NITROSOMONAS EUROPAEA AND NITROBACTER AGILIS: PURE CULTURE STUDIES, CELL ENUMERATION, AND THE EFFECT OF PESTICIDES ON NITRIFICATION

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ABSTRACT

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PURE CULTURE STUDIES, CELL ENUMERATION
AND THE EFFECT OF PESTICIDES ON NITRIFICATION

by Aline L. Garretson

Readily reproducible growth conditions in 250 ml stationary flask cultures were obtained for Nitrobacter agilis and Nitrosomonas europaea. For both organisms a minimal inoculum of 3.13% (by volume) was required for the initiation of growth in Medium A with a lag period of less than 48 hrs. N. agilis completely oxidized the energy substrate during the exponential growth phase. However, N. europaea cultures terminated the exponential growth phase leaving 12 to 24% of the NH_4-N unoxidized to NO_2-N, depending on the growth conditions. N. agilis grew equally well in Medium AA, containing no insoluble components, at a pH of 10.0 after autoclaving. N. europaea did not survive in this medium when $CaCl_2$ was added and NO_2-N was replaced by NH_4-N. The removal of amines from distilled water used in the preparation of media had no gross effect on the rate of nitrification by Nitrobacter.

Three bacterial and 2 streptomyces cultures were isolated from a contaminated N. agilis culture. These isolates were heterotrophic and incapable of utilizing NH_4-N, NO_2-N or NO_3-N. When grown along with pure cultures of either

N. agilis or N. europaea, they exerted no adverse effect on either growth or nitrification of the nitrifiers.

A membrane filter technique was attempted for the quantitation of cell populations by a modified colonial plate count method. However, colonial development was inadequate on the filter to permit easy enumeration and further work will be necessary to enhance colonial size or to facilitate visualization.

The nitrification process of N. agilis was sensitive to aldrin, lindane, and rhothane at final concentrations of 1 ug/ml. Complete inhibition for 14 days was obtained with rhothane, aldrin, and parathion at 10 ug/ml. Delayed nitrification occurred with baygon at 10 ug/ml and with lindane at 1 ug/ml. Malathion caused delayed nitrification at 1000 ug/ml. Lindane, malathion, and baygon were at least 100 times more toxic for Nitrosomonas cultures than for Nitrobacter.

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Aline L. Garretson

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To members of my family, for their constant encouragement, assistance, and understanding during the course of my master's program.

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INTRODUCTION

Soil nitrification has long been of interest to both the agronomist and theoretical microbiologist. This process is important in agriculture since nitrate or ammonium is the major nitrogen form assimilated by higher plants. Nitrogen assimilated in the inorganic state is then incorporated in the proteinaceous matter of all living tissue. The nitrogenous materials available in the soil exist largely in the organic form. The release of the organically bound element is accomplished by two separate and distinct microbiological processes, ammonification and nitrification. The latter process is limited to a relatively small number of nitrifying organisms. Their importance in the nitrogen cycle lies in their capacity to produce nitrate. The dominant microbial species concerned in the oxidation of ammonium to nitrite, and nitrite to nitrate are members of two genera of the family Nitrobacteriaceae, Nitrosomonas and Nitrobacter. Only two species of each genera are recognized, Nitrosomonas europaea, Nitrosomonas monocella, Nitrobacter winogradskyi, and Nitrobacter agilis.

Up to the present time few reports have appeared concerning metabolic studies on the chemolithotrophic nitrifiers. The carbon metabolism of these organisms still remains to be elucidated. The study of these bacteria not only has practical application to agriculture but also offers a stimulating challenge to biochemical investigations.

My initial objective was to study the effect of various chemical factors, including pesticides, on the nutrition and physiology of these organisms. Because of their unique properties ordinary microbiological methods were not readily utilizable and the methods applicable were cumbersome and time consuming.

The size of colonies formed on a solid medium is so small that visualization without optical magnification is not possible. Turbidimetric measurement of cell numbers is also impossible unless repeated supplementation of both nutrients and buffer are made to prolong the exponential growth phase. Therefore, these methods are not applicable for the preparation of large numbers of replicate cultures. With the exception of an analytical chemical analysis for the three states of nitrogen (NH_4^+, NO_2^-, or NO_3^-), no other assay method is available for the accurate quantitation of small numbers of microorganisms. Consequently, it was deemed advisable not only to develop an accurate method of cell quantitation but also to improve on cell culture conditions for faster rates of reproduction and more efficient utilization of metabolites.

During the course of this study, difficulties were encountered in the maintenance of pure cultures. Heterotrophic contamination therefore became an additional area for attention.

There subsequently evolved three major areas for research: (a) pure culture methods, (b) cell enumeration, and (3) the effect of pesticides on nitrification. Although

closely interrelated, each area represents a distinct phase of activity and the judgment was made to treat each as a separate unit in this dissertation.

The results described in this dissertation have established a necessary foundation for future study of the biochemistry and physiology of these microorganisms. The need for an effective cell enumeration method also warrants further study.

HISTORICAL

Biological Nitrification Concept

Despite its great economic importance in agriculture, the process of nitrification has received relatively little attention and then only spasmodically during the past eighty-five years. Although Pasteur in 1862 was the first to suggest that the formation of nitrate in soil was microbiological and perhaps similar to the conversion of alcohol to acetic acid it was not until 1877 that Schloessing and Müntz demonstrated that the oxidation of ammonia was indeed microbiological. The experimental evidence was obtained by passing sewage daily through a column of sterile sandy soil and chalk. After twenty days, the ammonia input appeared in the effluent almost completely as nitrate-nitrogen. This process after inhibition by the addition of chloroform or heating the column could be restored to its former activity by the addition of fresh garden soil washings.

Between 1890-1891 the oxidizing organisms were isolated apart from the soil by three independent workers (Frankland and Frankland, 1890; Warrington, 1891; Winogradsky, 1890a,b,c; 1891a,b). It followed from the work of Warrington that two kinds of special, aerobic organisms must be responsible, respectively, for the two stage oxidation, ammonia to nitrite and nitrite to nitrate. His attempts to subculture these organisms from enrichment cultures failed. Winogradsky, however, was able to isolate colonies on an inorganic medium

solidified with silica gel. Two types of nitrifiers were isolated, Nitrosomonas, oxidizing ammonia to nitrite, and Nitrobacter, oxidizing nitrite to nitrate.

Metabolic Activities

The next advance came in 1916 when Meyerhof extensively investigated the respiratory activity of these organisms and the effect of various inhibitors. By measuring oxidation rates the optimal pH of Nitrobacter was found to be about the same as that of Nitrosomonas, 8.5 to 8.8 (Meyerhof, 1916a,b). Meyerhof's early studies (1916a, 1917) also throw light on many other factors influencing the metabolism of these organisms. The extreme sensitivity of these organisms to hydrogen ion concentration requires a bicarbonate buffer. Growth inhibition of Nitrobacter occurs as nitrate accumulates or in the presence of free ammonia, while on the other hand Nitrosomonas is inhibited by both ammonia and nitrite. Both forms are inhibited by divalent metal ions and lowered oxygen pressure (Meyerhof, 1916; Amer and Bartholomew, 1951). This fact suggests that at least one stage of the oxidation involves an oxidase with much less affinity for O_2 than cytochrome oxidase. Meyerhof's investigations indicate that ions must freely enter the Nitrobacter cell, since at the optimal pH both nitrite and bicarbonate are very largely dissociated, and non-ionized-NO_2 and -NO groups in the form of metallic complexes or organic nitroso-compounds (Meyerhof, 1917) could not be used.

Glaring gaps in the literature on the metabolic activities of the nitrifiers continued until the advent of the first soil perfusion apparatus described by Lees and Quastel (1944) and subsequently modified by Audus (1946) and Lees (1947). In this apparatus, a metabolite is continuously percolated through a soil column, the repeated perfusion permitting direct study of the kinetics of soil nitrification under controlled environmental conditions. The subsequent use of this technique for the study of organic and inorganic conversions was published intermittingly in several reports from 1944 to 1954. It is to be expected that in soil, with its complex microflora and its special physicochemical conditions, the kinetics, possibly even the mechanism of nitrification, could differ materially from what takes place in pure cultures of the nitrifiers. A significant difference between growth in soil and in pure culture is the response to the effect of organic matter. Peptone is far less inhibitory in sand than in solution cultures (Wimmer, 1904), and cottonseed meal and ammonium sulfate both are more rapidly nitrified in soil than in solution. The presence of certain heterotrophs developing symbiotically in soil greatly influences the nitrification process (Desai and Fazal-un-Din, 1937; Pandalai, 1946). The stimulation by colloids in culture media on bacterial behavior (Albrecht and McCalla, 1937, 1938; Conn and Conn, 1940; Nommik, 1957; Zobel, 1943) suggests the possibility of similar effects in soils.

Nutrition

Although considerable attention has been given to the nutrition of the nitrifying chemolithotrophs these studies often have been incomplete and ambiguous. Many of the earlier investigations performed in impure media and in mixed culture should be verified under improved cultural conditions now available. Most studies were concerned with the influence of organic compounds on both growth and nutrition of the nitrifiers as a group, and the effect upon the overall oxidation of ammonium ion to nitrate (Bomeke, 1950; Lees and Quastel, 1946a,b,c,; Meiklejohn, 1951, 1952a; Quastel and Scholefield, 1949). Using pure culture methods in a defined medium recent studies have been sharply focused on the possible effects of organic supplements. In the light of these recent experiments no sugar, organic acid or other organic molecule will serve as sources of either carbon or energy for the growth of N. agilis (Delwiche and Finstein, 1965; Ida and Alexander, 1965). A stimulatory effect on nitrification and growth of this strain is obtained with supplements of yeast extract, Vitamin-Free Casamino Acids, and certain amino acids, while a strong stimulation limited to growth is obtained with acetate (Delwiche and Finstein, 1965). Both acetate and glycine contribute to cell carbon. These same workers demonstrated that the organism is permeable to certain organic acids. At least some of the enzymes for organic metabolism must also be present. No stimulation of nitrite production by N. europaea by either B vitamins or

amino acids (Gundersen, 1955a) has been found and similar negative responses have been obtained with N. agilis (Aleem and Alexander, 1960). Krulwich and Funk (1965), however, have more recently reported enhancement of both nitrite utilization and growth with biotin using four strains of N. agilis.

It is quite certain that Nitrosomonas and Nitrobacter do develop in inorganic solutions from which all traces of organic matter is removed. The sole energy sources supporting growth are nitrogenous, typically ammonium or nitrite salts. The nitrogen of amino acids, amides, proteins or urea is not oxidized by N. europaea although some of the nitrogen in certain purines may be converted to nitrite by some strains; the purines are likely deaminated prior to nitrification (Ruban, 1958). Engle and Alexander (1960) and Silver (1960) have demonstrated hydroxylamine and formic acid oxidation by this bacterium but there is no evidence that these compounds are energy sources for proliferation.

