NITRIFICATION--A POTENTIAL FOR HETEROTROPHIC NITRIFIERS FROM AN ACTIVATED SLUDGE SYSTEM

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CHAPTER I

INTRODUCTION

The process of nitrification occurs in a spectrum of richly diverse terrestrial, aquatic and sedimentary environment. It is of paramount importance to primary productivity, waste disposal, nutrient cycling and the quality of the environment.

The water quality problems caused by different forms of inorganic nitrogen are well documented. The major problem is eutrophication, excessive plant growth and/or algae "blooms" resulting from over-fertilization of rivers, lakes, and estuaries. Results of eutrophication include deterioration in the water quality, odor problems from decomposing algae, and a lower dissolved oxygen level which can adversely affect fish life.

The principal toxicity problem is from ammonia in the molecular form (NH_3). A slight increase in pH may cause a great increase in toxicity as the ammonium ion (NH_4^+) is transformed to ammonia (NH_3). Reported levels at which acute toxicity is detectable have ranged from 0.01 mg/ℓ to over 2.0 mg/ℓ (1) of molecular ammonia-nitrogen. Ammonium can be biologically oxidized to nitrite and then to nitrate in receiving waters and thereby add to the oxygen demand imparted by carbanaceous materials. The nitrate form, however, is the main concern associated with public health hazard. It is limited principally to groundwater where high concentration can occur. Nitrate in drinking water was first associated in 1945 with methemoglobinemia, a sometimes fatal blood disorder which affects infants

less than three months old.

Ammonia also increases the chlorine demand required for disinfection of wastewater effluents. In the past several years, the number of processes utilized in wastewater treatment has increased rapidly. Many of these processes have been developed with the specific purpose of transforming nitrogen compounds or removing nitrogen from the wastewater stream. Ammonia nitrogen can be removed from wastewater physically, chemically, biologically or by combination of any of the above methods. However, from a technical and economic viewpoint, biological nitrification is the most feasible method of removing ammonia from wastewater. Although all biological treatment methods such as activated sludge, trickling filter and rotating biological contactors can support nitrification, activated sludge has received more attention. Probably the most common method of removing nitrogen from wastewater is biological nitrification and denitrification process. The process basically consists of oxidizing all the ammonia to nitrates (nitrification) and then reducing the nitrates to nitrogen gas (denitrification) which is released to the atmosphere.

So far as is known,the microorganisms that have been implicated in nitrification are all chemoautotrophic bacteria. Although the family of Nitrobacteriaceae consists of at least five genera of ammonia-oxidizing bacteria and three genera of nitrite oxidizing bacteria. Most cultural and biochemical studies have been performed with Nitrosomonas and Nitrobacter; just how legitimately the data may be transposed to the ecological situation is highly questionable. Are the forms the same in soil, waters and sewage? Are those responsible for nitrification the classical autotrophs, are some as yet undescribed autotrophs, or are

some not autotrophic at all?

Reviews of the early literature, Waksman (169), Barrit (8); Stephenson (170) make it clear that many isolates were claimed as putative agents of nitrification and that a considerable amount of controversy was engendered in relation to those claims. To a limited degree some measure of controversy still exists. This is based on inability to explain the occurrence of nitrification in certain situations where the presence of the classical autotrophs would seem unlikely-based on their known physiology--or where autotrophic nitrifiers are either not detectable or detectable only at very low numbers (168).

Nitrosomonas europeae and Nitrobacter winogradskyi are the types most commonly isolated from soils, sewage, and the freshwater environment, and these organisms are accordingly often considered to be the most important nitrifiers, but their appearance may simply represent successful competition with other nitrifiers in isolation procedures. Furthermore, viable counts of nitrifiers in soils and sediments range from 10^3 - 10^5 organisms g^{-1} but much larger numbers (10^7 - 10^8 organisms g^{-1}) are generally described in high ammonium environments such as activated sludge (2). Both groups are roughly equally represented. It may, however, be true that current estimates of the numbers of some species are severe underestimates and are largely a reflection of cultural conditions used in the enumeration procedures. Independent estimates of nitrifier biomass may be made by relating data obtained from pure culture study to those observed from natural environments. In pure culture studies it has been estimated that a population of 2×10^8 Nitrosomonas and 4×10^7 Nitrobacter are needed to convert 1 mg of ammonium-nitrogen to nitrate-nitrogen (168). Tate (3), based on these estimates, found insufficient

populations of Nitrosomonas and Nitrobacter to account for the nitrate level observed in his soil samples. Estimates such as these confirm the view that conventional enumeration procedures considerably underestimate the number of nitrifiers present. An alternative explanation, however, is that considerable non-autotrophic nitrification could be responsible. Evidently, observations of soils inhibited with N-serve, a well known potent inhibitor of Nitrosomonas supported this view (3).

Studies on nitrification in activated sludge systems have also indicated the potential for heterotrophic nitrification. Stover and Kincannon (4), from their studies on biological nitrification, found that the limiting mean cell residence time required to attain complete nitrification in one-stage carbonaceous-nitrification system was affected by COD:NH_3-N ratio. Also, the autotrophic nitrifying microorganisms do not respond to spontaneous increases in nitrogen concentrations as well as the heterotrophic microorganisms respond to increases in organic carbon sources. Esfandi and Kincannon (5) also reported that shock loads of ammonia-nitrogen source did not affect nitrification in the activated sludge system, and rapid heterotrophic growth inhibited the nitrification process. From these observations, it could be argued that during rapid heterotrophic growth when organic carbon source is still available, the heterotrophs do not need other energy source such as NH_3-N for survival except for cell synthesis, hence nitrification is not essential for obtaining energy. When rapid growth ceases, or available carbon source is limited, they turn to available nitrogen for energy supply. This is why COD:NH_3-N greatly affects the nitrification process as reported by Stover and Kincannon (4). Moreover, Little (6) also reported that there exists a definite COD concentration at which nitrification will occur.

Hence, the level of organic carbon source, a limiting nutrient for heterotrophs, seems to have an important role in biological nitrification.

A great deal of the research on nitrification has dealt with either the process chemistry alone or the process chemistry in conjunction with microbiology in enrichment situation. Considerable studies have been done on the chemistry of nitrification, and it is easily followed, but the microbiology has been sharply limiting, even under enrichment conditions. Very little is known about the diversity of nitrifiers. The unfortunately still too common oversimplification that in equating Nitrosomonas and Nitrobacter to nitrification should be questioned based on existing evidence for the potential of heterotrophic nitrification.

As a first step towards a better understanding of the role of heterotrophs in nitrification, it was considered necessary to isolate the organism capable of oxidizing ammonia as well as utilizing organic carbon as carbon and energy source. A laboratory scale continuous flow activated sludge reactor with internal cell recycle was conducted to supply the inoculum for isolation. An enrichment culture was first developed to stimulate the growth of the nitrifying heterotrophs. Once isolated, growth characteristics were determined. The effect of C:N ratio on the nitrification rate was also investigated. Most pure culture studies involved high substrate concentration, and great gaps exist in information regarding the normally lower substrate concentration that exists in most treatment plants where available organic carbon is relatively low. Hence, it was necessary that both the effects of high and low C:N ratio on nitrification rate should be investigated. From this study, it is hoped that with accelerated the interest in the nitrifiers, a picture of morphologically and physiologically diverse bacteria will

emerge to replace the current Nitrosomonas-Nitrobacter stereotype. This will complicate the microbiological analysis problem but will contribute to a much better understanding of nitrification response to different environmental conditions.

CHAPTER II

LITERATURE REVIEW

Historical Background

One of the first instances of microbiological lore being applied to the many art of destroying one's enemies can be traced back to the Napoleonic era, during which time nitrate from biological nitrification for gunpowder was prepared in manure heaps maintained in a moist condition by water and urine addition. As nitrate production became more the concern of the scientific rather than the military mind, the oxidation became gunfodder in the less destructive warfare then proceeding between chemical and biological schools of thought. The chemical theorists, as was their wont, advanced the concept of a soil catalyst serving in the conversion of ammonium to nitrate. However, Pasteur in 1862 suggested a reevaluation of this view in the light of his evidence that vinegar resulted from a biological oxidation, one apparently similar to nitrification (7). Three years later, Liebig (7), in addition to other researchers upheld the view that nitrification was of chemical nature. As late as 1877, Pasteur's prediction of the biological nature of nitrification was eventually fulfilled by Storer in America, and Schloesing and Muntz in France (8). Schloesing and Muntz first demonstrated the association of living microorganisms with ammonia oxidation reaction. Sewage trickling through a sand column was found to produce nitrate with the removal of ammonia. The reaction could be stopped completely simply by exposing the column to chloroform vapor or by heating the column to greater than

100°C.

The next task, presumably a simple one, was to obtain the responsible microorganisms in pure culture. Students who study nitrification to the present day, often attempt the same feat, and rarely do even these modern investigators meet with success. Small wonder, therefore, that the pioneer microbiologists met with considerable difficulties. Since they had not yet been introduced to the concept of chemoautotrophy, the laboratory media then in vogue were not suited to support the colonial growth of such chemoautotrophs. But, in their failures, these pioneers had made many observations, eg., the toxicity of ammonia to nitrite oxidizers and the related accumulation of nitrite were reported by Warington in 1891 (9). He also discovered that soil must be used as an inoculum for nitrification process and the oxidation reaction actually occurred in two stages, with each stage being accomplished by different microorganisms.

Finally, in a series of studies which have been described too often to bear repetition, but never sufficiently often to diminish admiration, Winogradsky in 1890 (10) isolated the responsible autotrophs in monoculture. He described representatives of two small groups of specialized chemoautotrophic bacteria and clearly related the metabolism of each to the two corresponding stages of nitrification: the oxidation of NH_4^+ to NO_2^-, and the subsequent oxidation of NO_2^- to NO_3^-. Hence the strictly biological nature of nitrification was firmly established with the isolation of the "nitrifying bacteria" by Winogradsky.

The Nitrifying Microorganisms

Autotrophic Nitrifiers

The ease of initiating enrichments of nitrifying autotrophs had

been reported and observed by many soil scientists and microbiologists. By contrast, comparatively few individuals have isolated in pure culture the organisms carrying out the oxidation.

On the basis of the dominant nitrifiers in enrichments and the frequency of isolation of specific bacteria in pure culture, it appears that only two autotrophic genera are prominent in nitrification, Nitrosomonas and Nitrobacter. These two genera, the former containing ammonium oxidizers, the latter the nitrite oxidizers, are the gram negative chemoautotrophic nitrifying bacteria. Most of the ammonium-oxidizing isolates seem to be related to or identical with Nitrosomonas europaea, which is a rod-shaped bacterium, 0.9-1.0 x 1.1-1.8 μ with a single polar flagellum or occasionally one flagellum at each end of the cell. The common nitrite-oxidizing bacterium appears to be Nitrobacter winogradskyi, characterized as a short, gram negative, non-motile rod, 0.6-0.8 x 1.0-1.2 μ as described by Breed et al., (11). Nelson (12) described Nitrosomonas monocella as a short, ovoid, gram-positive rod which is motile by means of a single polar flagellum; his isolate of Nitrobacter agilis was reported to be similar to N. winogradskyi, but it had a single polar flagellum as locomatory organelle.

Some differences appear to exist between various isolates. Thus, some strains are gram negative and non-motile as reported by Lewis and Pramer (13). Some are gram positive (14) while others are weakly gram positive to gram negative and motile by a single polar flagellum or occasionally two flagella. The early finding by Stutzer (15), that at least some strains of Nitrobacter form buds has been confirmed by Zavarzin and Legunkova (16); and it appears that N. winogradskyi may have, in addition to a budding or a bud-like mechanism, both a flagellated and

a non-flagellated stage. The differences observed among various early investigators may be more apparent than real because of the suboptimal conditions provided to the cultures, and a reinquiry into the morphology of these bacteria is clearly warranted.

In addition to Nitrosomonas and Nitrobacter, indisputably the most abundant of the autotrophs, five other genera of nitrogen autotrophs are recognized by Breed et al. (11). These are the ammonium-oxidizing Nitrosococcus, Nitrosospira, Nitrosogloea and Nitrosocystis, and the nitrite oxidizer, Nitrocystis. Strains designated as members of these groups are not commonly encountered, but there are occasional reports of the isolation from soil of Nitrosogloea by Sims and Collins (17), Nitrosococcus by Sakai (18), Nitrosospira and Nitrosocystis by Maciejewska (19). No attempt will be made here to assess the validity of these genera, an issue first raised by the demonstration of Imshenetsky (20) and Grace (21) that myxobacteria occurring as contaminants in Nitrosomonas cultures may give rise to forms that appear to be new types of autotrophic nitrifiers.

At the present time, the only microorganisms linked directly to nitrification in natural environments are the gram negative chemoautotrophic bacteria comprising the family Nitrobacteriaceae. A listing is presented in Table I. This arrangement is that of Watson (22) as compiled for the 8th edition of Bergey's Manual, with the addition of two recently described species of NH_4^+ oxidizers Nitrosovibrio tenius by Harms et al., (23) and Nitrosococcus mobilis by Koops et al. (24). Nitrosovibrio constitutes a new genus.

TABLE I

CURRENTLY RECOGNIZED NITRIFYING CHEMOAUTOTROPHIC SPECIES

Genus	Species
	(Oxidize NH_3 to NO_2^-)
Nitrosomonas	europaea
Nitrosospira	briensis
Nitrosococcus	nitrosus
	oceanus
	mobilis
Nitrosolobus	multiformis
Nitrosovibrio	tenuis
	(Oxidize NO_2^- to NO_3^-)
Nitrobacter	winogradskyi
	(agilis)
Nitrospina	gracilis
Nitrococcus	mobilis

The brevity of Table I is surprising in light of the diversity of environments in which nitrification takes place, but not in the light of the limited research effort focused on the microbiology of nitrification during the past four decades, and the difficulties associated with such study.

Nitrification by Heterotrophs

The unique position of the autotrophic Nitrobacteriaceae in nitrification was challenged when it was first demonstrated that some heterotrophic organisms could also effect the oxidation of ammonia to nitrite (25, 26, 27). Since then there have been scattered claims of heterotrophic microorganisms capable of effecting nitrification reactions in

culture media.

Almost forty years after the description of autotrophic bacteria, the first report of a heterotrophic species which could effect nitrogen oxidation is that of Nelson in 1929 (12). Later, Cutler (28), Cutler and Mukerji (29), Crump (30) and Nechaeva (31) also published reports indicating the possibility of nitrification by heterotrophic bacteria. Such interest in this unique ability for NH_4^+ oxidation was renewed by the observation of Quastel and Scholefield (32) that pyruvic oxime was converted to NO_2^- in soil percolation columns. Several bacteria, identified as Achromobacter and Corynebacterium genera, were reported to be responsible (33, 34). Jensen (35) isolated three more groups of heterotrophic bacteria which formed NO_2^- from the oxime of pyruvic acid, including twenty-four strains of Nocardia corallina, one strain of Agrobacterium sp., and three strains of Alcaligenes sp. One N. corallina pure culture produced 40 µg NO_2^--N/ml in 8 days with pyruvic oxime as the only nitrogen source, and 70 µg NO_2^--N/ml in oxime plus peptone medium. All of these bacteria formed only NO_2^- as a nitrification product, but were of interest because of their relatively high activity in this regard. More data supporting the claims for heterotrophic nitrification were added by Fisher, Fisher and Appleman (36) and Hutton and ZoBell (37). Unlike the oxime oxidation by the heterotrophs, the amount of nitrogen oxidized from ammonium salts is small. Campbell (38), Eylar and Schidt (39) and Hirsch et al. (40) found that only a maximum of 5 ppm nitrite-nitrogen is produced from ammonium salts by a spore-forming, thermophilic bacillus, a number of bacteria and a variety of actinomycetes.

By contrast with bacteria and actinomycetes, which may form nitrite but rarely nitrate, certain fungi are capable of producing relatively large

quantities of nitrate from ammonia salts. The first instance of NO_3^- formation by heterotroph was reported by Sakaguchi and Wang (41). They observed that Aspergillus oryzae synthesized nitrate in a medium containing both ammonium and nitrite but was unable to do so in media containing the individual nitrogen sources. Schmidt (42) isolated a soil fungus, Aspergillus flavus capable of forming NO_3^- where grown in a yeast extract--peptone-glucose medium. He reported about 25-30 μg NO_3^--N/ml was formed in 7-14 days, with NO_2^--N rarely exceeding 1.5 μg/ml. Eylar and Schmidt (39) carried out a survey of microorganisms isolated from soil to determine the incidence of heterotrophic forms capable for producing NO_2^- and NO_3^-. Fifteen of the sixteen nitrate-forming isolates investigated were fungi. The ability to form NO_3^- was found to reside almost exclusively in the A. flavus isolates. Various bacteria and fungi, comprising about 7% of all the isolates, were able to form very low (0.2 - 1.0 μg/ml) concentrations of NO_2^-. In addition, Malavolta, de Camargo and Haag (43) found that A. wentii produce nitrite and nitrate. Aleem (62) also reported the generation of nitrite and nitrate when A. wentii hyphal extracts were supplied with hydroxylamine.

Nitrite also may be generated from compounds containing N at a level of oxidation higher than that found in ammonium. Thus, Castell and Mapplebeck (44) detected nitrite in cultures of Pseudomonas, Proteus and Microbacterium which had been grown in the presence of hydroxylamine, and Quastel and Scholefield (34) found that oximes of pyrivic, oxaloacetic, phenylpyruvic, and α-ketoglutaric acids were rapidly oxidized to nitrite when applied to soil. Nitrite is likewise released microbiologically from aromatic nitro compounds (45). Hence, the term nitrification was defined in its broadest sense by Alexander, Marshall and Hirsch (46),

i.e., "nitrification is a biological conversion of nitrogen in organic or inorganic compounds from a reduced to a more oxidized state." In the process of heterotrophic nitrification, both inorganic and organic nitrogenous substrates are metabolized (47), and the products known to be excreted in the reaction sequence, besides nitrite and nitrate, include hydroxamic acids (48), Oximes (49), nitroso compounds (50), nitro compounds (46), and amine oxides (52).

Most of the studies on heterotrophic nitrification were demonstrated in axenic cultures, very few have been shown to occur in natural environments. Studies by Verstraete and Alexander (47) on axenic cultures of a nitrifying strain of Arthrobacter revealed that this heterotroph excreted hydrocylamine, a hydramic acid, 1-nitrosoethanol nitrite and nitrate. On the basis of these findings with pure cultures, they initiated an investigation to determine whether the same type of nitrification could occur in natural environments of sewage, river water, lake water and soil. They found that a carbon source was needed for the occurrence of this pattern of nitrification. Of the carbon sources tested, only acetate and succinate supported this kind of nitrification. Studies by Etinger-Tulczynska (54) also provided good evidence for heterotrophic nitrification. He found that nitrification of added ammonium-N proceeded rapidly at 28^{o}C but was inhibited partially and in some soils completely when incubated at 37-40^{o}C. In contrast, nitrate formation from inherent soil-N proceeded better at 37-40^{o}C than at 28^{o}C. Bacteriological examination showed that a temperature of 37-40^{o}C had an injurious effect on the population of nitrifiers, especially the nitrate-forming bacteria. Nitrification by Nitrosomonas-Nitrobacter group in culture media was also markedly inhibited at 37^{o}C as compared with that at 28^{o}C. His studies also showed that chloromycetin and potassium chlorate, known inhibitors

for autotrophic nitrifiers, suppressed the formation of nitrate from the added ammonium-N, but did not exert any appreciable effect upon nitrate formation from inherent soil nitrogen. As a result of such marked differences in these two nitrification processes, he concluded that greater part of nitrate orginating from soil-N was produced by some process other than that which is responsible for nitrification of ammonium-N. Most recent data reported by Tate (3) also suggested heterotrophic population may be responsible for some of the nitrate produced in histols. He observed that populations of Nitrosomonas and Nitrobacter were insufficient to account for nitrate concentration in muck soil. When amended with sodium acetate and ammonium-nitrogen, a fourfold increase in the heterotrophic nitrifying Arthrobacter was observed.

Methane Oxidizing Bacteria

The methane oxidizing bacteria, or methylotrophs are a morphologically diverse group of bacteria capable of oxidizing CH_4 as their prime carbon and energy source. In 1953, Hutton and Zobell (37) showed that several CH_4 oxidizing bacteria were able to oxidize CH_4 to NO_2^- in a defined medium. Their data indicated that about 20 µg NO_2^--N were produced during 19 days of incubation.

The morphological and biochemical similarities between the obligate CH_4 oxidizers and the autotrophic nitrifying bacteria are noteworthy. Morphologically, both have extensive proliferations of internal membrane assemblies which appear to be invaginations of the cytoplasmic membrane. The membraneous systems are unproven as to function, but are generally considered to bind the enzymes responsible for oxidation of their respective substrates (55). Biochemically, both groups have an incomplete

tricarboxylic acid cycle and each oxidizes its energy source, CH_4 or NH_4^+, by mixed function oxidases. Methane oxidizers are able to oxidize NH_4^+ to NO_2^- and when they do so the CH_4 mono-oxygenase enzyme complex appears to be the same one which oxidizes NH_4^+. Oxidation of NH_4^+ by CH_4 oxidizers is considered by Whittenbury and Kelly (56) to be an instance of "co-oxidation" whereby an organism is able to oxidize a compound but is not able to grow on it as a single source of energy. It is not yet clear how NH_4^+ oxidation is to a co-oxidizing methylotroph.

In 1977, Dalton (57) examined the behavior of soluble extracts of Methylococcus capsulatus that oxidize CH_4 to methanol and NH_4^+ to NO_2^-. He found that the apparent Km value for NH_4^+ in cells and extracts was very high, which suggests that nitrification would be significant only when either the NH_4^+ concentration was high or the CH_4 concentration was low. Dalton thought it likely that such a situation could prevail in aquatic environments wherein high populations of CH_4 oxidizers may have built previously; they would then be the major nitrifying organisms. In reality, nothing is known as to what, if any, contribution the CH_4 oxidizers may make to nitrification, but in view of the features shared with autotrophic nitrifiers, the possibility is intriguing and bears examination. The possibility of co-oxidation of methane by NH_4^+ oxidizers is also intriguing as an unexplored factor in the survival of the autotrophic nitrifiers in nature.

Isolation and Cultivation

Isolation of Autotrophic Bacteria

Difficulties associated with the study of nitrifiers commence with their isolation in pure culture. Plating, even on strictly inorganic

media, is not suitable for their direct isolation from natural environment because organic materials introduced with the inoculum permit growth of heterotrophic contaminants. Most successful isolations of autotrophic nitrifiers have been preceded by extensive and careful serial enrichment procedures (77), but this approach has distinct disadvantage in diversity studies since a single isolate is likely to achieve dominance during enrichment. This selectivity may or may not favor a significant number of the population, and the same procedure may result in the isolation of very similar strains from different soils as reflected in the data of Fliermans,et al. (78). Procedures used by Soriano and Walker (79) in which dilutions are plated and individual colonies are subsequently picked for enrichment in liquid culture in attempts to overgrow contaminants. Belser and Schmidt (80) also avoided initial enrichment by transferring directly from positive MPN tubes into a dilution isolation series in liquid medium. This approach provides the possibility of obtaining isolates from different numerically abundant segments of a given nitrifying population.

Several procedures have been devised so that it is no longer a feat in itself to isolate the bacteria. One modification entails bubbling carbon dioxide through the enrichments to remove the cells from the carbonate particles onto which many contaminants adhere (81). Another technique involves the use of compounds inhibitory to many of the contaminants (82, 83). Lewis and Pramer (84) designed a procedure to bypass the perennial problem of adsorption of both contaminant and nitrifier by the carbonate particles; this problem was surmounted by means of a solution free of insoluble carbonates in which the pH was maintained relatively constant by regular additions of alkali. As long as the ratio of autotrophs to heterotrophs is sufficiently wide, there is no problem in

obtaining a heterotroph-free culture of autotroph. Once isolated the autotrophic nitrifiers are slow growing and yields are low. Biochemical studies have been hampered by these features and by the susceptibility of nitrifier cultures to contamination. This is especially true for studies with NH_4^+ oxidizers. With few pure culture isolates available, biochemical studies have been limited to Nitrosomonas and Nitrobacter, and usually to the same few strains of these genera.

Isolation of Bacteria From Sewage

It is generally acknowledged that no single medium or procedure is apt to be totally satisfactory for the enumeration and isolation of all the bacterial species which may be present in sewage. However, considerable effort has been spent in devising special media and in comparative studies to determine the most satisfactory medium.

Ferrer, Stapert, and Sokolski (85) developed on iron peptone agar and a procedure which employed membrane filtration rather than a poured agar plate. Incubation was conducted at room temperature for four days. These medium and procedure were found to yield higher counts of bacteria from water samples than those obtained by the use of Difco tryptone glucose agar.

Van Gils (86), in his studies on the bacteriology of activated sludge, employed tryptone glucose agar and sewage agar prepared from screened municipal sewage. The tryptone glucose agar yielded two to six times the number of colonies found on the sewage agar. In a study of the relative number of predominant bacteria isolated from the various activated sludges examined with sewage agar and a numeral medium agar with glucose and ammonium sulfate, a high percentage of species found on the sewage agar were not capable of growth on the other medium. The cultures were incubated

at 25°C for six days.

A preparation of autoclaved and neutralized (H_3PO_4) sewage solidified with agar was used by Dias and Bhat (87) to study the dominant bacteria in activated sludge. Some 150 isolates were obtained from raw sewage. Upon transfer to proteose peptone yeast extract agar about 91 percent were found to survive. A 10 to 15 day incubation period at 16°C to 27°C (room temperature) was employed.

Prakasam and Dondero (88) prepared a sewage agar and an activated sludge extract agar for comparative use with nutrient agar and five other counting media. They noted an apparent difference between the predominant populations of sewage and activated sludge and the suitability of each medium for the respective sampling source. For the enumeration of activated sludge bacteria, the activated sludge extract agar medium yielded the highest counts with nutrient agar a close second best. The enumeration of sewage bacteria was best performed (highest counts) with nutrient agar. The activated sludge extract agar produced results not significantly different from nutrient agar for some sewage inocula. Sewage agar was judged to be a poor medium for growth and isolation. Both sewage agar and the sludge extract agar were found to vary in efficacy, i.e., their comparative effectiveness with other media was not constant. Dondero et al. (89) presented various isolation techniques employing sewage agar supplemented with growth factors for the isolation of Sphaerotilus from activated sludge.

