1985

Response of a Nitrifying Biofilm to Copper

Denise Moldroski Mott
Eastern Illinois University

THESIS REPRODUCTION CERTIFICATE

TO: Graduate Degree Candidates who have written formal theses.

SUBJECT: Permission to reproduce theses.

The University Library is receiving a number of requests from other institutions asking permission to reproduce dissertations for inclusion in their library holdings. Although no copyright laws are involved, we feel that professional courtesy demands that permission be obtained from the author before we allow theses to be copied.

Please sign one of the following statements:

Booth Library of Eastern Illinois University has my permission to lend my thesis to a reputable college or university for the purpose of copying it for inclusion in that institution's library or research holdings.

February 22, 1985 — Denise Lynn Mott

Date — Author

I respectfully request Booth Library of Eastern Illinois University not allow my thesis be reproduced because _____

_____

_____

_____ — _____

Date — Author

m

Response of a Nitrifying Biofilm to Copper

(TITLE)

BY

Denise Moldroski Mott

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1985

YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
THIS PART OF THE GRADUATE DEGREE CITED ABOVE

May 10, 1985
DATE

ADVISER

May 10, 1985
DATE

COMMITTEE MEMBER

May 10, 1985
DATE

COMMITTEE MEMBER

May 10, 1985
DATE

DEPARTMENT CHAIRPERSON

Denise Mott, M.S. Eastern Illinois University. May 1985. Response of a Nitrifying Biofilm to Copper. Major Professor: Dr. William A. Weiler.

Abstract. In pure cultures of Nitrosomonas, copper is known to cause a stimulation of nitrification at low levels ($\leq$ 0.03 mg Cu^{+2}/l) and an inhibition affect at higher levels ($\geq$ 0.05 mg Cu^{+2}/l). Nitrifying activated sludges are much more tolerant of copper showing only 75% inhibition at 150mg Cu^{+2}/l. The purpose of this paper was to determine the copper sensitivity of a nitrifying biofilm grown in a continuous flow trickling filter system.

A continuous flow trickling filter system was built using limestone chips for the biofilm substrate. The biofilm was established from secondary effluent. After 1 month, a stable nitrification rate of 4 mg NO_3^--N/l/hr was obtained at a flow rate of 13.9 l/hr. A synthetic medium was then introduced and pH was stablized (6.5 - 7.5) with a polystaltic pump buffer drip system for the rest of the study.

Inhibition studies at various copper levels (0.005 - 1.0 mg/l Cu^{+2}) were initiated. Copper was introduced for 12 hours, the column was drained, flushed with new synthetic medium, and refilled with three liters fresh medium. Nitrification rate (mg NO_3^--N/l/hr) was monitored every 4 hours for 60 hours or until a return to the basal rate (12 - 14 mg NO_3^--N/l/hr) was noted. A peak nitrification rate occurred 12 hours after introduction of the stimulatory level of 0.10 mg Cu^{+2}/l. At the copper level of 1.0 mg Cu^{+2}/l, an immediate (90%) inhibition was apparent.

The nitrifying biofilm response to copper was comparable to that of pure cultures of Nitrosomonas and much more sensitive than that which was reported for the nitrifying sludges.

ACKNOWLEDGEMENTS

I would like to express my sincerest thanks to my advisor, Dr. William A. Weiler, for his ideas, guidance, and support during this investigation, during the manuscript preparation, and throughout my course of graduate study.

I would also like to thank the members of my graduate committee, Dr. John E. Ebinger, Dr. Richard D. Andrews, and Dr. Wesley C. Whiteside, for their constructive criticism in the preparation of this manuscript, and for their encouragement during my studies at Eastern Illinois University.

Finally, to my husband David and son Gavin, are extended my most heartfelt thanks for their love, encouragement, and overwhelming patience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Bacterial nitrification has been of interest to soil scientists and agriculture for the past 100 years. Recently, interest in this process has been increasing especially in the area of biological wastewater treatment. Discharge of nitrogenous compounds from wastewater facilities can have many deleterious effects on the environment.

Biostimulation of receiving waters due to excessive nitrogen discharge leads to eutrophication and excessive plant (algal) growth. This growth in turn is a causative factor in the deterioration of clear water, odor problems, and low D.O.(dissolved oxygen) levels which adversely affect fish and aquatic invertebrate life. Ammonia toxicity is another problem affecting fish population (Process Design Manual for N Control, 1975).

As a result of these environmental problems, the EPA currently has standards for ammonia discharge from treatment facilities and, therefore, more and more treatment plants have come to rely on biological nitrification as a component of wastewater processing. The increase in biological nitrification use in wastewater treatment has produced a need for more information on the causes of system breakdown and the optimization of recovery. This is especially important in areas of industrial concentration where incoming "shock loads" of nitrification-inhibiting substances are a real possibility. The purpose of this study is to compare the sensitivity to copper of a nitrifying biofilm to an activated sludge system and a pure Nitrosomonas culture.

REVIEW OF THE LITERATURE

BIOLOGICAL NITRIFICATION

Nitrification is a commonly used term for the process of oxidation of ammonia to nitrate. Actually, there are two major reactions involved: 1) Nitrosification - oxidation of ammonia to nitrite and 2) Nitrification - oxidation of nitrite to nitrate. (Unless so indicated, the common definition of nitrification will be utilized in this paper.) The autotrophic nitrifying bacteria all derive their cell materials from the reduction of CO_2. These bacteria can be separated into two groups based on what compound is utilized to produce the energy needed to assimilate the CO_2.

Those organisms capable of oxidizing ammonia to nitrite ($NH_4^+ + O_2$ ----> NO_2^- + Energy) include Nitrosomonas, Nitrosospira, Nitrosococcus, and Nitrosolobus; those organisms that oxidize nitrite to nitrate ($NO_2^- + O_2$ ----> NO_3^- + Energy) are Nitrobacter, Nitrospina, and Nitrococcus (Buchanan and Gibbons, 1974). It has been widely accepted and repeatedly verified in wastewater research that the oxidation of nitrite to nitrate by Nitrobacter species normally occurs much more rapidly and, consequently, the concentration of nitrite typically remains very low (less than 1.0 mg/l) (Randall and Buth, 1984). Many studies have been done on the "classic" group of nitrifying organisms (Nitrosomonas, Nitrobacter) which are thought to be the major converters of ammonia to nitrate (Lees, 1963), while very little has been done on the growth, metabolism, or inhibition of the other genera or "nonclassic" groups.

Growth requirements of the nitrifying bacteria include a

specific inorganic nitrogen source (NH_4^+ or NO_2^-). The obligatory specificity of the nitrifying bacteria for a N source, along with their relatively slow growth rates, can result in growth limitation due to competition in an environment with a high C:N ratio and a thriving heterotrophic population (Painter, 1970). Other requirements for growth are inorganic carbon cources, oxygen, and disputed amounts of trace elements. Nitrosomonas requires phosphorus, magnesium, iron, calcium, and copper (Lees, 1955). VanDroogenbroeck and Laudelout (1967) found 310 mg/l P to be the optimum concentration for highest growth rate and least lag time in Nitrosomonas. Sodium was found to be a necessary trace metal for a Nitrosomonas strain isolated from activated sludge (Loveless and Painter, 1968) in contrast to the findings of Engle and Alexander (1958). Necessary growth requirements of Nitrobacter are phosphorus, magnesium (Lees, 1955), iron, molybdenum, and copper (Kiesow, 1962).

The temperature and pH range for growth are similar for both genera: 5 - 40 °C and pH of 6.5 - 8.5 for Nitrosomonas, 5 - 30 °C and pH 5.8 - 8.5 for Nitrobacter (Buchanan and Gibbons, 1974). Buswell and coworkers (1954) reported optimum growth temperatures to be in the range of 30 - 36 °C for Nitrosomonas. An optimum growth temperature range of 34 - 35 °C for Nitrobacter was reported by Deppe and Engle in 1960 (in Painter, 1970), while Laudelout and Van Tichelen (1960) reported the optimum growth temperature to be about 42 °C.

BIOCHEMISTRY OF NITRIFICATION

Due, in part, to the difficulty of maintaining pure Nitrosomonas (or Nitrobacter) cultures, the complete biochemistry of ammonia or nitrite oxidation remains unclear. Various researchers have determined that hydroxylamine plays a role in ammonia oxidation (Hofman and Lees, 1952; Engle and Alexander, 1958; Hooper and Terry, 1973). Lees in 1954 (in Painter, 1970) concluded that there should be three stages in ammonia oxidation to nitrite each involving two-electron changes. The first stage has been postulated as: $2NH_4^+ + O_2$ -----> $2NH_2OH + 2H^+$. The second stage has been theorized to be hyponitrite, dehydroxyammonia, nitroxyl, or nitrohydroxylamine (Painter, 1970), although there is no substantiating evidence as yet. The proposed pathway for ammonia oxidation (at this time) is: NH_4^+ ---> NH_2OH ---> ? ---> NO_2^- (Lees, 1963).

It has been shown that a copper containing protein is necessary for the oxidation of ammonia (Nicholas et al., 1962). Nitrite oxidation by Nitrobacter is also known to involve an enzyme system requiring Fe^{+2} and Fe^{+3} (Aleem and Alexander, 1958). Because the specific chemistry of the nitrification process is still unclear, few publications are available relating to the modes of action of specific inhibitory substances in Nitrosomonas and Nitrobacter. Hooper and Terry (1973) have, however, implicated several biochemical processes in ammonia oxidation on the basis of reactions involving inhibitors and Nitrosomonas.