The mineral requirements, except for the carbon and energy sources, resemble those known for heterotrophs. The nutrient demand in laboratory studies is low since the number of cells is relatively small. Specific requirements for magnesium, phosphate, and nitrite have been reported (Bomeke, 1950; Meiklejohn, 1952b; Meyerhof, 1916a) but under questionable cultural conditions. The optimal iron concentration for growth of N. winogradskyi is reported to be about 6 ug/ml for the oxidation of 200 ug/ml nitrite-nitrogen (Meiklejohn, 1953) but this iron level exceeds the amount of cell carbon

formed (Meyerhof, 1916a). The use of well-defined culture methods has been recently initiated for nutritional studies of N. agilis (Aleem and Alexander, 1960). Using an alumina purified inorganic medium, the requirements for each nutrient is studied by omitting the nutrient under study and adding graded amounts to flasks in a standard series. Using these methods the optimal nutrient levels now appear to be approximately 5 ug/ml for both phosphorus and magnesium and at least 0.005 ug/ml for iron with N. agilis. These data conflict with the findings of Meiklejohn who reported the iron requirement as high as the P and K levels. It is suggested that the data of Meiklejohn may result from reactions with $CaCO_3$ rendering most of the iron unavailable to the microorganisms. The investigations of Aleem and Alexander (1960) also reveal that the effect of nitrate on rate of nitrite oxidation is one of retarding the initiation of growth, results which are similar to those obtained in soil (Stojanonic and Alexander, 1958). The toxicity of ammonium salts in alkaline environments seems to be a generic property since N. agilis is markedly inhibited as are other Nitrobacter isolates (Bomeke, 1950; Boullanger and Massol, 1903; Meyerhof, 1916b). Cell-mass development and concomitant nitrite oxidation is enhanced in freshly inoculated cultures if the medium contains small amounts of molybdenum (Finstein and Delwiche, 1965; Zavarzin, 1958). Zavarzin postulated that a molybdoflavoprotein is concerned in the energy yielding reaction of these autotrophs. According to investigations of Finstein

and Delwiche the enhancement may simply be a response to the development of greater cell mass and not a direct molybdenum function in enzymatic nitrite oxidations. A function of molybdenum in nitrate reductase (Nicholas, Nason and McElroy, 1953) does suggest, but does not establish, a function in the reverse direction. Winogradsky (1890a, 1891) used $MgCO_3$, instead of $CaCO_3$, in his original medium. When made up with purified reagents this medium gave poor growth and nitrification. Kingma Boltjes (1935) established Ca ion as the missing factor which was essential for Nitrosomonas but not necessary for Nitrobacter (Meyerhof, 1916a,b). However, Alexander (1965) believes there is no valid evidence for a requirement for significant quantities of calcium and cited several reports in support of his argument (Aleem and Alexander, 1960; Bomeke, 1950; Lees and Meiklejohn, 1948). Potassium (Welch and Scott, 1959) and sulfur are also required elements, and copper is reported to be stimulatory to Nitrobacter (Kiesow, 1962; Zavarzin, 1958).

Pure Culture Methods

After gelatin proved to be useless for the colonial development of the nitrifiers from enrichment cultures, Winogradsky (1891a,b), using an inorganic medium solidified by silicic acid, finally isolated Nitrosomonas and Nitrobacter in monoculture. Since Winogradsky's time the isolation and pure culture of nitrifying bacteria were repeatedly reported (Bisset and Grace, 1954; Bomeke, 1939; Gibbs, 1919; Gould and

Lees, 1960; Kingma Boltjes, 1935; Lees, 1952; Lewis and Pramer, 1958; Meiklejohn, 1954; Nelson, 1931). Considerable difficulty still exists, however, in obtaining monocultures free of contaminating microorganisms. These contaminants often remain undetected because of the morphological similarity to the predominant organism and some may be incapable of developing on conventional laboratory media. Common contaminants in the final enrichments include species of Pseudomonas, Hyphomicrobium, Mycobacterium, Flavobacterium and Serratia as well as an occasional myxobacterium (Gundersen, 1955b; Stapp, 1940; Ulyanova, 1960). A symbiotic relationship may exist which might explain the growth of the autotroph in an unfavorable soil environment.

Many methods have been described to separate the autotrophs from their contaminants. In some of these procedures, the autotrophic population is increased by addition of successive increments of nitrogen and thereby increasing the ratio of autotrophs to heterotrophs. The enrichment culture is then diluted, and pure cultures made by single-cell technique, either by plating or by tube dilution to a heterotroph free endpoint. Several modifications have been devised, one modification entails bubbling carbon dioxide through the cultures to remove the cells from the carbonate particles (Meiklejohn, 1950). Inhibitory compounds such as antibiotics and dyes are also employed (Gould and Lees, 1958, 1960; Prouty, 1929) for suppression of contaminating species.

Lewis and Pramer (1958) eliminated the problem of adsorption of contaminant and nitrifier on the carbonate particles by using soluble carbonates in their medium.

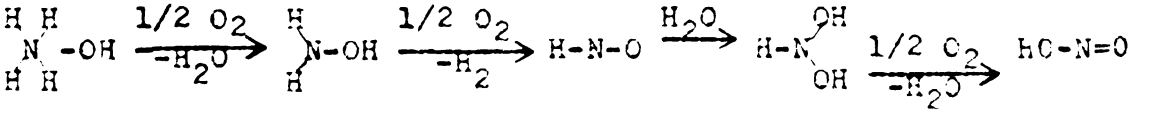
The problem of contaminants is not ended once a nitrifier is obtained in pure culture. The corollary problem of maintaining a culture contaminant free is perhaps more important when studying the biochemistry of these organisms. The recent study of Clark and Schmidt (1966) on the effect of mixed culture on N. europaea serves to emphasize this point, and indicates the ease in which stock laboratory cultures become contaminated with chemoorganotrophs.

Biochemistry of Nitrification

Until recently, effective investigations on the mechansim of nitrification were hindered because of the lack of cell culture methods for the production of large quantities of active cells which could be easily freed from insoluble medium ingredients. Improved culture methods are now available (Engle and Alexander, 1958a,b) for N. europaea in which this chemolithotroph grows in a medium free of insoluble carbonates with a relatively short generation time. The yield of metabolically active cells is 72.4 mg (dry weight) per liter when grown in a 25 L pyrex fermentor fitted with a stainless steel heating and sparging assembly. Similar apparatus for large scale aerated batch cultures are described for Nitrobacter (Aleem and Alexander, 1958; Butt and Lees, 1958; Gould and Lees, 1960; Lees and Simpson, 1957; Skinner and Walker, 1961).

Since the development of these large batch cultures, a number of investigations have been reported on the biochemical reactions of washed cell suspensions and cell-free preparations. These studies have resulted in numerous reports on the intermediary metabolism of nitrification.

All considerations of the nitrogenous intermediates in the mechanisms of nitrification originates with Kluyver and Donker (1926). The conversion of ammonia to nitrate involves a sequential conversion of nitrogen from the -3 to the +5 oxidation state. The compounds of nitrogen known for all the possible oxidation states in this sequence are: hydrazine (-2), hydroxylamine (-1), molecular nitrogen (0), hyponitrous acid or nitrous oxide (+1), nitric oxide (+2), nitrous acid (+3), and nitrogen dioxide (+4). Subsequent investigators have attempted to provide either confirmatory evidence in support of the proposed pathway by Kluyver and Donker, or evidence for a satisfactory alternative. Kluyver and Donker proposed a sequence consisting of a series of oxidations each representing a loss of two electrons:



Ammonium hydroxide	Hydroxyl-amine	Nitroxyl	Dihydroxy-ammonia	Nitrite

Hydroxylamine (NH_2OH) is commonly considered to be the initial product of ammonia oxidation. Some weak evidence in support of hydroxylamine as an intermediate is provided by several

investigators. Added hydroxylamine is oxidized by N. europaea cell suspensions to nitrite rapidly and stoichiometrically without any time lag (Engle and Alexander, 1958a). Hydroxylamine is identified as the accumulated nitrogenous compound when Nitrosomonas is incubated with ammonium sulfate in the presence of hydrazine (N_2H_4), which inhibits the oxidation of hydroxylamine (Yoshida and Alexander, 1964). N. europaea cells contain an enzyme catalyzing the oxidations of hydroxylamine (Burge, Malavolta, and Delwiche, 1963; Engle and Alexander, 1959; Nicholas and Jones, 1960). These enzyme preparations show no detectable oxidation of ammonium but in the presence of some suitable electron acceptor produced about three-fourths of the expected quantity of nitrite. In this reaction, neither nicotinamide adenine dinucleotide (NAD) nor nicotinamide adenine dinucleotide phosphate (NADP) is reduced.

The intermediate at the next higher oxidation state remains completely obscure. Although hyponitrous acid (HO-N=N-OH) is a possible intermediate, no microbial conversion of hyponitrite to nitrite is noted with either cell suspension or enzyme extracts prepared from Nitrosomonas (Lees, 1954; Nicholas and Jones, 1960).

Reduction of cytochrome c, in the absence of oxygen, takes place during hydroxylamine oxidations with cell-free extracts (Nicholas and Jones, 1960). In this process nitrous oxide is evolved which is probably a degradation product of the unknown intermediate formed between hydroxylamine and

nitrite (Falcone, Shug, and Nicholas, 1962). Aleem et al. (1962) propose that this intermediate is nitrohydroxylamine (NO_2•NHOH). These investigators observed that one mole of nitrite is formed for each mole of nitrohydroxylamine metabolized. It is suggested that nitrohydroxylamine is generated in a reaction between hydroxylamine and endogenous nitrite, and the resulting product is then oxidized by the bacterium to 2 moles of nitrite.

$$NH_2OH + HNO_2 \xrightarrow[-H_2O]{1/2\ O_2} NO_2{\bullet}NHOH \xrightarrow{1/2\ O_2} 2HNO_2$$

Anderson (1964) reports negligible amounts of nitrite formed from hydroxylamine anaerobically in the presence of Nitrosomonas extract and methylene blue. Nitric oxide and nitrous oxide are produced in amounts equivalent to the hydroxylamine added.