Berg,et al. (90) utilized the pour plate method with various media to determine the bacterial populations present in primary wastewater effluent. Nutrient agar was found to yield higher counts than either brain-heart infusion agar or trypticase soy agar. Nutrient agar was also found to give higher counts than those obtained on yeast-extract agar or

tryptone glucose yeast-extract agar in studies on the aerobic bacteria in waste stabilization ponds by Gann, et al. (91).

Bacterial isolates were obtained from waters of two rivers by Stumm-Zollinger (92) using three media for one river and nutrient broth agar for the other river. In the comparison of the three media, a medium consisting of casitone, sodium caseinate, starch, glycerol, potassium phosphate, and filter-sterilized river water was found to be superior for bacterial enumeration to brain-heart infusion medium or a medium of yeast extract, casein hydrolysate, and potassium phosphate. Nutrient agar provided about 50 isolates which were propagated in a nutrient broth, glucose, and sodium oleate medium for further studies on mixed microbial communities.

Lighthart and Oglesby (93) reviewed wastewater treatment plant ecology and noted the following: a) there has been no standardization of an analytical method for the isolation of bacteria from wastewater or its treatment processes; b) there are many types of organisms associated with the various treatment processes; and c) to understand the functional capabilities of the bacteria and classify them taxonomically, a large number of organism characteristics must be determined. They selected tryptone-glucose-meat and yeast extract agar as the most satisfactory of the eight tested media for enumeration, differentiation, and isolation. However, the data which they presented indicated that nutrient agar also appeared to be satisfactory for enumeration, with activated sludge extract agar being slightly less satisfactory. They tested 50 to 100 colonies selected at random without regard to predominant colony types. A 10 day incubation at $20^{o}C$ was employed.

Process Chemistry and Biochemistry of Nitrification

Autotrophic Nitrogen Oxidation

Both the conversion of ammonium to nitrite and the transformation of the latter to nitrate are exothermic reactions. For the chemoautotrophic bacteria, the oxidation is physiologically the equivalent of the heterotrophic conversion of organic carbon to carbon dioxide, both processes providing the respective groups of microorganisms with the energy required for biosynthetic reactions. In addition, a portion of the energy released in the autotrophic oxidation of the nitrogen must be coupled with the reduction of carbon dioxide to the oxidation level characteristic of the protoplasmic constituents of the cell. The heterotrophs may put to use part of the energy liberated in its nitrogen oxidation, but more likely it squanders the energy with no appreciable coupling of nitrification with phosphorylation.

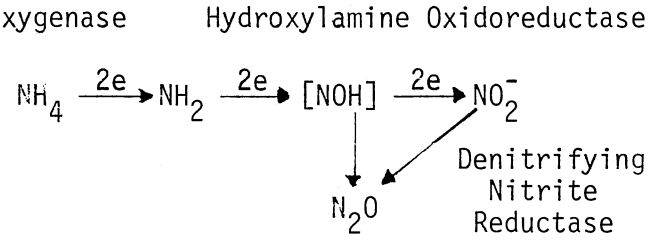
Biochemistry of NH_4^+ Oxidation

NH_4^+ is oxidized to NO_2^- by Nitrosomonas according to the equation:

$$NH_4^+ + 1\tfrac{1}{2}\ O_2 \xrightarrow{6e} NO_2^- + H_2O + 2H^+ \qquad \Delta F = -65 \text{ kcal}$$

All the energy for biosynthesis and maintenance is provided by this reaction. Hofman and Lees (58) showed that hydroxylamine is a likely first intermediate. If it is so, the change in free energy in the initial step is quite small, namely -0.70 kcal per mole. There is a valency change of N from -3 (NH_4^+) to +3(NO_2^-). This would suggest at least one other intermediate. Nicholas (59) had suggested nitroxyl (NOH) to be the most probable second intermediate, although it is unstable and there is

no direct evidence in favor of it. The pathway of NH_4^+ oxidation as depicted by Nicholas (59) is shown below.



Little is known of the mechanism of the first step of oxidation, NH_4 to NH_2OH. This is because of the low activity of cell-free extracts of N. europaea (60). The oxidation of NH_2OH to NO_2^- is carried out by the enzyme hydroxylamine oxidoreductase. This occurs only in the presence of a suitable electron acceptor. Two electrons from the dehydrogenation of NH_2OH, forming [NOH], are thought to pass to a membrane-associated terminal electron transport chain involving cytochrome a_2 (61). Synthesis of ATP is coupled to that reaction and reduction of pyridine nucleotide is coupled to ATP hydrolysis (62). Oxidation of [NOH] to NO_2^- takes place with the net addition of an atom of oxygen derived from O_2. Hooper (63) has proposed that this step occurs by an internal mixed function oxygenase mechanism. Features of the electron transport components were reviewed by Sujuki (60), Hooper (63) and Nicholas (59).

N_2O evolved during NH_4 oxidation as depicted above could arise from interaction of hydroxylamine oxidoreductase and nitrite reductase, influenced by conditions of aerobiosis (59). Data obtained by Ritchie and Nicholas (64) with ^{15}N-labeled and unlabeled NH_2OH, and with ^{15}N-labeled and unlabeled NO_2^- further suggested that some N_2O may form from reduction of NO_2^- via [NOH]. In light of recent interest in mechanisms leading to the generation of N_2O, more attention should be given in both biochemical

and ecological studies to the generation of N_2O by NH_4^+ oxidizers other than N. europaea and to the regulatory role of O_2 tensions.

Carbon Metabolism by NH_4^+ Oxidizers

Nitrifiers can synthesize all of their cell constituents by fixing C from CO_2 through the Calvin reductive pentose phosphate cycle. ATP and reducing equivalents are required for the pathway. For every three molecules of CO_2 fixed, there is a gain of one C_3 molecule, phosphoglyceric acid and subsequently converted to hexoses. Some phosphoglyceric acid also serves for synthesis of alamine and serine (59).

Nitrifying bacteria have an incomplete tricarbocylic cycle which further decreases their already limited metabolic versatility (65). The absent enzyme, α-ketoglutarate dehydrogenase prevents tricarboxylic and cycle to function as an energy generating mechanism, but operates instead in a purely biosynthetic role.

The sensitivity of NH_4^+ oxidizers to inhibition by trace amounts of numerous organic compound is well known and was emphasized by Winogradsky (66). Growth of these autotrophs in nature in the presence of organic matter has generally been considered to reflect indifference to or tolerance of such organic matter except as it may provide substrate NH_4^+. Studies using labeled compounds have shown, however, that Nitrosomonas, at least, could incorporate certain organic compounds readily. In a study of growth stimulation of N. europaea by a heterotrophic contaminant, Clark and Schmidt (67) were able to mimic the stimulation effect by addition of pyruvate to a pure culture of the nitrifier. Pyruvate-2-^{14}C

was incorporated readily and becamse widely distributed in cell fractions. In a later study, Clark and Schmidt (68) found that each of 14 labeled amino acids presented in trace amounts was taken up by growing cells and metabolized. This was true even for those amino acids which were inhibitory at higher concentrations. It seems most probable that nitrifiers can and do utilize many organic metabolites when growing in natural habitats, and respond favorably or adversely depending on the kind and concentrations of the metabolites.

Biochemistry of NO_2^- Oxidation

The oxidation of NO_2^- to NO_3^- by *Nitrobacter* is as follows (59):

$$NO_2^- + 1/2\ O_2 \xrightarrow{2e} NO_3^- \qquad \Delta F = -17.8 \text{ kcal}$$

With only a 2e shift in oxidation state from +3 to +5, the reaction poses no obvious problem of intermediates. However, NO_2^- oxidizers are probably among the least favored of bacteria since their high energy requirement for biosynthesis is linked to oxidation of a low energy-yielding substrate. The reaction is carried out by a NO_2^- oxidase system with electrons carried to O_2 via cytochromes leading to the generation of ATP. Part of the ATP is consumed in a reversal of the $NADH_2$-cytochrome and reductase reaction that is conventional for heterotrophs, to account for the generation of reducing power (69). Thus, the synthesis of NADH at the expense of ATP and reduced cytochrome further limits the energy efficiency of NO_2^- oxidizers, but it does solve a problem of providing an E_o value low enough to participate in the reduction of CO_2. All NO_2^- oxidizing and associated ATP synthesizing systems are located in the membrane, and a particularly extensive cytomembrane system is a feature of *Nitro-*

bacter; the cytomembrane arrangement probably provides the large number of NO_2^- oxidizing sites seemingly needed in order to compensate for the low yielding oxidations, by making possible high turnover rates.

Nitrobacter has an interesting array of assimilatory reductase enzymes. Nicholas (59) reviewed earlier work in his laboratory which showed that Nitrobacter cells incorporated ^{15}N from NH_2OH, NO_2^-, NH_4^+ and NO_3^- into cellular N. Presumably, the assimilatory NO_3^- reductase system is the main reductase system operative during normal growth, and accounts also for the inhibitory action of the chlorate ion which is reduced to the toxic chlorite ion.

Carbon Metabolism by NO_2^- Oxidizers

Carbon dioxide is fixed in NO_2^- oxidizers by the Calvin reductive pentose cycle as in the NH_4^+ oxidizers. Shively et al. (70) reported that phage-like polyhedral bodies sometimes observed in electron micrographs of Nitrobacter are carboxysomes which enclose particles of the key CO_2 fixing enzyme ribulose diphosphate carboxylase. The carboxysomes may aid in the survival of Nitrobacter, since particles isolated from cells starved of NO_2^- retain a high specific activity for the enzyme (70).

Nitrobacter differs from the NH_4^+ oxidizers in that it may be facultative rather than obligate autogroph. The distinction is based on demonstrations that certain strains can be induced to grow heterotrophically using organic C and organic energy source in the absence of NO_2^- (71, 72, 73). Acetate, formate, pyruvate, yeast extract, peptone and casein hydrolyzate, all in low concentrations, are assimilated in the absence of NO_2^-. The heterotrophic mode is clearly not preferred. A Nitrobacter strain examined by Bock (73) required 6 months acclimation before heterotrophic

growth commenced with extremely long generation time of 70 hours as compared to about 14 hours for autotrophic growth. The long heterotroph lag, slow heterotrophic growth, and the ability to interchange from autotrophic to heterotrophic growth and vice versa has been confirmed with Bock's strain. It seems unlikely that Nitrobacter may use the heterotrophic mode in soil for other than mere survival. The use of organic compounds as auxiliary C and energy sources in the presence of NO_2^- (mixotrophic growth) sometimes stimulates Nitrobacter (74). This has interesting implications that should be studied for the natural environments.

Heterotrophic Nitrogen Oxidation

The physiological or biochemical characteristics associated with nitrogen oxidation by heterotrophs are unknown. Although much work has been done in pure cultures, surprisingly little attention has been given to the enzymes that are concerned in catalyzing the heterotrophic nitrogen oxidation. One of the most significant observations is that of Kuznetsov (75), who noted that all 4 of his heterotrophic nitrifying bacteria possessed peroxidase. He felt that the peroxidative process was a side-reaction in the course of the bacterial activities. A peroxidase was also found in a nitrate-forming A. flavus strain by Alexander, Marshall and Hirsch (46). Nevertheless, the precise role of one or both of these enzymes in the nitrification reaction sequence remains obscure.

In the earlier part of the literature review, intermediates in the conversion of ammonium to nitrate were mentioned. The pathway may involve solely inorganic substances such as hydroxylamine, hyponitrite and nitrite; alternatively, organic intermediates may be produced during the microbial oxidation of ammonium, amino-, or amide-nitrogen.

An organic pathway may include aromatic or aliphatic intermediates.

Chemical precedents for the interconversions of various of these substances are many, and certain oxidizing agents convert nitrogen in organic combination from lower to higher oxidation states; e.g., specific oxidizing agents from N-alkyl hydroxylamines and oximes from amines, microalkanes from oximes and hydroxamic acids from amides.

Biological precedents for reactions of the type that may be concerned in heterotrophic nitrification are few. Thus, hydroxamic acids can be formed from hydroxylamine and either amides or fatty acids, and hydroxamic acids may be hydrolyzed with the liberation of free hydroxylamine (76). Oximes of a number of organic acids are rapidly converted to nitrite when perfused through soil, and Achromobacter sp. brings about a similar conversion of pyruvic oxime to nitrite in culture (34).

Nitrification in Wastewater Treatment

Nutritional Aspect of Nitrogen in Waste Treatment

It is widely recognized that the removal of organic matter from wastewaters is accomplished primarily by the bacteria which use the organic matter as a source of carbon and energy. Various other essential nutrients are needed, but nitrogen plays a vital role since it is required for the synthesis of proteins and nucleic acids. If nitrogen is lacking totally in biological wastewater treatment system, net synthesis of new cells is prevented. It is therefore a common practice in wastewater treatment operations to maintain the nitrogen supply at a BOD:N ratio of 20:1 by addition of nitrogen in the incoming waste stream (141).

The biochemistry of nitrogen and the effect of nitrogen efficiency have been studied by Symons and McKinney (142). They also studied the effect of the concentration and form of nitrogen on the growth of sludge.

They postulated that when the cell dies, it lyses and releases the nitrogenous material into solution. This organic bound nitrogen is then available for further use by other bacteria for synthesis. It was found that from COD removal point of view, the nitrogen requirement is 1.17 lb per 150 lb of COD, which corresponds to COD:N ratio of 85:1. These conclusions were based on batch studies with 10 percent recycling of sludge, using sodium acetate as the sole source of carbon.

Hattingh (143) suggested that a value of BOD:N of 19:1 for maximum nitrogen content in the sludge. Later (144) he also found that when BOD:N ratio was greater than 37:1, bulking of sludge occurred. Simpson (145) found that nitrogen requirement could be reduced to one-fifth of the amounts proposed by Sawyer (146) in extended aeration systems. Eckenfelder and McCabe (147) found that pulp and paper mill waste can be treated with no nitrogen supplements in aerated lagoons due to extended period of aeration which permitted reuse of nitrogen after auto-oxidation. Eckenfelder and Weston (148) have summarized the chemical equations for the synthesis of cellular material, oxidation of organic substrate, and auto-oxidation of the cellular material. Ludzack, Schaffer, and Ettinger (149) found an increase in ammonia concentration in the effluent when detention time or temperature or mixed liquor suspended solids were increased.

Work by Bechir and Symons (150) shows that efficiency of organic matter removal declines as the nitrogen content of the mixed liquor volatile solid decreases. This correlation was found to be influenced very slightly by type of substrate and solids retention time, but was found to be dependent on the type of nitrogen fed for supplement. They believed that this relationship could be expressed as follows:

% organic nitrogen x (% soluble COD remaining)$^{1/2}$ = 14 for ammonia fed system.

Komolrit (157)observed that nitrogen-deficient systems had lower capability of withstanding qualitative shock loads. He indicated the necessity of the presence of nitrogen for acclimation purposes. The preferential substrate utilization or substrate interaction phenomenon was found to be more apparent in nitrogen-limited system.

Krishnan and Gaudy (139) found that in many systems utilizing a single substrate such as glucose, considerable amount of COD remained after glucose, as measured by anthrone or glucose oxidase, had been removed. Further analysis showed that volatile acids, primarily acetic acid, may constitute a large fraction of this COD (152). These metabolic intermediates or end products were found during active growth or under nonproliferating conditions. If adequate nitrogen was available they were metabolized after the original carbon source was removed. Similar results of partial oxidations of glucose were reported by Clifton in pure cultures studies with Bacillus megaterium (153) and Escherichia coli (154).

By observing cell growth in flasks with different amounts of ammonium sulfate but the same amount of organic carbon source, Komolrit (151) estimated that approximately 150 mg/ℓ ammonium sulfate would be required for metabolizing 500 mg/ℓ of either glucose or sobitol. This corresponds to a C:N ratio of approximately 6:1 or COD:N of 16.6:1. He also observed that the nitrogen-limited conditions did not seem to affect the growth rate of the systems, but affect the total yield of the population. However, the smallest concentration of nitrogen used by him corresponded to a COD:N ratio of 50:1.

Factors Affecting Nitrification

The main factors which limit nitrification are substrate NH_4^+, DO, CO_2, pH, and temperature.

Substrate and Product Inhibition

Assuming tentatively that nitrification in soil is largely an autotrophic phenomenon, it is likely that rarely would any nutrient other than the energy substrate be limiting for the activity of the population. Both Nitrosomonas sp. and Nitrobacter sp. are sensitive to their own substrates or products (94, 95, 96) and to the substrate of the others (97). According to Anthonisen (98), the degree of inhibition depends upon the ammonia-ammonium and the nitrite-nitrous acid equilibria. Studies with pure cultures have shown that free ammonia and undissociated nitrous acid are more inhibitory than NH_4^+ or NO_2 (99) and studies with mixed culture have suggested the same thing (100).

Wide ranges of ammonium and nitrite ion concentrations can be oxidized by the nitrifiers. Huang et al. (101) reported a range of 2.5-110 mg/ℓ of NH_3-N from a mixed culture of a film reactor, but a much higher range of 100-1000 mg/ℓ has been reported by Adams (102) from bench-scale study with activated sludge system. Normal ammonia and nitrite ion concentrations in domestic wastewaters (103) are not inhibiting. However, substrate and product inhibition are of significance in treatment of industrial, poultry and agricultural wastes.

Effect of D.O.

In activated sludge system for carbonaceous removal, supplying of O_2 in excess presents no problems in heterotrophic systems because the value

of K_s for oxygen is generally small in comparison to the concentration of D.O. in the reactor, thereby making the rates independent of DO. For nitrification systems, on the other hand, K_s for DO is relatively high, having been estimated to be 2.0 mg/ℓ (104). Focht and Chang (94) have estimated values as low as 0.5 mg/ℓ, but the factors affecting the magnitude of K_s for DO are not yet well established. Consequently, for illustrative purposes, K_s for DO is assumed to be 1.3 mg/ℓ (95). This relatively high value is quite important in light of the low K_s values for NH_4^+-N, because it means that unless special precautions are taken, the concentration of DO, rather than the NH_4^+-N or NO_2^--N concentrations, will control the rate of nitrification.

Wild et al. (105) found that DO at levels greater than 1 mg/ℓ would have no inhibition on nitrification. On the other hand Nagel and Haworth (104) found that increasing the DO concentration above 1 mg/ℓ would increase the ammonium oxidation rate. The results of the study by Nagel and Haworth (104) seems to contradict the well established concept of critical oxygen concentration, and as such, some plausible explanation for these two different observations is warranted.

The limiting concentration of oxygen which permits nitrification has been reported as 0.5 mg/ℓ (106, 107), but less than 0.3 mg/ℓ has been reported by Downing and Scragg (108).

Effect of pH

The effect of pH on nitrification has long been recognized. Nitrification causes a destruction of alkalinity and thus there is a potential for a drastic drop in pH. Moreover, nitrifying bacteria are very sensitive to changes in pH. Hence, buffering is required for any nitrification system (109).

Studies on the effect of pH for both pure and mixed cultures have been reported. Barrit (8), Buswell et al. (110), Lees (111), Engel and Alexander (112), Loveless and Painter (113) reported pH optima for pure cultures of Nitrosomonas in the range of 7.2-8.6. On the other hand, at pH 5.5 or 9.2, complete inhibition of the pure cultures was observed (8). Metcalf and Eddy (114) reported a pH optima of 8.0-8.8 for lab scaled activated sludge system while Rimer and Woodward (115) reported an optima of 8.4-8.5 for two stage activated sludge pilot plant.

Painter (116), in his review on inorganic nitrogen metabolism, had summarized two sources of error that should be recognized in Nitrification study, i) when an adjustment is made to the pH level of a culture, local "overshooting" of hydrogen ion can occur, ii) if the pH of the system is not buffered, acid produced during nitrification would lower the pH and if it is buffered, the anion of the buffer used might have an influence on culture activity. This discrepency can be attributed to bacterial acclimation or population selection as suggested by some researchers.

During experimental studies, Stankewich (117) and Haung and McCarty (118) have observed that even though nitrifying bacteria initially prefer a pH near 8, they are capable of acclimating to lower environmental pHs wherein they will reestablish their maximum growth rate. Approximately 10 days of acclimation is required for a pH shift from 6.0 - 7.0 (118).

Effect of Temperature

Temperature has a strong effect upon the growth rate of nitrifying bacteria just as it has upon the heterotrophs. It has been shown that nitrification reaction follows Van't Hoff-Arrhenius law up to $30^{o}C$ (114) while Wong-Chong and Loehr (119) have observed that the effect of

temperature on μ_{max} fits the equation over the physiological range. They found that deviation of Nitrobacter occurred at lower temperatures than did deviation of Nitrosomonas.

The relationships between growth rate and temperature have been reported by many researchers. Stankewich (117) has summarized the results of several authors with the general equation:

$$\mu_{MT} = \mu_{M15} e^{K(T-15)}$$

where μ_{MT} is the maximum specific growth rate at any temperature T(^{o}C) and μ_{M15} is the rate at 15^{o}C. However, the overall optimum temperature for the growth of the nitrifying bacteria appears to be in the range 28^{o}-38^{o}C, although higher temperatures up to 42^{o}C have been reported for Nitrobacter by Painter (116).

Inhibitors

The autotrophic nitrifying bacteria have been the subject of many investigations designed to determine the effect of inhibitory substances. This is because of their unique physiology, their importance in the nitrogen cycle and their marked sensitivity to toxic compounds. Direct regulation of nitrification in soil with the nitrifiers as the target organisms became feasible with the introduction of 2-chloro-6-(trichloromethyl)pyridine (nitrapyrin), patented as a specific inhibitor of the NH_4^+ oxidation stage of nitrification (120, 121). Campbell and Aleem (122, 123) reported the sensitivity of N. europaea to inhibition by nitrapyrin at concentrations of 0.2 µg/ℓ, and the lack of sensitivity of Nitrobacter at concentrations as high as 50 µg/ml. The great interest that this compound has attracted arises from its ability, at least

in certain circumstances, to diminish the loss of available nitrogen from the soil through a retardation in the production of nitrate, the highly leachable anion which is also a focal point in nitrogen volatilization. This specific inhibitor has been very useful and is of considerable potential for experimental study of the nitrification process, the ecology and biochemistry of the nitrifying bacteria since it suppresses autotrophic, but not heterotrophic nitrogen oxidation. Bundy and Bremner (124) found that nitrapyrin was effective in retarding NH_4^+ oxidation derived from urea. Two soils that accumulated more than 160 ppm NO_2^--N after urea application with no inhibitor yielded little or no NO_2^- when inhibitors was added with urea. The results could be significant in view of the increasing importance of urea as a fertilizer in world agriculture. Numerous chemicals have been used in attempts to achieve these objectives progress has been summarized in several reviews (125, 126, 127). The most comprehensive comparative testing of nitrification inhibitors was reported by Bundy and Bremner (128) who used 24 compounds applied to three soils at the rate of 10 µg/µg to observe effects on the oxidation of 200 µg/µg NH_4^+-N. He found that ten of the compounds had essentially no effect on the nitrification process and nitrapyrin was ranked as the most effective of all the compounds tested.

Experimenters concerned with the selective inhibition of the NO_2^- oxidizers are limited to relatively few reagents. The Na^+ and K^+ salt of chlorate was shown by Lees and Simpson (129) to be a sensitive and specific inhibitor of Nitrobacter. This inhibitor can be a potential for use in nitrification activity determination so that NO_2^- accumulation can serve as a more sensitive and convenient measure of nitrification rate than NO_3^- accumulation.

Kinetics of Biological Nitrification

Kinetics of Growth and Substrate Utilization

The kinetics of growth and substrate removal for autotrophic bacteria can be expressed by the same equations for the heterotrophic bacteria. The most popular model utilized to describe the specific growth rate of Nitrosomonas and Nitrobacter with NH_4^+-N and NO_2^--N as the respective grow-limiting substrate is that of Monod (130):

$$\mu = \mu_{max} \frac{S}{K_s + S}$$

where:

μ = growth rate of microorganism, day^{-1}.

μ_{max} = maximum growth rate, day^{-1}.

K_s = half velocity constant = substrate concentrate, mg/ℓ, at half the maximum growth rate and

S = Growth limiting substrate concentration, mg/ℓ.

Monod originally developed this equation to fit the declining phase of bacterial growth for batch studies. It has been subsequently found to adequately represent batch and continuous culture (131, 132, 133). Applied to nitrogen oxidation by nitrifying bacteria, (134, 135, 96, 136, 137) the Monod expression provides for a continuous transition between first and zero order kinetics based on substrate concentration. The growth limiting nutrient, S in the equation has generally been considered as the energy source, and somewhat less often, the electron acceptor (dissolved oxygen). The carbon source as a limiting nutrient, an approach to studies of heterotrophic bacteria, has not been considered widely in

nitrification study. Most studies on the applicability of Monod expression do not involve carbon source limitation for nitrification. Nevertheless, the possibility should be checked as has been suggested by Loveless and Painter (113), particularly if air is the only source of carbon for the nitrifiers.

Exponential Growth and Exponential Substrate Utilization

The increase of microorganisms in a system is generally exponential, when the amount of substrate is very large compared to the amount of microorganisms present, such as in the early stages of cultivation starting with a small inoculum. In this log growth phase, bacterial growth can be represented by the following equation:

$$\frac{dx}{dt} = \mu x \tag{1}$$

$$\mu t = \ln(x/x_o) \tag{2}$$

where $\frac{dx}{dt}$ is the rate of change of cell concentration, μ is the specific growth rate ($time^{-1}$), x_o and x are the concentrations of cells initially and at time t.

The decrease of substrate is exponential, when the amount of microorganisms is very large compared to the amount of substrate, such as in the later stages of cultivation or in the case where a large inoculum is used. During this phase, the reaction is first order with respect to S and is expressed as:

$$-\frac{ds}{dt} = K_1 \cdot S \tag{3}$$

$$K_1 t = \ln(S_o/S) \quad (4)$$

where $\frac{ds}{dt}$ is the rate of substrate utilization, K_1 is the specific substrate removal rate, S_o and S are the concentrations of substrate initially and at time t.

As the available food supply is exhausted, a negative acceleration phase exists. Usually, this phase is called the "declining" growth phase, in which the growth rate and the substrate removal rate are still expressed by a first order reaction. But Equations (1) and (2) become:

$$\frac{dx}{dt} = C \cdot X \quad (5)$$

$$C \cdot t = \ln(X/X_o) \quad (6)$$

where C is a variable function of remaining food, while substrate removal is expressed by the following relationship:

$$-\frac{ds}{dt} = K_2 \cdot X \cdot S$$

$$K_2 \cdot X \cdot t = \ln(S_o/S)$$

where K_2 is logarithmic substrate removal rate when the growth rate becomes substrate dependent.