Ammonia oxidation was effectively halted by the addition of the metal-binding compounds DTC (diethyldithiocarbamate) or KCN to

Nitrosomonas cells (Table 1). When the inhibited cell suspension was treated with $CuCl_2$, nitrite synthesis increased from 6 - 41% (in DTC inhibited cells) and 14 - 30% (in KCN inhibited cells). Full recovery of NH_4^+ oxidation was not expected as the authors found that Cu^{+2} inhibited cell-free PMS (electron acceptor phenasine methosulfate)-dependent nitrite synthetase (Hooper and Terry, 1973).

P-460, a novel cytochrome from Nitrosomonas, was found to have a high affinity for CO and to bind with NH_2NH_2 (Erikson and Hooper, 1972). The sensitivity of ammonia oxidation to SKF 525, Lilly 18947, and CO (Table 2) suggests dependence on a factor similar to cytochrome P-450 of mammalian microsomes (Hooper and Terry, 1973). It was also found that several strong, nonpermeable enzyme inhibitors were not inhibitory to ammonia or hydroxylamine oxidation indicating that the ammonia oxidation system is not located on the extreme outer cell surface of Nitrosomonas (Hooper and Terry, 1973).

Harold, in 1970, found that the compounds DNP (dinitrophenol), duPont 1799 (aa´ bishexafluoroacetonylcyclohexane), and tetrachlorosalicylanilide (Table 3) appear to inhibit oxidative phosphorylation, ATP - dependent NADP reduction, protein pumping, and ion transport in bacteria. These compounds were also found to be specific inhibitors of ammonia oxidation (Hooper and Terry, 1973), which suggested to the authors that one or more of these processes may be in some way involved with ammonia oxidation.

Short chain alcohols (Table 4) specifically inhibit ammonia (but not hydroxylamine) oxidation (Hooper and Terry, 1973). Alcohols have been shown to react with catalase (Nicholls and Schonbaum, 1963) and it has been proposed that these short-chain alcohols react with some

Table 1. Proposed metal chelating substances which inhibit nitrification. (Reference numbers refer to the citation section)

Compound	Inhibition Concentrations	% Inhibition of Ammonia Oxidation	Reference
Allyl thiourea	10^{-6} M	82%	18,47
2,2´ bipyridine	10^{-4} M	100%	16,18
Diethyldithiocarbamate	10^{-5} M	100%	18
Dimethyl gloxime	140 mg/l	50%	16
Dithiol	10^{-3} M	100%	18
8-Hydroxyquinoline	10^{-5} M	100%	18,43
KCN	5×10^{-6} M	78%	18
NaN	10^{-3} M	90%	18
Na S	10^{-4} M	100%	18
o-Phenathroline	5×10^{-5} M	100%	18

Table 2. Enzyme and heme protein-binding compounds (Hooper and Terry, 1973).

Inhibitor	Concentration (M)	% Inhibition of Ammonia Oxidation
Aminoguanidine	10^{-3}M	74%
3-Aminotriazole	10^{-3}M	100%
2-Chloro-6-trichloromethylpyridine (N-Serve)	5×10^{-5}M	86%
CO (95% O : 5% CO)	0.05M	92%
2,4-Dichloro(6-phenylphenoxy)ethyl-aminehydrobromide (Lilly 53325)	10^{-4}M	96%
2,4-Dichloro-6-phenylphenoxyethyl-diethylamine (Lilly 18947)	10^{-4}M	96%
β-Diethylaminoethyldiphenylpropyl-acetate (SKF 525)	5×10^{-5}M	65%
Diphenylthiocarbazone	3×10^{-5}M	50%
Ethyl xanthate	10^{-4}M	100%
1-Isonicotinyl-2-isopropyl-hydrazine (Iproniazid)	0.01M	88%
Nil NH	2×10^{-3}M	84%
Thiosemicarbazide	10^{-5}M	95%

Table 3. Uncouplers and inhibitors of electron transport (Hooper and Terry, 1973).

Inhibitor	Molar Concentration	% Inhibition of Ammonia Oxidation
Phenazine methsulfate	5×10^{-4}M	100%
m-Chlorocarbonylcyanidephenylhydrazone	10^{-5}M	83%
2,6-Dichlorophenolindephenol	10^{-3}M	100%
N,N'-Dicyclohexylcarbodiimide	5×10^{-5}M	60%
2,4-Dinitrophenol	2×10^{-4}M	73%
Methylene blue	10^{-4}M	100%
aa'-bis(Hexafluoroacetonyl)cyclo-hexanone (duPone 1799)	2×10^{-5}M	73%
Tetra chlorosalicylanilide	10^{-5}M	59%

Table 4. Alcohols which may react with a peroxide-metabolizing enzyme or as free-radical trapping agents (Hooper and Terry, 1973).

Compound	Inhibition Concentrations	% Inhibition of Ammonia Oxidation	Reference
Allyl alcohol	19.5 mg/l	75%	43
Amino ehtanol	0.2 M	43%	18
n-Butanol	0.11 M	100%	16,18
t-Butanol	0.11 M	35%	18
Ethanol	0.09 M	100%	18,43
Methanol	5×10^{-3} M	100%	18
n-Propanol	0.33 M	100%	18
i-Propanol	0.13 M	91%	18

peroxide-metabolizing enzyme or possibly act as free-radical trapping agents (Hooper and Terry, 1973). They also suggest that short chain amines compete with ammonia for a permease-type enzyme, as demonstrated with methylamine in fungi (Hacket et al., 1970). In summary, the authors (Hooper and Terry, 1973) feel that ammonia oxidation in Nitrosomonas is dependent on 1) some metal ion such as copper, 2) a CO binding-factor, 3) a P-450 like protein, 4) a functionally intact membrane, and 5) a role for an H_2O_2 or OH radical-producing, -utilizing, or -detoxifying system.

INIBITION OF NITRIFICATION

Inhibition of the nitrifying process in general can occur in either of two areas: 1) inhibition of a primary oxidation step or 2) inhibition of the CO_2 reduction-assimilation step. Many researchers have added to the list of nitrification inhibitors both physical and chemical factors.

Physical Inhibition

Nitrifying bacteria have a narrow pH range in which they maintain an active metabolism. Kholdebarin and Oertli (1977) reported that outside the range of 7.0 - 9.5, less that 50% of the optimum rate of nitrification occurs. Wild and colleagues (1971) indicated that 90% of the maximum nitrification rate occurs in the range of pH 7.8 - 8.9 and that outside a range of pH 7.0 - 9.8, less than 50% optimum rate occurs. Engle and Alexander (1958) reported an optimum rate of ammonia oxidation in Nitrosomonas at pH 6.2 - 9.6. It was suggested that the inhibition of nitrification at high pH values is caused mostly by the injurious effect of excess NH_3 and

NH_2OH present (Stojanovic and Alexander, 1958; Aleem and Alexander, 1960).

Temperature does affect the rate of nitrification and the rate increases throughout a range of 5 - 30 °C (Wild et al., 1971). In contrast, Borchart (1966, in Wild et al., 1971) indicated that nitrification was fairly constant in the temperature range of 15 - 35 °C in activated sludge. Temperature changes have a greater effect on the rate of nitrate formation than on the rate of nitrite formation (Randall and Buth, 1974), therefore causing a potential toxic nitrite buildup in activated sludge processes when the temperature drops below 12 °C.

Hooper and Terry (1973) found that illumination of Nitrosmonas at 420 lux resulted in complete and irreversible inactivation of ammonia oxidation with no effect on hydroxylamine oxidation. The ratio of oxygen uptake in the dark to that in light (4000 lux) at 25°C was 1:22 for Nitrosomonas and 1:5 for Nitrobacter (Ulken, 1963 in Painter, 1970). Lees (1963) suggested that the inhibition seen in Nitrobacter by earlier researchers may be related to some light-induced malfunctioning of the cytochrome pigment system used in nitrite oxidation.

Chemical Inhibition

During a study to determine the effect of variable shock loads of BOD on the rate of nitrification in sewage effluents, Wild and coworkers (1971) observed that there was no apparent inhibition at BOD concentrations of 5 mg/l, 11 mg/l, or 45 mg/l. They proposed that at a sustained high BOD loading, nitrification would eventually

cease due to increased sludge production and the washing-out effect that sludge wasting would have on nitrifiers (a direct result of their relatively slow reproductive rate).

Ulken in 1963 (in Painter, 1970) reported that Nitrosomonas was less sensitive to oxygen depletion than Nitrobacter due to the greater complexity of the ammonia oxidizing system in Nitrosomonas. The Michaelis constant (Km) for Nitrosomonas at 20° C was found to be 0.3 mg/l (Schoberl and Engle, 1964 in Painter, 1970; Loveless and Painter, 1968). Using trickling filter effluent run through a pilot nitrification unit, Wild and coworkers (1971) found that there was apparently no inhibition of nitrification occurring at DO levels greater than 1.9 mg/l. Randall and Buth (1984) found in activated sludge mixed liquor that concentrations in excess of 2.0 mg/l DO were not rate limiting.

