sufficient for assignment to the genus Alcaligenes.

Assignment to species of the isolates is not currently possible.

While the isolates exhibit some of the qualities attributable to A. faecalis and A. eutrophus, none of the Alcaligenes species currently described are appropriate. Alcaligenes faecalis does not utilize carbohydrates and has a G + C content of 58.9 moles %. A. eutrophus utilizes glucose, frustose, phenol, benzoate, is capable of chemolithotrophic growth and has a G + C content of 66.3 - 66.8 moles %. Thus, the isolates, while clearly identified as members of the genus Alcaligenes, are not presently assignable to any of the delineated species of the genus.

TABLE 34

Physiological characteristics of the PO nitrifiers OS1, GS1, and OS3.

Physiological or Biochemical Test	OS1	GS1	OS3
Catalase	+	+	+
Oxidase	+	+	+
Indole (Kovak's Test)	-	-	-
SIM: Sulfur (H_2S)	-	-	-
Indole	-	-	-
Motility	+	+	+
TSI: Sugars Fermented	-	-	-
Tolerance of Anaerobiosis	-	-	-
FeS Precipitation	-	-	-
Flourescence	+ (Dull yellow-green)	+ (Dull yellow-green)	+ (Dull yellow-green)
Phenazine Production	+ (Much less than *P. aeruginosa*)	+ (Much less than *P. aeruginosa*)	+ (Much less than *P. aeruginosa*)
O_2 Not Required for Growth (Hugh-Leifson)	-	-	-
O_2 Sensitivity (Ferment Glucose)	-	-	-
Denitrification	+	+	+
Arginine Dihydrolase	-	-	-
2-ketogluconate	-	-	-
3-ketolactose	-	-	-

TABLE 34 CONTINUED

Physiological or Biochemical Test		OS1	GS1	OS3
Heat-Tolerance (80C-15 min.)		-	-	-
pH Tolerance		4.5-10.5	4.5-10.5	4.5-11.0
Chemolithotrophic Growth (H_2,CO_2,N_2, and O_2)		-	-	-
Growth Temperature:	12C	-	Minimal	Minimal
	25-36C	+	+	+
	40C	-	-	-
D.N.A.-G&C Content		66.1 moles %		

TABLE 35

Staining characteristics of the PO nitrifiers OS1, GS1, and OS3

Type of stain	OS1	GS1	OS3
Gram	Negative Coccoidal-Rod	Negative Coccoidal-Rod	Negative Coccoidal-Rod
Spore	-	-	-
Negative (Dorner's Nigrosin)	Small, Curved Rod	Short Rod	Short Rod
Acid-Fast	-	-	-
Flagellation (Transmission Electron Microscopy)	+(3.6)	+(5.0)	+(4.7)
Lipid	+	+	+
Capsule	+	+	+

TABLE 36

Use of selected carbon and nitrogen sources by the PO nitrifiers OS1, GS1, and OS3

Carbon, Nitrogen, or Carbon-Nitrogen Source	OS1	GS1	OS3
Acetate	+	+	+
Pyruvate	+	+	+
Succinate	+	+	+
Benzoate	+	+	+
Citrate	+	+	+
Butyrate	+	+	+
Propionate	+	+	+
Glucose	+	+	+
Rhamnose	-	-	-
Mannitol	-	-	-
Starch	-	-	-
Fructose	-	-	-
Xylose	-	-	-
Ribose	-	-	-
Ribotol	-	-	-
Cellobiose	-	-	-
Sucrose	-	-	-
Maltose	-	-	-
L-Arabinose	-	-	-

TABLE 36 CONTINUED

Carbon, Nitrogen, or Carbon-Nitrogen Source	OS1	GS1	OS3
L-Alanine	+	+	+
L-Arginine	+	+	+
L-Histidine	+	+	+
DL-Asparagine	+	+	+
DL-Aspartate	+	+	+
Acetamide	-	-	-
Glutathione	+	+	+
NH_4^+	+	+	+
NO_3^-	+	+	+
N_2 Fixation (Stanier, et al., 1966)	±	±	±
N_2 Fixation (Burke's Medium)	±	±	±
N_2 Fixation (Acetylene Reduction)	-	-	-
Plate Gelatin	-	-	-
Nutrient Gelatin	-	-	-
Casein	-	-	-
Cellulose	-	-	-
Chitin	-	-	-
Agar	-	-	-
Phenol	-	-	-
Ethanol	+	+	+

TABLE 36 CONTINUED

Carbon, Nitrogen, or Carbon-Nitrogen Source	OS1	GS1	OS3
Glycerol	-	-	-
Litmus Milk	Alkaline	Alkaline	Alkaline