Linear Growth and Linear Substrate Utilization

Fujimoto (138) has proposed that when a substrate of low solubility is supplied, the growth rate is determined by diffusion of substrate in the medium. If the supply of such a substrate is sufficient, the amount of the actual useful substrate in the medium is kept constant throughout

the cultivation without any relation to the microorganisms. For this case the following equation was derived.

$$\frac{dx}{dt} = \mu \frac{X}{1 + kx}$$

where k is a constant. If the amount of microorganism is very large, the $\frac{X}{1+kx}$ is held constant and therefore the cell growth and also the substrate consumption is linear and the rate is zero order.

According to Krishnan and Gaudy (139) the substrate consumption in batch system follows zero order kinetics under three sets of conditions; in systems with high solids and an adequate nitrogen supply; in systems with no external nitrogen supply; in systems in which protein synthesis is inhibited. Zero order consumption of substrate may be expressed as:

$$-\frac{ds}{dt} = k$$

where the substrate consumption rate is independent of substrate concentration.

Kinetic Constants for Nitrifying Bacteria

Comparison of the values of μ_{max} for nitrifiers given in Table II with typical values for heterotrophs shows K_s for both nitrifiers to be at least an order of magnitude smaller than that of the heterotrophs. The importance of these low K_s values can be seen by examining the Monod equation. Experimentally determined values of K_s for nitrification are much smaller than the ammonia concentrations usually found in wastewater. Under these conditions, the model would reduce to a zero order expression. Such zero-order kinetics have also been observed by a number of workers (101, 114, 119).

TABLE II

SOME TYPICAL VALUES OF MONOD CONSTANTS

Constant	Nitrosomonas	Nitrobacter	Mixed Cultures		Heterotrophs From Activated Sludge With Glucose Substrate
			MH_4^+-N Substrate	NO_2^--N Substrate	
μ_{max} day^{-1}	0.45-2.2	0.28-1.44	0.014-0.027	0.006-0.035	7.2-17.0
K_s mg/ℓ	0.06-5.6	0.06-8.4	0.6-3.6	1.1-2.1	<1 - 187

The other parameters of importance are true cell yield and the specific decay rate. Poduska and Andrews (96) and Stankewich (117) have summarized observed yields from the literature and have reported them to vary from 0.03 to 0.13 mg cells/mg NH_4^+-N oxidized for Nitrosomonas and from 0.02 to 0.07 mg cells/mg NO_2^--N oxidized for Nitrobacter. Consequently, the true cell yield lies within the same ranges. There have been relatively few values of decay rate reported in the literature. However, Poduska and Andrews (96) have estimated it to be 0.005 hr^{-1} for both Nitrosomonas and Nitrobacter. From theoretical considerations on the thermodynamics of growth, Stratton and McCarty (140) estimated yield values to be 0.29 and 0.084 for Nitrosomonas and Nitrobacter respectively.

Nitrification in Activated Sludge Systems

Biological control of nitrogen in wastewater nitrification is an essential process to reduce the oxygen demand of ammonia and organic nitrogen or as the preliminary step to complete nitrogen removal by denitrification.

Nitrification with cell recycle can be done in either two ways: in combination with carbon oxidation, or in a separate system following carbon oxidation. What are the relative merits of combined versus separate carbon-oxidation-nification? The question has been considered by Stall and Sherrad, (4, 155) also, through a mathematical model based on the parameter sludge age, θ_c and continuous culture theory (156). It has been shown experimentally by Stover and Kincannon (4) that one-stage combined carbonaceous-nitrification systems could achieve degrees of nitrification comparable to two-stage separate systems when designed and operated

properly. This seems to hold even for treatment of wastewaters with high COD:NH_3-N ratio of 500:50. But Chick (161), while comparing the performance of these two systems with filters, found the separate stage units did not perform as well, for reasons difficult to identify. Stover and Kincannon (4) also compared the amount of sludge produced from the two systems and found that sludge produced in the separate system is greater than the combined system. If sludge disposal is a problem, the latter scheme is preferred. On the other hand, the effect of unpredictable variations in organic or toxicant loading may make the combined system more vulnerable than the separate system (4). Also, if the wastewater contains a very high concentration of NH_4^+-N (100, 157), where the formation of NO_2^--N or NO_3^--N can be high enough to cause inhibition, then a multistage system should be selected where denitrification is used to destroy the end products of the first stage nitrification unit; thereby allowing complete nitrification in the final unit (100, 157).

Sawyer (158) stated that removal through nitrification followed by denitrification represents the most promising method. It has the advantage of returning nitorgen to the atmosphere in its natural form. If this is a preferred system in some situations, a large part of the normal BOD will have to be removed before the wastewater enters the nitrification unit. Sawyer believes that a BOD of 40 or 50 mg/ℓ can be tolerated in the feed to nitrification unit. However, Esfandi and Kincannon (5), from their study on nitrification inhibition, concluded that rapid heterotrophic growth in one-stage system and shock loads of organic carbon source would inhibit the nitrification process. But such an inhibition is only temporary, and nitrification is restored when the system recovers from the consequences. These effects have also been observed in biological towers (6).

Many discrepencies exist in the literature regarding the conditions necessary for nitrification. Stover (159), from his investigations on the removal of nitrogenous oxygen demand in wastewaters, has concluded that at 20°C nitrification ceases in biological processes at a mean cell residence time of approximately three days or less. Hence, a six-day or greater mean cell residence time is required for a two-stage system. For one-stage system, it was suggested that a 10-day or greater mean cell residence time is necessary due to the effect of COD:NH_3-N ratio. Downing, et al. (134) have outlined basic requirements for nitrification, i.e., at least 0.5 mg/ℓ of DO, 8 hr detention time, and a low organic loading.

Nitrification in Attached-Growth System

The incorporation of ammonia-nitrogen into cellular matter and the oxidation of ammonia nitrogen to nitrate-nitrogen can be equitably compared in the fluidized and fixed bed reactor system. Studies by Stover (159) have demonstrated that biological filters and rotating biological filters are excellent processes for the removal of ammonia-nitrogen from wastewaters.

A study by Sirikun (160) has shown that nitrification and carbonaceous oxidation can take place simultaneously in trickling filters, but oxidation of ammonia proceeded best in the lower sections of the tower. Chick (161) demonstrated that complete nitrification of domestic sewage was readily maintained on solid surfaces in columns filled with uniformly sized coke. This study was significant in showing that consistent nitrification of domestic waste was possible on fixed supports. Since the nitrifying organisms are capable of attaching to solid surfaces, fixed

film reactors take advantage of long solid retention times which are inherent in this system. Another study by Grantham, et al. (162) reported that low rate trickling filters successfully maintain high degrees of nitrification. Typical results showed nitrification versus depths for a variety of media. They found that at an organic loading higher than 1600 lb BOD/acre/day, the degree of nitrification dropped below 75 percent for an eight-foot filter depth. Little (6) reported that there exists a definite COD concentration at which nitrification will begin in a biological tower; and in the presence of rapid heterotrophic growth, nitrification did not occur. He also found that under shock loading conditions, COD removal rates could be increased.

The effect of competition for space between the heterotrophs and autotrophs in a trickling filter has been observed by Stover and Kincannon (163). They observed that when a wastewater containing both organic matter and NH_4^+-N is applied to a packed tower, only a fraction of the tower height will be available for nitrification, and the magnitude of that fraction will depend upon both the absolute and relative concentration of the two substrate, organic carbon and ammonia nitrogen. Hence, by varying the COD:NH_3-N ratio, the depth for nitrification to take place could be controlled. In other words, nitrification could be controlled at any depth by controlling the COD:NH_3-N ratio in the incoming waste, as has also been observed by Little (6).

The Rotating Disc System for carbon oxidation and nitrification has several of the assets of suspended growth systems (164, 165, 166). It has been proven to be capable of high carbon and ammonia removals. Stover and Kincannon (165) treated a synthetic waste with a COD:NH_3-N ratio of 250:27.6 and found a 90% COD and almost complete ammonia-N removal.

In a combined carbon oxidation/nitrification rotating biological contactor, Antonie (162) found that nitrification begins when the BOD_5 approaches 30 mg/ℓ. This implies that in a multi-stage system such as the RBC, nitrification will only predominate in the later stages where significant amount of BOD_5 has been removed. Stover and Kincannon (165) have presented data that support this observation. In their study, a six-stage system with five 23.25 in. diameter polystyrene disks in each stage was employed. Results of their study shows that COD removal is virtually completed in the first stage, whereas ammonium oxidation is completed only after the fifth stage. Ammonium oxidation rate has been observed to decrease after the first stage. This is attributed to the fact that nitrogen for heterotrophic cell synthesis is required mainly in the first stage. In the subsequent stages, ammonium removal is due almost entirely to nitrification.

CHAPTER III

MATERIAL AND METHODS

Activated Sludge Nitrification Process

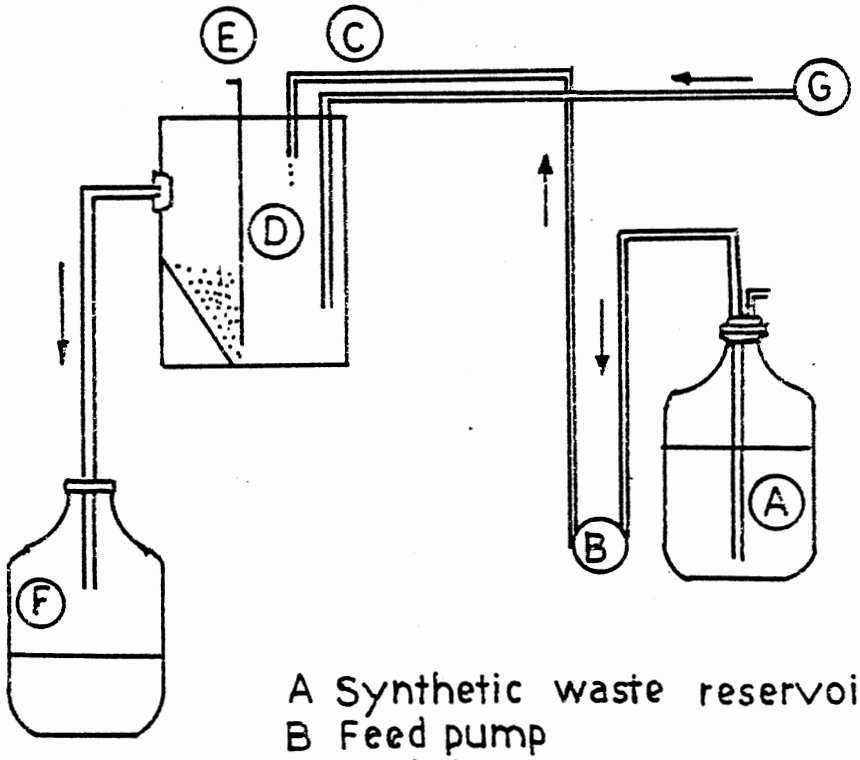
The activated sludge system employed in this study was designed to accomplish both carbonaceous removal and nitrification in the same reactor. A plexiglass pilot plant unit with total internal cell recycle (Figure 1) was used. The reactor consisted of an aeration and settling chamber which were separated by an adjustable plexiglass baffle. Oxygen supply and mixing for the biological solids were supplied by compressed air through two diffusers in the aeration chamber. This arrangement also provided "suction" for recycling the settled biological solids from the settling chamber into the aeration chamber. The reactor temperature was maintained at a room temperature of approximately $20^{o}C \pm 2^{o}C$.

The total volume of the reactor was four liters (approximately three liters aeration chamber and one liter settling chamber). A variable speed Cole-Parmer Master-flex tubing pump (Model No. 7013) provided continuous flow of wastewater to the system at a rate of 5.8 ml/min. This provided an overall detention time of 11.2 hours (approximately eight hours aeration and three hours settling).

The effluent flowed by gravity from the settling compartment to the holding tank, where it was collected.

Sludge from Tulsa municipal wastewater treatment plant was used to provide the initial inoculum for the reactor.

Figure 1. Schematic Flow Diagram of a Laboratory-Scale Continuous Flow Activated Sludge System With Total Internal Recycle



A Synthetic waste reservoir
B Feed pump
C Feed line
D Aeration chamber
E Baffle
F Effluent reservoir
G Air supply

Composition of the synthetic wastewater applied during continuous-flow operation is shown in Table III. Glucose was used as the carbon source since it is easily metabolized, and it provides an excellent carbon and energy source. The nitrogen source was provided in the form of ammonium sulfate. Concentrated stock solutions were used to provide the desired concentration of feed. These solutions were prepared for ease of handling and storage, and were kept at a temperature of $1^{o}C$ in closed sterile containers to avoid contamination and growth. Whenever the feed solution was changed, the container was cleaned with chromic acid cleaning solution and rinsed with tap water to minimize microbial growth. The feed line and pump were also disinfected by pumping five-percent clorox solution for a few minutes and tap water before being used for pumping the feed solution to the reactor.

After acclimation, the unit was operated at a slow growth rate, i.e., a high mean cell residence time of 10 days. The growth rate was controlled by the daily wasting of sludge. The growth rate is defined as

$$\mu_n = \frac{F_W X_R + (F - F_W)X_e}{VX}$$

and the sludge waste flow rate is

$$F_W = \frac{\mu_n VX - FX_e}{X_R - X_e}$$

where:

F_W = waste sludge flow rate

μ_n = observed growth rate

V = volume of reactor

F = influent flow rate

TABLE III

COMPOSITION OF SYNTHETIC WASTEWATER

Constituents	Amount
Glucose	500 mg/ℓ
Ammonium Sulfate $(NH_4)_2SO_4$	250 mg/ℓ
Magnesium Sulfate, $MgSO_4$-$7H_2O$	50 mg/ℓ
Ferric Chloride, $FeCl_3 \cdot 6H_2O$	0.25 mg/ℓ
Manganous Sulfate, $MnSO_4 \cdot H_2O$	5.0 mg/ℓ
Calcium Chloride, $CaCl_2$	3.75 mg/ℓ
1 M Phosphate Buffer Solution, pH 7.0	5 ml/ℓ
Tap Water	100 ml/ℓ

X_e = effluent suspended solids

X_R = waste solids concentration

X = aeration tank solids

Samples were collected every other day and analyzed for:

1. Influence COD and NH_3-N.
2. Effluent COD, NO_2^--N and NO_3^--N.
3. Mixed liquor suspend solids.
4. Effluent suspended solids.

Isolation and Purification of Pure Cultures

Development and Preparation of Enrichment Media

The enrichment media used for these studies consisted of minimal salt medium with variable carbon source and supplementary substrate. The constituents of the enrichment medium is shown in Table IV. Carbon sources chosen are glucose and soidum acetate. Nitrogen source in the form of ammonium sulfate was in excess relative to assimilatory need. Supplementary substrates, yeast-extract, bacto-peptone and beef-extract are excellent stimulators of bacterial growth (171). Nitrapyrin (N-Serve) was supplied at 20 mg/ℓ so as to inhibit autotrophic nitrification by Nitrosomonas. Acti-Dione, at 100 mg/ℓ effectively inhibited all fungi (172).

All equipment, glassware and media used in this experiment that might have introduced contaminating microorganisms to the cultures were sterilized by autoclaving for 15 minutes to 120 minutes at 15 psi and 121°C or by hot air oven for at least two hours at 160°C. The carbon source and supplementary substrate were sterilized separately from the salt medium and added aseptically.

TABLE IV

CONSTITUENTS OF THE ENRICHMENT MEDIUM

$(NH_4)_2SO_4$	2350 mg/ℓ
$MgSO_4 \cdot 7H_2O$	200 mg/ℓ
$CaCl_2 \cdot 2H_2O$	100 mg/ℓ
$NaHCO_3$	50 mg/ℓ
$Fe\ Cl_3 \cdot 6H_2O$	0.5 mg/ℓ
$Cu\ SO_4 \cdot 5H_2O$	0.5 mg/ℓ
$Zn\ SO_4 \cdot H_2O$	0.5 mg/ℓ
$Mn\ SO_4 \cdot H_2O$	0.5 mg/ℓ
K_2HPO_4	1400 mg/ℓ
KH_2PO_4	800 mg/ℓ
Carbon Source[a]	Variable
Supplementary Substrate[b]	Variable

[a]Glucose or sodium acetate.

[b]Yeast-extrate, Bacto-peptone, or beef-extract.

Isolation of Pure Cultures

In each 250 ml flask with 60 ml of enrichment medium, 2 ml of seed from the bench-scaled activated sludge unit were added and shaken on a rotary shaker at 120 revolutions/min. at room temperature for one week. Duplicate flasks were prepared for each carbon source and supplementary substrate. Each culture flask was tested quantitatively for NO_2^--N and NO_3^--N at the end of the week. Because active formation of NO_3^--N was observed from the culture flasks with sodium acetate and yeast-extract as the substrates, this combination of carbon source and supplementary substrate was later used for routine isolation of the nitrifying heterotroph.

Aliquots of the enrichment cultures grown on sodium acetate and yeast-extract were diluted in a ten fold series to a 10^{-10} dilution. One ml of each dilution was plated out on the same medium solidified by the addition of 1.7% agar. The plates were incubated at 30^oC for as long as 4 days if growth was found to be scanty. The solidified medium was also streaked with the enrichment and incubated at 30^oC until growth appeared.

Separate colonies which appeared to be distinctive were picked and individually tested for nitrifying activity by growing them axenically in the liquid acetate medium of the same composition as before but with the yeast-extract omitted. Nitrogenous components of the yeast-extract could be oxidized (32) and give a positive result to the nitrogen tests. Uninoculated controls were carried out for all the routine isolation procedures, and duplicate flasks were prepared for each isolate.

Since the problem of purity of cultures is of prime importance, great care was taken to be sure there was no contamination. This was done by streaking serially on nutrient agar and also on the solidified medium used for isolation. Each pure culture flask and the uninoculated

control were analyzed for NO_2^--N and NO_3^--N at the end of the week.

Once the responsible bacteria was isolated, stock cultures were maintained separately in the media with the same constituents as before but with sodium acetate and glucose as carbon sources. Daily transfers of about 2 ml of the cultures were made to fresh sterile media. Purity checks were made by spread plate technique at each transfer period. NO_2^--N and NO_3^--N were again tested quantitatively from the stock cultures.

To confirm heterotrophic nitrification, the bacterium was grown in the same medium as previously described with and without the specific inhibitor, Nitrapyrin. Special precautions are taken in the use of this inhibitor (124). The stock solution of nitrapyrin was stored at low temperature to minimize abiological hydrolysis and in a glass bottle with a glass stopper.

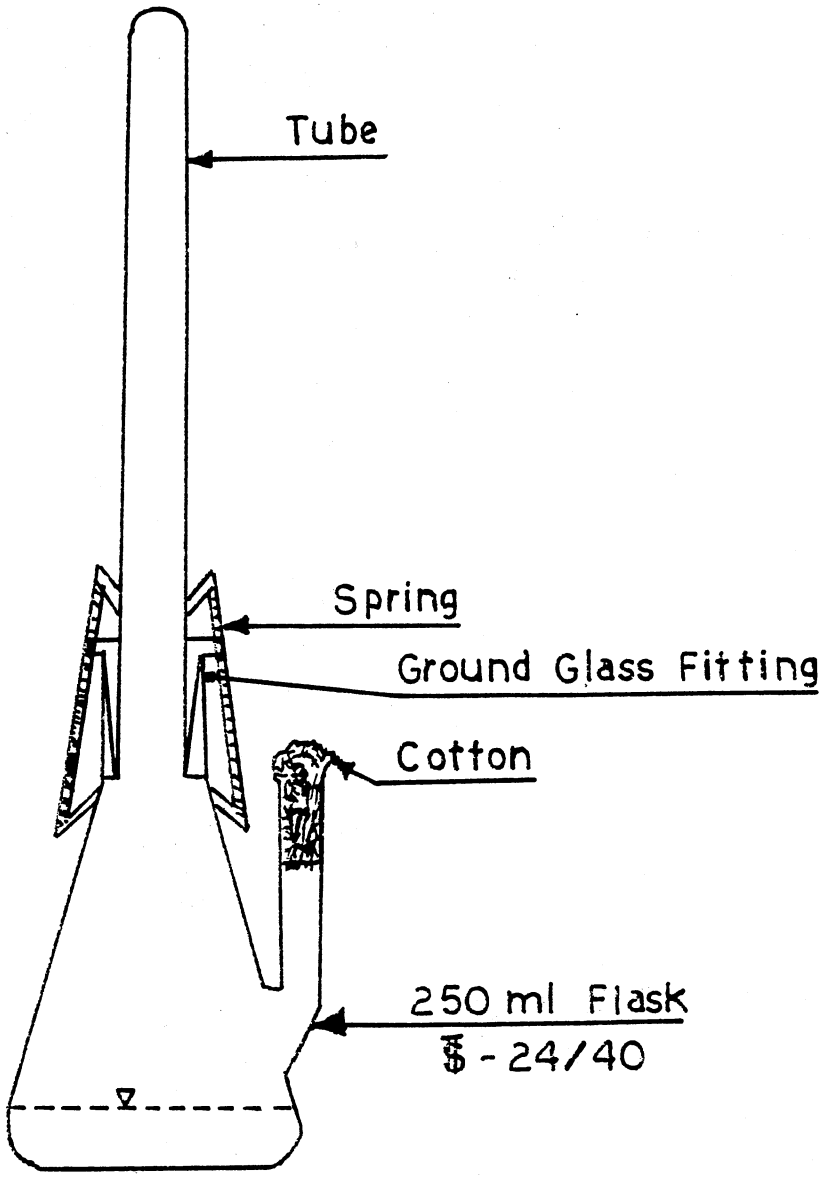
10 mg/ℓ of 2-chloro-6-(trichloromethyl) pyridine (Nitrapyrin) was supplied for the direct inhibition of nitrifying activity with Nitrosomonas as the target organisms. Nitrapyrine was patented as a specific inhibitor of the NH_4^+ oxidation stage of nitrification (120, 121). It was a gift from the Dow Chemical Co., Midland, Michigan, under the trade name, N-Serve.

Pure Culture Batch Studies

Shaken Flask Method

Flasks of special design as shown in Figure 2 were used to culture and measure growth rate by optical density. The flasks were 250 ml capacity Erlenmeyer type with 24/40 $\overline{S}$ tops fitted with optically matched test tubes. A side-arm was attached to the side of the flask. Cotton was used to plug the side arm to maintain sterile flask contents or to

Figure 2. Diagram of Shaken Flask Reactor


Tube
Spring
Ground Glass Fitting
Cotton
250 ml Flask
$ - 24/40

protect the culture from contamination. The optical density of the reaction liquor was determined by inverting the flask and inserting the test tube into the port of the spectrophotometer.

The culture, after acclimation with glucose as carbon source was used for growth study. The glucose concentrations used were 100, 300, 500, 700 and 1000 mg/ℓ. The growth flasks were seeded with 2 ml of acclimated culture which had been transferred to fresh medium 18 to 24 hours prior to being seeded. Duplicate flasks were prepared for each substrate concentration. The initial optical density (O.D.) of each growth flask with the medium was determined at a wavelength of 540 mμ with Bausch & Lomb Spectronic 20. A sterile medium sample was used as a blank (O.D. = 0). Optically density values were measured at least hourly or sometimes at 30 minutes interval until little or no change in O.D. occurred between readings. The purity of the cultures was checked by plating a sample of the suspension on nutrient agar following the run. Upon confirming the purity of the culture, an inoculum from the plate was made into sterile liquid growth medium to acclimate for use in the next study or onto a sterile nutrient agar slant in a culture tube, labeled, grown and then stored at $4^{o}C$ for future use and reference.

The kinetic constants μ, μ_{max}, and K_s were determined experimentally on glucose as carbon source. The method used was that proposed by Monod (130) with a comparative check by the method of Lineweaver and Burk (59) where:

μ = the exponential growth rate.

μ_{max} = the maximum rate of growth.

K_s = the "saturation constant" numerically equal to the substrate concentration S, at which the growth rate, μ, is one half the maximum value, μ_{max}.

A representation of these values is shown in Figure 3.

The value of μ for each substrate concentration, S, was determined from a semi-logarithmic plot of optical density, O.D., versus time, T, in hours. The straight line of the plot with O.D. increading with T represents the exponential growth phase which was used for the calculation of μ as follows:

$$\mu = \frac{\ln 2}{T} = \frac{0.693}{T}$$

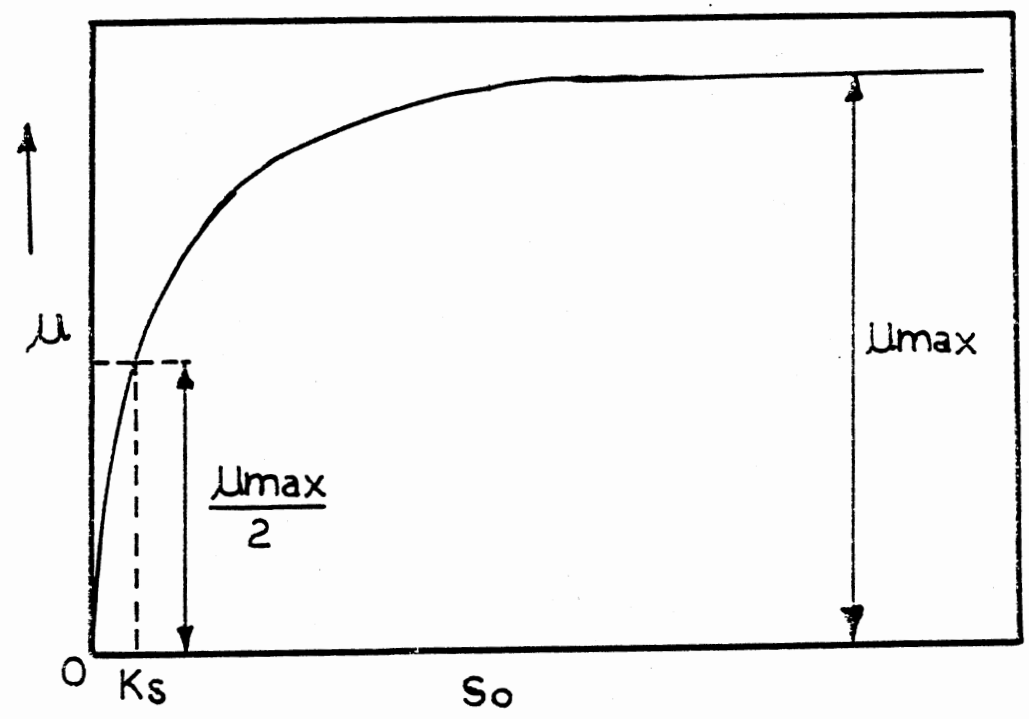
where T = the doubling time or generation time as determined by a two-fold increase in O.D.