Early researchers (reviewed by Lees, 1963) found that ammonia was inhibitory to Nitrobacter. Ulken (1963, in Painter, 1970) demonstrated an increase in lag period and a decrease in the growth rate of Nitrobacter at 8 and 16 mg NH_4^+-N/l, respectively. Aleem (1959, in Painter, 1970) reported that Nitrobacter is extremely sensitive to ammonium salts, especially at alkaline pH values, whereas the nitrite oxidizing enzyme system of the organism is not. Ammonia toxicity in Nitrobacter was also noticed as increasing with increasing pH (Stojanovic and Alexander, 1958). In contrast, Kholdebarin and Oertli (1977) reported that nitrite oxidation is stimulated by the presence of NH_4^+- N. Wild et al. (1971) found that the nitrification process was not inhibited by ammonia nitrogen concentrations of less than 60 mg/l, but since he measured rate of

nitrification by the change in the initial NH_4^+-N concentration, the affect on NO_2^- oxidation by Nitrobacter was not determined.

Nitrite nitrogen has been found to inhibit Nitrosomonas (Oginsky and Umbriet, 1959). Meyerhof (1916, in Painter, 1970) found a 36% inhibition of oxygen uptake in Nitrosomonas at nitrite levels of 1400 mg NO_2^--N and complete inhibition at 4200 mg NO_2^--N/l. Lewis (1959) and Pokallus (1963) (both in Painter, 1970) found that nitrite was more toxic to Nitrosomonas than to Nitrobacter during logrithmic growth. Substantial concentrations of nitrite have been found to be toxic under the low pH conditions that result as alkalinity is destroyed during the nitrification process (Randall and Buth, 1984).

Nitrosomonas was not inhibited by a concentration of 3g ammonium sulfate per liter and Engle and Alexander (1958) inferred that higher concentrations of ammonia -N would also be without effect. In contrast, Hockenbury and coworkers (1977) reported that ammonia levels greater than 50 mg/l exert substrate inhibition effects on its own oxidation by Nitrosomonas. Engle and Alexander (1958) found that 60 μmoles of nitrite per Warburg vessel was slightly inhibitory to Nitrosomonas. Oxygen uptake by Nitrobacter was inhibited 40% when nitrite levels were 1400 mg NO_2^-- N/l and Boon and Laudelout (1962) felt that the inhibition was due to undissociated nitrous acid. Loveless and Painter (1968) found "limitation" of Nitrosomonas growth at 2500 mg NO_2^-- N/l.

Meiklijohn (1954, in Painter, 1970) compiled a list of metal chlorides which completely or substantially inhibited respiration in Nitrosomonas (Table 5).

Table 5. Metal chlorides causing substantial inhibition to respiration in Nitrosomonas (Meiklejohn, 1954, in Painter, 1970).

Metal	Inhibitory Concentration	
Na, K, Mg	0.5M	(11,500 - 19,500 - 12.000 mg/l resp.)
Ca, Sr, Ba	0.3M	(8,000 - 17,600 - 27,400 mg/l resp.)
Fe, Al, Cu	0.01M	(560 - 170 - 630 mg/l respectively)
Zn, Pb, Mn	0.01M	(650 - 2080 - 550 mg/l respectively)
Co	0.001M	(59 mg/l)
Ni	0.0002M	(12 mg/l)
Hg	1×10^{-5}M	(2mg/l)
Ag	2.5×10^{-6}M	(0.25 mg/l)

Skinner and Walker (1961, in Painter, 1970) found Fe^{+2} up to 2 mg/l had no effect on growing cultures of Nitrosomonas while 0.25 mg/l of Ni and Cr completely inhibited growth. Co and Mn up to 1 mg/l had no effect but Cu was completely inhibitory at 0.5 mg/l. Randall and Buth (1984) found that there was a synergistic inhibitory effect between temperature and Ni toxicity for nitrification in activated sludge systems. Beg and colleagues (1982) using a fixed film reactor model found Cr caused 50% inhibition of nitrification reaction at 50 mg/l. Loveless and Painter (1968) tested several metal ions individually over a range of concentrations for evidence of biological activity in a Nitrosomonas culture isolated from activated sludge at pH 7.3 and 7.9 (Table 6).

In activated sludge studies, Tomlinson et al.(1966) found that concentrations of metals necessary to inhibit nitrification were much higher than those required to give the same effect in pure cultures. Up to 150 mg $Cu^{+?}$/l (copper sulfate) were required to produce 75% inhibition in activated sludge nitrification compared to 4 mg Cu^{+2}/l in pure culture. Many very potent inhibitors of nitrification are metal-chelating, sulfur-containing compounds which inhibit enzymes requiring metals for activation (Lees, 1963; Downing, 1964) (Table 1). Certain chelating agents (thioureas, allyl-thiourea, 8-hydroxyquinoline, salicyladoxime, histidine) were toxic to the primary oxidation reaction of Nitrosomonas (Lees, 1952) but the theory that they act in chelating some essential element was not substantiated.

Organic compounds in general (glucose, glycerol, acetate,

Table 6. Effects of various metal ions on the biological activity of a Nitrosomonas strain isolated from activated sludge (Loveless and Painter, 1968).

Element	Range Tested mg/l	Concentration at which effect observed (mg/l) Stimulation	Inibition
Cu	0.005 - 0.56	0.005 - 0.03	0.05 - 0.56
Cu*	0.005 - 0.48	none	none
Ca	0.5 - 20	none	none
Ca*	0.5 - 20	0.5 - 10	none
Mg	12.5 - 100	12.5 - 50	50 - 100
Zn	0.005 - 0.5	none	0.08 - 0.5
Co	0.005 - 0.5	none	0.08 - 0.5
Al	0.005 - 1.0	none	none
Sr	0.005 - 0.05	none	none
Pb	0.005 - 0.05	none	none
B	0.005 - 0.05	none	none

* in presence of 5 mg/l EDTA

butyrate) were not toxic to Nitrosomonas, although mannose was found to be very growth-inhibitory (Jensen, 1960). Jensen (1960) also reported glycine, alanine, and asparagine to be strongly inhibitory to growth of a strain of Nitrosomonas (Table 7) but found aspartic acid, glutamic acid and urea to be comparatively non-toxic toward cells. Hockenbury and Grady (1977) found that aliphatic or aromatic amines do not act as competitive inhibitors of ammonia oxidation. In contrast, Hooper and Terry (1973) proposed that amines compete with ammonia for enzyme sites. Tomlinson and coworkers (1966) studied nitrification inhibition in activated sludge and produced a list of inhibitory substances that includes some industrially significant compounds. Two of these compounds, chloroform and phenol, are general inhibitors of bacterial metabolism. The three most potent inhibitors of ammonia oxidation studied by Hockenbury and Grady (1977) were dodecylamine, aniline, and n-methyl aniline, all causing 50% inhibition at concentrations less than 1 mg/l. Table 8 includes a listing of tested nitrification inhibitors which are also industrially significant chemicals. A summary of all known inhibitor substances is included as Table 9.

The great number of known nitrification inhibitors and the fact that many of them are industrially significant chemicals illustrates one of the problems facing wastewater treatment today. Much more time and effort needs to be expended in this area to greatly reduce the possibility of system malfunctions in sewage facilities utilizing biological nitrification as a phase of wastewater treatment.

Table 7. Nitrification inhibitors containing free amine groups.

Compound	Reference *	Compound	Reference *
Allyl thiourea	18	L-Histidine	10,16
Amino ethanol	16,18	Hydrazine	18,43
Amino quanidine	18	L-Lysine	10
p-Amino propiophenone	16	L-Methionine	10
3-Amino triazole	18	Methylamine	18
Aniline	16	Methylamine HCl	43
L-Arginine	10	Methyl thiourea	47
Asparagine	19	1-Naphthylamine	16
Aspartic Acid	19	Naphthylethylenediamine-diHCl	16
Benzidine diHCl	16	NH_2 NH_2	16
Benzocaine	16	p-Nitroaniline	16
Benzylamine	16	8-Nitrourea	43
Dicyandiamide	43	p-Phenylazoaniline	43
Diquanide	43	L-Threonine	10
Dithiooxamide	43	Thioacetamide	43
Dodecylamine	43	Thiosemicarbazide	18,43
Ethylene diamine	16	Thiourearea	43,47
Guanidine carbonate	43	L-Valineazoaniline	10
Hexamethylene diamine	16		

* refers to citation section

Table 8. Industrially significant chemicals that are inhibitory to nitrification.

Compound	Concentration	% Inhibition of Ammonia Oxidation	Reference *
Acetone	0.14 M	100%	18
Aniline	100 mg/l	86%	16
n-Butanol	0.11 M	100%	16,28
Carbon disulfide	35 mg/l	75%	43
Chloroform	18 mg/l	75%	43
Ethanol	18 mg/l	100%	18,43
Ethylacetate	0.23 M	100%	18
Ethylene diamine	100 mg/l	73%	16
Hexamethylene diamine	100 mg/l	52%	16
Methanol	5×10^{-3} M	100%	18
Phenol	5.6 mg/l	75%	43
n-Propanol	0.33 M	100%	18
i-Propanol	0.13 M	91%	18

Table 9. Alphabetical listing of chemical substances inhibitory to nitrification.