Nitrite is rapidly metabolized by extracts of N. agilis cells with complete conversion to nitrate and the consumption of oxygen is essentially equal to the theoretical amount expected according to the following equation:

$$NO_2^- + 1/2\ O_2 \longrightarrow NO_3^-$$

The enzyme is associated with the particulate constituents of the cell (Aleem and Alexander, 1958). Laudelot and Van Tiechelen (1960) report the Michaelis constant, Km, to be 6.7×10^{-4} M at 32°C. Aleem and Nason (1959) report the enzymic oxidation of nitrite is catalyzed by a cytochrome-containing electron transport particle via cytochrome c and

cytochrome oxidase-like components. This oxidation is coupled with the generation of high-energy phosphate bonds identified as adenosine triphosphate, ATP (Aleem and Nason, 1960). The overall reaction is :

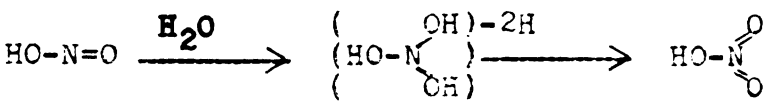
$$NO_2^- + 1/2\ O_2 + nADP + nPi \xrightarrow[\text{transport chain}]{\text{cytochrome electron}} NO_3^- + nATP$$

The energy liberated by the above equation is available for the reduction and assimilation of carbon dioxide (Aleem, 1965) and for the reduction of pyridine nucleotides essential for the operation of the carbon reduction cycle (Aleem, 1965; Aleem, Lees and Nicholas, 1963). This process of chemosynthesis appears to be analogous to photosynthesis.

As in photosynthesis, water must be the hydrogen donor for the pyridine nucleotide reduction. Aleem, Hoch, and Varner (1965) report that water, and not molecular oxygen participates in the nitrite oxidation and that the hydrogen donor for the concomitant reduction of pyridine nucleotides is also water:

$$NO_2^- + H_2O^{18} + A \rightleftharpoons NO_3^{18} + AH_2$$

From their results it appears that a hydrated nitrite molecule, or some activated form of it, may be the substrate for a dehydrogenation:



Iron, but not zinc, molybdenum, magnesium or copper, is stimulatory to nitrite oxidation by enzyme preparations (Aleem and Alexander, 1958). This observation suggests a role for iron in this reaction.

Pesticides

Since World War II the use of synthetic organic biocides has resulted in a marked increase in crop production and decrease in incidence of insect-borne diseases. More recently, however, there has been a growing concern with the effects of their distribution in the environment. This interest has been mainly centered on the presence of pesticides, in or on foods for human consumption, and the contamination of air and surface waters. The pesticide residues in soil have been well studied but relatively few investigations have been directed toward their effect on soil microbial interactions.

Among the possible toxic effects of pesticides on the normal microbial flora in the soil, in addition to the inhibition of specific members of the population, there are secondary effects changing microbial dominance and concommitant alteration of the nitrogen cycle. Since nitrate is the major source of nitrogen assimilated by higher plants one would expect that an imbalance in the microbial population could ultimately affect the rates of nitrification and influence plant nutrition.

Approximately 60,000 pesticidal products have been registered in the United States. A minimum of 490 basic

chemicals are on the market, 200 of which are of first-rate importance. These economic poisons include the insecticides, herbicides, fungicides, rodenticides, and nematocides. These substances fall into one of several groups of chemical compounds: chlorinated hydrocarbons, organic phosphates, carbamates, dinitrophenols, organic mercurials, organic sulfur compounds, natural products, and inorganics.

A beneficial use of inhibitors is the prevention of nitrogen losses following fertilization. Surveys have been aimed at finding non-phytotoxic chemicals which would selectively inhibit the nitrifying bacteria and thereby maintain the fertilizers in a reduced form for longer periods. One such compound, 2-chloro-6-(trichloromethyl)-pyridine, causes a marked deleterious effect on nitrifying organisms (Goring, 1962a,b) and this action was not noted with other groups of organisms (Shattuck and Alexander, 1963).

EXPERIMENTAL

PART I. MAINTENANCE AND PROPAGATION IN PURE CULTURE

Introduction

Methods for the continuous propagation of pure cultures of N. europaea and N. agilis in stationary culture have received little attention in the past and the more recent publications on the growth of these nitrifiers have been limited to large scale aerated batch cultures, shake flask cultures or soil perfusions.

Since none of these culture systems was applicable to our intended investigations requiring large numbers of repetitive cultures, it was first necessary to establish readily reproducible growth conditions. Small volume, stationary flask cultures were utilized in which pH, aeration, and substrate nitrogen closely approximated the optimal conditions for nitrification in the soil.

Materials and Methods

Culture strains. Cultures of N. europaea were received on two different occasions, 7/25/65 and 4/15/66, from Dr. E. L. Schmidt. This strain was identified as ATCC 12248. N. agilis was obtained as follows: from Dr. I. Fischer a garden soil isolate of Dr. C. C. Delwiche, and an unidentified culture from Dr. David Pramer. All of these cultures were maintained at 25-30 C by continuous subculture in fluid media.

Growth media. For cell culture maintenance and experimentation the organisms were cultivated in 250 ml flasks containing 50-75 ml of the appropriate growth medium. This resulted in an air space to culture fluid volume ratio ranging from 2 to 4.

N. europaea was grown in a basal medium, referred to as Medium A in the text, supplemented with 0.3 or 0.5 g $(NH_4)_2SO_4$/liter. The concentration of NH_4-N in the complete medium was 63.6 and 106 ug/ml, respectively. Medium A is described in detail in the appendix (page 83) and is a modification of the medium of Pramer and Schmidt (1965).

N. agilis was propagated in one of two media: either Medium A, just described, but containing 102 ug NO_2-N/ml in the form of $NaNO_2$ instead of $(NH_4)_2SO_4$, or a modification of the medium described by Aleem and Alexander (1960). The latter basal medium, designated as Medium AA in the text, is also described in the appendix (page 83). The complete medium contained 0.6 KNO_2/ liter (98.8 ug NO_2-N/ml).

All of the components of the media were reagent grade. Distilled, deionized water was routinely used throughout the course of this study. A further reduction of water impurities was effected in most of the experiments by the passage of distilled water through a mixed cation and anion resin

(Barnstead Still and Sterilizer Co., Boston, Mass.) and then a cation removal resin (Barnstead Still and Sterilizer Co., Boston, Mass.). This second resin was for the removal of amines known to be commonly present in the condensed "boiler" water.

The pH of all fluid media prior to sterilization ranged from 8.0 to 9.0 depending upon the specific reagents in the medium. The pH was independent of the quality of the water used to dissolve the reagents.

Sterilization of the complete medium was accomplished by autoclaving unless otherwise indicated. In certain experiments sterilization was accomplished by passage through sterile membrane filters (Millipore Filter Corp., Bedford, Mass.) with a pore size of 0.45 u. Stock cultures were incubated at 25-30 C, and experimental cultures were incubated at 28-30 C.

Nitrification analysis. Analysis of culture fluids was routinely made on the basis of the appearance of nitrite in the case of Nitrosomonas, or the disappearance of nitrite in the case of Nitrobacter, by the alpha-naphthylamine-sulfanilic acid procedure of Rider and Mellon (1946) which was performed spectrophotometrically at a wave length of 520 mu.

Ammonia was determined spectrophotometrically at 410 mu by a direct nesslerization procedure (American Public Health Association, [illegible]).

Culture purity. The presence of heterotrophic contaminants in autotrophic cultures was routinely evaluated by the addition of a 5 to 10% inoculum to each of the following organic fluid media: Nutrient Broth, Lactose Broth, Czapek Dox Broth, and Thioglycollate Broth. Incubations were carried out at 28 to 30 C for 1 month. The absence of turbidity was considered an indication of culture purity.

RESULTS

Rates of Nitrification

It was noted in initial experimentation that the rates of nitrification differed greatly with the two types of chemolithotrophs. In order to evaluate these differences accurately, duplicate experiments for Nitrobacter agilis (Pramer) and Nitrosomonas europaea (Schmidt) were conducted in Medium A. A standard series of flasks for each strain was inoculated at twofold dilutions ranging from 25 to 1.56% (by volume). Samples of 1 ml were taken daily over a 7 day incubation period from the Nitrobacter cultures and over an 8 day incubation period from the Nitrosomonas cultures. The rates of nitrification were determined by chemical analysis. The % NO_2-N oxidized by various levels of inoculum was plotted against time for Nitrobacter (Fig. 1). The % NO_2-N produced by the 3.13 and 12.5% inocula is similarly represented (Fig. 2) for the Nitrosomonas series. The 25 and 6.25% inocula were omitted from this graph as they approximated the 12.5 and 3.13% inocula, respectively.

The results of both experiments demonstrated a short lag period of oxidation, or growth, followed by a rapid period of exponential growth with the 3.13% inoculum. In the case of the Nitrobacter cultures, this was the minimum level of inoculum initiating exponential growth within 48 hours.

The Nitrobacter culture series effected the complete conversion of NO_2-N to NO_3-N during the exponential growth