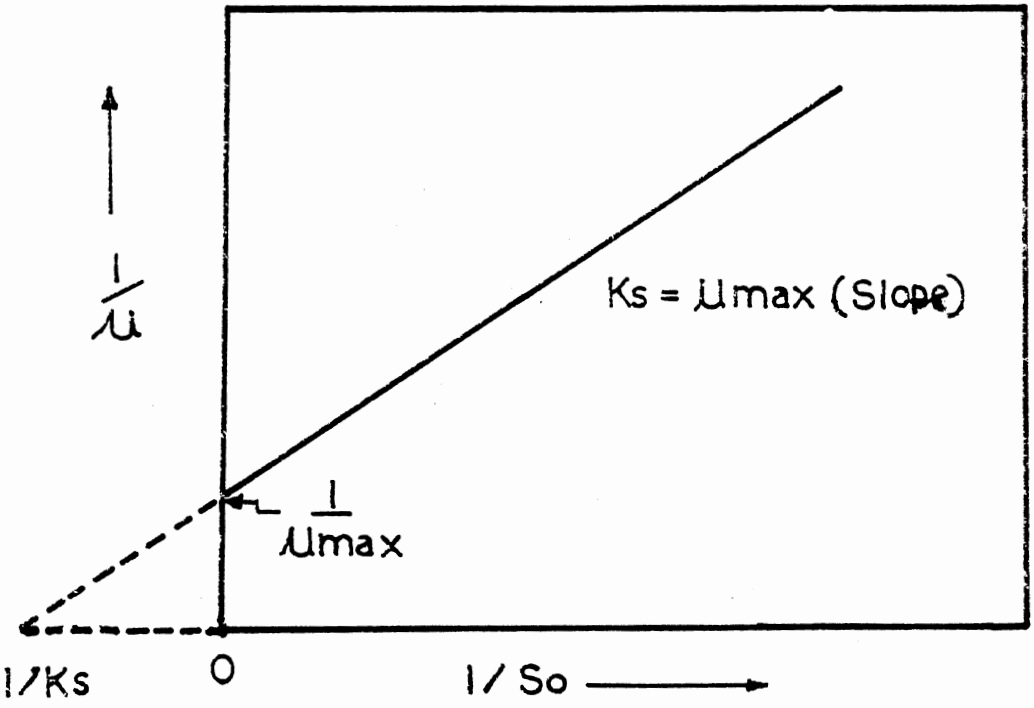
In order to establish a range of C:N ratios for studying the substrate removal characteristics later in the batch bubble aeration reactor, the growth parameters μ_{max}, and K_s were determined in which the limiting growth factor was nitrogen. Shaker flask experiments as described before were used. Each flask was supplied with identical growth media and seed, but differing amounts of nitrogen source to obtain C:N ratios of 1.5:1, 3:1, 5:1, 10:1, 20:1, 100:1 and 200:1. Glucose concentration in each flask was supplied at 500 mg/ℓ, giving a TOC of 200 mg/ℓ.

Bubble Aeration Batch Study

The same procedure for culture acclimation as described for the shaken flask method was used to prepare the inoculum for this type of batch growth study. The major differences between this method and the shaken flask method are the volume of culture, the method of aeration and mixing, the sampling methods, and the sample analysis.

Figure 3. A Presentation μ, μ_{max} and K_s


μ
μmax
μmax/2
0
Ks
So


1/μ
Ks = μmax (Slope)
1/μmax
1/Ks
0
1/ So

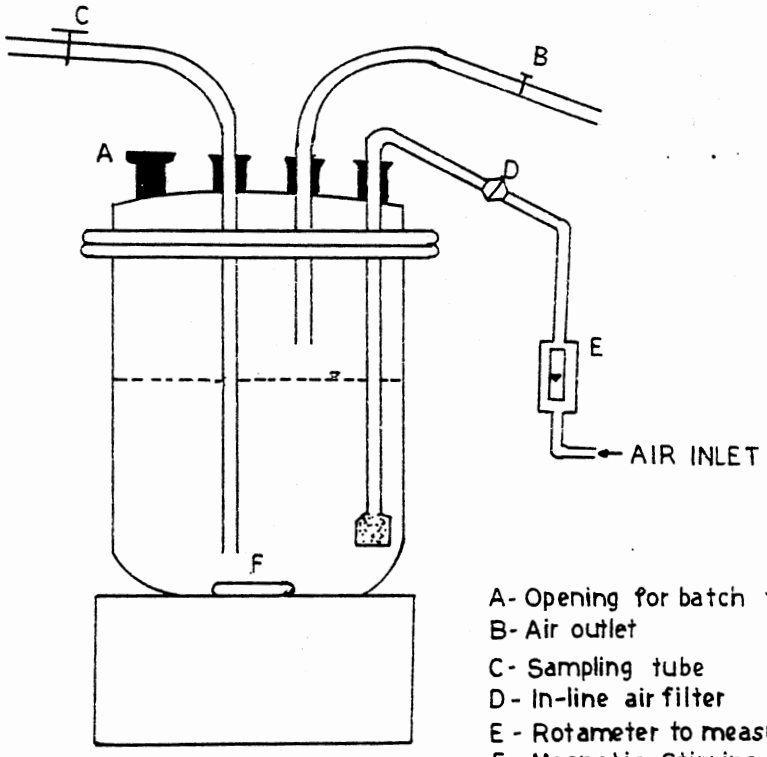
The reactor used was a two-liter cylinderical glass resin kettle having a slightly flattened bottom. The top of the reactor had a ground glass flange which was fitted with a matching cover. The cover contained four openings which were utilized for the necessary attachments for feeding, aeration and sampling (Figure 4).

The medium used was of the same composition as the shaken flask method but with varying C:N ratio. Initial volume of the medium was 1.5 liter. The aeration rate was 2 liters per minute (check by a rotameter). The air was filtered through two tubes packed with glass wool (of which had been autoclaved with the medium reservoir and reactor assembly) before being discharged through a diffuser into the medium in the reactor. Additional mixing of the reactor content was accomplished by magnetic stirrer to drive a Teflon coated metal bar at the bottom of the reactor. The reactor was operated at room temperature of approximately $20^{o}C \pm 2^{o}C$.

Sampling was performed at various time intervals until the formation of NO_3^--N with the removal of NH_3-N has been observed. The samples were removed from the reactor by closing the air outlet and opening a sampling tube which extended vertically into the reactor, thereby causing air pressure increase in the reactor to force a desired volume of the reactor contents up and out through the sampling tube. When the desired volume of sample had been withdrawn, the sampling tube was closed and the air outlet reopened.

The inoculum volume was 15 ml. The purity of the culture was checked by spread plate technique or by streaking on nutrient agar. A specimen colony of the culture was inoculated into sterile liquid growth medium to acclimate for use in the next study or onto a nutrient agar slant in a culture tube, labeled, grown, and then stored at $4^{o}C$ for future use.

Figure 4. Bubble Aeration Batch Reactor



A- Opening for batch feed
B- Air outlet
C- Sampling tube
D- In-line air filter
E - Rotameter to measure flow
F - Magnetic Stirring bar

Samples were analyzed for O.D., pH and biological solids. The sample filtrate (0.45 μm membrane filter) was analyzed for TOC, NH_3-N, NO_2^--N and NO_3^--N. The correlation between O.D. and biological solids is shown in Figure 5.

Analyses and Techniques

Chemical Oxygen Demand

The chemical oxygen demand (COD)of both influent and filtered effluent of the nitrification activated sludge system were determined by using Hach chemicals (Hach Chemical Co., Ames, Iowa).

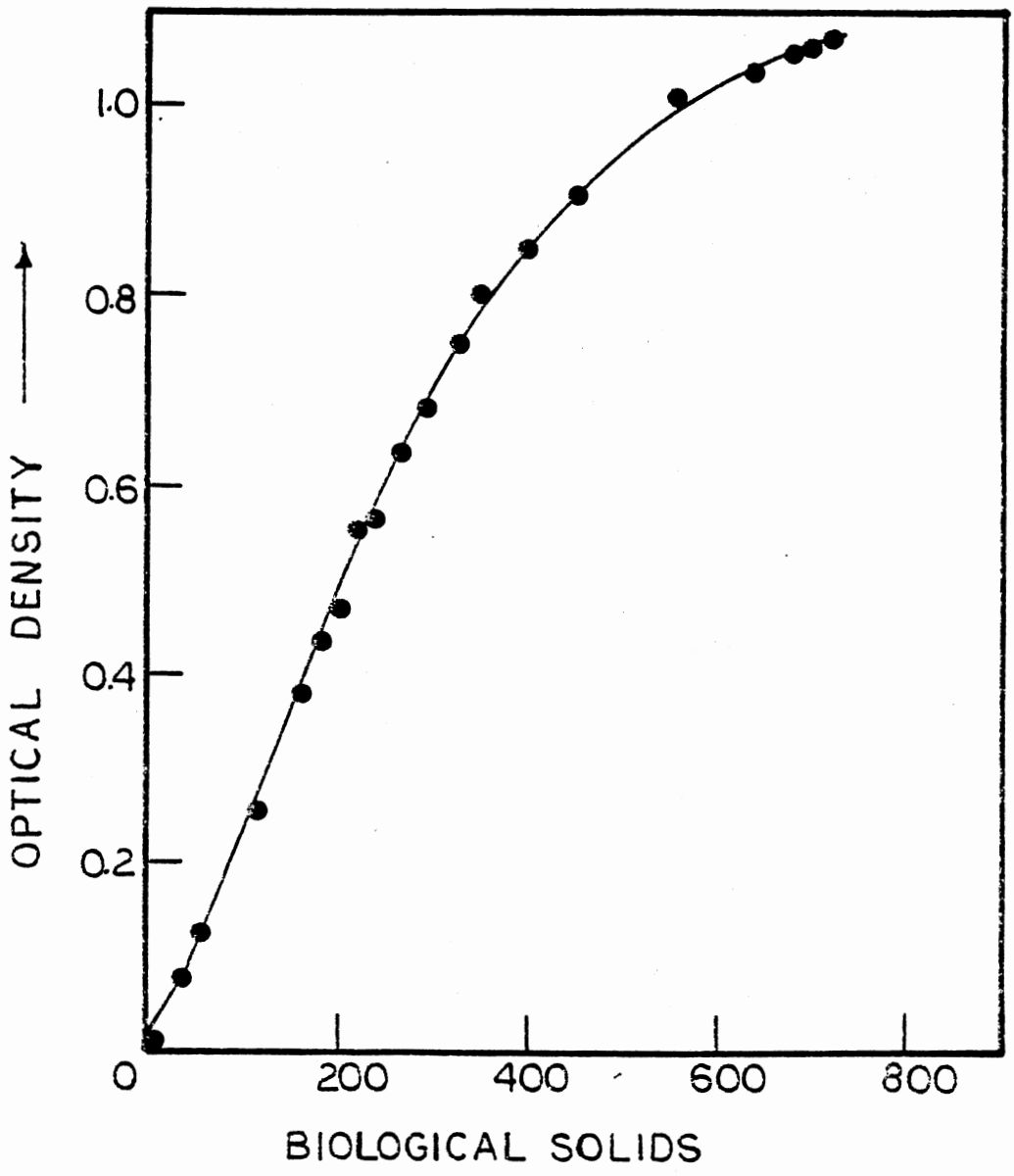
Total Organic Carbon

The Beckman Model 915 Carbon Analyzer was used to determine the TOC concentration of the filtered samples.

Biological Solids

A Sorvall Superspeed Centrifuge, Type SS-IA (Ivan Sorvall, Inc., Bristol, Conn.) was used. Samples were centrifuged at a rate of 10,000 rpm for five minutes. The supernatant was filtered through 0.45 μm pore size membrane filter (Millipore Filter Corp., Bedford, Mass.), and the filtrate was put in cold storage for later analyses of nitrogen contents. The pellet of solids was removed from the centrifuge tube and place on the membrane filter for solids determination. Solids retained on the filter through which the supernatant was filtered and that of the pellet were placed in a 103^{o}C oven for a period of two hours. The samples were then removed and cooled in the desiccator, and weighed again. The biological solids were determined from the difference between initial and final weighing.

Figure 5. Correlation Between Biological Solids and Optical Density


OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0
200
400
600
800
BIOLOGICAL SOLIDS

Measurements of biological solids were also performed indirectly by determining the optical density of a suspension of solids at 540 mμ with a spectrophotometer (Bausch and Lomb, Spectronic 20).

The relationship between optical density and biological solids concentration is given in calibration curve shown in Figure 5.

Spread Plate Technique

A total volume of 0.20 ml of diluted sample was inoculated onto the agar surface in the Petri dish. The inoculated dish was placed on a turntable and rotated while the inoculum was spread evenly over the agar with a bent glass rod. The rod was sterilized prior to spreading the inoculum by inversion in isopropyl alcohol, flaming and cooling.

After inoculation, the plates were covered and allowed to stand until the liquid from the inoculum appeared to have been absorbed into the medium. The plates were then inverted and incubated at $30^{o}C$ for 48 hours.

pH

The pH of the activated sludge unit as well as the batch reactor was monitored as often as every hour, utilizing an Orion pH/MV Meter which was standardized at pH 7.0 and pH 4.0 before use.

Ammonia-Nitrogen, Nitrite-Nitrogen and Nitrate-Nitrogen

All the nitrogen contents of the activated sludge unit and the batch reactor were determined by using Hach Chemicals (Hach Chemical Co., Ames, Iowa).

CHAPTER IV

RESULTS

Operational Performance of One-Stage Nitrifying Activated Sludge System

Performance data for the one-stage activated sludge system operated to achieve both carbonaceous removal and nitrification in the same reactor is presented in this section. The system was operated by controlling the daily amount of sludge wasted so as to maintain a desired mean cell residence time of 10 days. As shown in Figure 6, the system when operated at COD:NH_3-N ratio of 500:50 throughout the study was able to achieve more than 90 percent COD removal. The percent ammonia-nitrogen removed from the system that was converted to nitrate-nitrogen was about 80 percent. A summary of the COD removal characteristics along with the degree of nitrification achieved is shown in Table V. Only weekly averages for all the parameters shown are plotted on the performance flow chart in Figure 6.

This system was operated two months before the intensive isolation of the nitrifying heterotroph and throughout the period from September, 81 to June 82 for supplying the seeds required for isolation.

Isolation, Purification and General Properties of Pure Cultures

In an attempt to isolate a heterotroph capable of nitrifying,

Figure 6. Operational Characteristics of a Continuous Flow Nitrifying Activated Sludge System With Total Internal Cell Recycle at Glucose Concentration of 500 mg/ℓ and Ammonia Concentration of 50 mg/ℓ Operating at a Constant Mean Cell Residence Time of 10 Days

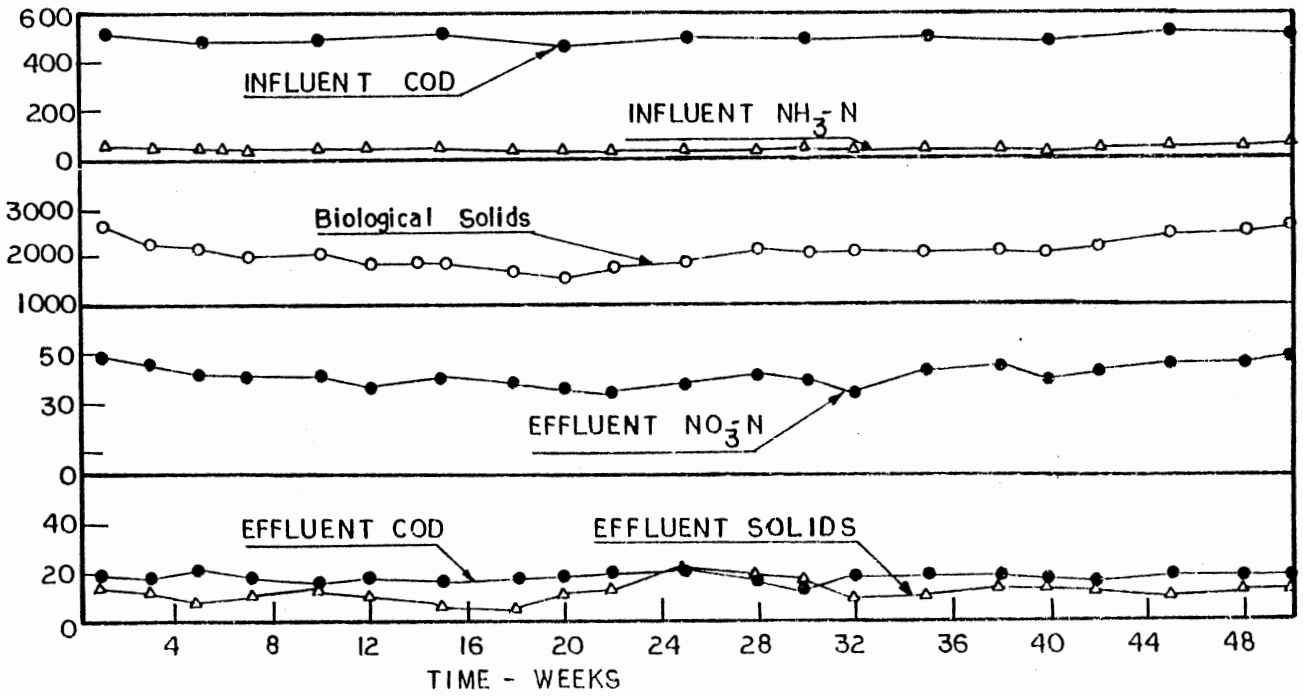

600
400
200
0
INFLUENT COD
INFLUENT NH_3-N
3000
2000
1000
Biological Solids
50
30
0
EFFLUENT NO_3-N
40
20
0
EFFLUENT COD
EFFLUENT SOLIDS
4
8
12
16
20
24
28
32
36
40
44
48
TIME - WEEKS

TABLE V

SUMMARY OF PERFORMANCE DATA FOR NITRIFYING ACTIVATED SLUDGE SYSTEM

Weeks	Si mg/ℓ	Se mg/ℓ	NH_3-Ni mg/ℓ	NH_3-Ne mg/ℓ	NO_2^--N mg/ℓ	NO_3^--N mg/ℓ	pH	θc Days	X mg/ℓ	Xe mg/ℓ
1	510	28	54	0	0.2	48	7.42	11.4	2590	16
2										
3			53	0	0.5	46	7.38	11.8	2340	16
4										
5	480	30	52	0	0.6	42	7.41	14.0	2220	6
6										
7			50	0	0.4	40	7.21	12.0	2090	4
8										
9			50	0	0.2	42	7.12	11.2	2140	10
10	500	20								
11			48	0	0.3	35	7.81	11.6	1908	8
12										
13			52	0	0.8	40	7.13	12.8	1950	4
14										
15	520	26	50	0	0.6	38	7.25	9.6	1710	22
16										
17			46	0	0.4	36	7.42	13.6	1550	20
18										
19			48	0	0.4	34	7.18	15.6	1870	18
20	460	30								
21			50	0	0.6	38	7.36	13.2	1940	26
22										
23			50	0	0.2	42	7.52	13.6	2280	24
24										
25	500	40	50	0	0.8	40	7.12	10.4	2760	40
26										
27			48	0	0.5	34	7.44	8.4	2810	72
28										
29			52	0	0.1	42	7.12	13.5	3120	63

TABLE V (Continued)

Weeks	Si mg/ℓ	Se mg/ℓ	NH_3-Ni mg/ℓ	NH_3-Ne mg/ℓ	NO_2^--N mg/ℓ	NO_3^--N mg/ℓ	pH	θc Days	X mg/ℓ	Xe mg/ℓ
30	500	20								
31			54		0.3	45	7.11	11.8	2590	44
32										
33			52	0	0.2	40	7.41	10.8	2180	28
34										
35	520	18	50	0	2.4	42	7.51	13.4	2230	16

enrichments were prepared as shown in Table VI. Glucose and sodium acetate were supplied at 3800 mg/ℓ and 8500 mg/ℓ respectively. The supplementary substrates, yeast extract, Bacto-peptone and beef extract were supplied at 500 mg/ℓ, 500 mg/ℓ and 300 mg/ℓ respectively to the mixed enrichment cultures. The combination of sodium acetate and yeast extract favored nitrate production after one week of incubation. As shown in Table VI when glucose is used solely as a carbon source, nitrate-nitrogen levels detected were less than 1.0 mg/ℓ while with sodium acetate only, the levels detected were less than 2.0 mg/ℓ. When supplied with supplementary substrates, much higher level of nitrate-nitrogen were detected. The combination of sodium acetate and yeast-extract gave the highest nitrate-nitrogen levels of 3.82 mg/ℓ and 3.64 mg/ℓ from the duplicate cultures. This combination of substrates was later used for routine isolation from the period September 81 to June 82. Table VII shows the descriptions of colony monphology for the bacteria isolates obtained from the enrichment cultures. The pure culture isolates were designated by the month, year and number, S-81-1 for that period they were isolated and tested for NO_2^--N and NO_3^--N production. A total number of 70 isolates were studied. Table VIII shows the residual TOC, NH_3-N and formation of NO_2^--N and NO_3^--N from the pure cultures. Throughout the study, purity of the cultures was checked by spread plate technique before being analyzed for TOC, NH_3-N, NO_2^--N and NO_3^--N. Samples which were susceptible to contamination usually showed a change in color in the culture flask. One type of isolate, the yellow microorganism, was usually present when cultures were contaminated. Once contaminated, the isolation procedure was repeated and the desired colony was picked and grown in the liquid medium again. Due to the ease of contamination and difficulties of maintaining pure cultures, only seven cultures with duplicate growth

TABLE VI

INFLUENCE OF CARBON SOURCE AND SUPPLEMENTARY SUBSTRATES ON MIXED ENRICHMENT CULTURES

Substrates	mg Nitrogen/ℓ Formed							
	Substrate Only		Substrate + YE		Substrate + BP		Substrate + BE	
	NO_2^--N	NO_3^--N	NO_2^--N	NO_3^--N	NO_2^--N	NO_3^--N	NO_2^--N	NO_3^--N
Glucose (3,800 mg/ℓ)	0.16	0.53	0.42	1.53	0.57	1.23	0.32	1.82
	0.12	0.32	0.28	1.20	0.21	1.89	0.16	0.86
Sodium Acetate (8,500 mg/ℓ)	0.32	1.24	0.98	3.82	0.92	1.51	0.28	1.21
	0.10	1.86	0.85	3.64	0.24	0.88	0.12	1.08

YE = Yeast-extract (500 mg/ℓ).

BP = Bacto-peptone (500 mg/ℓ).

BE = Beef-extract (300 mg/ℓ).