Compound	Reference	Compound	Reference
Acetone	18	2,4-Dichloro-6-phenylphenoxy-ethyldiethylamine(Lilly18947)	18
$AlCl_3$	29,30	Dicyandiamide	43
Allyl alcohol	43	N,N´Dicyclohexylcarbodiimide	16,18
Allyl chloride	43	β-Diethylaminoethyldiphenyl-propylacetate (SKF525)	18
Allyl isothiocynate	43	Diethyldithiocarbamate	18
Allyl thiourea	18,47,48	Diguanide	43
Amino ethanol	16,18	Dimethylgloxinebazone	16
Amino guanidine	18	Dinitrophenol	18
p-Amino propiophenone	16	Diphenylthiocarbazone	18
3-Amino triazole	18	Dithiolamine	18
Asparagine (1HOH)	19	Dithiooxamide	43
L-Arginine	10	Dodecylamine	43
Aniline	16	Ethanole diamine	18,43
Aspartic acid	19	Ethylacetatete	18
Barium chloride	30	Ethylene diamine	16
Benzidine diHCl	16	Ethyl xanthate	18
Benzocaine	16	Guanidine carbonate	43
Benzothiazole disulfide	43	Hexamethylene diamine	16
Benzylamine	16	L-Bistidine	10,16
2,2´ Bipyridine	16,18	Hydrazine	18,43
aa´Bis(hexafluoroacetonyl)-cyclohexanone(DuPont1799)	18	Hydroxyquinoline	18,43
Bisphenol (Na salt)	47	$FeCl_2$	10
Bromodichloropropane	47	1-Isonicotinyl-2-isopropyl-hydrozine (Iproniazid)	18
n-Butanol	16,18	$PbCl_2$	29,30
t-Butanol	18	L-Lysine	10
Calcium chloride	29,30	$MgCl_2$	29,30
Carbon disulfide	43	$MnCl_2$	30
Chlorocarbonylcyanidephenyl-Hydrazone	18	Mercaptobenzothiazole	43
Chloroform	43	$HgCl_2$	30
3-chloro-6-trichloromethyl-pyridine (N-Serve)	9,16,18	Methanoline	18
$CoCl_2$	29,30	L-Methionine	10
O-Crèsol	18	Methylamine	18
$CuCl_2$	29,30	Methylamine hydrochloride	43
Diallyl ether	43	n-Methylaniline	16
1,2 Dibromoethane	47	Methylene Blue	18
2,3 Dibromopropan-1-ol	47	Methyl isothiocyanate	43
Dichlorohexylcarbodiimide	16	Methyl thiourea	47
Dichlorophenolindophenol	16,18	Na_2N	18
2,4-Dichloro-6-phenylphenoxy-ethylhydrobromide (Lilly 53325)	18	1-Naphthylamine	16
		Naphthylethylenediamine diHCl	16
		NaS	18

Table 9. continued

NH_2NH_2	18	Na methyldithiocarbamate	43
$NiCl_2$	30	$SrCl_2$	29,30
Ninhydrin	16	Tannic acid	16
p-Nitroaniline	16	2,3,5',6'tetrabromobesphenol-(sodium)	47
8-Nitrourea	43	Tetrachlorosalicylanilide	18,47
Pentabromophenol (Na salt)	47	Tetramethylammonium chloride	27
o-Phenanthroline	18	Tetramethylthiuramdisulfide	43
Phenazine methosulfate	18	Thioacetamide	43
Phenol	43	Thiosemicarbazide	18,43
p-Phenylazoaniline	16	Thiourea	43,47
KCl	30	L-Threonine	47
KCN	18	Tribromophenol	47
Potassium thiocyanate	43	Trichloromethyl pyridine	47
n-Propanol	18	Triethylamine	16
Quinacrine	49	L-Valine	10
AgCl	30		
Skatol	43		
NaCl	30		
Na dimethyldithiocarbamate	43		

MATERIALS AND METHODS

The Apparatus. A continuous flow trickling filter system (Fig. 1) was constructed from a 66.0 cm section of 4.0 cm inside diameter PVC pipe, tygon tubing, rubber stoppers, stainless steel diffuser plate, 4-liter glass reservoir, air pump and airstone, and Gorman-Rupp Industries pump (Model #14925-001). The PVC pipe was filled with crushed limestone collected from an east-central Illinois stone quarry and sieved through screens to obtain an average stone size of 6.5 mm^3. Prior to placement in the PVC pipe, the stone was washed in hot water, cold water, and lab pure water. The recycled medium, aerated by an airstone in the large glass reservoir, was pumped from the reservoir to the top of the PVC pipe and flowed by gravity through the gravel bed back into the reservoir. The glass reservoir the screened with aluminum foil to minimize algal growth. A Powerstat variable autotransformer (Model #116B-1159) was used to regulate the current to the GRI pump, allowing manual regulation of the medium flow rate through the system.

Biofilm Establishment. Secondary effluent from the Charleston, Illinois, Wastewater Treatment Plant was used initially to establish a nitrifying biofilm. Initial flow rate was 13.9 l/hr. Ammonium sulfate solution (200 mg/l NH_4^+-N final concentration) was added to each 3 l of fresh secondary effluent as a nitrogen source. The medium was replaced every 24-48 hours.

When the nitrate concentration approached 200 mg/l, due to ammonia conversion to nitrate and biofilm growth, more NH_4^+-N feed

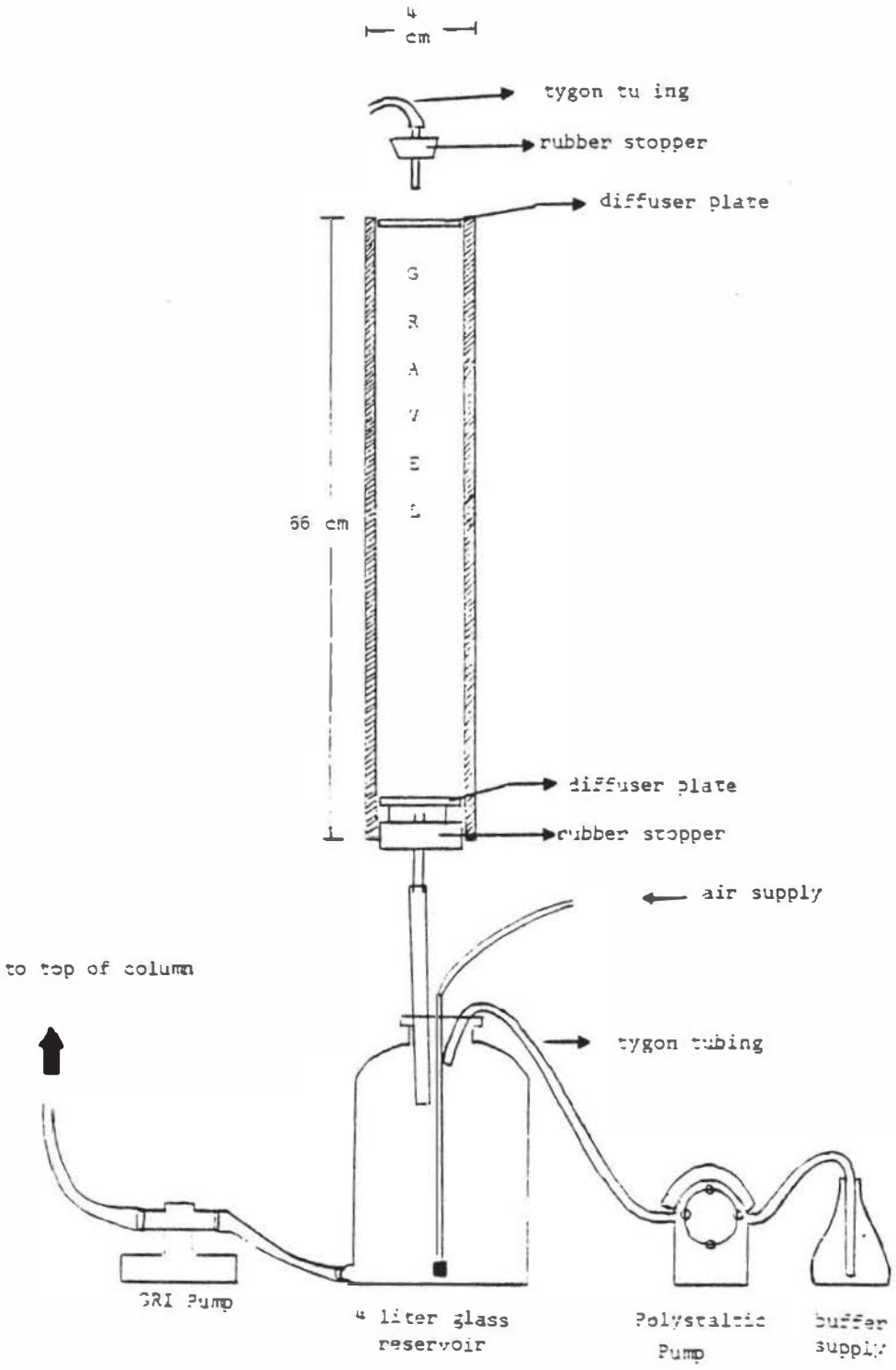

Figure 1. Continuous Flow Trickling Filter System.

solution was added (up to 500 mg/l total feed). Once the biofilm appeared stable with regard to its nitrification rate, an analysis was made to determine the most efficient flow rate. Nitrate levels were determined using an Orion Research Ionalyzer (Model #407A) and an Orion Nitrate Selective ion electrode (Model #90-02) at four different flow rates and the most efficient flow rate was used throughout the rest of the experiment. The pH of the medium remained constant at 7.2 throughout the flow rate tests.