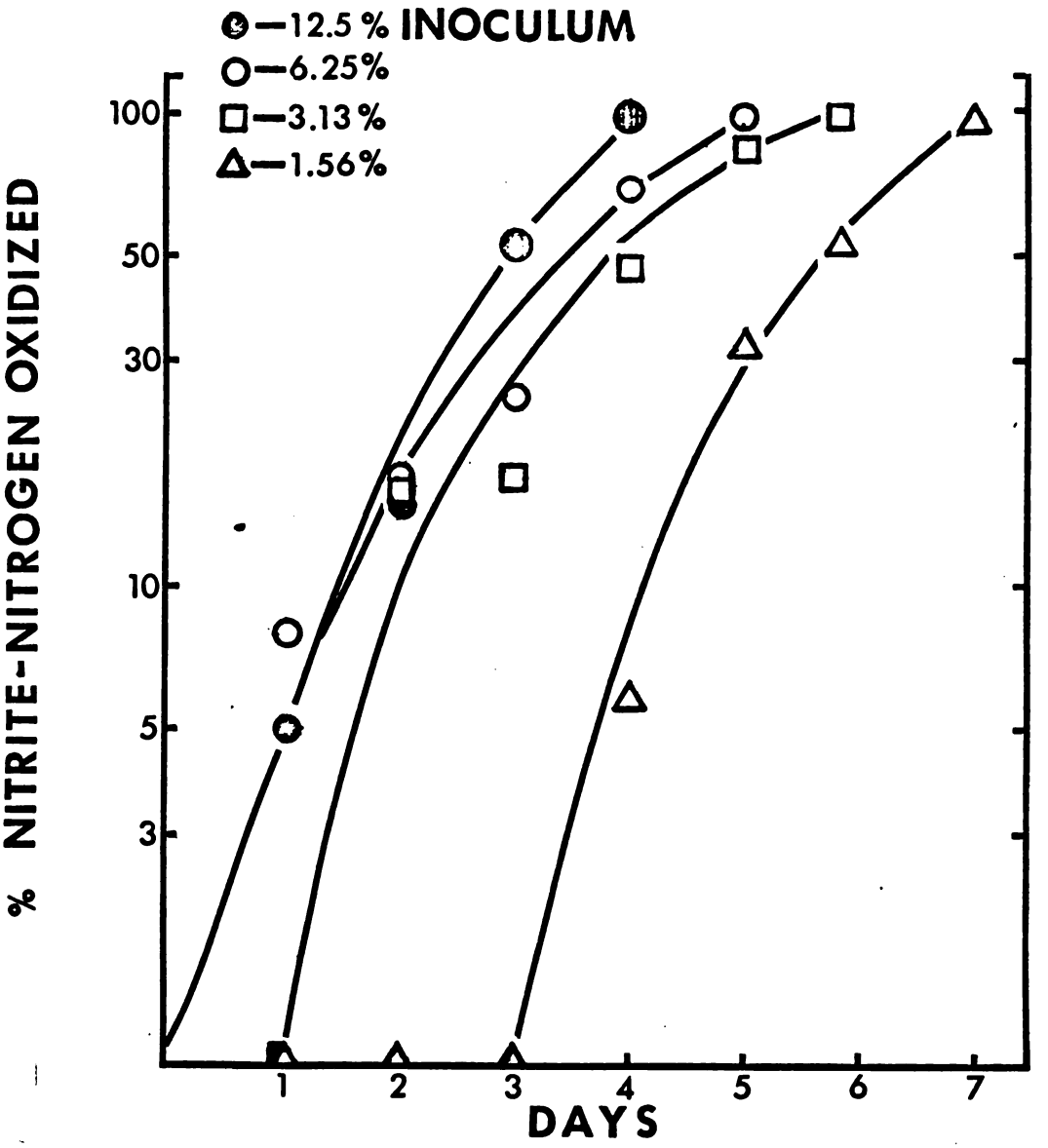


Fig. 1. Logarithmic plot of % nitrite-nitrogen oxidized by various % inocula of Nitrobacter agilis (Pramer).

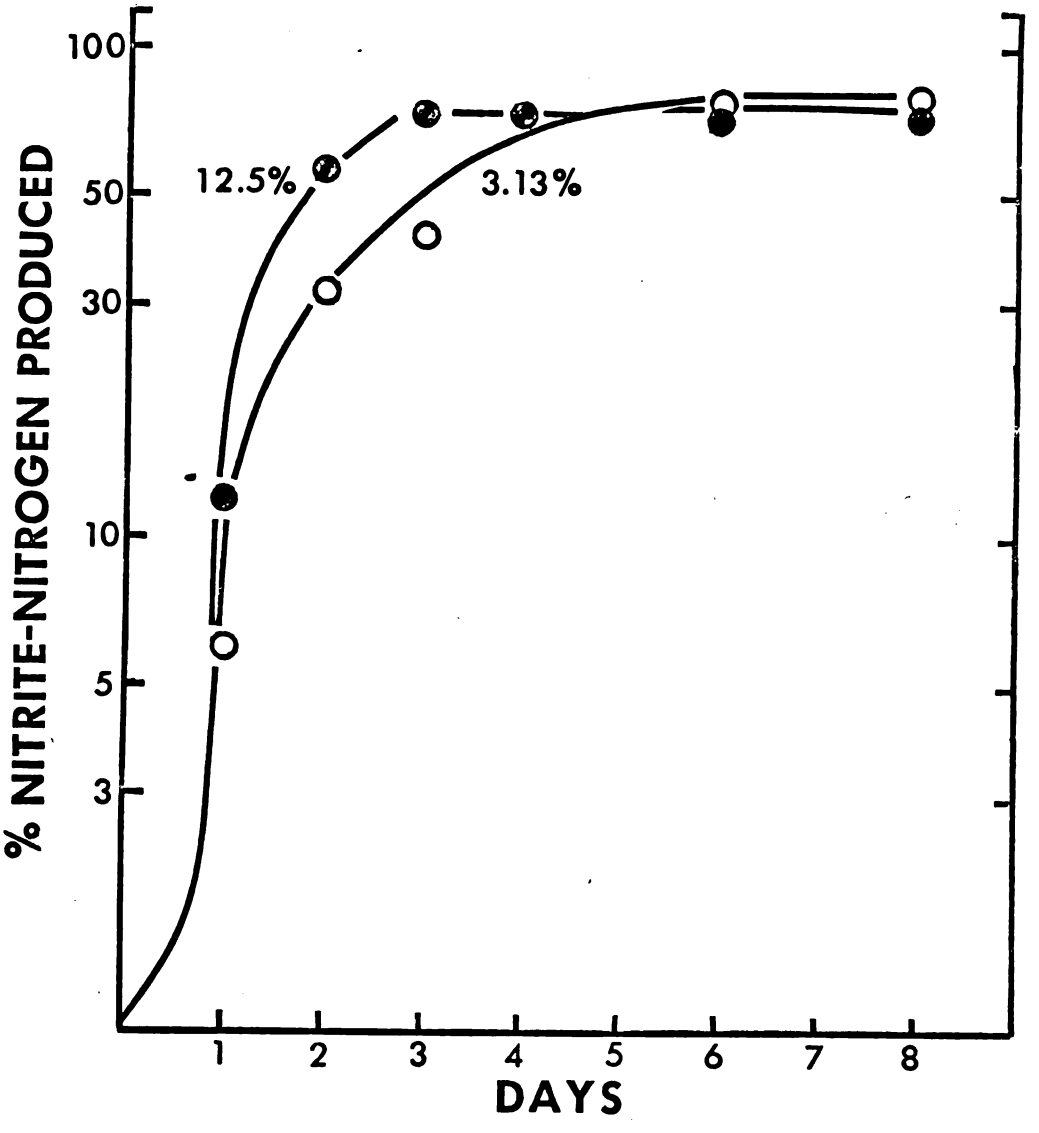


Fig. 2. Logarithmic plot of % nitrite-nitrogen produced by 3.13 and 12.5% inocula of Nitrosomonas europaea (Sohmidt).

phase. A similar response was also obtained with the Fischer strain of N. agilis. For Nitrosomonas, however, the exponential growth phase was terminated when 70 to 80% of the theoretical amount of NH_4-N appeared in the form of NO_2-N. These patterns of nitrogen oxidation have been repeatedly encountered in this laboratory.

Incomplete Oxidation by Nitrosomonas

A more detailed analysis of the data obtained in the previous growth experiment showed that approximately 14.8% (average value) of the NH_4-N was not converted to NO_2-N after 10 days incubation (Table 1). Although slightly higher % values were obtained with the 25 and 12.5% inocula, this was not considered significant. The amounts of NO_2-N initially present in the media were considerably lower than concentrations reported to be inhibitory to Nitrosomonas (Engle and Alexander, 1958).

In order to determine if NH_4-N became inaccessible during the incubation period another series of duplicate cultures was seeded in Medium A containing 66.3 ug NH_4-N/ml. After a 7 day incubation period assays for both NO_2-N and NH_4-N were conducted on the culture fluids (Table 2). The combined values for NH_4-N and NO_2-N after 7 days incubation were similar to the initial concentration of NH_4-N for the two inocula of 6.25 and 3.13%. These results demonstrate that most of the NH_4-N not converted to NO_2-N was still available in the medium for the ultimate oxidation to NO_2-N.

Table 1. The incomplete utilization of NH_4-N in Medium A by Nitrosomonas europaea (Schmidt) after 10 days incubation

Inoculum (%)	Oxidative state of nitrogen			
	Initial		After 10 days incubation	
	NO_2-N from inoculum[a] (ug/ml)	NH_4-N[b] (ug/ml)	NO_2-N produced[a] (ug/ml)	NH_4-N not converted to NO_3-N (%)
25	50.0	82.5	70.0	15.2
12.5	25.8	93.3	77.2	17.3
6.25	11.3	97.8	84.7	13.3
3.13	5.90	103.0	90.1	12.6

[a] Values obtained by assay.

[b] Calculations based on the dilution of the medium which contained 106 ug NH_4-N/ml.

Table 2. The availability of NH_4-N for conversion to NO_2-N in Medium A during the late exponential growth phase of Nitrosomonas europaea (Schmidt)

Culture description		Oxidative state of nitrogen			
Inoculum (%)	Growth phase	Initial		After 7 days incubation	
		NH_4-N	NH_4-N + NO_2-N	NH_4-N	NH_4-N + NO_2-N
			(ug/ml)		
6.25	Late exponential	62.3	66.6	19.8	63.3
3.13	Early exponential	64.3	66.9	53.8	62.5

Media Studies

Medium A was routinely used for the maintenance of stock cultures of both nitrifiers, the only composition difference being in the nitrogen source. This medium contained a large amount of a fine precipitate which settled out rapidly and under certain conditions aggregated into larger particles.

For pure culture isolation and plate counting methods the even dispersal of microorganisms is an essential requirement. Since the nitrifiers as well as possible heterotrophic contaminants tend to adhere to the insoluble particles, uniform dispersion and separation of the microorganisms are not possible in this type of medium. A completely soluble medium is also desirable for the preparation of cell suspensions free of media constituents.

Growth of these microorganisms was therefore attempted in Medium AA, which contained no insoluble components and which was originally described by Aleem and Alexander (1960) for the growth of N. agilis in flasks incubated on a 285-rpm rotary shaker. The results obtained in this laboratory with N. agilis are given in Table 3. Equal, if not slightly better rates of oxidation were obtained in Medium AA with two isolates. In spite of the fact that the inoculum consisted of microorganisms grown in Medium A, no period of adjustment to the new Medium AA was observed.

Attempts to culture N. europaea in Medium AA (Table 4) containing 63.4 ug NH_4-N/ml in place of NO_2-N and with

Table 3. Similar rates of nitrification by two *Nitrobacter agilis* cultures in Medium A and Medium AA

Medium	Culture	NO_2-N remaining (ug/ml)[a]		
		Days		
		2	3	4
A	Pramer	97.0	68.0	0
	Fischer	96.5	66.0	0
AA[b]	Pramer	79.5	51.5	0
	Fischer	75.5	33.0	0

[a] 105.0 ug NO_2-N/ml present initially in both media as determined by chemical analysis.

[b] Medium AA sterilized by filtration through a membrane filter.

Table 4. Dissimilar rates of nitrification by Nitrosomonas europaea (Schmidt) in Medium A and Medium AA

Medium	NO_2-N produced[a] (ug/ml)						
	Days						
	3	4	5	11	14	17	21
A	0	8.8	10.5	18.0	28.5	34.5	-
	0	10.2	16.5	33.0	41.5	-	-
AA[b]	0	6.4	7.0	12.5	13.0	13.5	13.0
	0	7.6	10.8	12.5	15.0	15.0	16.0

[a] NH_4-N contained in medium was approximately equal to 63.5 ug/ml.