TABLE VII

DESCRIPTION OF COLONY MORPHOLOGY FOR THE BACTERIA ISOLATES OBTAINED FROM THE NITRIFYING ACTIVATED SLUDGE SYSTEM FOR THE PERIOD SEPTEMBER, 81 TO JUNE, 1982

Culture	Configuration	Margin	Elevation	Optical Characteristics	Remarks
S-81-1	round	entire	crateriform	transparent	white
S-81-2	round	entire	convex	transparent	light, milky color
S-81-3	irregular	lobate	flat	translucent	grey-white
S-81-4	round	entire	convex	opaque	pink dot in a chain
S-81-5	concentric	wavy	raised	transparent	grey center-slightly greenish margin
S-81-6	filiform	lobate	convex	opaque	colorless edge, slight pink center
S-81-7	round	entire	flat	opaque	colorless
S-81-1	irregular	wavy	drop-like	transparent	white
O-81-2	wrinkled	entire	umbonate	transparent	light orange
O-81-3	round	entire	raised	transparent	colorless to white
O-81-4	irregular	erose	flat	opaque	whitish
O-81-5	rhizoid	wooly	convex	translucent	light brown
O-81-6	round	entire	flat	transparent	slight yellow
O-81-7	round	entire	convex	translucent	beige, round dot in a chain
N-81-1	round	entire	convex	translucent	colorless to white
N-81-2	circular	entire	flat	transparent	hard, white like tiny volcanoes
N-81-3	round	entire	flat	transparent	brown to almost black color

TABLE VII (Continued)

Culture	Configuration	Margin	Elevation	Optical Characteristics	Remarks
N-81-4	circular	entire	convex	translucent center transparent margin	pink purple center and lighter outer margin
N-81-5	circular	entire	convex	transparent	greenish yellow
N-81-6	irregular	lobate	pulvinate	translucent	almost white, but greyish
N-81-7	spindle	undulate	curvex	translucent	colorless
D-81-1	filiform	lobate	raised	opaque	deep blue green
D-81-2	punctiform	undulate	convex	transparent	white at first, then developed copious slime
D-81-3	circular	entire	convex	translucent	beige-white
D-81-4	round	smooth	umbonate	translucent	off-white
D-81-5	circular	curled	convex	transparent	almost yellow
D-81-6	irregular	wavy	convex	translucent center transparent margin	white margin beige at center
D-81-7	wrinkled	smooth	convex	transparent	light brown and shinny
Ja-82-1	round	entire	convex	transparent margin opaque center	colorless margin, white center
Ja-82-2	round	entire	convex	translucent to opaque	yellow-white
Ja-82-3	round	entire	convex	opaque	white
Ja-82-4	round	entire	raised	transparent	almost colorless to very light brown
Ja-82-5	round	entire	flat	transparent	bright yellow
Ja-82-6	round	irregular	drop-like	transparent margin translucent center	colorless to white at center

TABLE VII (Continued)

Culture	Configuration	Margin	Elevation	Optical Characteristics	Remarks
Ja-82-7	round	entire	drop-like	translucent	white
F-82-1	round	entire	convex	translucent iridescent	colorless to very light beige
F-82-2	round	irregular	umbonate	transparent margin opaque center	colorless edge white center
F-82-3	round	entire	drop-like	transparent	colorless
F-82-4	round	entire	convex	translucent iridescent	colorless
F-82-5	round	entire	umbonate	transparent margin translucent center	colorless edge bright yellow center
F-82-6	round	scalloped	convex	translucent edge opaque center	flat colorless edge pink convex center
F-82-7	round	entire	convex	translucent	light brown to almost beige
Mr-82-1	round	entire	flat	transparent	off-white
Mr-82-2	round	entire	convex	transparent	very light brownish yellow
Mr-82-3	irregular	wavy	raised	translucent	greenish blue
Mr-82-4	round	entire	flat	transparent	pink center with slightly white margin
Mr-82-5	wrinkled	entire	convex	translucent margin opaque center	slightly greyish white
Mr-82-6	irregular	lobate	convex	translucent	grey-white
Mr-82-7	round	entire	raised	translucent	colorless to almost white
A-82-1	round	entire	flat	translucent	slightly orange

TABLE VII (Continued)

Culture	Configuration	Margin	Elevation	Optical Characteristics	Remarks
A-82-2	concentric	lobate	flat	transparent margin translucent center	white margin slight blue center
A-82-3	irregular	entire	convex	transparent	orange, almost red
A-82-4	irregular	erose	pulvinate	transparent	slightly purplish blue
A-82-5	round	entire	convex	opaque	almost yellow
A-82-6	round	entire	flat	transparent	colorless
A-82-7	round	entire	convex	transparent	deep pink
M-82-1	irregular	endulate	convex	slightly transparent	colorless
M-82-2	irregular	wavy	convex	transparent	dark green to almost black
M-82-3	irregular	entire	raised	almost opaque	colorless
M-82-4	round	entire	convex	transparent	colorless, almost same color as agar
M-82-5	round	entire	flat	translucent	grey-white
M-82-6	irregular	entire	flat	translucent	colorless
M-82-7	wrinkled	wavy	convex	transparent	very light bluish green
Ju-82-1	round	lobate	raised	opaque center almost transparent margin	almost brown center colorless margin
Ju-82-2	round	entire	convex	translucent	very light beige, almost same color as agar
Ju-82-3	round	entire	umbonate	translucent	almost milky white
Ju-82-4	irregular	curled	convex	transparent	light blue
Ju-82-5	round	entire	convex	translucent	slightly yellow

TABLE VII (Continued)

Culture	Configuration	Margin	Elevation	Optical Characteristics	Remarks
Ju-82-6	irregular	entire	convex	translucent	almost brownish
Ju-82-7	round	entire	raised	transparent	colorless to light pink

TABLE VIII

RESIDUAL TOC, NH_3-N AND FORMATION OF NO_2^--N OR NO_3^--N FROM PURE CULTURES OF BACTERIA OBTAINED FROM THE NITRIFYING ACTIVATED SLUDGE SYSTEM AFTER INCUBATION FOR 7 DAYS IN SHAKER FLASKS WITH TOC CONCENTRATION OF 1500 mg/ℓ AND NH_3-N OF 500 mg/ℓ

Culture	Residual TOC (mg/ℓ)	Residual NH_3-N (mg/ℓ)	NO_2^--N (mg/ℓ)	NO_3^--N mg/ℓ
S-81-1	22	250	0.00	0.00
S-81-1	25	255	0.00	0.00
S-81-2	20	230	0.00	0.00
S-81-2	18	228	0.00	0.00
S-81-3	22	188	0.00	0.00
S-81-3	20	202	0.00	0.00
S-81-4	18	180	0.48	0.00
S-81-4	16	192	0.42	0.00
S-81-5	20	242	0.07	0.12
S-81-5	22	248	0.08	0.10
S-81-6	21	250	0.00	0.00
S-81-6	24	256	0.00	0.00
S-81-7	27	224	0.51	0.11
S-81-7	30	232	0.48	0.13
0-81-1	21	186	0.00	0.00
0-81-1	18	192	0.00	0.00
0-81-2	16	198	0.06	0.12
0-81-2	18	188	0.08	0.12
0-81-3	30	204	0.54	0.00
0-81-3	32	198	0.50	0.00
0-81-4	22	220	0.58	0.16
0-81-4	21	226	0.52	0.14
0-81-5	20	202	0.00	0.00
0-81-5	18	200	0.00	0.00
0-81-6	23	196	0.00	0.00
0-81-6	25	192	0.00	0.00
0-81-7	20	168	0.72	0.11
0-81-7	22	164	0.76	0.12
N-81-1	25	186	0.00	0.00
N-81-1	21	188	0.00	0.00
N-81-2	28	192	0.06	0.12
N-81-2	26	196	0.08	0.11
N-81-3	18	182	0.50	0.00
N-81-3	16	180	0.54	0.00
N-81-4	20	230	0.00	0.00
N-81-4	16	228	0.00	0.00
N-81-5	30	224	0.00	0.00
N-81-5	26	220	0.00	0.00
N-81-6	21	200	0.45	0.16
N-81-6	22	204	0.42	0.12
N-81-7	26	212	0.00	0.00
D-81-1	26	208	0.05	0.00

TABLE VIII (Continued)

Culture	Residual TOC (mg/ℓ)	Residual NH_3-N (mg/ℓ)	NO_2^--N (mg/ℓ)	NO_3^--N mg/ℓ
D-81-1	28	204	0.08	0.00
D-81-2	18	180	0.00	0.00
D-81-2	16	186	0.00	0.00
D-81-3	22	210	0.62	0.00
D-81-3	24	216	0.65	0.00
D-81-4	14	182	0.00	1.2
D-81-4	17	180	0.00	1.4
D-81-5	14	189	0.20	0.14
D-81-5	16	186	0.24	0.18
D-81-6	21	210	0.00	0.00
D-81-6	20	214	0.00	0.00
D-81-7	26	230	0.00	0.00
D-81-7	28	236	0.00	0.00
Ja-82-1	21	232	0.00	0.00
Ja-82-1	18	229	0.00	0.00
Ja-82-2	24	240	0.00	0.20
Ja-82-2	26	236	0.00	0.18
Ja-82-3	20	210	0.52	0.00
Ja-82-3	24	214	0.55	0.00
Ja-82-4	20	180	0.00	0.00
Ja-82-4	20	182	0.00	0.00
Ja-82-5	20	186	0.12	0.00
Ja-82-5	21	180	0.08	0.00
Ja-82-6	22	216	0.48	0.00
Ja-82-6	24	218	0.42	0.00
Ja-82-7	28	220	0.09	0.00
Ja-82-7	26	226	0.12	0.00
F-82-1	28	205	0.70	0.00
F-82-1	28	210	0.74	0.00
F-82-2	30	220	0.12	0.00
F-82-2	30	226	0.06	0.00
F-82-3	32	218	0.00	1.06
F-82-3	34	222	0.00	1.10
F-82-4	22	196	0.00	0.00
F-82-4	18	194	0.00	0.00
F-82-5	24	210	0.08	0.00
F-82-5	24	214	0.08	0.00
F-82-6	28	232	0.00	0.00
F-82-6	26	230	0.00	0.00
F-82-7	25	220	0.62	0.12
F-82-7	22	218	0.66	0.18
Mr-82-1	22	240	0.52	0.00
Mr-82-1	20	240	0.54	0.00
Mr-82-2	20	220	0.08	0.12
Mr-82-2	18	216	0.06	0.10
Mr-82-3	22	200	0.00	0.00
Mr-82-3	20	206	0.00	0.00

TABLE VIII (Continued)

Culture	Residual TOC (mg/ℓ)	Residual NH_3-N (mg/ℓ)	NO_2^--N (mg/ℓ)	NO_3^--N mg/ℓ
M5-82-4	26	198	0.40	0.21
M5-82-4	30	202	0.42	0.18
M5-82-5	21	232	0.00	0.00
M5-82-5	22	228	0.00	0.00
M5-82-6	24	204	0.48	0.00
M5-82-6	26	200	0.44	0.00
M5-82-7	22	210	0.21	0.00
M5-82-7	24	216	0.18	0.00
A-82-1	28	230	0.00	0.00
A-82-1	24	236	0.00	0.00
A-82-2	25	180	0.08	0.22
A-82-2	26	184	0.12	0.18
A-82-3	22	200	0.00	0.00
A-82-3	20	204	0.00	0.00
A-82-4	22	190	0.00	0.00
A-82-4	24	196	0.00	0.00
A-82-5	26	240	0.00	0.00
A-82-5	26	228	0.00	0.00
A-82-6	21	180	0.41	0.00
A-82-6	22	186	0.44	0.00
A-82-7	22	200	0.00	0.00
A-82-7	20	209	0.00	0.00
My-82-1	20	230	0.08	0.00
My-82-1	18	234	0.12	0.00
My-82-2	22	210	0.00	0.00
My-82-2	24	212	0.00	0.00
My-82-3	26	224	0.00	0.00
My-82-3	24	228	0.00	0.00
My-82-4	30	224	0.09	0.00
My-82-4	29	220	0.06	0.00
My-82-5	26	210	0.00	0.00
My-82-5	27	216	0.00	0.00
My-82-6	26	218	0.00	0.00
My-82-6	26	210	0.00	0.00
My-82-7	22	180	0.23	0.00
My-82-7	24	202	0.18	0.00
Ja-82-1	20	215	0.00	0.00
Ja-82-1	22	212	0.00	0.00
Ja-82-2	18	180	0.62	5.84
Ja-82-2	14	182	0.64	5.62
Ja-82-3	20	210	0.00	0.11
Ja-82-3	20	212	0.00	0.14
Ja-82-4	20	180	0.00	0.00
Ja-82-4	22	180	0.00	0.00
Ja-82-5	26	232	0.00	0.00
Ja-82-5	25	228	0.00	0.00
Ja-82-6	20	216	0.42	1.08

TABLE VIII (Continued)

Culture	Residual TOC (mg/ℓ)	Residual NH_3-N (mg/ℓ)	NO_2^--N (mg/ℓ)	NO_3^--N mg/ℓ
Ja-82-6	23	208	0.48	1.24
Ja-82-7	22	203	0.00	0.00
Ja-82-7	18	206	0.00	0.00

flasks for each culture and a control could be studied at a time.

Of the 70 cultures tested for NO_2^--N and NO_3^--N production, only 13 were capable of producing NO_2^--N in excess of 0.4 mg/ℓ which is 10 times the maximum value encountered in the uninoculated control media, and 5 were able to form NO_2^--N in excess of 0.6 mg/ℓ. In terms of NO_3^--N production, only D-81-4, F-82-3 and Ju-82-6 were able to form NO_3^--N in excess of 1.0 mg/ℓ (10 times the maximum encountered in the uninoculated control); while only one culture, Ju-82-2 had proved capable of forming NO_3^--N in excess of 5.0 mg/ℓ. Culture J -82-2 was thus chosen for study in the subsequent experiments. Generically, culture Ju-82-2 has not been identified.

To confirm heterotrophic nitrification, nitrapyrin and sodium bicarbonate were used with the carbon source, glucose and sodium acetate. As shown in Table IX Nitrapyrin had no effect on the NO_2^--N and NO_3^--N production but when supplied with sodium bicarbonate alone without any organic carbon source, the medium did not promote any growth of the heterotrophs.

Pure Culture Batch Systems

Shaken Flask Growth Studies

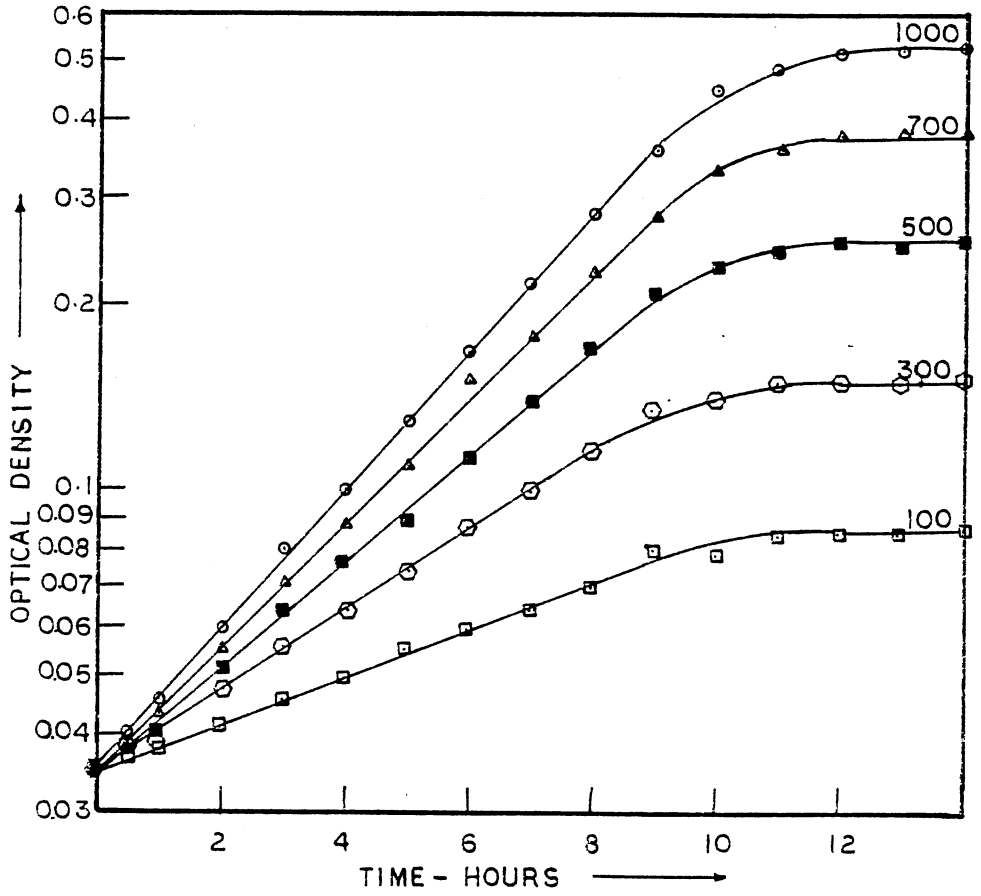
Cultures Ju-82-2 were grown in shaker flasks previously described. The medium used was of the same composition as before but with glucose as the carbon source at concentrations of 100, 300, 500, 700 and 1,000 mg/ℓ. These concentration ranges were sufficient to establish values for μ_{max} and K_s as described before. Growth curves obtained are shown in Figure 7, a semi-logarithmic plot of optical density versus time during the growth period. The initial substrate concentration, S_o, employed for each culture flask is shown on the curves.

TABLE IX

CONFIRMATION TESTS FOR HETEROTROPHIC NITRIFICATION BY CULTURE Ju-82-2

Carbon Source	NO_2^--N mg/ℓ	NO_3^--N mg/ℓ
Glucose only	0.12	2.02
	0.08	2.85
Glucose + Nitrapyrin	0.14	2.12
	0.06	2.64
Sodium Acetate only	0.68	5.12
	0.65	5.04
Sodium Acetate + Nitrapyrin	0.62	6.02
	0.66	5.84
Sodium Bicarbonate only	No Growth	No Growth

Figure 7. Batch Growth Curves at Various Initial Substrate Concentrations of Glucose at 100, 300, 500, 700 and 1,000 mg/ℓ


0·6
0·5
0·4
0·3
0·2
0·1
0·09
0·08
0·07
0·06
0·05
0·04
0·03
OPTICAL DENSITY
1000
700
500
300
100
2
4
6
8
10
12
TIME - HOURS

Figures 8 and 9 illustrate the Monod plot and Lineweaver-Burk plots for the determination of μ_{max} and K_s. These values were found to be in close agreement for the culture when calculated from the two types of plots. The μ_{max} and K_s obtained from the Monod plot were 0.37 hr^{-1} and 220 mg/ℓ while from the Lineweaver-Burk plot, these values were 0.39 hr^{-1} and 232 mg/ℓ, respectively.

Growth studies were also carried out by keeping the glucose concentration constant at 50 mg/ℓ and varying the ammonium sulfate concentrations. Optical density readings did not show substantial variations among the flasks. It was felt that growth ceases after the utilization of glucose, and nitrification occurs after the growth phase might require some form of inorganic carbon. Hence, sodium bicarbonate at 50 mg/ℓ was supplied at the various nitrogen level concentration as before with constant glucose concentration in each flask. As observed before, no obvious difference in optical densities could be read from the growth flasks.

In the later part of the growth study experiments, growth parameters μ_{max} and K_s were determined at a constant glucose concentration of 500 mg/ℓ and varying amounts of nitrogen to give C:N ratios of 1.5:1, 3:1, 5:1, 10:1, 20:1, 100:1, and 200:1. The growth curves are presented in Figure 10. It is seen that although the same concentration, 500 mg/ℓ, of glucose was supplied to each flask, the biological growth obtained was not the same in each flask. Since all the constituents of the medium were identical except for the NH_3-N concentration, the growth in the flasks with C:N ratios of 20:1 or more was limited by nitrogen concentration. The maximum solids concentrations in the flasks containing C:N ratios of 20:1, 100:1, and 200:1 were 150 mg/ℓ, 60 mg/ℓ and 30 mg/ℓ; whereas in the rest of the flasks with C:N ratios of 10:1, 5:1, 3:1, and

Figure 8. Determination of μ_{max} and K_s for Culture Ju-82-2 by Monod Plot

μ_{max}	0.37	hr^{-1}
K_s	220	mg/ℓ

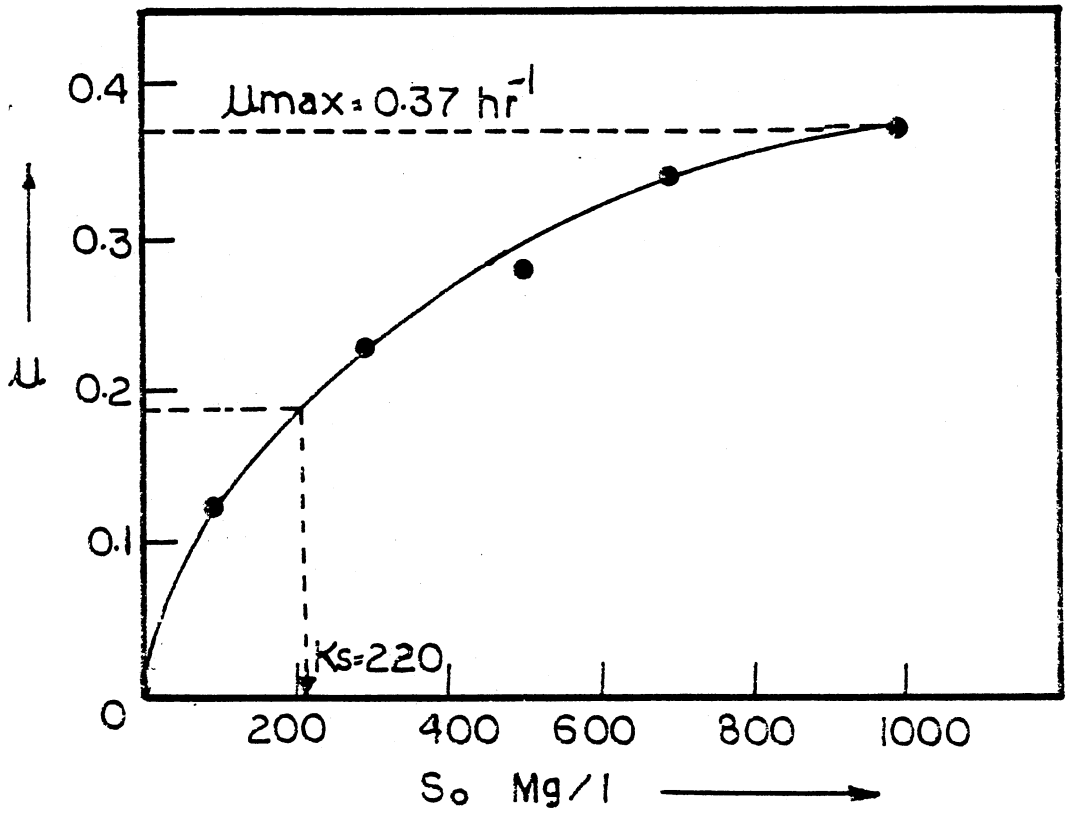

0.4
μmax = 0.37 hr⁻¹
0.3
μ
0.2
0.1
Ks=220
0
200
400
600
800
1000
So Mg/l

Figure 9. Determination of μ_{max} and K_s for Culture Ju-82-2 by Lineweaver-Burk Plot

μ_{max}	0.39	hr^{-1}
K_s	232	mg/ℓ

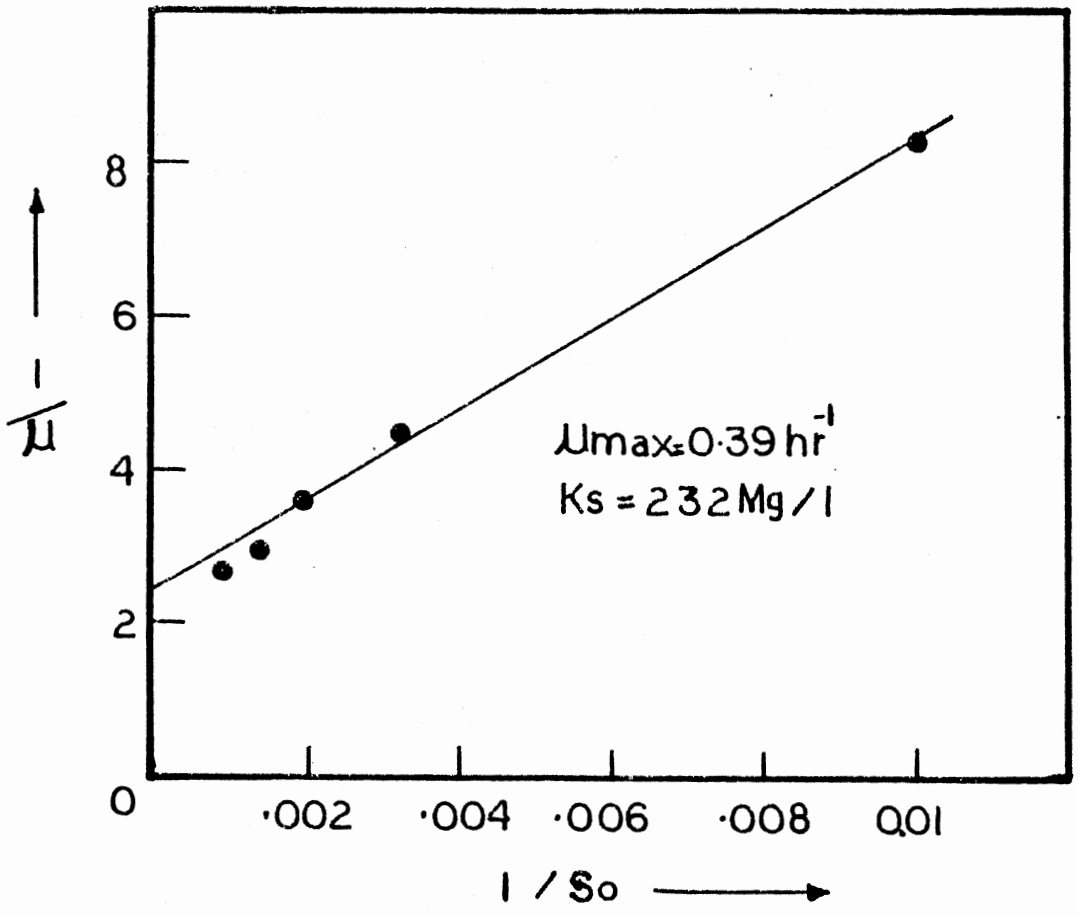
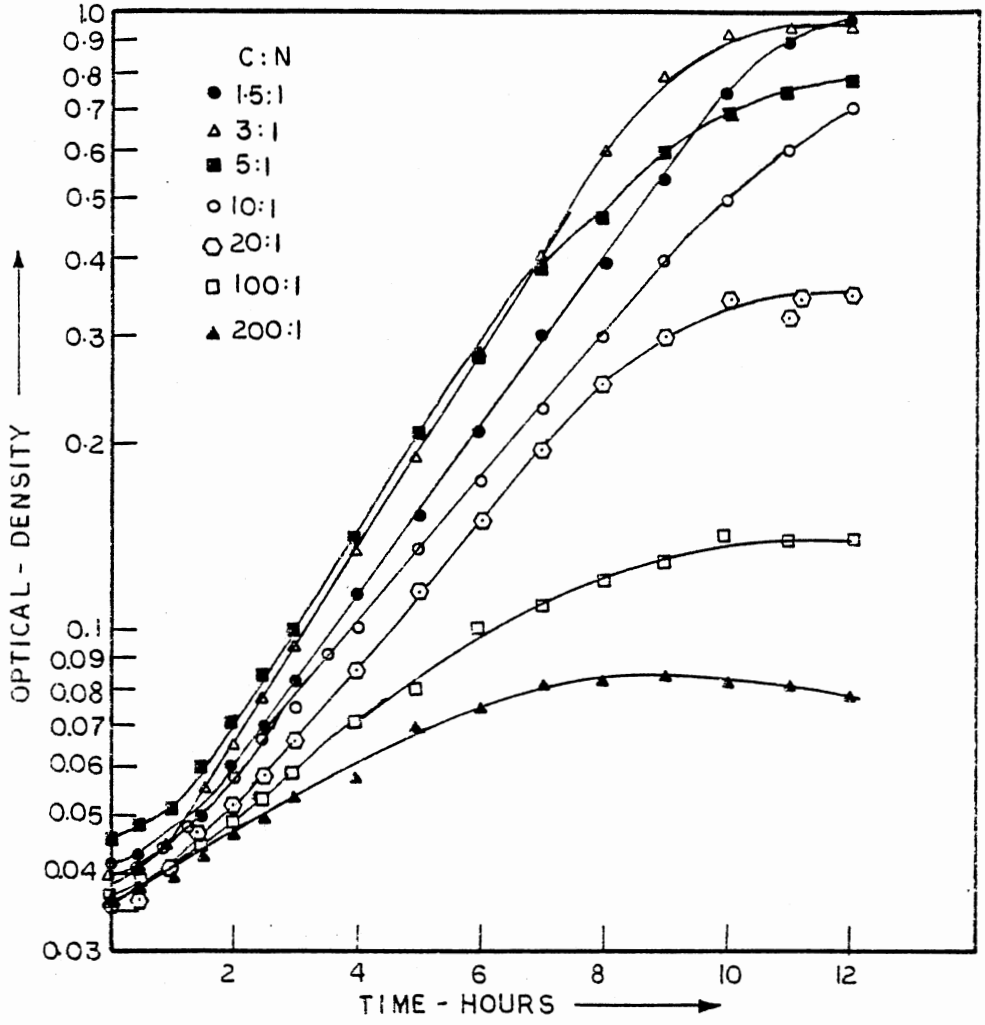

8
6
4
2
0
1/μ
μmax=0·39 hr⁻¹
Ks = 232 Mg/l
·002
·004
·006
·008
·01
1/So

Figure 10. Batch Growth Curves at Various C:N Ratios of 1.5:1, 3:1, 5:1, 10:1, 20:1, 100:1, and 200:1


C : N
1·5 : 1
3 : 1
5 : 1
10 : 1
20 : 1
100 : 1
200 : 1
OPTICAL - DENSITY
TIME - HOURS
1.0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0·03
2
4
6
8
10
12

1.5:1, the maximum solids concentrations were 330 mg/ℓ, 300 mg/ℓ, 510 mg/ℓ and 560 mg/ℓ respectively. These data show that provision of C:N ratios of 10:1 or less would ensure adequate unhindered growth. The specific growth rates observed for C:N ratios of 1.5:1, 3:1, 5:1, 10:1, 20:1, 10:1 and 200:1 were 0.31, 0.37, 0.34, 0.28, 0.27, 0.17 and 0.14 hr^{-1} respectively. μ_{max} and K_s obtained from Monod plots were 0.37 hr^{-1} and 12 mg/ℓ respectively (Figure 11). These values were in close agreement with the Lineweaver-Burk plot for which μ_{max} and K_s obtained were 0.37 hr^{-1} and 11 mg/ℓ respectively (Figure 12).