Nitrification in Synthetic Media. Once the biofilm was established, the synthetic medium of Beg and Atiquallah (1973) was introduced. The Beg and Atiquallah medium contained a $NaHCO_3/Na_2CO_3$ buffer system, which when actually tested, had an initial pH of 9.8 (not the reported 8.5). In an attempt to alleviate this high pH problem, the Na_2CO_3 component was omitted and the $NaHCO_3$ concentration was increased (to 3 g/l) until an initial pH of 8.0 was reached. Upon initial use, however, pH stability rapidly deteriorated; after 50 hours, the pH rose to 9.6.

A second modification was to reduce the $NaHCO_3$ concentration to 1 g/l. This $NaHCO_3$ level provided an initial medium with a pH of 7.4, but after 10 minutes of recycling, the pH had increased to 7.9.

A different synthetic medium (Randall and Buth, 1984) was next used which included a $K_2HPO_4/KH_2PO_4/Na_2CO_3$ buffer system (Table 10). This system provided a medium with an initial pH of 7.3, but the medium soon became acidic (pH 4.4-5.4) as nitrification progressed. To solve this problem, a Buchler Instruments Polystaltic pump (model #30233) was introduced to provide a slow "drip-feed" of buffer to the glass reservoir. This medium and its modification successfully

Table 10. Synthetic medium for nitrifying biofilms (Randall and Buth, 1984).

Medium Component	Concentration, mg/l
$MgSO_4(7H_2O)$	44.0
$MnSO_4(H_2O)$	4.4
$FeSO_4(7H_2O)$	0.56
$CaCl_2$	3.3
K_2HPO_4	590.0
KH_2PO_4	290.0
Na_2CO_3	320.0

maintained the pH at 6.5 - 7.5 throughout the course of nonstressed nitrification. Additional buffer was added during the inhibition studies as needed to control the downward shift in pH due to the large increase in nitrification rates.

Inhibition Studies. Changes in nitrate-N production or ammonia-N utilization were used to indicate inhibition or stimulation of the biofilm after known inhibitory substances were added. Originally, 20 mg/l Cr^{+2} and 1000 mg/l F^{-} were used as inhibitors. The response of the biofilm system, however, indicated a possible ion exchange reaction occurring within the column (F^{-} for NO_3^{-}, Cr^{+2} for NH_4^{+}), masking true nitrification rates. Consequently, copper (Cu^{+2}) at concentrations of 0.005 - 1.0 mg/l were used instead.

The inhibitory substance (Cu^{+2}) was added to fresh synthetic medium, and nitrate-N production was monitored on a 4-hour basis for 12 hours. The column was drained, rinsed with 500 ml of synthetic medium to remove residual Cu^{+2}, and the system refilled with 3 l of fresh medium. A 24 - 48 hour recovery period was monitored at 4 hour intervals and the pH buffer and feed solution ($(NH_4)_2SO_4$) were added as necessary to prevent nutrient depletion and gross pH changes over prolonged periods. Calculations of nitrate levels were corrected for dilution by added feed and buffer solutions.

RESULTS AND DISCUSSION

The nitrifying biofilm was established in secondary effluent (Fig. 2). After approximately 1 month, at a flow rate of 13.9 l/hr, the nitrification rate had leveled out at about 4.0 mg/l NO_3^--N/l/hr. At this time, the biofilm appeared healthy, exhibiting the typical orange-brown color, gram negative bacilli, and associated sludge worms.

A flow rate study, to determine the most efficient flow rate to use for the remainder of the inhibition study, utilized medium flow rates of 11.5 l/hr, 13.9 l/hr, 23.6 l/hr, and 26.4 l/hr. The resultant 23.8 l/hr rate was found to be the most efficient for nitrification (Fig. 3). Nitrification peaked at 9.0 mg/l/hr. An equally efficient rate of 22.6 l/hr was the rate eventually used during the inhibition study. At rates lower than 11.5 l/hr, the recirculating pump was not able to return medium to the top of the column.

Synthetic medium (Randall and Buth, 1984) was next introduced, and a problem with pH instability was immediately evident. During the nitrification process, alkalinity is destroyed and the pH decreases, which causes problems when working with a small volume recirculating system. The Randall and Buth (1984) medium was modified with addition of a polystaltic pump and buffer system which resulted in a relatively controllable pH. As can be seen in Fig. 4, pH variability was greatly reduced after the buffer modification. Other advantages of the use of the synthetic medium and its modification were also apparent. The more than adequate nutrient content of the synthetic medium allowed the nitrification rate to

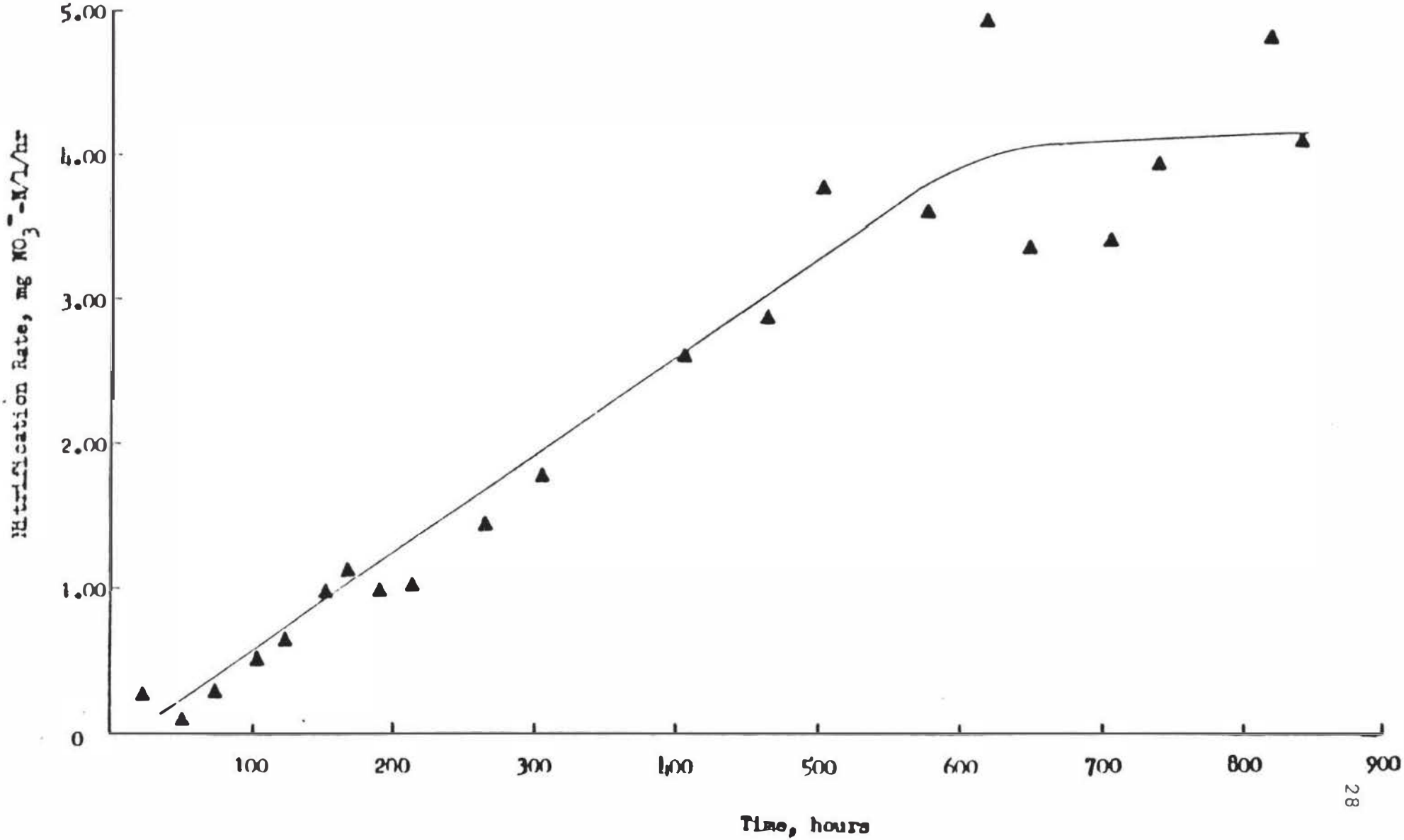

Figure 2. Establishment of the Nitrifying Biofilm.