[b] See text (page 29) for modification of Medium AA.

calcium chloride added (0.136 g/L), were unsuccessful. In Medium A, the end of the exponential growth phase was reached at about 14 days after the conversion of most of the NH_4-N to NO_2-N. In Medium AA, however, only 15 ug/ml NO_2-N were detected after a similar incubation period, and no further oxidation was noted after a subsequent period of 7 days.

Influence of pH

Meyerhof (1916a,b) clearly defined the effect of pH by the bicarbonate ion upon the respiration of Nitrosomonas and Nitrobacter. The rate of respiration reached a maximum at pH 8.5 to 8.7 for both microorganisms and a steep decrease in rate was initiated on the alkaline side at a pH value slightly above these values. Complete inhibition of oxygen uptake occurred between pH 9.5 and 10.5 for Nitrosomonas and Nitrobacter. Less dramatic drops in rate of respiration were observed on the acid side.

Since the effect of pH would thus appear to be very critical, studies were made on the influence of pH on Nitrobacter in Medium AA. The pH of this medium was found to vary considerably depending on various physical factors. The initial pH at time of media preparation was 9.0 but upon refrigeration at 10°C for 18 hours the pH dropped to 8.53. Attempts to lower the initial pH by the addition of KH_2PO_4 were made and replicate cultures prepared (Table 5). The observed rates of nitrification over a 3 day growth period were equal for the three levels of KH_2PO_4 added to the medium.

Table 5. Influence of pH on nitrification by Nitrobacter agilis (Fischer) in Medium AA

Initial pH	pH Post autoclave	Amount KH_2PO_4 added (g/L)	NO_2-N remaining (ug/ml)[a]	
			Days	
			2	3
8.53	10.0	-	59.0	0
8.05	10.0	0.08	62.0	0
7.63	10.0	0.20	59.0	0

[a] 105.0 ug NO_2-N/ml in medium initially.

The added amounts of KH_2PO_4 had no measurable effect on the final pH since after autoclaving the pH rose to 10.0 in each case. It appears that the buffering capacity of this medium was too great to be altered by the relatively small amounts of added KH_2PO_4. It is interesting to note that in spite of the high pH at the time of inoculation no inhibition of Nitrobacter occurred.

Further attempts to regulate the pH nearer to 8.0 by the use of increased amounts of NaH_2PO_4 were also unsuccessful. When the pH was adjusted to 7.6, 8.0 and 8.50 by variations in the amount of NaH_2PO_4, the resulting pH after overnight exposure to air, increased to 8.9--9.0.

It is apparent that the pH rises as the bicarbonate dissociates with the concommitant loss of CO_2, and the phosphate buffer system is ineffective in its presence. A more easily controlable buffer system would be required for the proper evaluation of pH effects.

Removal of Amines from Distilled Water

Because of the method of cleaning commercial steam distillation apparatus contaminating amines are frequently present in the distillate. The high pH of the distilled water encountered in this laboratory indicated that such contamination was probably taking place. Values of pH as high as 8.5 were not unusual. Because of the known sensitivities of the nitrifiers to amines, measures were undertaken either to remove the amines from the distilled water or

eliminate their presence entirely by the use of glass distilled tap water. A growth experiment was then performed to determine if the absence of amines grossly affected the rate of nitrification (Table 6). From the results obtained no such unfavorable effect was evident.

Heterotrophic Contamination

Periodic checks for microbial contaminants were routinely made since it had been our experience that both Nitrosomonas and Nitrobacter fluid cultures readily became contaminated with heterotrophic bacteria, as well as with species of molds commonly encountered in microbiological laboratories. Frequently, contaminants went unnoticed in the stock cultures of the nitrifiers because of insufficient growth in the inorganic medium. In most cases of contamination, however, growth was initiated within 1 to 2 days in all subcultures made in test broths except Czapek Dox.

A particular stock culture of N. agilis was found to contain a variety of bacterial contaminants when plated on membrane filters (refer to Part II of this thesis). Since these contaminants had no apparent toxic effect on the Nitrobacter, studies were undertaken to determine the role of these organisms in the growth and nitrification of the chemolithotroph. Preliminary isolation of the contaminants was accomplished by transfer of a portion of the membrane filter containing representative types of the various colonies to fresh Medium AA containing 106 ug-N/ml. After 14 days, 2 successive subcultures were made in Nutrient Broth followed

Table 6. The influence of amine-free water on nitrification by Nitrobacter

Media	Water source	Culture	NO_2-N remaining (ug/ml)			
			Days 0	3	4	5
A[a]	Reg.[b]	Pramer	99.0	97.0	38.0	0.0
		Fischer	103.0	74.4	26.6	0.0
A[a]	Glass[c]	Pramer	99.0	90.0	43.2	0.1
		Fischer	102.0	73.0	30.0	0.0
AA	Reg	Pramer	104.0	78.4	12.0	0.0
		Fischer	105.0	74.4	4.9	0.0
AA	Glass	Pramer	100.0	74.4	22.6	0.0
		Fischer	104.0	56.4	3.4	0.0

[a] Calcium chloride omitted.

[b] Regular distilled water passed over standard deionizer.

[c] Glass distilled tap water passed over standard deionizer.

by cross streaking on Nutrient Agar. Eight individual colonies were then isolated. Upon later examination only 4, or perhaps 5, different morphological types were recognized, including 2 different streptomyces.

Nutrient Agar plates were then prepared from broth suspensions of the bacterial isolates and individual colonies were again cultivated. When cross streaking of these isolates on agar gave colonies of a single type, subcultures were made on Nutrient Agar slants. These cultures were designated isolates 34-1 to 34-7. At this time all of the isolates differed morphologically. Three bacterial and 2 streptomyces species were readily distinguishable while the remaining 2 isolates were only slightly different from the bacterial isolates. The bacteria were gram negative rods of varying size. With the exception of isolate 34-3, all remained viable after repeated subculture in Nutrient Broth.

Preliminary Characterization. In an attempt to distinguish among the various isolates, their oxygen requirements and growth characteristics in Nutrient Broth were investigated (Table 7). Further characterization of the isolates was attempted by growth in various media (Table 8). Additional tests on the bacterial isolates were performed using Phenol Red Broth Base Redi-Disc (Pennsylvania Biological Laboratories, Inc., Philadelphia, Penn.) with 23 different carbohydrates (Table 9). In all media excluding Nutrient Broth growth was slow and sparse

Table 7. Cultural characteristics of heterotrophs isolated from Nitrobacter agilis (Fischer) and grown in nutrient broth at 30 C for 48 hrs

Isolate No.	Surface growth	Sediment	Turbidity	Media appearance	Relative growth
34-1	None	Pellets	None	Brown	Sparse
34-2	+	+	+	Cloudy	Abundant
34-4	+	+	+	Cloudy	Abundant
34-5	None	Pellets	None	Unchanged	Sparse
34-6	None	+	+	Cloudy	Abundant
34-7	None	+	+	Cloudy	Abundant

Table 8. Characterization of heterotrophs isolated from Nitrobacter agilis (Fischer) in various media[a]

Media	Growth and reaction isolate					
	34-1	34-2	34-4	34-5	34-6	34-7
Nutrient Broth (24 hr)						
30 C	Wk	+	+	-	+	+
37 C	Wk	+	+	-	+	+
0.5% Inulin (Phenol Red Broth)	-	-	-	-	-	-
0.5% Sorbitol (Phenol Red Broth)	-	-	-	-	-	-
Litmus Milk						
2 day	-	-	-	-	-	-
10 day	-	+	+	-	+	+
Simmons Citrate Agar						
Growth	Wk	-	+	Wk	+	+
Reaction	-	-	-	-	+	+
4% Gel. in N.B.						
Growth	Wk	+	+	-	+	+
Liquifaction	-	+	-	-	-	-
4% Gel. in BHI						
Growth	+	+	+	Wk	+	+
Liquifaction	-	+	-	-	-	-

[a] All results based on a 48 hour test period at 30 C unless otherwise indicated.

Table 9. Growth at 14 days of heterotrophic bacteria isolated from Nitrobacter agilis (Fischer) without carbohydrate fermentation at 37 C

Carbohydrate	Isolate		
	34-2	34-6	34-7
Dextrose	+	-	+
Sucrose	+	-	-
Sorbitol	+	-	-
Lactose	+	-	-
Salicin	+	-	-
Mannitol	+	-	-
Xylose	+	+	Slight
Maltose	+	Slight	"
Rhamnose	+	-	"
Dulcitol	+	Slight	"
Arabinose	+	"	"
Galactose	+	"	"
Raffinose	+	"	Slight
Levulose	+	+	+
Inositol	+	Slight	Slight
Trehalose	+	"	"
Adonitol	+	"	"
Mannose	+	"	"
Sorbose	+	-	"
Starch	+	Slight	"
Inulin	+	"	"
Melibiose	+	"	"
Melizitose	+	+	"

for the bacterial isolates compared to the abundant growth obtained in Nutrient Broth using an identical inoculum. The Streptomyces isolates 34-1 and 34-5 even grew poorly in Nutrient Broth. For this reason the carbohydrate fermentation study (Table 9) was not utilized for the streptomyces isolates.

From the foregoing tests certain basic similarities among the heterotrophs were recognized. Maximum growth was obtained in Nutrient Broth at 30 or 37 C within 48 hrs and growth in Phenol Red Broth containing either 0.5% inulin or 0.5% sorbitol was sparse even after 10 days. As one would suspect the streptomyces isolates grew poorly in all of the media tested, and the mycelia formed pellets in the liquid media. A positive response in Litmus Milk was obtained with the bacteria and a negative response was obtained with the streptomyces at 10 days.

Distinction between 2 of the bacterial isolates was made on the basis of the following:

Isolate 34-2 Gelatin liquifaction at 48 hours, negative test on Simmons Citrate Agar, surface growth on Nutrient Broth, and growth in 23 carbohydrate media;

Isolate 34-6 No gelatin liquifaction, positive test on Simmons Citrate Agar, no surface growth in Nutrient Broth, and varied growth response in carbohydrate media.

Isolates 34-4 and 34-7 were similar to 34-2 and 34-6, respectively. Isolates 34-1 and 34-5 were readily distinguishable on the basis of morphology.

It was clearly evident that a minimum of 4 different heterotrophs had been isolated from the contaminated Nitrobacter stock culture. Isolate 34-7 was suspected of containing a mixture of bacteria.

These cultures were subsequently rechecked microscopically for purity using the Gram stain. Isolates 34-4 and 34-7 were indeed found to be mixed with the 34-2 and 34-6 types. Pour plate colony isolations were again made in an attempt to confirm the purity of the 34-2 and 34-6 isolates and to separate the bacteria in the mixed cultures. Isolates 34-2 and 34-6 had remained pure while 34-4 was found to be a mixture of these two. Isolate 34-7 contained the 34-6 type in small amounts as well as a type differing from the pure cultures. The 3 different bacterial isolates are described in Table 10. Gram stains of these colonies confirmed the purity of the 34-2 and 34-6 cultures and the impurity of the 34-7 isolate. The 34-2 culture consisted of very short gram negative rods, less than 0.7 u in length. The 34-6 gram negative rods were considerably larger. Isolate 34-7 contained a mixture of gram negative rods and the predominant type was larger than those of the 34-6 isolate. It was impossible to determine if this new colony type still contained a mixture of bacteria or if the rods were pleomorphic.