Bubble Aeration Systems

To determine the influence on nitrification of the C:N ratios of components in the medium, a factor generally affecting the magnitude of nitrification, the concentrations of glucose and ammonium sulfate were varied to give a range of C:N ratios. Substrate utilization characteristics at both high acetate TOC of 1000 mg/ℓ and low glucose TOC of 200 mg/ℓ respectively were studied. By supplying the same TOC concentrations to the growth media, NH_3-N concentrations were varied accordingly to achieve C:N ratios of 3:1, 5:1 and 10:1.

Effects of C:N Ratios

Substrate removal characteristics for which high acetate TOC concentrations of about 1,000 mg/ℓ and different nitrogen levels corresponding to C:N ratios of 3:1, 5:1, and 10:1 are shown in Figures 13, 14, and 15, respectively. These systems were operated at the corresponding nitrogen levels for a period four days. As seen from the linear TOC removal patterns, these systems were able to maintain heterotrophic growths, however, the time taken by these systems for TOC removal was significantly different. At time 26 hours, the 3:1 system was able to bring the TOC level down

Figure 11. Determination of μ_{max} and K_s for Culture Ju-82-2 by Monod Plot

μ_{max}	0.37	hr^{-1}
K_s	12	mg/ℓ

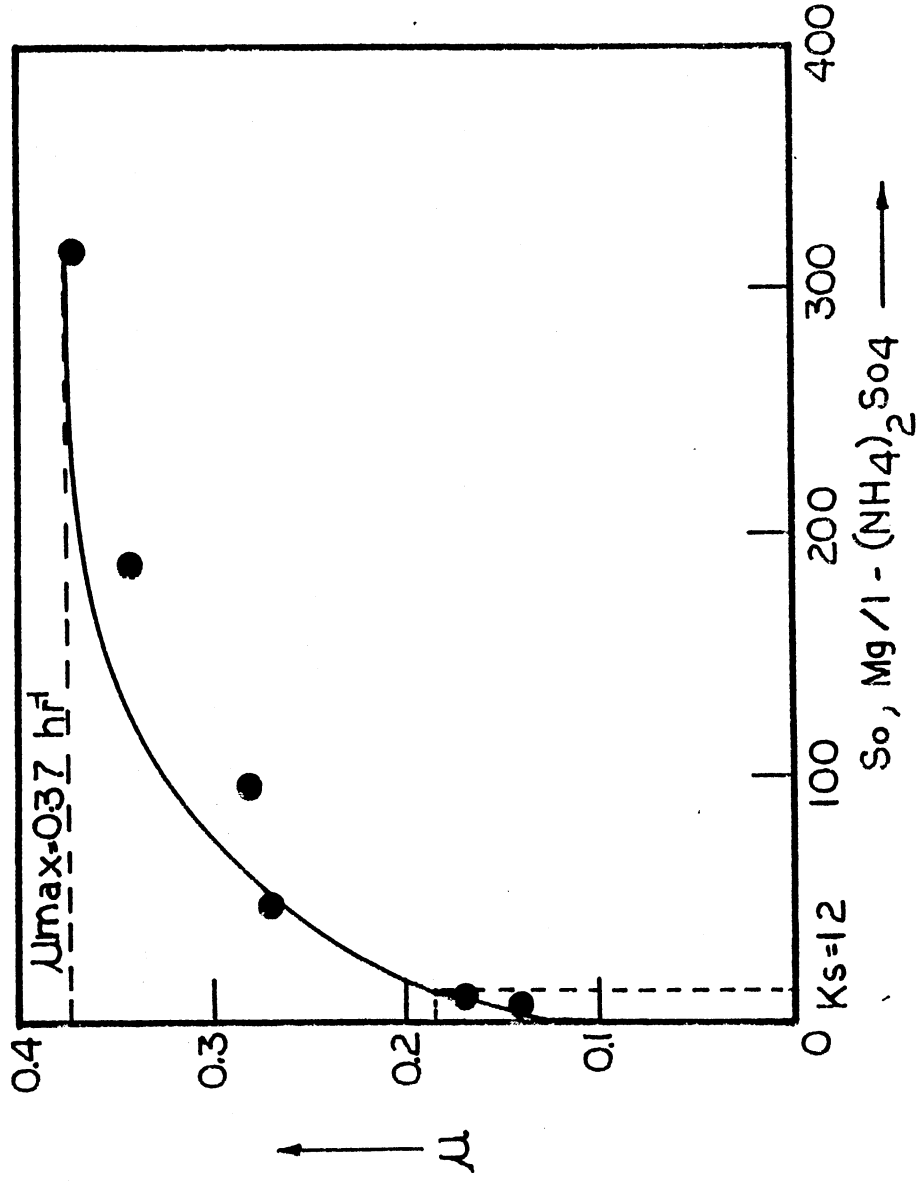

μmax=0.37 hr⁻¹
0.4
0.3
0.2
0.1
μ
0
Ks=12
100
200
300
400
So, Mg/l - (NH4)2SO4

Figure 12. Determination of μ_{max} and K_s for Culture Ju-82-2 by Lineweaver-Burk Plot

μ_{max}	0.37	hr^{-1}
K_s	11	mg/ℓ

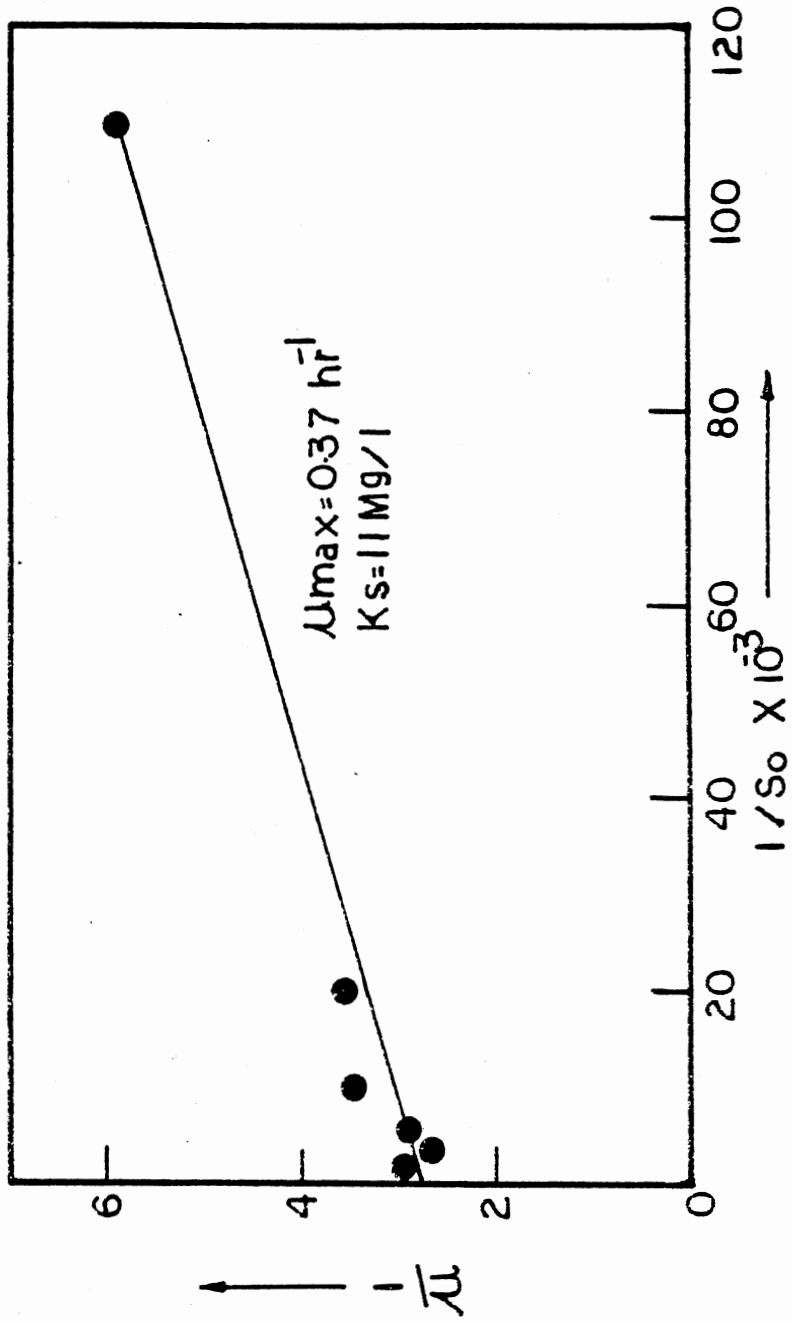

μmax=0.37 hr⁻¹
Ks=11 Mg/l
1/μ
1/So X 10⁻³
0
2
4
6
20
40
60
80
100
120

Figure 13. Substrate Removal Characteristics for Acetate Carbon at C:N Ratio of 3:1

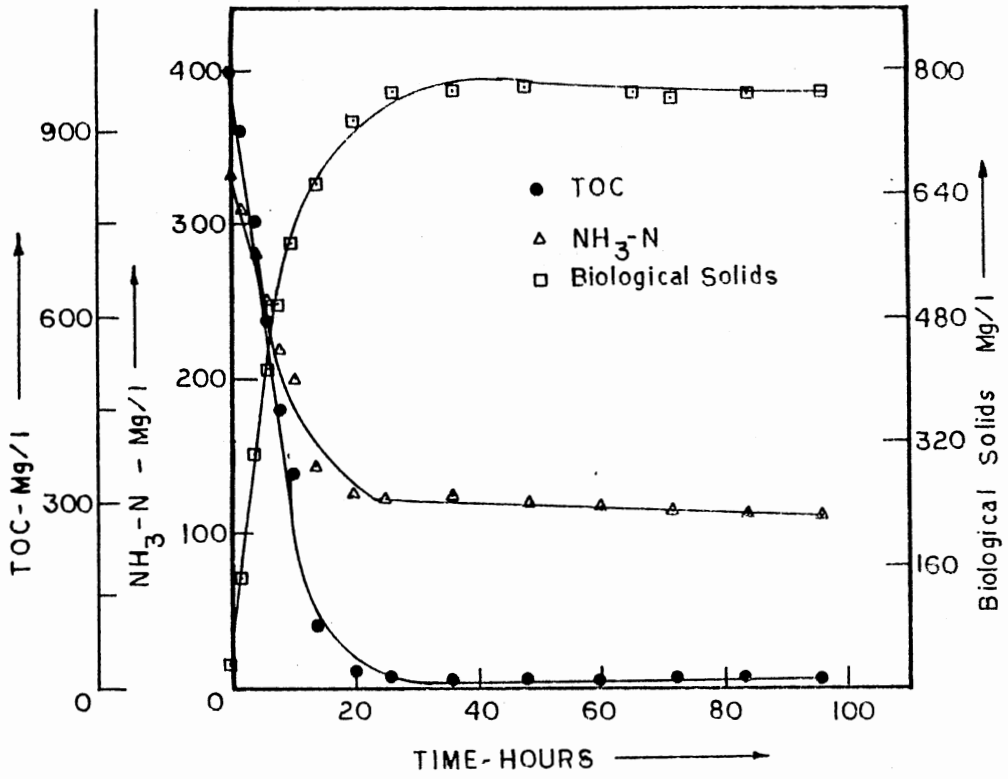

TOC
NH3-N
Biological Solids
TOC - Mg/l
NH3-N - Mg/l
Biological Solids Mg/l
TIME - HOURS
0
300
600
900
100
200
300
400
160
320
480
640
800
20
40
60
80
100

Figure 14. Substrate Removal Characteristics for Acetate Carbon at C:N Ratio of 5:1

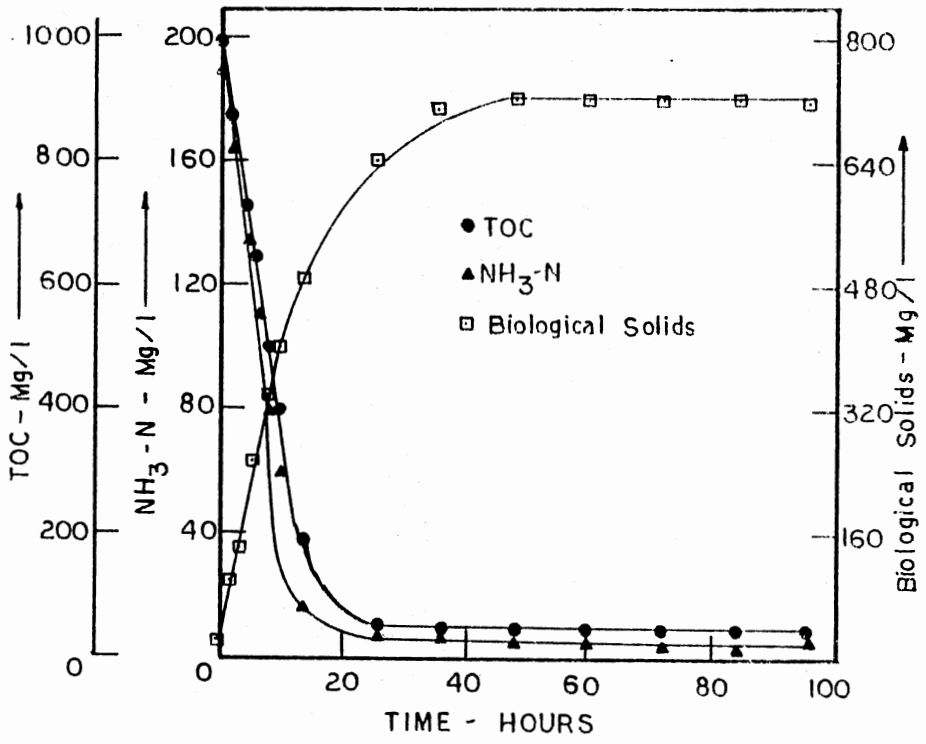
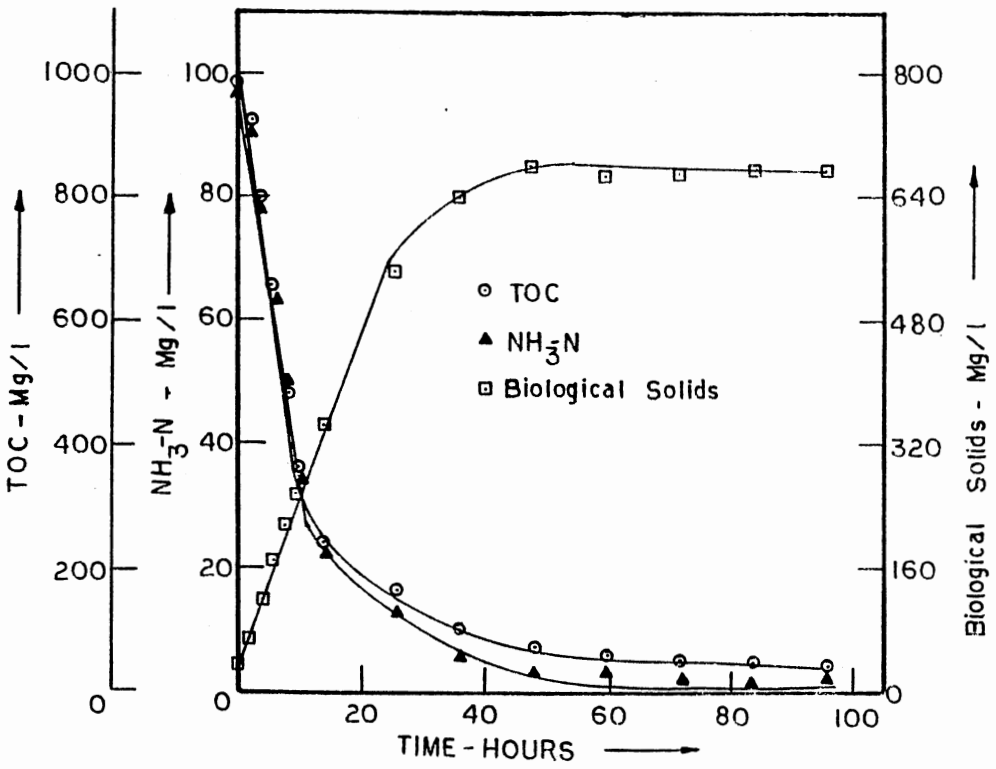

TOC - Mg/l
NH_3-N - Mg/l
Biological Solids - Mg/l
TIME - HOURS
● TOC
▲ NH_3-N
⊡ Biological Solids
0
200
400
600
800
1000
40
80
120
160
200
320
480
640
800
20
40
60
80
100

Figure 15. Substrate Removal Characteristics for Acetate Carbon at C:N Ratio of 10:1


TOC - Mg/l
NH_3-N - Mg/l
Biological Solids - Mg/l
TIME - HOURS
TOC
NH_3-N
Biological Solids
0
200
400
600
800
1000
20
40
60
80
100
160
320
480
640

to 20 mg/ℓ, whereas the 5.1 and 10:1 systems brought the level down only to about 60 mg/ℓ and 160 mg/ℓ respectively. TOC removal rates for these systems are 64, 57 and 53 mg/ℓ/hr respectively (Figure 16).

NH_3-N removals for these systems also follow linear relationships with the removal rates being 13, 12 and 5 mg/ℓ/hr for the 3:1, 5:1 and 10:1 system respectively. Figure 17 shows the NH_3-N removal patterns for these three systems. However, the 3:1 system with NH_3-N supplied in excess relative to assimilatory need, was able to promote nitrification after 26 hours. The appearance of NO_3-N in relation to bacterial growth can be observed from Table X. The concentration of NO_3^--N increased after the growth phase when TOC was brought down to a non-biodegradable concentration of 20 mg/ℓ. Concomitant with the disappearance of NH_3-N after the growth phase, the appearance of NO_3-N became evident. After 4 days, the system was able to nitrify, giving rise to 11.04 mg/ℓ NO_3^--N. Had the system been run for a longer period, a much higher level of NO_3^--N would have been obtained by this system. However, the 5:1 and 10:1 systems had demonstrated only heterotrophic growth without nitrification being taken place. Tables X, XI, and XII show the details of substrate removal characteristics for 3:1, 5:1 and 10:1 system respectively.

Substrate removal characteristics for which low glucose TOC concentrations of about 200 mg/ℓ and nitrogen levels corresponding to C:N ratio of 3:1, 5:1 and 10:1 are shown in Figures 18, 19, and 20 respectively. These systems were operated for a period of 34 hours. These systems were able to maintain heterotrophic growth as the high TOC system previously described, but the time taken to remove TOC was significantly different. At time 6 hours, the 3:1 system was able to lower

Figure 16. Total Organic Carbon Removal for Systems 3:1, 5:1 and 10:1

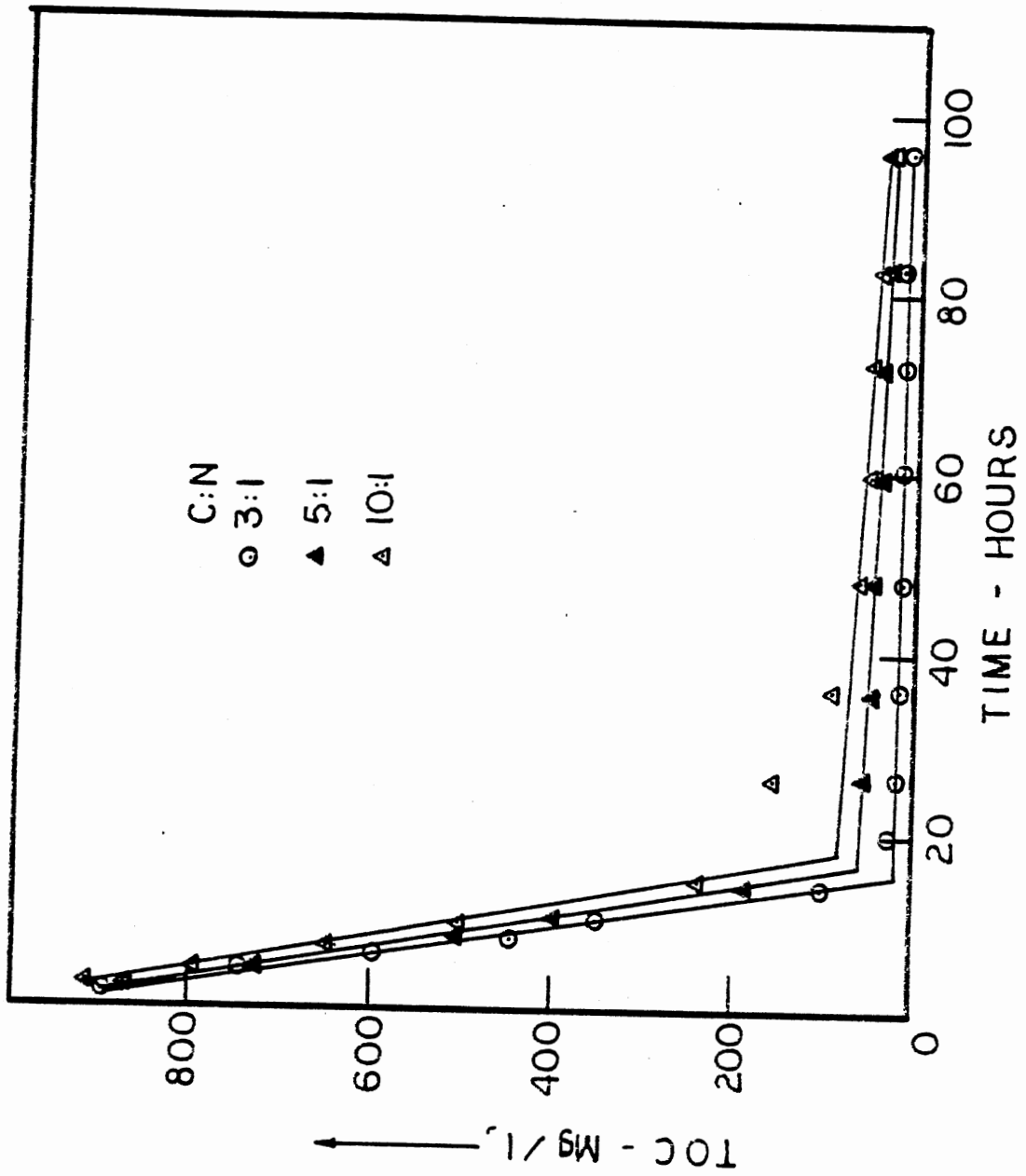

C:N
3:1
5:1
10:1
TOC - MG/L
0
200
400
600
800
TIME - HOURS
0
20
40
60
80
100

Figure 17. Ammonia-nitrogen Removal for Systems 3:1, 5:1 and 10:1

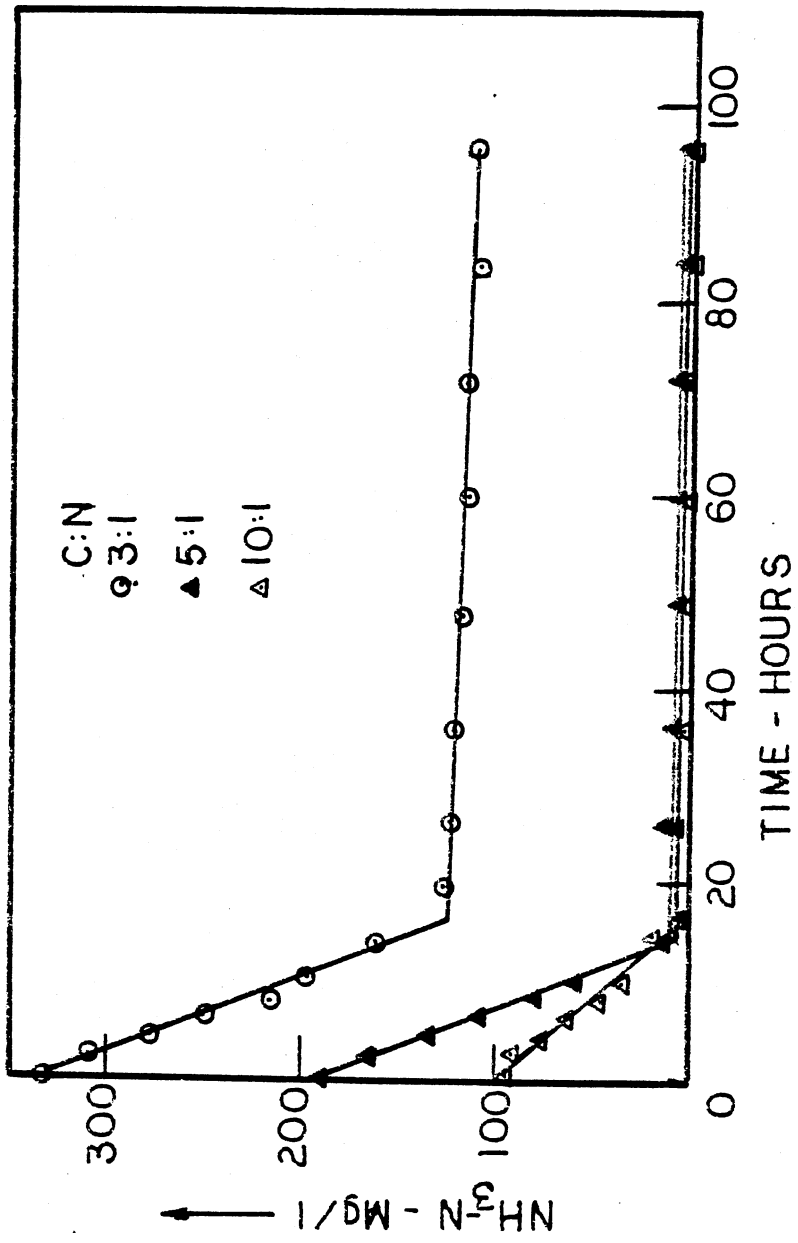

C:N
3:1
5:1
10:1
300
200
100
0
20
40
60
80
100
TIME - HOURS
NH3-N - MG/1

TABLE X

SUBSTRATE REMOVAL CHARACTERISTICS FOR ACETATE CARBON AT C:N RATIO OF 3:1

Time Hours	TOC mg/ℓ	NH_3-N mg/ℓ	NO_3^--N mg/ℓ	pH	O.D.	Biological Solids mg/ℓ
0	998	330	0.00	7.12	0.08	30
2	900	310	0.00	7.15	0.30	130
4	750	280	0.00	7.10	0.70	300
6	600	250	0.00	7.08	0.86	410
8	450	218	0.00	7.10	0.95	500
10	350	196	0.00	7.16	1.00	560
14	100	144	0.00	7.18	1.04	650
20	30	128	0.06	7.09	1.07	720
26	20	125	0.50	7.11	1.08	770
36	20	123	1.80	7.12	1.08	770
48	20	120	3.65	7.16	1.09	780
60	18	118	5.40	7.10	1.09	780
72	18	116	7.25	7.06	1.09	780
84	18	113	8.98	7.02	1.09	780
96	18	110	11.04	7.11	1.09	780