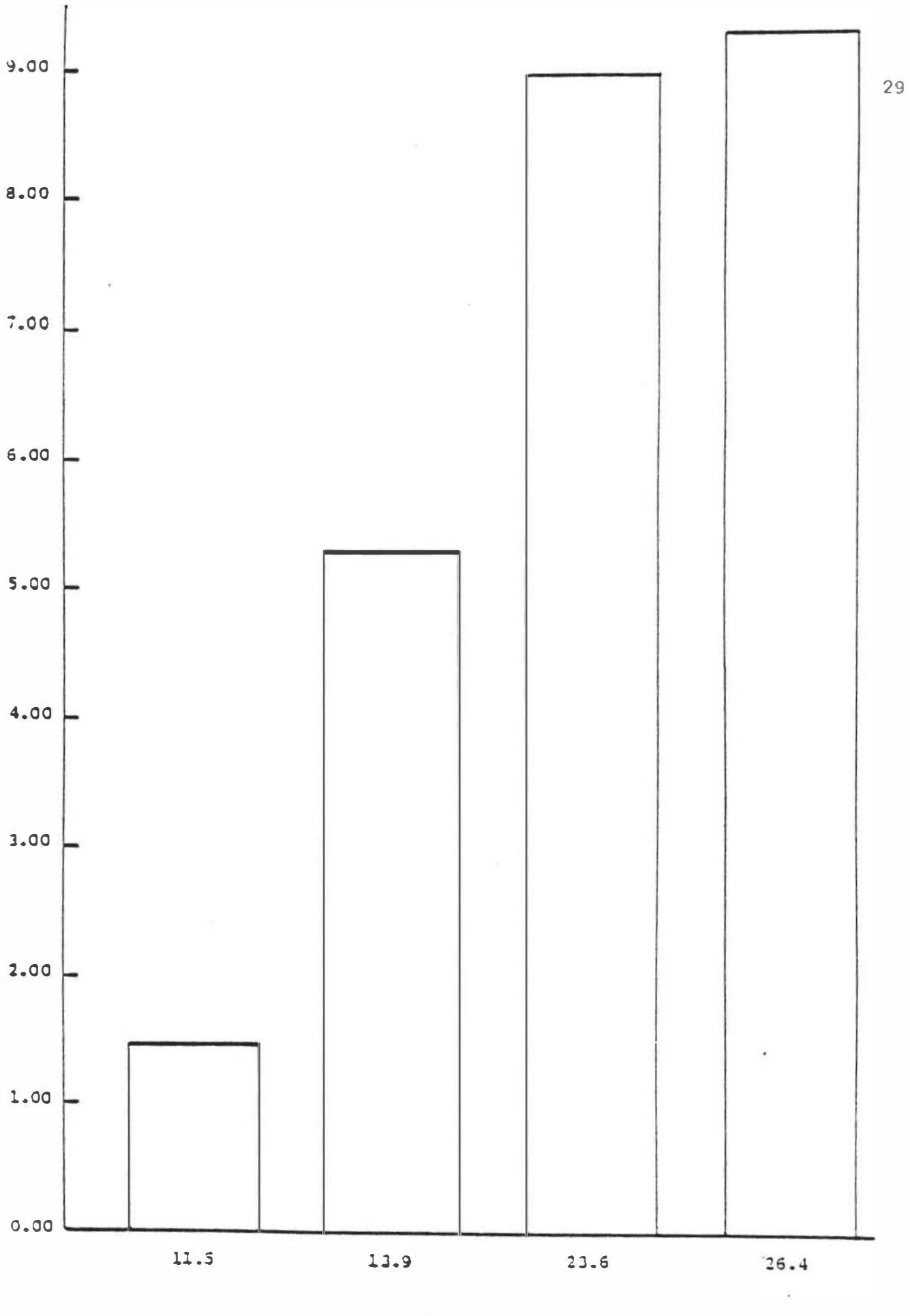

Figure 3. The effect of 4 different flow rates on the rate of nitrification.

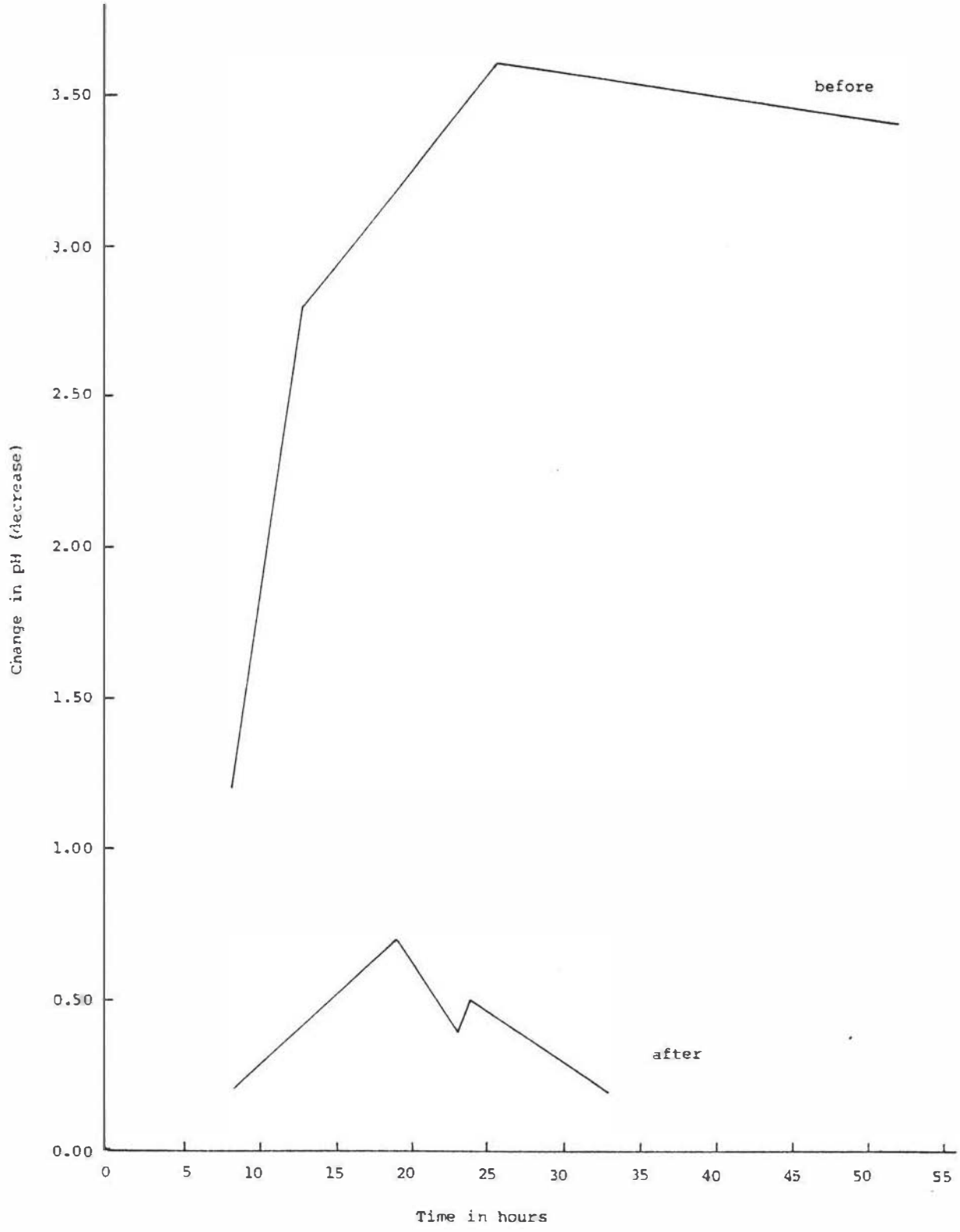

Figure 4. pH Changes before and after use of a polystaltic pump buffer drip.

reach a much higher level (12.0 - 14.0 mg NO_3^--N/l/hr) than when secondary effluent was utilized (6 - 7 mg NO_3^--N/l/hr).

The choice of copper as an inhibitor was made for several reasons. The use of copper allowed for comparison with previous activated sludge and pure Nitrosomonas culture nitrification inhibition studies, and it also minimized earlier problems encountered when the biofilm presumably acted as an ion-exchange column upon addition of F^- and Cr^{+2} inhibitors. Copper is also known to be utilized by the biofilm in the ammonia oxidase system of Nitrosomonas (Nicholas et al., 1962).

Previous research has indicated that the presence of low levels of Cu^{+2} are stimulatory to ammonia oxidation in a pure Nitrosomonas culture isolated from activated sludge (Loveless and Painter, 1968). At higher levels, Cu^{+2} is a metabolic inhibitor and has been utilized in inhibition studies with activated sludge (Tomlinson et al.,1966) and with Nitrosomonas culture (Loveless and Painter, 1968; Skinner and Walker,1961, Meiklijohn, 1954, both in Painter, 1970). There have been no previous copper inhibition studies available for nitrifying biofilms. On the basis of the Nitrosomonas cultures isolated from activated sludge, however, a stimulatory effect was expected at concentrations of 0.005 - 0.03 mg Cu^{+2}/l while an inhibitory effect was expected at concentrations greater than 0.05 mg Cu^{+2}/l (Loveless and Painter, 1968).