Table 10. Colonial characteristics on nutrient agar of heterotrophic bacteria isolated from Nitrobacter agilis (Fischer)

Colonial characteristic	Isolate 34-2	Isolate 34-6	Isolate 34-7
Surface colonies			
Size	10-16 mm	2-4 mm	5-7 mm
Form	Circular	Circular	Circular
Surface	Smooth (Radiately ridged)	Smooth	Rough
Edge	Entire	Entire	Undulate
Elevation	Flat	Convex	Flat
Optical characters	Opaque	Transparent	Transparent
Lustre	Dull	Glistening	Glistening
Consistency	Membranous	Membranous	Butyrous
Color	Milky white (reverse-large yell. center)	Tan	Tan
Deep colonies	+	+	-
Size	Extend to surface	1 mm	
Form		Ellipsoidal	
Pigmentation (21 days)	-	Pink	Golden brown

Non-utilization of NH_4-N and NO_2-N. Since the heterotrophic contaminants had no apparent toxic effect on the cultivation of N. agilis various studies were undertaken to determine if they played a role in the nitrification process.

The heterotrophic isolates were seeded singly or combined in the Nitrobacter medium (Medium AA containing 106 ug NO_2-N/ml) in the absence of Nitrobacter. After 13 days, no loss in NO_2-N content was detected (Table 11). This fact clearly indicated that the inorganic form of nitrogen remained unchanged.

No effect on the rate of nitrification by a pure culture of N. agilis was demonstrated when the contaminants were added individually or all together (Table 12).

The continuous presence of one or more of these contaminants in the stock culture of Nitrobacter was followed over a 7 month period. No change in the culture behavior or rate of nitrification was observed during this entire period.

The effect on the oxidation of NH_4+ by N. europaea was also investigated under similar experimental conditions. The heterotrophic isolates were not only unable to convert the NH_4^+ to NO_2^- during a 14 day incubation period (Table 13) but their presence had no overall effect on the oxidation by Nitrosomonas (Table 14). The presence of two of the isolates, 34-1 and 34-2, may have slightly delayed the oxidation process. However, other factors could have been responsible for the slight difference in the results.

Table 11. Failure of heterotrophic contaminants to oxidize NO_2-N in Medium AA containing 106 ug NO_2-N/ml (after 13 days)

Heterotrophic isolate	Glucose added (%)	NO_2-N remaining (ug/ml)
34-1	None	107
	1.0	102
34-2	None	106
	1.0	105
34-5	None	109
	1.0	100
34-6	None	104
	1.0	100
34-7	None	114
	1.0	104
All	None	110
	1.0	106

Table 12. Failure of heterotrophic contaminants to effect NO_2-N oxidation by N. agilis (Fischer) in Medium AA containing 106 ug NO_2-N/ml

Heterotrophic isolate	NO_2-N (ug/ml)	
	Days	
	4	5
None	34.8	6.1
34-1	3.5	0.0
34-2	13.5	0.0
34-5	34.5	1.7
34-6	4.0	0.0
34-7	34.0	3.2
All[a]	27.0	0.7
Nutrient broth[b]	31.5	2.8

[a] All above heterotrophic isolates added.

[b] Equivalent amount of Nutrient Broth added as in heterotroph inoculations.

Table 13. Failure of heterotrophic contaminants to oxidize NH_4-N in Medium A containing 66 ug NH_4-N/ml (after 14 days).

Heterotroph isolate	(%) Glucose	NO_2-N produced (ug/ml)
34-1	None	0.25
	1.0	0.25
34-2	None	0.0
	1.0	1.0
34-5	None	0.0
	1.0	0.75
34-6	None	0.0
	1.0	0.0
34-7	None	0.0
	1.0	0.0
All	None	0.0
	1.0	0.0

Table 14. Influence of heterotrophic contaminants on NH_4-N oxidation by Nitrosomonas europaea (Schmidt) in Medium A containing 66 ug NH_4-N/ml

Heterotrophic isolate	NO_2-N produced (ug/ml)			
	Days			
	6	7	9	14
None	34.5	39.4	41.0	42.0
34-1	9.6	9.8	11.5	41.0
34-2	6.1	5.5	7.0	37.3
34-5	38.0	45.8	46.0	46.5
34-6	32.5	37.5	44.0	45.5

Test tube experiments in Nutrient Broth containing nitrate were negative in all cases for nitrate reductase activity. Therefore, none of the isolates were capable of reducing nitrate to nitrite, when tested in a medium giving optimal growth.

DISCUSSION

For lack of a better method of estimation of cell growth, the rate of oxidation of the energy substrate was regularly determined colorimetrically using the a-naphthylamine and sulfanilic acid method. Engle and Alexander (1958a) presented evidence that the rates of nitrification by N. europaea paralleled cell synthesis as estimated by viable cell counts by the dilution tube method. It is assumed that a similar relationship between viable cell count and rate of oxidation also exists for Nitrobacter. Consequently, the rate of oxidation of the energy substrate was considered synonymous to cell growth, and all growth studies were made using the criterion of oxidation rate.

Short term stationary cultures prepared in media containing limiting amounts of energy substrate were used throughout the course of this study. This type of culture system permitted the handling of large numbers of replicate cultures with a minimum amount of manipulation. This cell culture method, however, did impose serious restrictions in the use of cell enumeration methods which could have been employed had larger cell populations been produced. These methods include turbidimetric and direct microscopic cell counting techniques. Since no such method of cell enumeration was applicable, inoculum size had to be quantitated on a % basis. This was done for both nitrifiers.

The results indicated that a 3.13% inoculum taken from a culture which had recently completed the oxidation of 100 ug (NH_4-N or NO_2-N)/ml was the minimal inoculum for both nitrifiers for the initiation of growth within a reasonably short lag period.

Recent growth studies cited in the literature have all been for large scale continuous culture systems in which amounts far greater than 100 ug N/ml were oxidized. The growth curves contained in Fig. 1 and 2, however, are typical of the growth responses obtained by other investigators, except for the incomplete utilization of NH_4-N by Nitrosomonas.

The % utilization of energy substrate by N. europaea differed markedly from N. agilis. Unlike Nitrobacter, Nitrosomonas cultures regularly terminated the exponential growth phase prior to 100% conversion of NH_4-N to NO_2-N. In fact, cessation of the exponential phase occurred when 12.6 to 31.7% of the NH_4-N still remained in the unoxidized form. Because of this wide range among various experiments it would appear that multiple factors were involved.

The experimental data in Table 2 precluded the inaccessibility of NH_4^+ for oxidation to nitrite. Only 3.3 and 3.4 ug N, for the 2 inocula levels respectively, could not be accounted for as NH_4-N and NO_2-N. These amounts of nitrogen may have been present as an intermediate oxidative state and not detected by NH_4-N and NO_2-N assay. In the case of the cultures in the late exponential phase of growth (6.25% inoculum), 24.7% of the initial NH_4-N added still remained

unoxidized.

Engle and Alexander (1958a) reported a slight diminution in the rate of nitrite formation at the end of the exponential growth phase in a 25 L pyrex fermentor culture with a final nitrite concentration of 1162 ug NO_2-N/ml. A perfunctory re-analysis of their data showed that the logarithmic phase for nitrite production was maintained for approximately 70 hours during which time only 700 ug NO_2-N/ml were detected. In view of the results obtained in this laboratory concerning the incomplete oxidation of NH_4-N, it is possible that their results were, at least partly, a reflection of a similar phenomenon.

Comparisons were made on the growth of 2 cultures of N. agilis in Medium A containing a large amount of a fine precipitate, and Medium AA, containing no insoluble components. Rates of nitrification were identical for both cultures in the 2 media. A similar comparison was made for N. europaea (Schmidt) in the same basal media containing NH_4-N in place of NO_2-N. Medium AA did not support the growth of this nitrifier even with $CaCl_2$ added.

The growth of N. agilis was not affected by initial pH as high as 10.0. This contrasts markedly with the findings of Meyerhof (1916a,b) who demonstrated a steep decrease in rate of respiration for Nitrobacter on the alkaline side of pH 8.7. It is apparent that the effect described by Meyerhof is not obtained in fluid cultures of N. agilis during active growth.

Deionized distilled water used in the preparation of media was routinely passed over a cation removal resin for the removal of amines. This was done as a precautionary measure since continuous monitoring of the distilled water supply was not feasible, and it was decided to eliminate the possibility of the presence of inhibitory levels of amines.

The use of impure strains of the chemolithotrophs, although widely practiced in the past, is extremely unwise, particularly for biochemical investigations. Nevertheless, only a few recent studies have specifically cited the use of pure culture techniques.

The use of an unsterilized 48 L carboy containing unsterilized deionized tap water also was reported (Anderson, 1964). Another investigator reported the use of a Nitrobacter culture containing known quantities of heterotrophic contaminants (Fischer, 1965).

Since methods for obtaining pure cultures of the nitrifiers have been available for some time, there is no good reason for the use of impure strains. An attempt was made in this laboratory to define the role of heterotrophic contaminants which had no apparent toxic effect on Nitrobacter.

The heterotrophs found as contaminants in our stock culture of N. agilis and previously described in detail, were unique in several respects. They produced no toxic effect on either the Nitrobacter or on one another. They were maintained at a low optical density in Nitrobacter cultures

for a period of 7 months, and they were incapable of utilizing inorganic nitrogen in the form of NH_4-N, NO_2-N, or NO_3-N. Their nitrogen requirements must have been satisfied indirectly. The presence of these organisms also had no effect on the growth or nitrification of the Nitrobacter.

One can readily appreciate from these interactions with the nitrifier, that any biochemical investigations, aside from the nitrification process itself, performed on this mixed culture could lead to possible erroneous interpretation.

So far as the identity of the bacterial heterotrophs is concerned, insufficient differential tests were performed for accurate classification, and further testing is beyond the scope of this thesis. It is most probably that the bacterial contaminants represent different species of Pseudomonas.

PART II. ATTEMPT TO DEVELOP AN IMPROVED CELL ENUMERATION METHOD USING MEMBRANE FILTERS.

Introduction

It can not be assumed that cultures oxidizing an equal amount of an inorganic nitrogen substrate have the same activity since it has been shown by several investigators that growth and oxidative capacity are not simultaneously inhibited. Meyerhof (1916a) and Gould and Lees (1958) demonstrated nitrate inhibition of growth first and then oxidation of Nitrobacter. Lees (1952) found that streptomycin at concentrations inhibitory to growth had no direct effect on ammonia oxidation.