TABLE XI

SUBSTRATE REMOVAL CHARACTERISTICS FOR ACETATE CARBON AT C:N RATIO OF 5:1

Time Hours	TOC mg/ℓ	NH_3-N mg/ℓ	NO_3^--N mg/ℓ	pH	O.D.	Biological Solids mg/ℓ
0	998	190	0.00	7.09	0.06	25
2	870	164	0.00	7.06	0.22	95
4	730	134	0.00	7.06	0.32	135
6	650	110	0.00	7.04	0.60	250
8	500	80	0.00	7.08	0.77	340
10	400	60	0.00	7.14	0.85	400
14	190	16	0.00	7.14	0.95	490
26	60	8	0.00	7.12	1.02	600
36	50	6	0.00	7.18	1.06	710
48	48	6	0.00	7.12	1.07	720
60	48	5	0.00	7.16	1.07	720
72	47	5	0.00	7.12	1.07	720
84	46	4	0.00	7.10	1.07	720
96	46	3	0.00	7.09	1.06	710

TABLE XII

SUBSTRATE REMOVAL CHARACTERISTICS FOR ACETATE CARBON AT C:N RATIO OF 10:1

Time Hours	TOC mg/ℓ	NH_3-N mg/ℓ	NO_3^--N mg/ℓ	pH	O.D.	Biological Solids mg/ℓ
0	992	98	0.00	7.22	0.08	30
2	920	91	0.00	7.20	0.16	70
4	800	78	0.00	7.18	0.28	120
6	650	64	0.00	7.11	0.40	170
8	500	49	0.00	7.16	0.50	210
10	360	34	0.00	7.15	0.59	250
14	240	22	0.00	7.09	0.78	350
26	160	14	0.00	7.12	0.99	550
36	100	6	0.00	7.15	1.04	640
48	70	3	0.00	7.09	1.06	680
60	55	3	0.00	7.08	1.06	680
72	50	2	0.00	7.12	1.06	680
84	50	2	0.00	7.11	1.05	670
96	48	2	0.00	7.16	1.05	670

Figure 18. Substrate Removal Characteristics for Glucose Carbon at C:N Ratio of 3:1

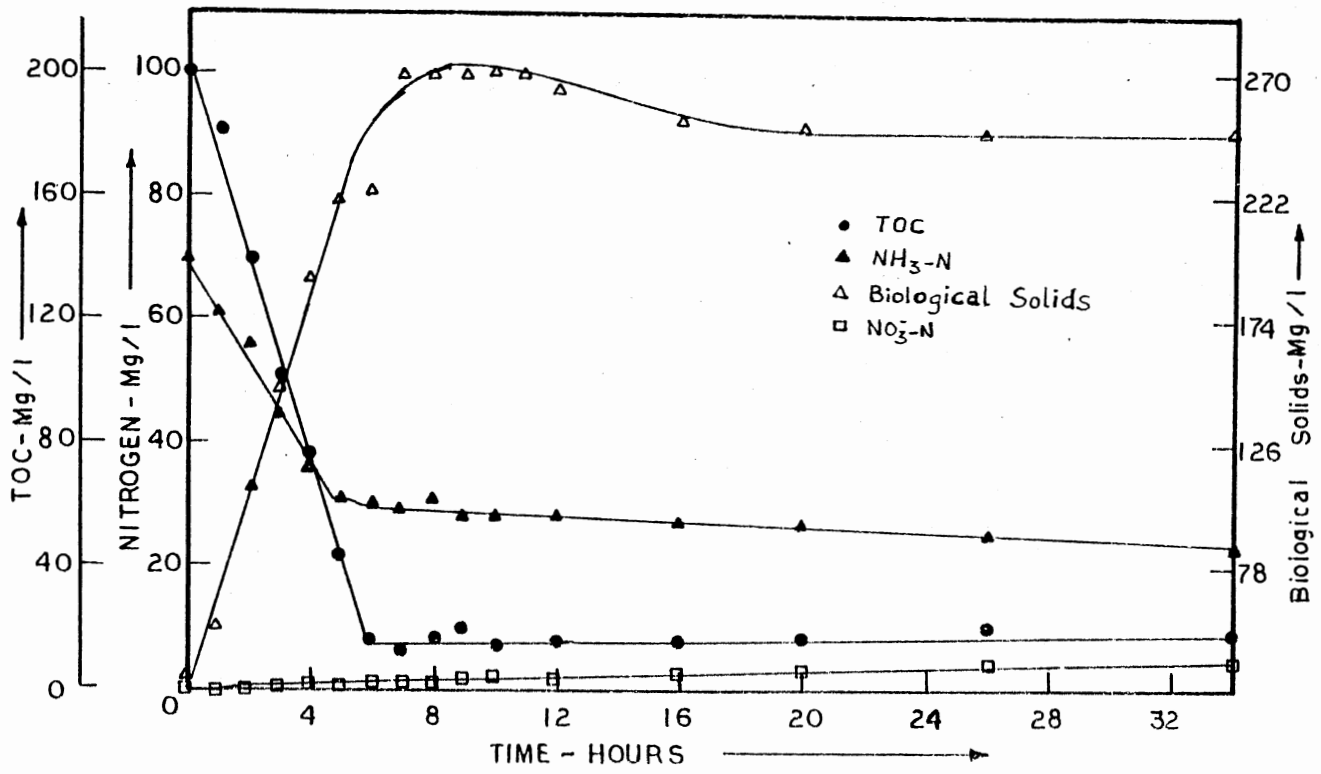

TOC - Mg/l
NITROGEN - Mg/l
Biological Solids - Mg/l
TIME - HOURS
TOC
NH_3-N
Biological Solids
NO_3^--N
0
40
80
120
160
200
20
60
100
126
174
222
270
4
8
12
16
20
24
28
32

Figure 19. Substrate Removal Characteristics for Glucose Carbon at C:N Ratio of 5:1

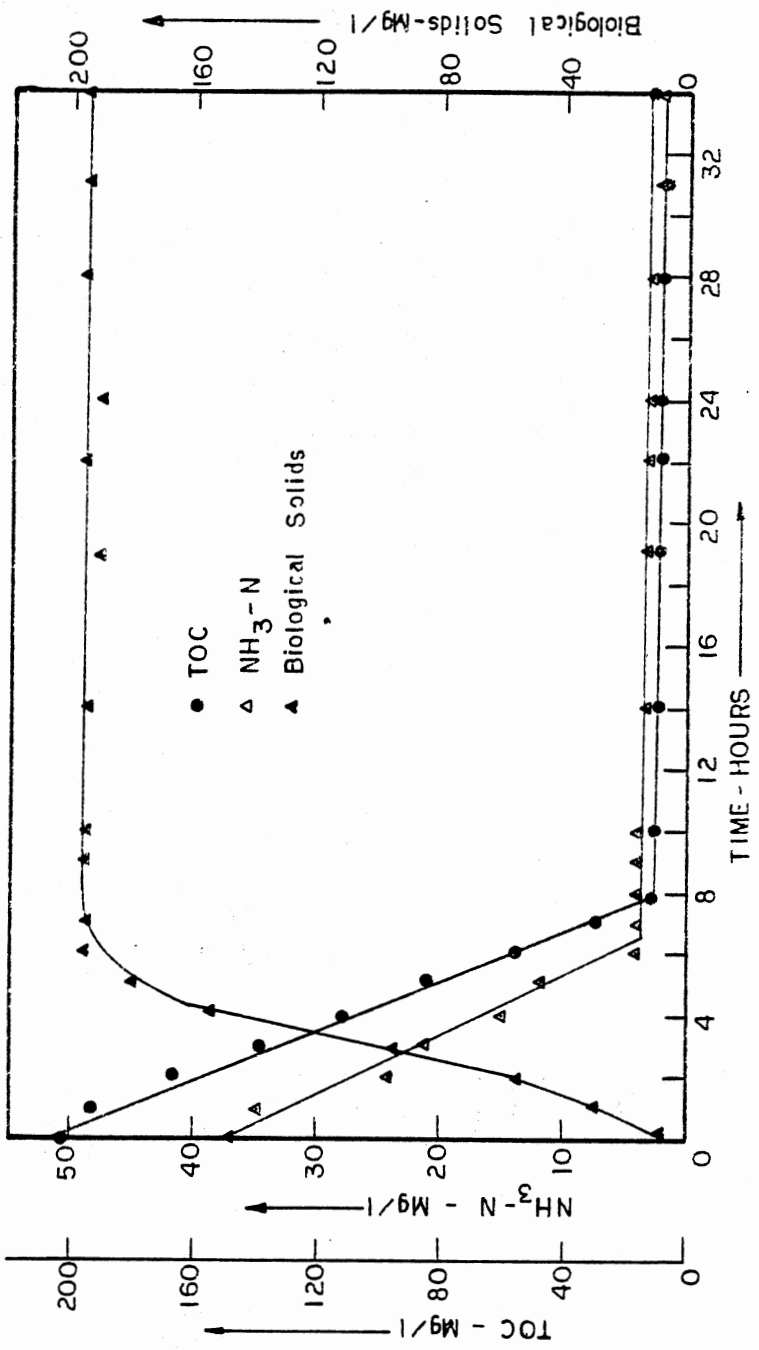
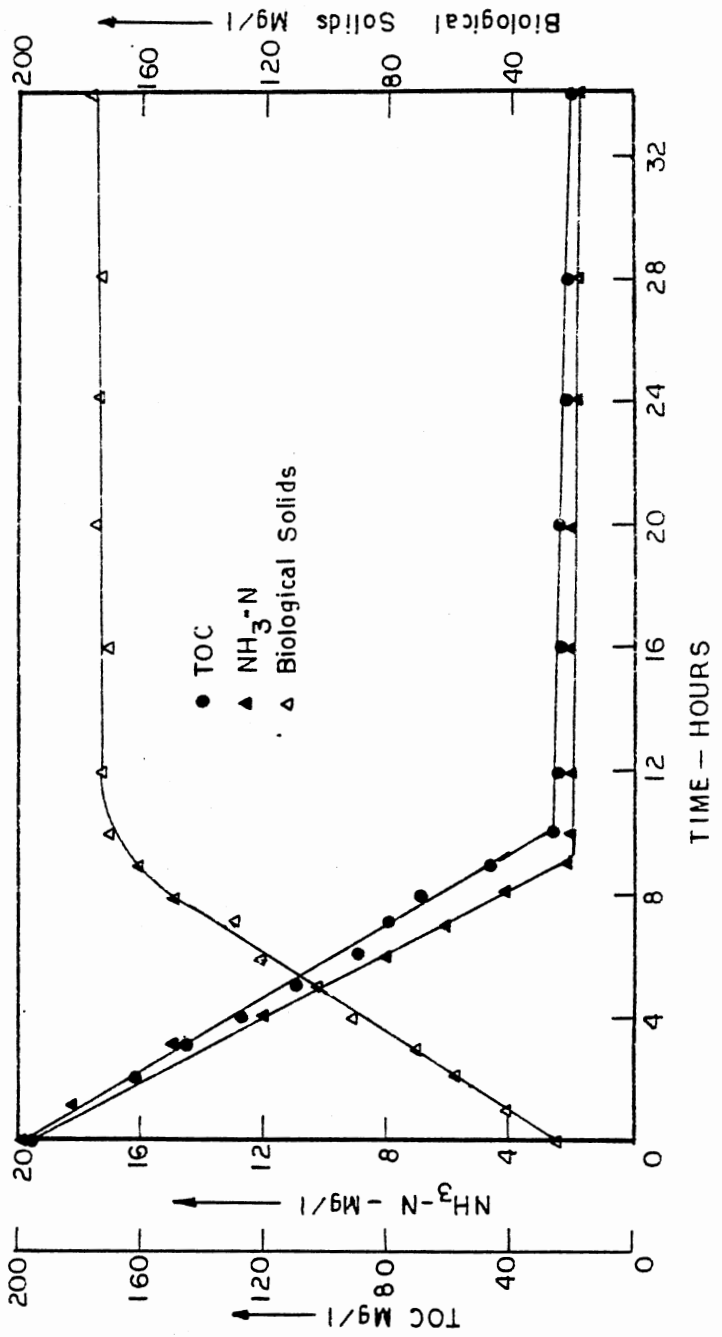

TOC - Mg/l
NH_3-N - Mg/l
Biological Solids-Mg/l
TIME - HOURS
TOC
NH_3-N
Biological Solids

Figure 20. Substrate Removal Characteristics for Glucose Carbon at C:N Ratio of 10:1


Biological Solids Mg/l
200
160
120
80
40
TOC Mg/l
200
160
120
80
40
0
NH3-N - Mg/l
20
16
12
8
4
0
TIME – HOURS
0
4
8
12
16
20
24
28
32
● TOC
▲ NH3-N
△ Biological Solids

the TOC level to 16 mg/ℓ whereas the 5:1 and 10:1 system brought the levels down to only 56 mg/ℓ and 90 mg/ℓ respectively. Consequently, TOC removal rates obtained from these systems are 30, 24 and 17 mg/ℓ/hr as shown in Figure 21. A comparison of NH_3-N removal rates is shown in Figure 22. The removal rates obtained from 3:1, 5:1 and 10:1 system are 6.5, 5.5 and 2.0 mg/ℓ/hr, respectively. However, the 3:1 system with excess NH_3-N was able to promote nitrification as early as 4 hours of incubation. Trace amount of NO_3^--N at 0.2 mg/ℓ was observed at this time and as TOC diminishes with time, nitrification was observed to increase gradually with the formation of 4.80 mg/ℓ NO_3^--N at 34 hour. The other two systems, operating at C:N ratios 5:1 and 10:1 did not promote nitrification even towards the end of the experiment at 34 hours. Tables XIII, XIV, XV show details of substrate removal characteristics with time for the three systems discussed above.

Effects of Glucose Shock Loading

The bubble aeration study was repeated for this study. The system was initially run at C:N of 3:1 since this condition had demonstrated nitrification potential by culture Ju-82-2. The initial TOC and NH_3-N concentrations were 208 mg/ℓ and 72 mg/ℓ respectively. The removal characteristics are depicted in Figure 23. Heterotrophic growth was observed from time zero until time 16 hours. When TOC was brought down to 12 mg/ℓ, the system started to nitrify with the formation of 0.12 mg/ℓ of NO_3^--N. At this point, NO_3^--N increased steadily. At 48 hours, NO_3^--N detected from the system was 5.20 mg/ℓ,while TOC and NH_3-N were 8 mg/ℓ and 25 mg/ℓ respectively. Two hours later, at time 50 hour, the system was shock loaded with glucose, giving a TOC concentration of 78 mg/ℓ.

Figure 21. Total Organic Carbon Removal for Systems 3:1, 5:1 and 10:1

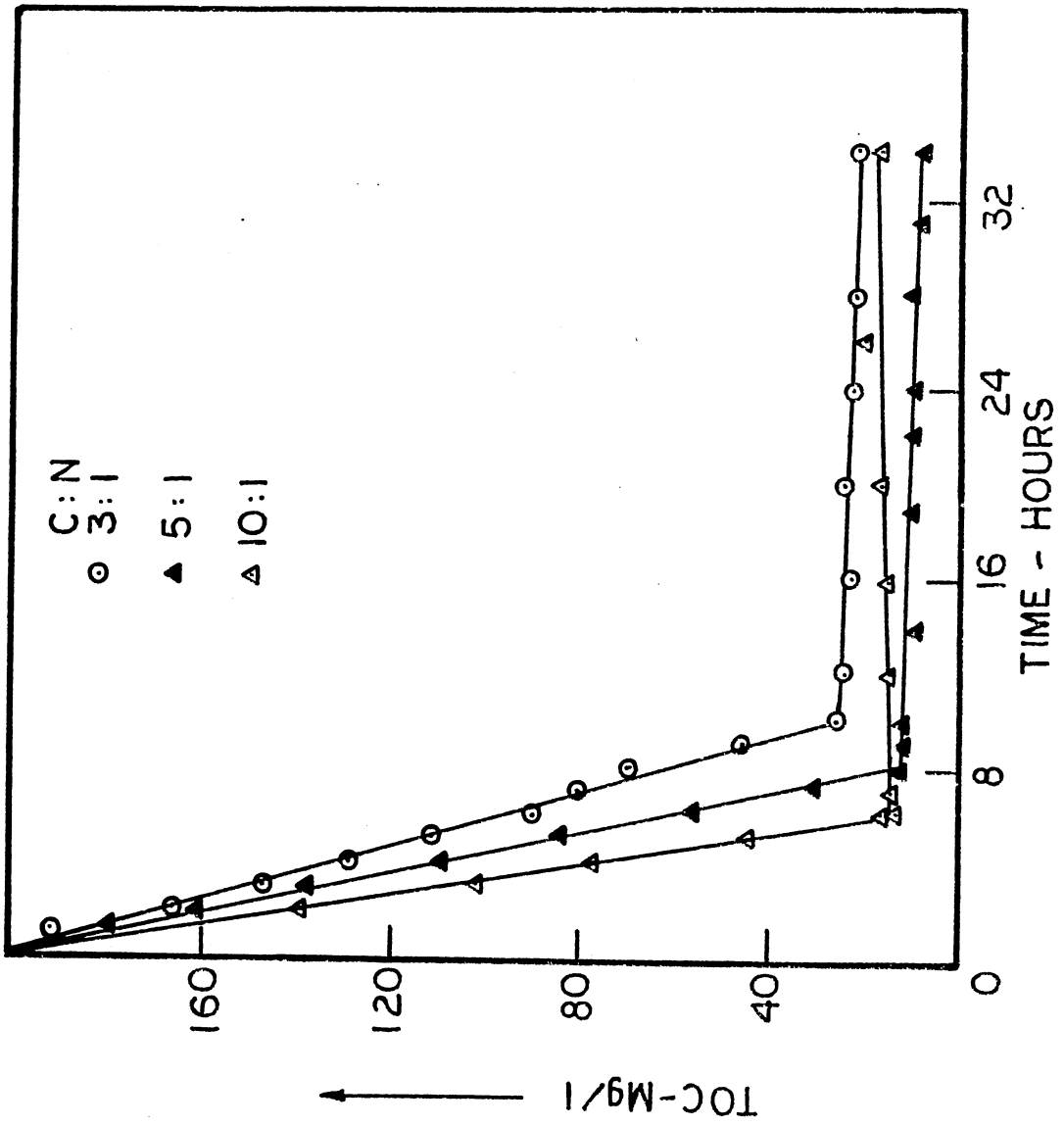

C:N
3:1
5:1
10:1
TOC-Mg/l
160
120
80
40
0
8
16
24
32
TIME - HOURS

Figure 22. Ammonia-Nitrogen Removal for Systems 3:1, 5:1 and 10:1

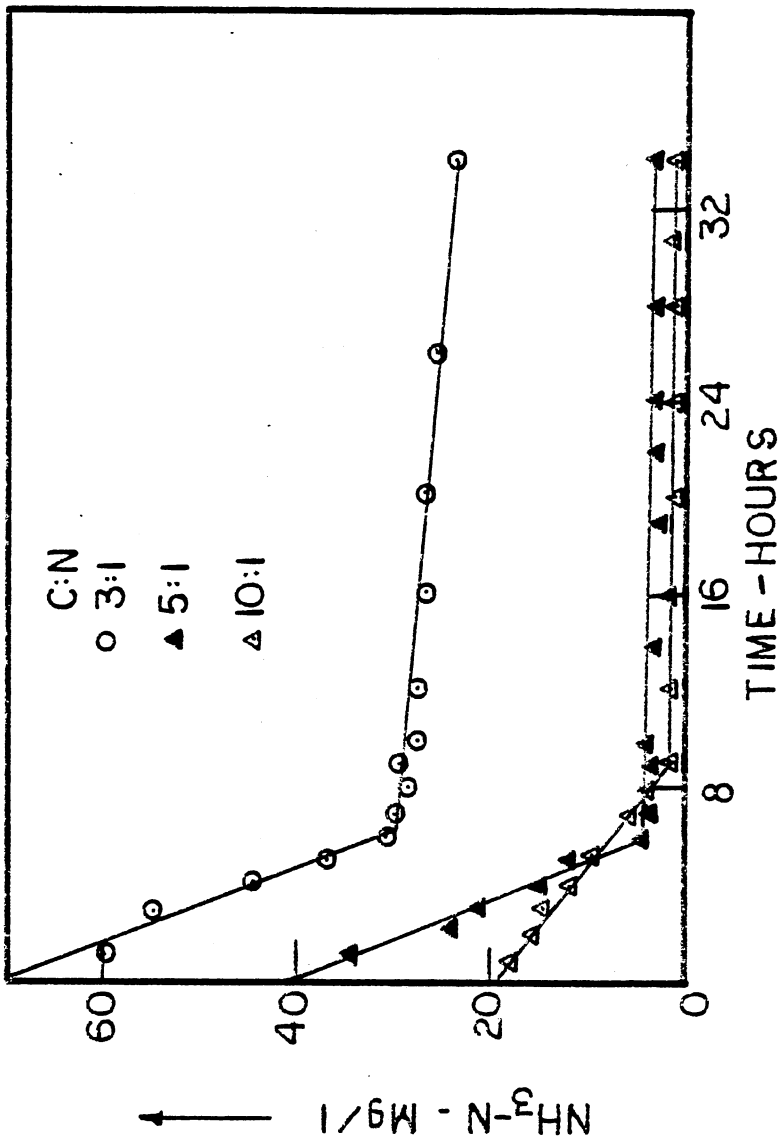

C:N
o 3:1
▲ 5:1
△ 10:1
0
20
40
60
8
16
24
32
TIME - HOURS
NH3-N - Mg/l

TABLE XIII

SUBSTRATE REMOVAL CHARACTERISTICS FOR GLUCOSE CARBON AT C:N RATIO OF 3:1

Time (hr)	TOC (mg/ℓ)	NH_3-N (mg/ℓ)	NO_3^--N (mg/ℓ)	pH	O.D.	Biological Solids (mg/ℓ)
0	200	70	0.00	7.48	0.09	35
1	182	60	0.00	7.45	0.13	55
2	140	57	0.00	7.39	0.17	70
3	102	55	0.00	7.42	0.37	150
4	77	45	0.20	7.33	0.48	195
5	44	37	0.50	7.44	0.55	225
6	16	31	0.50	7.21	0.57	235
7	14	30	0.70	7.25	0.66	270
8	14	29	0.50	7.28	0.66	270
9	13	30	1.00	7.39	0.64	260
10	13	28	1.20	7.21	0.66	270
12	16	28	1.50	7.19	0.64	260
16	16	27	2.10	7.20	0.63	255
20	17	27	2.70	7.18	0.62	250
26	20	26	3.70	7.15	0.63	255
34	18	24	4.80	7.12	0.63	255

TABLE XIV

SUBSTRATE REMOVAL CHARACTERISTICS FOR GLUCOSE CARBON AT C:N RATIO OF 5:1

Time (hr)	TOC (mg/ℓ)	NH_3-N (mg/ℓ)	NO_3^--N (mg/ℓ)	pH	O.D.	Biological Solids (mg/ℓ)
0	203	37	0.00	7.25	0.02	10
1	192	35	0.00	7.22	0.08	30
2	166	24	0.00	7.21	0.13	55
3	138	21	0.00	7.24	0.23	95
4	110	15	0.00	7.09	0.38	155
5	84	12	0.00	7.11	0.44	180
6	56	4	0.00	7.12	0.48	195
7	30	4	0.00	7.14	0.48	195
8	12	4	0.00	7.12	0.48	195
9	12	4	0.00	7.14	0.48	195
10	11	4	0.00	7.21	0.48	195
14	10	3	0.00	7.24	0.48	195
19	10	3	0.00	7.16	0.46	190
22	10	3	0.00	7.21	0.48	195
24	10	3	0.00	7.11	0.46	190
28	10	3	0.00	7.09	0.48	195
31	8	2	0.00	7.06	0.48	195
34	8	3	0.00	7.06	0.48	195

TABLE XV

SUBSTRATE REMOVAL CHARACTERISTICS FOR GLUCOSE CARBON AT C:N RATIO OF 10:1

Time (hr)	TOC (mg/ℓ)	NH_3-N (mg/ℓ)	NO_3^--N (mg/ℓ)	pH	O.D.	Biological Solids (mg/ℓ)
0	198	20	0.00	7.46	0.06	25
1	180	18	0.00	7.46	0.09	40
2	162	16	0.00	7.45	0.13	55
3	146	15	0.00	7.42	0.16	70
4	128	12	0.00	7.31	0.21	90
5	110	10	0.00	7.12	0.27	115
6	90	8	0.00	7.21	0.28	120
7	80	6	0.00	7.19	0.31	130
8	70	4	0.00	7.15	0.36	150
9	46	2	0.00	7.11	0.38	160
10	26	2	0.00	7.12	0.40	170
12	25	2	0.00	7.16	0.40	170
16	24	2	0.00	7.18	0.40	170
20	24	18	0.00	7.21	0.40	170
24	22	18	0.00	7.32	0.40	170
28	22	18	0.00	7.24	0.40	170
34	22	18	0.00	7.22	0.40	170

Samples after shock loading were collected at two-hour intervals. For the first two hours a lag was observed with only 4.0 mg/ℓ of TOC being removed. Solids concentration only increased by 5 mg/ℓ. After this point, TOC and NH_3-N removals with the build up of solids were observed. However, the system did not nitrify during this period of heterotrophic growth as can be observed by the constant NO_3^--N concentration of 5.8 mg/ℓ. However, at time 60 hours, TOC level was brought down to 12 mg/ℓ while removal of NH_3-H was accompanied by nitrification, giving rise to a NO_3-N reading of 6.35 mg/ℓ. The system then gradually nitrified throughout the rest of run with a reading of 9.86 mg/ℓ of NO_3^--N being observed at time 72 hour. Table XVI shows the details of substrate removal at this shock loading condition.