At a copper level of 0.005 mg/l, a stimulatory effect was found (Fig. 5) both while the copper-laced medium was in direct contact with the biofilm for 12 hours and after the inhibitor was removed and replaced with new medium. The nitrification rate peaked at

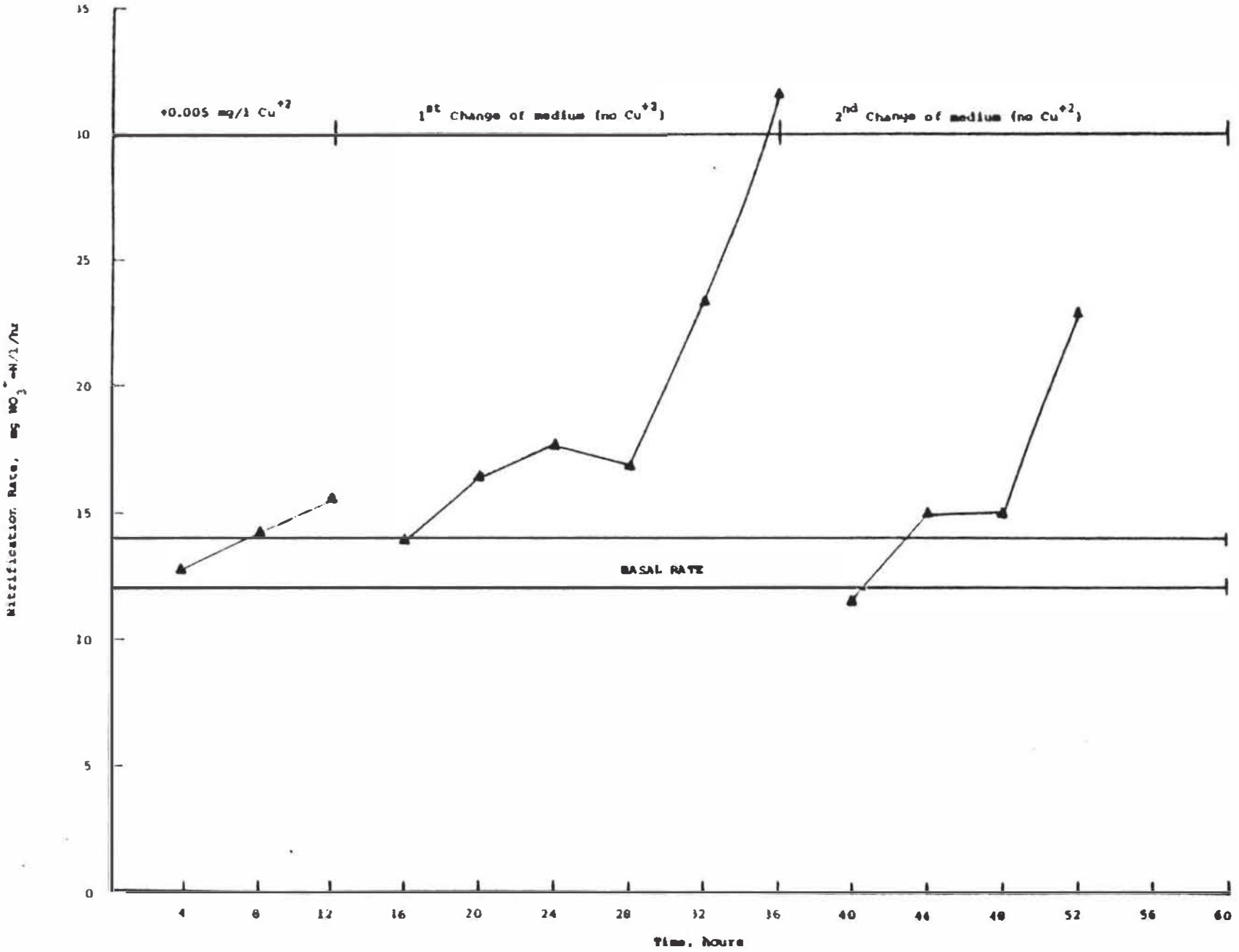

Figure 5. Nitrification rate vs. time at a copper concentration study of 0.005 mg Cu^{+2}/l.

31.7 mg NO_3^--N/l/hr (nearly 3 times that of the control) 24 hours after the inhibitor was replaced with fresh medium. In fact, the stimulatory effect was still noticeable, although at a lower level, 40 hours after the inhibitor was removed. An apparent leveling-off of the rate of ammonia conversion was noticed while the copper was in contact with the biofilm. This rate-stabilization was noticed at 8 hours and again at 20 and 44 hours. These static phases in the fresh medium immediately preceded peaks in the nitrification rate at 28 and 48 hours, respectively. The reason for the apparent rate stabilization and subsequent peak is unkown.

At a copper concentration of 0.01 mg/l, similar results were noted (Fig. 6) with rate stabilization again found at 16 and 32 hours after initial copper exposure. The highest nitrification rate reached at this copper concentration was 25.6 mg/l/hr 24 hours after the removal of copper. At this concentration, 72 hours after introduction of the copper medium, a drop back to the normal (original) nitrification rate was seen. In comparison, a return to the control rate of nitrification occurred 66 hours after treatment with copper at 0.005 mg/l.

At 0.05 mg Cu^{+2}/l, nitrification also was stimulated, reaching a peak level of 29.5 mg NO_3^--N/l/hr while Cu^{+2} was in contact with the biofilm. This time, however, there was no marked static phase during the 12 hour exposure to copper (Fig. 7). In addition, there was little, if any, static phase evident after replacement of copper medium with fresh medium, but a marked decrease in nitrification rate at 24-28 hours did occur prior to another modest increase in rate. A return to the normal nitrification rate occured between 52-56 hrs.

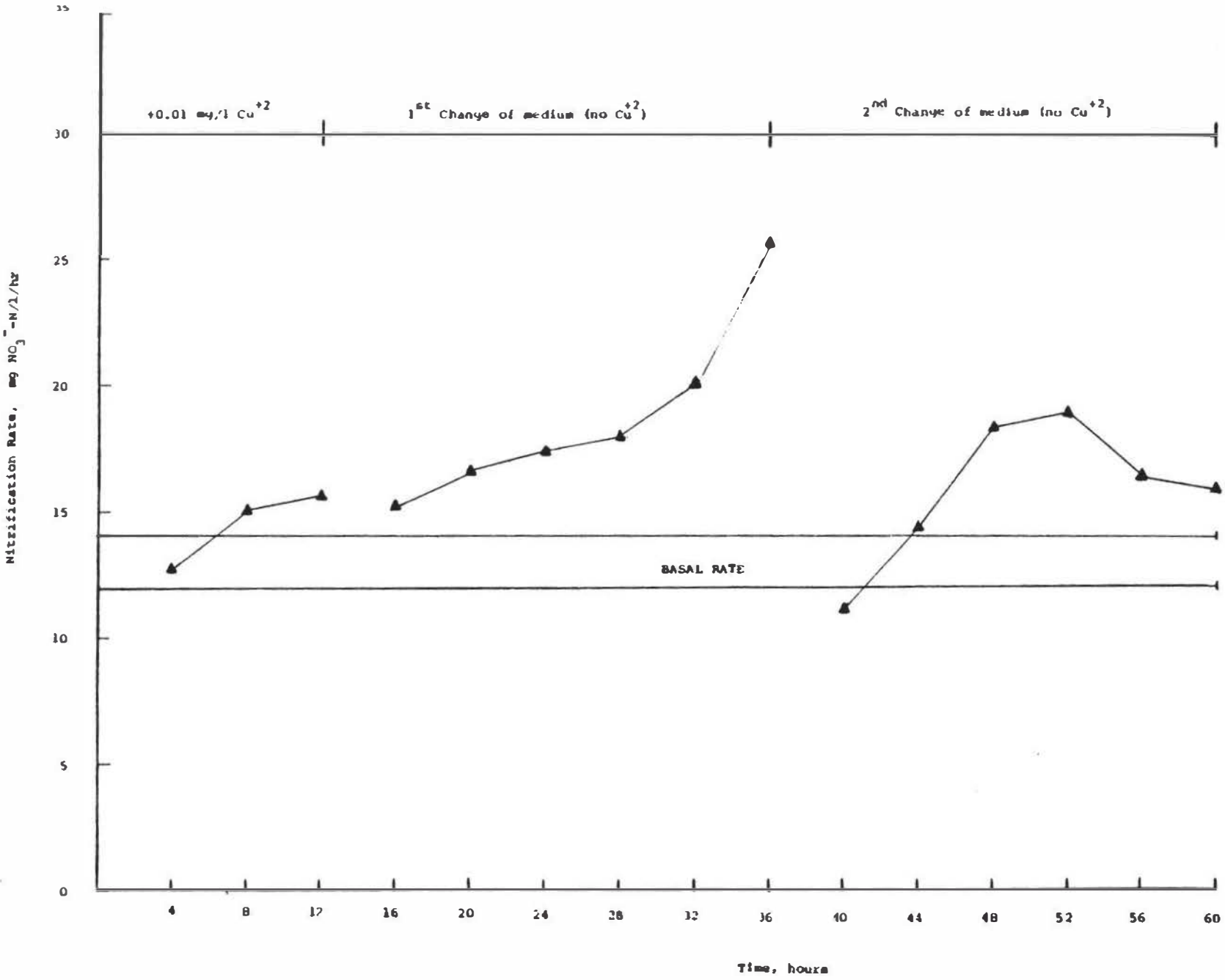

Figure 6. Nitrification rate vs. time at a copper concentration study of 0.01 mg Cu^{+2}/l.