Such dual type of inhibition could be extremely complicated. A direct method of growth evaluation in addition to the quantitative estimation of products of oxidation would be desirable in the study of the effects of various inhibitory or stimulatory substances.

The methods available for the quantitative detection of cellular growth of nitrifiers in liquid media are seriously restricted due to the nature of these microorganisms. Colony growth on solid media is limited to silica gels (Allen, 1957; Funk and Krulwich, 1964; Pramer, 1958; Temple, 1949; Winogradsky, 1891a), or an inorganic medium supplemented with washed agar such as Noble's Agar (Difco) (Gould and Lees, 1960; Kingma-Boltjes, 1935; Meiklejohn, 1950). The silica gels are unsuitable as a rapid means of obtaining plate

counts. The use of Noble Agar is at present the only reliable means for colony growth but does not allow macroscopic identification of these organisms. The colonies formed are so minute, less than 0.1 mm, that they must be viewed under low power magnification of 20 to 30 X. Having to employ a magnification device seriously hampers the use of the plate dilution technique, by requiring standardization of the microscopic field and the plate area observed.

The use of a membrane filter was therefore investigated. Membrane filters offered the advantage over agar plates in that they could be obtained with grid markings facilitating the counting of colonies with the aid of an ordinary dissecting microscope. The ease with which the membrane filters could be stained, and indicators applied also offered additional advantages over colony identification on an agar surface. Furthermore, the filtration of low bacterial populations permits the concentration of organisms on the membrane. And lastly, the stained membranes could be easily preserved and stored for later evaluation. The methods developed for the use of membrane filters are described and evaluated in this section.

Materials and Methods

Membrane filters. MF-Millipore filters of HA type (0.45 u pore size) and 47 mm in diameter were obtained presterilized and grid-marked. Each grid-square (3.1 mm^2) equals 1/100th of the 9.6 cm^2 effective filtering area of a

47 mm filter used in the Millipore filter holder.

Base media. Fluid media identical to that used in the maintenance of stock laboratory cultures (Part I) served as the source of nutrients. The filters were supported by either an absorbent pad saturated with 1.8 to 2.0 ml of fluid media or Noble Agar medium consisting of 1.5 per cent agar in the culture media just described.

Plating procedure. Serial dilutions were prepared in growth media from fluid cultures of the nitrifiers in the exponential phase of growth. Colony growth directly on the agar surface was obtained by spreading 0.1 to 0.2 ml of the culture dilution over the agar surface. In the membrane filter technique 5 to 10 ml of the diluted cell suspension was filtered over the membrane filter previously washed by the addition of 10 ml of fresh culture medium. The sides of the filtration funnel were then washed with 10 ml of fresh medium and the membrane filter transferred to either an agar plate or a petri dish containing an absorbent pad saturated with fluid medium. After incubation at 28 to 30 C for 8--12 days the membrane filter was then observed microscopically for colony formation, stained and colony counts made.

Preparation of methylene blue stain and indicators. Methylene blue chloride (National aniline Co.) solution was prepared by dissolving 1.5 gm of dye in 150 ml of ethyl alcohol and mixed with 500 ml KOH solution (0.01% Wt/Vol). The membrane was stained for 3 minutes by placing the filter

on an absorbent pad saturated with 1.8 ml of the staining solution.

TTC (2, 3, 5-triphenyl-2H-tetrazolium chloride, Eastman Organic Chemical) was prepared as a 2% solution in culture medium.

Resazurin solutions were prepared by dissolving 58 mg in 100 ml of M/15 phosphate buffer solution, pH 7.0 at 100°C.

Results

Development of Technique

A series of plating experiments was devised to determine the efficacy of using grid-marked membrane filters for the enumeration of cells in suspension culture. In the first experiment, a nine day old suspension culture of N. agilis (Fischer) was used. Suitable portions of dilutions ranging from 1/10 to 1/1280 were applied directly to the surface of agar plates and to the filters which were incubated over an agar or absorbent pad. After 12 days the plates contained several types of colonies and attempts were made to identify those formed by the Nitrobacter. Plates seeded with a 1/1280 dilution were too heavily populated on the agar surface for the estimation of colony number. The colony types present were morphologically of the same type obtained on the filter. Examination revealed at least 4 colony types, two of submacroscopic size and two readily visible without the aid of a microscope.

Staining of membrane filters with methylene blue greatly enhanced visualization of the colonies both macroscopically and microscopically. All 4 colony types clearly stood out as darkly stained areas on a lightly stained background.

Within minutes after the application of the TTC solution to membrane filters only a few colonies appeared bright red while most appeared unchanged. Further observations over a 24 hour period showed no additional changes except for a decrease in color intensity in the colonies originally reducing the tetrazolium salt.

The bacteria did not produce a detectable change in the resazurin.

Quantitation Procedure

After staining with methylene blue, colony counts were obtained by counting the number of colonies within one or more 3.1 mm squares. Approximatedly 60 colonies were counted per filter. The number of cells in the undiluted sample was calculated from the average number of cells observed at each dilution by applying the formula:

$$\text{Cells/ml} = \frac{(100)\ (\text{organisms counted})\ (\text{reciprocal of dilution})}{(\text{grid-squares exam.})\ (\text{ml of sample})}$$

Using this method of calculation the data from experiment I was evaluated on the basis of the numbers of macroscopic and microscopic colonies present in the original culture. The results are included in Table 15.

Table 15. Quantitation of various types of micro-organisms in a fluid culture of Nitrobacter agilis (Fischer) by membrane filter technique

Type colony	Nutrient base	Dilutions evaluated	Average[a] no. of cells/ml
Microscopic	Agar	1/320,1/640 1/1280	5.8×10^5
	Absorbent pad		6.0×10^5
Macroscopic	Agar	1/20, 1/40, 1/80	1.35×10^3
	Absorbent pad		1.04×10^3

[a] The average of cell counts on 3 dilutions run in triplicate.

A maximum of 60 colonies/filter grid-square (3.1 mm) could be accuratedly counted when the colony size was that of the nitrifiers, around 0.1 mm. As many as 6000 such colonies could be effectively evaluated on a single membrane filter using this technique.

The same plating procedure was repeated using a second subculture of the same N. agilis stock culture. Identical results were obtained as in the first experiment, that is the presence of four colony types was again noted. It appeared that 3 or even 4 different contaminants were present. The possibility that both of the microscopic type colonies were other than N. agilis was considered.

To determine the presence of viable N. agilis cells on the filter membrane, filter strips were taken after colony development, by aseptically cutting the filter, and were transferred to fresh nutrient media in test tubes. This type of subculture was prepared from areas of the membrane filter in which the macroscopic colonies were absent. Within a 14 day period nitrification occurred in 21 out of 21 such subcultures. The ability of N. agilis to grow on membrane filters was thus established.

The use of the membrane filter technique was then applied to contaminant-free N. agilis and N. europaea fluid cultures. When these cultures were plated on a suitable medium containing Noble Agar colonies developed within 8 days. No further increase in colony size was obtained after 8 days. Parallel studies were performed on the membrane filters but

the colony size was much smaller than those obtained growing directly on the agar surface. In fact, the size of the colonies was so close to that of media debris that distinction was not possible.

Discussion

A reliable membrane filter technique would greatly facilitate the enumeration of small numbers of viable cells in fluid cultures of Nitrobacter and Nitrosomonas. Evidence was obtained for the growth of N. agilis either on the filter surface directly or in the thin film of medium on the filter surface.

The reason for not obtaining colonies like those which appear on an inorganic medium solidified with Noble Agar is not understood. The possibility exists that Noble Agar contains certain substances conducive to growth enhancement. Several factors which increase colony size on washed agar are known. Trace amounts of peptone (Fred and Davenport, 1921) caused enlarged colonies of both nitrifiers, while biotin was found to accelerate colony formation by Nitrosomonas (Gunderson, 1955a). These as well as other compounds reported to be stimulatory to growth or nitrification should be investigated as potential stimulants of colony growth.

The inhibitory effect of membrane filters on colony formation should not be overlooked. Membrane filters produced by several manufactures are reported to contain 2 to 3% of their dry weight as detergent (Cahn, 1967). The same investigator found that the use of tissue culture medium filtered

through unwashed Millipore filters, containing Triton X-100 or a similar detergent, considerably lowered the plating efficiency of clonal cultures of cartilage cells *in vitro*.

The use of membrane filters in many areas of sterility testing is widely accepted, but the more specific application for fastidious microorganisms has been reported infrequently. The continuation of efforts leading to the application of this technique to the nitrifiers is warranted.

PART III. EFFECT OF PESTICIDES ON NITRIFICATION.

Introduction

The persistence of pesticides in soils for many months or even years may affect the role of the nitrifying bacteria in the maintenance of soil fertility.

The results of many investigators have indicated that many herbicides and insecticides when applied at the recommended field rates generally have no harmful effects upon the microscopic population or upon its biochemical activities (Alexander, 1964).

At the present time, however, little information is available on the effect of various pesticides on nitrification in pure cultures of Nitrosomonas and Nitrobacter. Caseley and Luckwell (1965) have shown that monuron, a substituted-urea herbicide, inhibits Nitrobacter at concentrations similar to those applied to soils. Campbell and Aleem (1965a,b) have shown that another substance, N-Serve, inhibits Nitrosomonas at extremely low concentrations. Studies on the effects of other pesticides on nitrification by both of these chemolithotrophs were undertaken in this laboratory.

Materials and Methods

Replicate cultures for pesticide assays were prepared as previously described under Part I of this thesis. Each culture flask contained a final volume of 50 ml after the appropriate additions of both test material and inoculum.

Medium A containing 64 ug NH_4-N/ml was used for the N. europaea (Schmidt) experiments while Medium AA containing 100 ug NO_2-N/ml was used for the N. agilis (Fischer) experiments.

Solutions of the pesticides were prepared in the appropriate solvent, either acetone or ethyl alcohol, and sterilized by passage through a sterile UF sintered glass filter. These preparations were then added aseptically in the appropriate volume to their respective assay flasks at the time of culture inoculation. Cultures in all experiments were incubated at 28-30 C for a period up to 14 days.

Results

The Effect of Pesticides on Nitrobacter agilis

The effect of pesticides at final medium concentrations ranging from 10^{-1} to 10^3 ug/ml on the growth of this organism was followed by determining the decrease in nitrite in the culture medium. Nitrite determinations were made as described previously.

N. agilis was sensitive to all 3 chlorinated hydrocarbons at concentrations as low as 1 ug/ml (Table 16). Lindane appears to be the least toxic and only produced a delay in nitrate production even at the highest level tested, 1000 ug/ml. Both rhothane, the most toxic, and aldrin caused complete inhibition over a 14 day period at concentrations of 10 ug/ml. Delayed nitrate production over the same period resulted from exposure to 0.1 and 1.0 ug/ml.