Effect of NO_2^--N on Nitrification

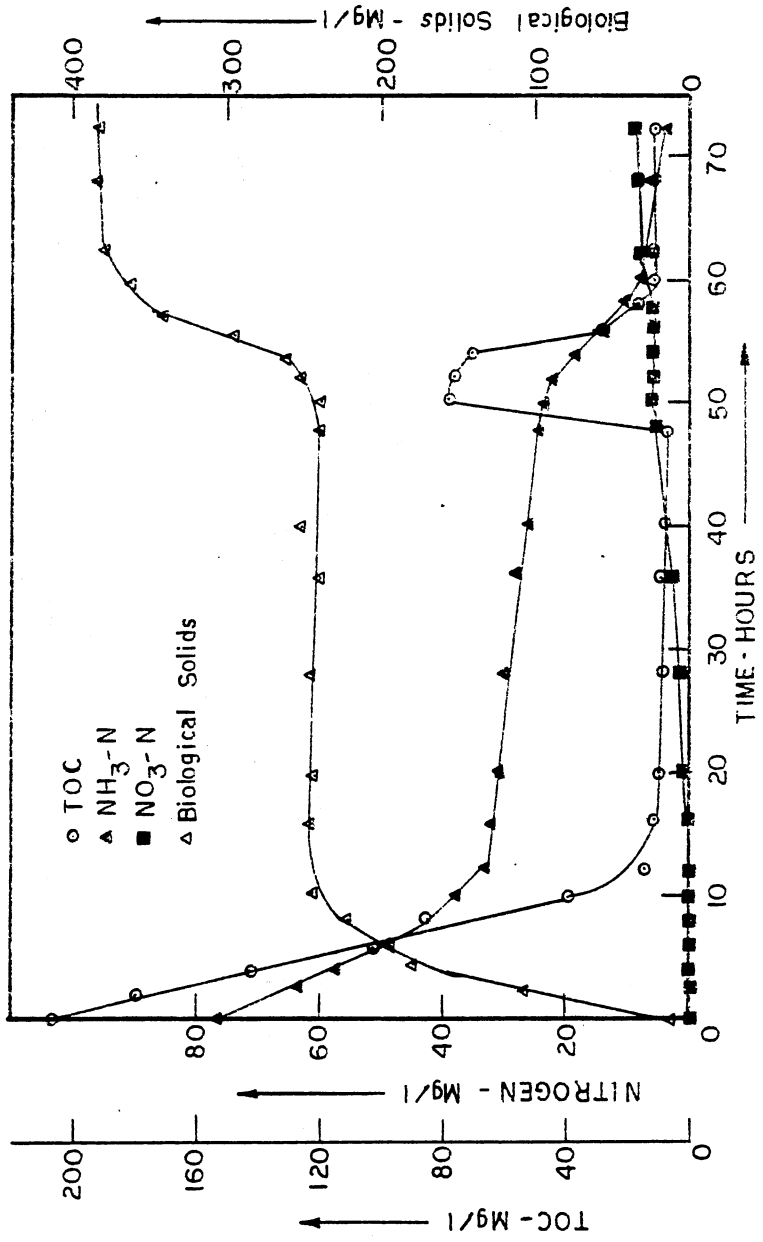
The bubble aeration system operating at C:N of 3:1 was repeated and studied as before, but 10 mg/ℓ of NO_2^--N was supplied in addition to the medium as previously described. Nitrification was observed after the active growth phase; but NO_2^--N supplied in the beginning of the experiment was not utilized, as 10 mg/ℓ was recovered at the end of the experiment. It was thought that since the 3:1 system was supplied with NH_3-N in excess of assimilatory need, the NH_3-N available after the growth phase could prevent the oxidation of the available NO_2^--N to NO_3^--N by the microorganisms. Hence, another system operating and C:N of 5:1 was set up to find out if the microorganisms could oxidize the available NO_2^--N to NO_3^--N if NH_3-N was not available after growth. Results obtained from this system were essentially the same as before, demonstrating normal heterotrophic growth until the depletion NH_3-N. NO_2^--N supplied to the

TABLE XVI

SUBSTRATE REMOVAL CHARACTERISTICS FOR GLUCOSE SHOCK LOADING AT 50 HR

Date	Time (hr)	TOC (mg/ℓ)	NH_3-N (mg/ℓ)	NO_3^--N (mg/ℓ)	pH	O.D.	Biological Solids (mg/ℓ)
7/10	0	208	72	0.00	7.52	0.06	25
	2	180	64	0.00	7.50	0.20	88
	4	142	58	0.00	7.48	0.42	180
	6	100	52	0.00	7.62	0.46	195
	8	87	42	0.00	7.50	0.52	220
	10	40	38	0.00	7.40	0.58	245
	12	14	34	0.00	7.46	0.58	245
	16	12	32	0.12	7.40	0.56	240
7/11	20	10	31	0.48	7.25	0.58	245
	28	9	30	1.30	7.09	0.58	245
	36	10	28	2.89	7.21	0.56	240
7/12	40	8	26	4.50	7.19	0.59	250
	48	8	25	5.20	7.10	0.56	240
7/13	50	78	24	5.80	7.12	0.56	240
	52	74	23	5.82	7.12	0.58	245
	54	60	19	5.84	7.14	0.64	265
	56	28	14	5.80	7.16	0.69	295
	58	16	10	5.80	7.14	0.77	340
	60	12	8	6.35	7.12	0.81	365
	62	13	7	7.25	7.09	0.82	380
7/14	68	12	6	8.15	7.10	0.84	385
	72	10	4	9.86	7.06	0.84	385

Figure 23. Substrate Removal Characteristics for Glucose Shock Loading at 50 Hr


TOC - Mg/l
NITROGEN - Mg/l
Biological Solids - Mg/l
TIME - HOURS
o TOC
▲ NH_3-N
■ NO_3-N
△ Biological Solids

system was not oxidized at the end of the experiment since the initial amount supplied, 10 mg/ℓ was recovered and NO_3^--N was not detected as had been observed before with the 3:1 system.

CHAPTER V

DISCUSSION

One-Stage Activated Sludge Nitrification System

The one-stage activated sludge nitrifying system was opearted throughout the isolation period from September 1981 to June 1982. During this period, inoculum required for the isolation experiments was taken from this system. Hence, it was necessary to maintain a controlled environmental condition for nitrification in this system throughout the study.

The synthetic wastewater used for this system consisted of a minimal salts medium with glucose as carbon source. The system was operated at constant COD:NH_3-N of 500:50. This combination of minimal salts, carbon source and buffer solutions is commonly used in the Bioenvironmental Engineering Laboratories of Oklahoma State University (159, 51). It is important to maintain this synthetic wastewater composition so as to provide growth of heterogenous population for nitrification. This heterogenous population was initially developed from sewage seed from the municipal sewage treatment plant at Tulsa, Oklahoma. The hydraulic flow rates were carefully controlled and the system was maintained at a θ_c of 10 days by daily wasteing of the biological solids. Essentially complete nitrification was obtained by this system at this operating condition as reported by Stover and Kincannon (4). They found that the degree of nitrification decreased with decreasing sludge age below 10 days. At a sludge age of

around three days, nitrification was completely prevented in the system. Hence, throughout this study, the system was maintained at a sludge age of at least ten days.

Stover and Kincannon (4) also reported that the COD to NH_3-N ratio greatly affects the degree to which nitrification can be achieved in the one-stage system. The system, when operated at low influent COD and high ammonia-nitrogen concentrations was as efficient as the two-stage system which they studied for comparison purpose. In this study, a constant COD:NH_3-N of 500:50 was maintained in the synthetic waste feed. Performance data and operational characteristics were presented in the preceeding chapter.

Isolation, Purification and General Properties of Pure Cultures

Prior to the extensive isolation of the nitrifying heterotroph, a study was made to evaluate enrichments with different supplementary substrates so that an enrichment technique could be designed to change the environment in such a way that the heterotroph capable of nitrifying could successfully compete against all other organisms and hence become the dominant population in the cultures.

The carbon sources tested were glucose and sodium acetate. Since glucose is easily metabolized by heterotrophs and the most common carbon source used in culture study. It was felt that data obtained from this study could then be compared to other studies. Sodium acetate was chosen on the basis of the reported study by Esfandi on the enhancement of the substrate on Nitrification (51). Supplementary substrates used were yeast extract, Bacto-peptone and beef extract. To avoid possible substrate toxicity, the organic compounds used were supplied at low concentrations

of 500, 500 and 300 mg/ℓ respectively for yeast extract, Bacto-peptone and beef extract. Yeast extract is an excellent stimulator of bacterial growth and is an excellent source of B-complex vitamins. Both beef extract and Bacto-peptone were recommended as good sources of nitrogenous compounds (171).

Of the carbon sources tested, sodium acetate when used as a sole source of carbon, promoted a higher level of nitrification than did the enrichments with glucose as the sole carbon source. Several studies also revealed that sodium acetate when used as a sole source of carbon was able to satisfy the requirement for heterotrophic nitrification. Fisher et al. (36) reported that as much as 87% of the removed NH_3-N could be accounted for as NO_2^--N formed from the heterotrophs isolated from soil samples. However, NO_3^--N was not produced from the cultures studied. Verstraete and Alexander (47) also reported that one isolate was particularly active and nitrified when grown with 1.69% sodium acetate as the carbon source and 0.4% $(NH_4)SO_4$ as nitrogen source. Glucose, however when used as a sole source of carbon, produced less than 1.0 mg/ℓ of NO_3^--N from the duplicated enrichment cultures. The ability to produce nitrite and or nitrate could not be judged from the results of this test in that organisms which could not effect a high degree of conversion to nitrite and/or nitrate could possibly do so with supplementary nitrogenous compounds. It was believed that a better assessment would be made by incubating supplementary substrates, yeast extract, peptone or beef extract with the heterotrophic cultures.

Results from the enrichment culture studies revealed that with supplementary substrate a higher level of nitrification could be achieved especially from the cultures grown on sodium acetate. The combination of sodium acetate and yeast extract produced 3.82 and 3.64 mg/ℓ of NO_3^--N from

the duplicate cultures studied. Hence, this combination of substrates was used for the routine isolation in the later part of the experiment.

Pure cultures of bacteria isolated from the enrichments were grown on acetate media of the same composition as before but yeast extract was omitted. Several studies have shown that the complex organic nitrogen present in yeast extract could be oxidized by the heterotrophs capable of nitrifying (32, 45, 40). Hence, it was omitted for these pure culture studies. Another reason for its exclusion in the pure culture media is that organic nitrogen present in the media could complicate the analysis procedures for nitrogen and total organic carbon. Moreover, the major concern in this study involved the different forms of nitrogen (ammonia, nitrite and nitrate) which were measureable in the cultures. Hence, an inorganic nitrogen source in the form of ammonium sulfate was employed in these pure culture studies. Like the carbon source, the ammonium sulfate can be made up in concentrated form and the carbon to ammonia-nitrogen ratio can be varied by the amounts of each applied to the media whenever necessary. Since the carbon source employed contained no nitrogen, the only source of nitrogen for bacterial grwoth and energy was the ammonium sulfate being supplied. Hence, the use of glucose or acetate as carbon source and ammonium sulfate as energy source gives an ideal combination for this investigative research.

Of the 70 isolates studied, only 22 were able to form NO_2^--N and/or NO_3^--N. The production of NO_2^--N and/or NO_3^--N is shown in Table XVII. The inability to effect a complete transformation to NO_3^--N was shown by the production of only NO_2^--N by some of the pure cultures. In the others, D-81-4 and F-82-3, a direct transformation to nitrate without nitrite formation appeared to have taken place. Only one isolate, Ju-82-2 was capable of both NO_2^--N and NO_3^--N production in excess of 0.6 mg/ℓ and 5.0 mg/ℓ

TABLE XVII

PRODUCTION OF NO_2^--N AND/OR NO_3^--N FROM PURE CULTURES INCUBATED IN SHAKER FLASKS FOR ONE WEEK

Isolates Which Produced NO_2^--N in Excess of		Isolates Which Produced NO_3^--N in Excess of	
0.4 mg/ℓ	0.6 mg/ℓ	1.0 mg/ℓ	5.0 mg/ℓ
S-81-4	O-81-7	D-81-4	Ju-82-2
S-81-7	D-81-3	F-82-3	
O-81-3	F-82-1	Ju-82-6	
O-81-4	F-82-7		
N-81-3	Ju-82-2		
N-81-6			
Ja-82-3			
Ja-82-6			
Mr-82-1			
Mr-82-4			
Mr-82-6			
Mr-82-6			
Ju-82-6			
TOTAL			
13	5	3	1

respectively. Generically, Ju-82-2 was not identified due to the tedious and complicated experimental procedures involved. Since the main objective of this study was to isolate a heterotroph capable of nitrifying. Confirmation tests for heterotrophic nitrification would suffice for such a study.

Confirmation tests for heterotrophic nitrification were carried out by using Nitrapyrin and sodium bicarbonate. Results from Table IX show that Nitrapyrin had no effect on cultures Ju-82-2 and sodium bicarbonate, when supplied as the sole carbon source, did not promote any growth. Nitrapyrin, a potent inhibitor of ammonia oxidation, has been shown to suppress autotrophic but not heterotrophic oxidation. The concentration of this chemical required to cause a marked inhibition of ammonia oxidation has been shown to vary from 0.05 to 20 ppm, depending upon the organic matters in the samples (120). Since only glucose and sodium acetate were used as the sole sources of carbon without any other supplementary substrates, a much lower concentration of nitrapyrin was required in this study. Besides, studies on pure cultures of Nitrosomonas (123) had shown that concentrations as low as 1.0 mg/ℓ could cause complete inhibition of ammonia oxidation. Hence, 10 mg/ℓ of Nitrapyrin was employed in this test. The overall effect of Nitrapyrin upon the chemoautotrophic metabolism of Nitrasomonas had been shown by Campbell et al. (123) to involve two things: (1) the inhibition of chemosynthetic reactions dependent upon ammonia oxidation coupled to phosphorylation and concomitant CO_2 reduction, and (2) a binding or chelating effect upon a metal component of the enzymes involved in ammonia oxidation. These workers believed that a metal involved in the substrate oxidation could well be copper at a concentration of 6×10^{-4} M Cu^{2+} which was found to be effective in a 50-70 percent reversal of Nitrapyrin inhibition of ammonia oxidation.

In view of the fact that nitrapyrin has no effect on heterotrophic nitrification as compared to its known inhibiting effect on autotrophic nitrification, it can be suggested that the mechanism involved in heterotrophic nitrification might involve a totally different pathway from that of the autotrophs. Hence, by employing Nitrapyrin and sodium bicarbonate, it was confirmed that nitrification by Ju-82-2 was heterotrophic in nature.

Pure Culture Batch Studies

Shaken Flask Growth Studies

Growth studies in shaker flasks were conducted so as to define a range of C:N ratios for the bubble aeration systems in the later part of this study. Growth parameters μ_{max} and K_s obtained under carbon-limiting conditions were 0.37 hr^{-1} and 220 mg/ℓ respectively. However, μ_{max} and K_s determined under nitorgen-limiting condition when the same glucose concentration was supplied to each culture flask were 0.37 hr^{-1} and 12 mg/ℓ respectively. It is observed that μ_{max} obtained from both conditions above were essentially the same. Growth of the heterotrophic microorganisms does not depend on the ammonia nitrogen alone. Organic carbon source is the factor that has to be considered for heterotrophic growth together with ammonia nitrogen for biosynthesis. K_s, under nitrogen limiting condition is, however, substantially different from that of the carbon limiting condition. The value of the shape factor K_s has an effect on the change in μ. The higher the value of K_s, the flatter is the curve; thus, when K_s is small, as in the case of nitrogen limiting condition, μ is dependent on ammonia nitrogen only at very low concentrations of S.

From the growth study results, a range of C:N ratios were selected

TABLE XVIII

SUBSTRATE REMOVAL AND GROWTH CHARACTERISTICS FOR PURE CULTURE Ju-82-2 GROWN IN SHAKER FLASKS AT VARIOUS NH_3-N CONCENTRATION AND AT SAME GLUCOSE CONCENTRATION OF 500 mg/ℓ

C:N Ratio	Initial Concentrations		Concentrations After Cessation of Growth		Specific Growth Rate
	TOC mg/ℓ	NH_3-N mg/ℓ	TOC mg/ℓ	NH_3-N mg/ℓ	$Hour^{-1}$
15:1	200	134	18	98	0.31
3:1	200	67	12	36	0.37
5:1	200	40	15	85	0.34
10:1	200	20	20	2.0	0.28
20:1	200	10	67	1.2	0.27
100:1	200	1.9	158	0	0.17
200:1	200	1.0	165	0	0.14

for the batch studies on substrate removal characteristics. These ratios were 3:1, 5:1 and 10:1. C:N ratio of 1.5:1 was omitted due to lower μ obtained as compared to C:N ratio of 3:1. At this low C:N ratio with high NH_3-N, the biological growth could be inhibited to a some extent. Table XVIII shows the substrate removal and growth characteristics determined after the cessation of growth for each C:N ratio.

Bubble Aeration Systems

The bubble aeration systems were studied at C:N ratios of 3:1, 5:1, and 10:1 for substrate sodium acetate TOC at 1,000 mg/ℓ and glucose TOC at 200 mg/ℓ. All removal rates found by this investigation followed zero order kinetics. These systems were able to maintain heterotrophic growth, however, the 3:1 systems were able to promote nitrification by cultures Ju-82-2. Provided that the nitrogen supply was not insufficient to meet the assimilatory demands of the microorganisms, nitrification could be promoted after these demands are met. Hence, a definite relationship exists between the C:N ratios and nitrification. The lower the ratio, that is, the higher the NH_3-N concentration being supplied in excess relative to assimilatory needs, the better it is for the system to promote nitrification.

The role of chemoautotrophic bacteria, primarily Nitrosomonas and Nitrobacter, in the process of nitrification is well known. Energy for growth and cell synthesis of these bacteria is obtained from the oxidation of ammonia or nitrite. Hence, it is not surprising that the growth rate of these bacteria parallels the oxidation rate of these inorganic nutrients. In this study, cultures Ju-82-2, on the other hand, did not nitrify until after the active growth phase. Results from both the ace-

tate and glucose systems with C:N ratio of 3:1 showed that nitrate did not appear during the active growth phase. The 3:1 system grown on acetate did not begin to nitrify until the TOC had been removed from 998 mg/ℓ to as low as 30 mg/ℓ. Soon after depleting the acetate TOC down to a level of 20 mg/ℓ, nitrate begin to accumulate and after 96 hours, 11.04 mg/ℓ was detected. During this period of nitrification, the biological solids were maintained at a maximum level of 780 mg/ℓ. The glucose 3:1 system, however, nitrified much earlier than the acetate system since the TOC supplied was only 200 mg/ℓ. NO_3^--N was detected as early as four hours. During this period of nitrification, biological solids decreased from a maximum of 270 mg/ℓ to 255 mg/ℓ.

The oxidation of ammonia-nitrogen from cultures Ju-82-2 after the active growth phase seems to indicate that it is not growth linked as in the autotrophic nitrifiers. The energy derived from the oxidation, therefore, is not required for growth of the bacteria, but no evidence is available to indicate whether this energy can be linked to biosynthetic reactions of the microorganism. However, the energy derived from the oxidation process could be used to maintain growth as in the case of the acetate system where the maximum solids level of 780 mg/ℓ was maintained throughout the period of nitrification. On the other hand, the delayed accumulation of NO_3^--N may be associated with cellular lysis, since active oxidation of NH_3-N did not begin until after a decrease in biological solids was observed as in the glucose 3:1 system.

Throughout the study, NO_2^--N was detected only in very low concentrations and in some instances, it was not at all detectable. The NO_2^--N appearing in low concentrations may originate from an oxidative pathway in which reduced forms of nitrogen are converted to nitrite, or it may be pro-

duced by the reduction of nitrate. If the former hypothesis is true, then nitrite is either an intermediate in nitrate formation or it may be foumed from some other intermediate in the process of nitrification by cultures Ju-82-2. On the other hand, if nitrite is synthesized from nitrate, then it is possible that the microorganisms are capable of growth on nitrate as sole source of nitrogen, and probably, they possess some kind of adaptive enzyme, nitrate reductase. However, it seems unlikely that nitrate reductase would be formed in a nitrifying culture, since the substrate, nitrate, did not appear until after the active growth phase. The fact that significant amounts of nitrate appeared only after there were no longer increases in biological solids may indicate that an extracellular substance or a compound released upon autolysis was acted upon by the microorganism to form nitrate. Whether the relatively low concentration of NO_2^--N detected in the systems were subsequently oxidized to nitrate is still not clear, but from the experiment with NO_2^--N medium, the initial amount of NO_2^--N supplied was recovered and NO_3^--N was not produced at the end of the experiment. However, Malavolta et al. (43) by growing cultures of A. flavus isolated from soil, were able to present evidence for the oxidation of nitrite to nitrate.

It is generally expected that the substrate acetate exerts a greater degree of species selectivity than the common substrate, glucose. The fact, that culture Ju-82-2 in this study was capable of utilizing acetate as a growth substrate indicates the inducement of two adaptive enzymes of the glyoxalate cycle, malate synthetase and Isocitratase. This rather specialized genetic capability is possessed by far fewer species than those capable of methabolizing simple carbohydrates such as the hexoses. The stimulatory effect of acetate on nitrification had also been observed by Esfandi (51). In batch experimental study, it was observed that acetate

enhance the ammonia removal rate and higher quantities of nitrate were detected in batch reactors containing the acetate.

A considerable body of knowledge now exists about the effects of various environmental factors, such as temperature, pH and dissolved oxygen concentration, upon the kinetics of nitrification. Much less information is available, however, about the effects of other factors, such as the presence of organic compounds. It is widely believed that the presence of organic matter and/or heterotrophic microorganism in some way inhibits the nitrification process. The specific nature of inhibition by organic matter has never been identified and fully understood. Studies by Stover and Kincannon (4) showed that quantitative shock loads of organic carbon inhibited nitrification. The degree of inhibition was observed to depend on the concentration and duration of the shock load. They observed that inhibition was temporary and nitrification was restored when the system recovered from the consequences of the shock load. In this study, the glucose 3:1 system was repeated and when rapid nitrification was observed after the active growth phase, the system was shock loaded with glucose, a lag was observed for the first two hours after the shock with only 4.0 mg/ℓ of TOC being removed. Nitrification was inhibited as TOC removal was observed with the build up of biological solids. However, when rapid heterotrophic growth ceased with the depletion of TOC, the system began to nitrify. The studies by Stover and Kincannon showing the disruption of nitrification by heterotrophic growth are certainly reinforced by the behavior of growing culture of Ju-82-2 in this sysgem.

A majority of the investigators suggest low organic loadings in nitrification systems in order to promote the nitrification process. In a Technology Transfer Publication (158), the Environmental Protection Agency

recommends that carbonaceous BOD_5 be removed to levels of about 50 mg/ℓ for the nitrification process to occur, however, a BOD_5 of 20 mg/ℓ was suggested by Little from his study with a nitrifying tower (6). At low organic loadings, a relatively inactive heterotrophic population will predominate, which will stimulate nitrification. Of immediate interest, however, is the evidence from this study suggesting that heterotrophic cultures of Ju-82-2 may be implicated in nitrification.

Because of the great abundance of the heterotrophic types, it is tempting to postulate a role for them. However, in constrast with the autotrophs, nitrification is not obligately associated with development of the heterotrophs so that the frequency of these microorganisms indicates merely a potential for activity rather than the occurrence of an actual transformation.

CHAPTER VI

CONCLUSIONS

From the results of this investigation, the following conclusions can be made:

1. An organism isolated from a nitrifying activated sludge system is capable of metabolizing organic carbon as carbon and energy source and nitrifying after the depletion of the organic carbon.

2. The growth constants μ_{max} (0.37 hr^{-1}) and K_s (220 mg/ℓ) obtained under carbon-limiting conditions are very typical of heterotrophic microorganisms.

3. The fact that the organism is incapable of growing in an inorganic media and nitrapyrin has no effect on its nitrifying activity clearly suggests that nitrification by this organism is heterotrophic in nature.

4. The insensitivity of organisms to the specific autotrophic inhibitor suggests physiological differences between heterotrophic and autrotrophic nitrifiers.

5. Under appropriate conditions of carbon and nitrogen supply especially when nitrogen is in excess of assimilatory needs, heterotrophic nitrification could be the predominant mode of nitrification in biological nitrification systems.

6. The inability in the past to explain the performance of nitrifying systems such as activated sludge, biological towers, and RBC's under organic shock load conditions can now be explained by the behavior of this

microorganism. Shock load of organic carbon source inhibits nitrification by this organism. However, inhibition is temporary and nitrification is recovered when the consequences of the shock load is restored. Hence, inhibition of nitrification occurs simultaneously with rapid heterotrophic growth when the organism is utilizing the organic carbon source.

CHAPTER VII

SUGGESTION FOR FUTURE STUDY

Based on the findings of this study, the following are some suggestions for future investigations involving heterotrophic nitrification.

1. Study of mixed cultures composed of autotrophic nitrifiers with the heterotrophic nitrifiers to show possible competition for substrate or commensal and sometimes mutualistic interaction.

2. A comparative investigation of mechanism of conversion of ammonia to nitrate by heterotrophic and autotrophic bacteria using labelled nitrogen technique might be rewarding.

3. The mechanism of heterotrophic nitrification requires further study by investigating the responsible enzyme and its activities.

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VITA 2

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