Table 16. Inhibition of nitrification by Nitrobacter agilis (Fischer) in Medium AA containing chlorinated hydrocarbons

Pesticide		NO_2-N remaining (ug/ml)[a]			
Name	Final conc. (ug/ml)	Days 3	4	6	14
Aldrin	10^3	88.8	90.0	83.0	98.5
	10^2	86.8	85.5	83.3	100.0
	10^1	83.8	79.0	87.5	80.0
	10^0	73.5	64.3	34.5	0.0
	10^{-1}	1.5	0.0	-	-
Lindane	10^3	71.3	62.0	69.0	44.5
	10^2	69.3	62.0	56.8	0.0
	10^1	69.9	69.0	65.5	0.0
	10^0	44.2	19.9	0.3	-
	10^{-1}	18.8	0.4	-	-
Rhothane	10^3	87.5	90.0	88.8	100.0
	10^2	75.0	81.3	90.0	103.0
	10^1	85.0	83.0	87.5	96.5
	10^0	80.3	35.0	81.3	88.0
	10^{-1}	42.3	31.5	0.0	-
Control	-	18.0	0.0	-	-

[a] Medium contained 100 ug NO_2-N/ml.

Of the two organic phosphate compounds (Table 17) malathion was the less toxic, in fact the least toxic of all the compounds tested, while parathion was as toxic as the chlorinated hydrocarbon, aldrin. Baygon, a methyl carbamate, had lower toxicity (Table 17), and was similar to lindane as it only caused delayed nitrification.

The Effect of Pesticides on Nitrosomonas europaea

Three of the same pesticides tested against N. agilis were also tested against N. europaea using the identical pesticide stock solutions. Each substance was representative of one of the three types of chemical compounds included in the previous study. With these compounds, lindane, malathion, and baygon, the sensitivity of this bacterium (Table 18) was greater than that of N. agilis. Based on complete inhibition dosage levels for a 14 day period, N. europaea was at least 100 times more sensitive to each pesticide than N. agilis. At all the concentrations tested, this bacterium gave an all or none response, that is either no inhibition, or complete inhibition was obtained. Delayed nitrification was not evident under these test conditions.

Discussion

It is evident from these studies and those reported by others that certain pesticides have a selective action on the nitrifiers (Campbell and Aleem, 1965a,b; Caseley and Luckwell, 1965; Goring, 1962a,b). In the case of lindane, malathion, and baygon they are more toxic to Nitrosomonas then to Nitrobacter.

Table 17. Inhibition of nitrification by Nitrobacter agilis (Fischer) in Medium AA containing organic phosphates and a-methylcarbamate.

Pesticide		NO_2-N remaining (ug/ml)[a]				
		Days				
Name	Final conc. (ug/ml)	3	4	6	9	14
Malathion	10^3	54.5	39.5	13.5	9.3	0.0
	10^2	17.7	0.0	-	-	-
	10^1	37.3	0.0	-	-	-
	10^0	18.0	0.0	-	-	-
	10^{-1}	23.9	0.0	-	-	-
Parathion	10^3	81.3	82.0	80.3	39.5	92.0
	10^2	79.0	83.3	85.3	82.5	95.0
	10^1	76.3	75.5	74.5	79.0	75.0
	10^0	0.0	-	-	-	-
	10^{-1}	1.0	-	-	-	-
Baygon	10^3	82.5	82.0	83.3	55.3	44.5
	10^2	74.5	70.5	55.3	28.5	0.0
	10^1	52.3	31.2	0.0	-	-
	10^0	28.5	2.0	-	-	-
	10^{-1}	14.3	0.0	-	-	-
Control	-	14.7	0.0	-	-	-

[a]Medium contained 100 ug NO_2-N/ml.

Table 18. Inhibition of nitrification by Nitrosomonas europaea (Schmidt) in Medium A containing various pesticides.

Pesticide		NO_2-N produced (ug/ml)[a]			
Name	Final conc. (ug/ml)	Days			
		7	8	11	14
Lindane	10^3	1.8	1.5	4.0	1.8
	10^1	2.5	1.8	3.8	1.0
	10^0	38.3	40.8	43.0	-
	10^{-1}	41.3	43.8	49.3	-
Malathion	10^2	4.0	3.8	5.3	4.0
	10^1	2.5	3.0	3.8	1.0
	10^0	28.8	31.3	34.0	30.3
	10^{-1}	40.3	44.3	48.3	-
Baygon	10^3	2.0	2.5	4.0	2.3
	10^2	4.3	6.8	5.3	7.0
	10^1	1.8	2.8	4.0	2.5
	10^0	35.0	36.5	40.8	40.5
	10^{-1}	38.5	42.5	41.0	40.0
Control	-	41.0	45.5	43.5	46.5

[a] Medium contained 66 ug NH_4-N/ml.

The level of toxicity causing complete inhibition of Nitrosomonas was 10 ug/ml for lindane, malathion and baygon. Nitrobacter was similarly inhibited with equal amounts of parathion, aldrin, and rhothane. The application of these pesticides at rates of 1-10 pounds/acre would result in similar concentrations in the soil which could cause inhibition or even death to the nitrifiers.

Casely and Luckwill (1965) reported that monuron applied at rates of 1 and 3 lbs/acre delayed the start of nitrification by 6 and 14 days respectively, while application at 10 lbs/acre completely inhibited nitrification. Their pure culture studies showed monouron to be an inhibitor of N. europaea at 100 ug/ml and of N. agilis at 25 ug/ml. It is conceivable therefore that those compounds showing inhibitory properties at concentrations of 25 ug/ml or less could have a detrimental effect on the rates of nitrification in the soil. All six of the compounds included in this study were within this concentration range for the inhibition of one or the other nitrifier. On the basis of results obtained from pure culture studies, one could predict those compounds likely to cause inhibition of nitrification under field conditions in the absence of such factors as the absorption on soil colloids and degradation of the inhibitor. It is also conceivable that degradation products might be even more toxic than the original substance, as in the case of aldrin which degrades to dieldrin. It has also been shown (Caseley and Luckwill, 1965) that the formulated products in

pure culture experiments are far more toxic to Nitrosomonas and Nitrobacter than the pure unformulated herbicides. These observations were attributed to the wetting agents ability to also inhibit nitrification.

SUMMARY

This dissertation has been primarily concerned with the development of methods for the study of the effects of various chemical factors, including pesticides, on the nutrition and physiology of the nitrifiers: Nitrobacter agilis, and Nitrosomonas europaea.

Readily reproducible growth conditions using 250 ml stationary flask cultures were obtained in an inorganic medium (Medium A) by inoculation with a 3.13% (by volume) inoculum. Inoculations were made from liquid cultures which had completed the exponential phase of growth in a medium containing 100 ug of energy substrate per ml.

During the exponential growth phase, N. agilis cultures effected 100% oxidation of NO_2-N. For N. europaea, however, the exponential growth phase was terminated when 70 to 80% of the theoretical amount of NH_4-N appeared in the form of NO_2-N. The incomplete oxidation of NH_4-N by Nitrosomonas cultures could not be attributed to either the spontaneous loss of NH_3, or a binding of NH_4 ions. Nor was it attributable to intermediate oxidation states as all of the NH_4-N supplied to the medium initially could be accounted for as either NH_4-N or NO_2-N.

Both the Pramer and Fischer cultures of Nitrobacter agilis grew equally well in Medium AA, a modification of the medium described by Aleem and Alexander (1960), which contained no insoluble components. This same basal medium

but with NH_4-N replacing NO_2-N, did not support the growth of N. europaea. Quantitative, but not qualitative, differences in the substances present in the medium probably were the cause of the differences in growth response of the two nitrifiers. N. europaea may have been less tolerant to a high hydrogen ion concentration (pH 10).

The removal of amines from distilled water through the use of a cation exchange resin had no gross effect on the rate of nitrification by Nitrobacter.

All stock cultures of the nitrifiers were repeatedly checked for the presence of contaminants. From one of the stock cultures of N. agilis (Fischer), 2 streptomyces and 3 bacterial strains were obtained. All except 1 bacterial strain was isolated in pure culture. The bacterial isolates were tentatively identified as various species of Pseudomonas.

An attempt was made to quantitate fluid culture populations using a Millipore membrane filter in a modified colonial plate count method. Growth of N. agilis was observed in or on the filter membrane but there was no easily recognizable colonial development.

An evaluation was made on the effect of various pesticides on nitrification by both N. agilis (Fischer) and N. europaea (Schmidt). N. agilis was sensitive to all 3 chlorinated hydrocarbons, aldrin, lindane, and rhothane at final medium concentrations of 1 ug/ml. Complete inhibition after 14 days was obtained with both rhothane and aldrin at concentrations of 10 ug/ml. Lindane was considerably less

toxic, causing only a delay in nitrate production over a concentration range of 1 to 1000 ug/ml. Malathion and parathion, two organic phosphate compounds, differed widely in their toxicity for Nitrobacter. Malathion was the least toxic of all the compounds tested causing only delayed nitrification at 1000 ug/ml. Parathion, as toxic as aldrin, gave complete inhibition at 10 ug/ml. Baygon, a methyl carbamate, delayed nitrification by Nitrobacter at concentrations of 10 ug/ml or greater.

Lindane, malathion, and baygon, were also tested against N. europaea. All three of these compounds were at least 100 times more toxic for the Nitrosomonas cultures than for Nitrobacter. Delayed nitrification was not evident in Nitrosomonas cultures as either no inhibition, or complete inhibition was obtained.

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APPENDIX

Formulas for basal media[a]

Components	Medium A[b]	Medium AA[c]
	g/liter	
$CaCl_2$	0.136	
NaCl		0.1
$MgSO_4 \cdot 7H_2O$	0.20	0.20
$FeSO_4 \cdot 7H_2O$	3.5×10^{-5}	3.5×10^{-5}
KH_2PO_4	0.7	
Na_2HPO_4	13.5	
K_2HPO_4		0.175
$NaHCO_3$	0.5	
$KHCO_3$		1.5
$NaMoO_4 \cdot 2H_2O$		2.5×10^{-5}
Deionized distilled water	q.s. liter	q.s. liter

[a] Does not contain nitrogen. For the addition of nitrogen to the basal medium consult the text (page 20).

[b] Modification of the medium described by Pramer and Schmidt (1965) which contained 0.1 g $MgSO_4 \cdot H_2O$, 0.014 g $FeCL_3 \cdot 6H_2O$ (no $FeSO_4$), and distilled H_2O.

[c] Modification of the medium described by Aleem and Alexander (1960) which contained 0.175 g $MgSO_4 \cdot 7H_2O$, and no $NaMoO_4$.