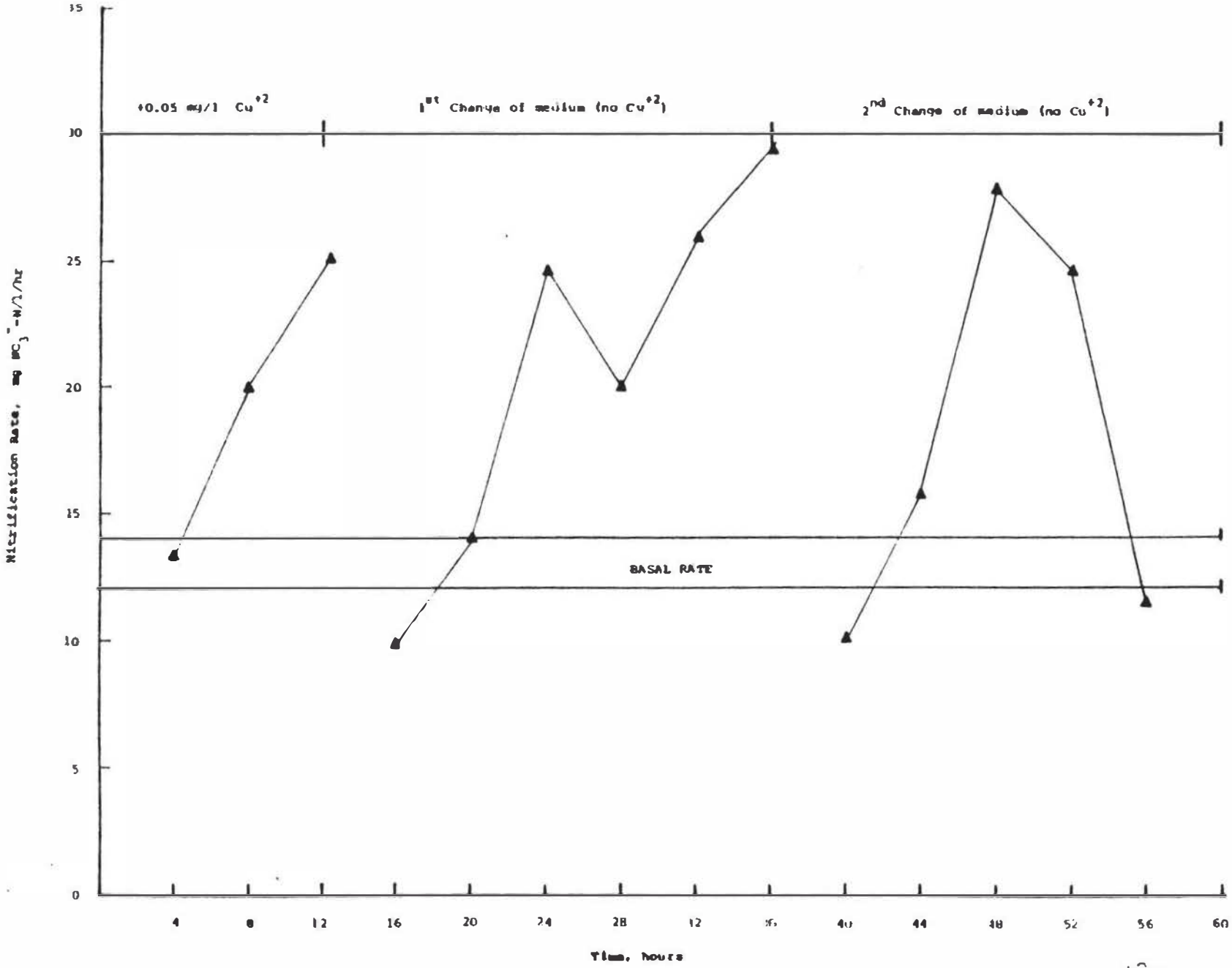

Figure 7. Nitrification rate vs. time at a copper concentration study of 0.05 mg Cu^{+2}/l.

0.1 mg Cu^{+2}/l, the stiumlatory effect resulted in the fastest rate of ammonia conversion recorded during the course of analysis, 33.5 mg NO_3^--N/l/hr, after 12 hours of contact with the copper-containing medium (Fig. 8). A nearly straight line relationship between the time of exposure and the nitrification rate occurred while the biofilm was in contact with the copper medium. Upon replacement with fresh medium, a peak nitrification rate of 31.3 mg NO_3^--N/l/hr was reached in 24 hours. At 40 hours, however, the rate again peaked (23.7 mg NO_3^--N/l/hr). The start of a possible downward trend in the nitrification rate was also noted at 52 hours (40 hours after copper removal). At this level of copper, again, a very slight slow-down in rate increase occurred at about 24-28 hours following copper medium replacement.

A stimulatory effect was also seen at copper levels of 0.5 mg/l (Fig. 9), although the rate while the biofilm was in direct contact with the copper medium only reached 21.6 mg NO_3^--N/l/hr (after 8 hr). Upon replacement of copper medium with fresh medium, the nitrification rate reached 28.6 mg NO_3^--N/l/hr at 32 hours. By 52 hours, the nitrification rate was back to normal. There was some evidence of a static phase from 20-28 hours, but these results could also have been directly affected by a somewhat lower than usual pH (5.6-6.0), causing a depressed effect on nitrification (Engle and Alexander, 1958).

At 1.0 mg Cu^{+2}/l medium, inhibition of nitrification was immediately apparent (Fig. 10). After 4 hours of direct exposure to the 1.0 mg Cu^{+2}/l level, a rate of 3.6 mg NO_3^--N/l/hr (compared to 12-14 mg NO_3^--N/l/hr in the control tests) was reached and nitrifi-

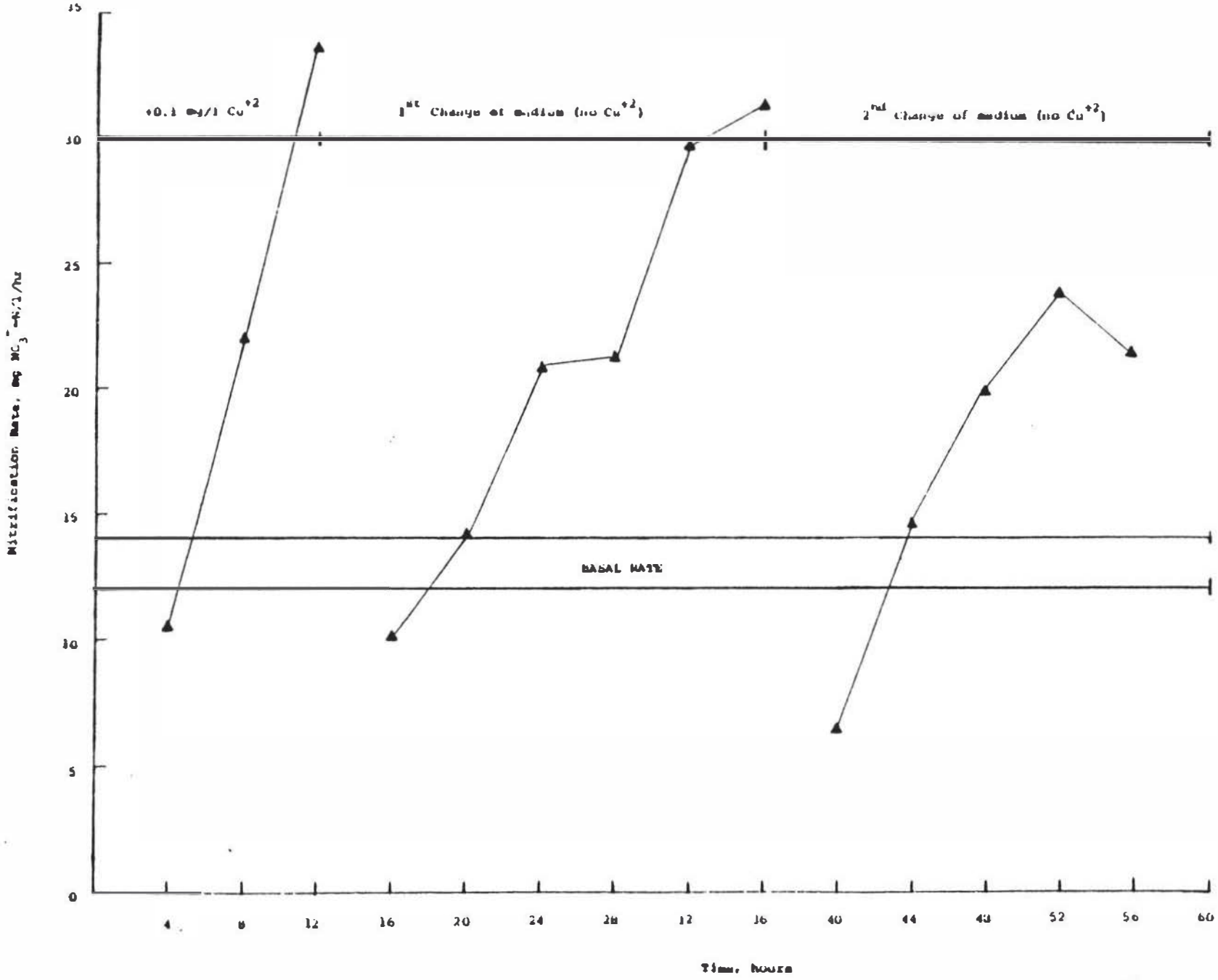

Figure 8. Nitrification rate vs. time at a copper concentration study of 0.10 mg Cu^{+2}/l.

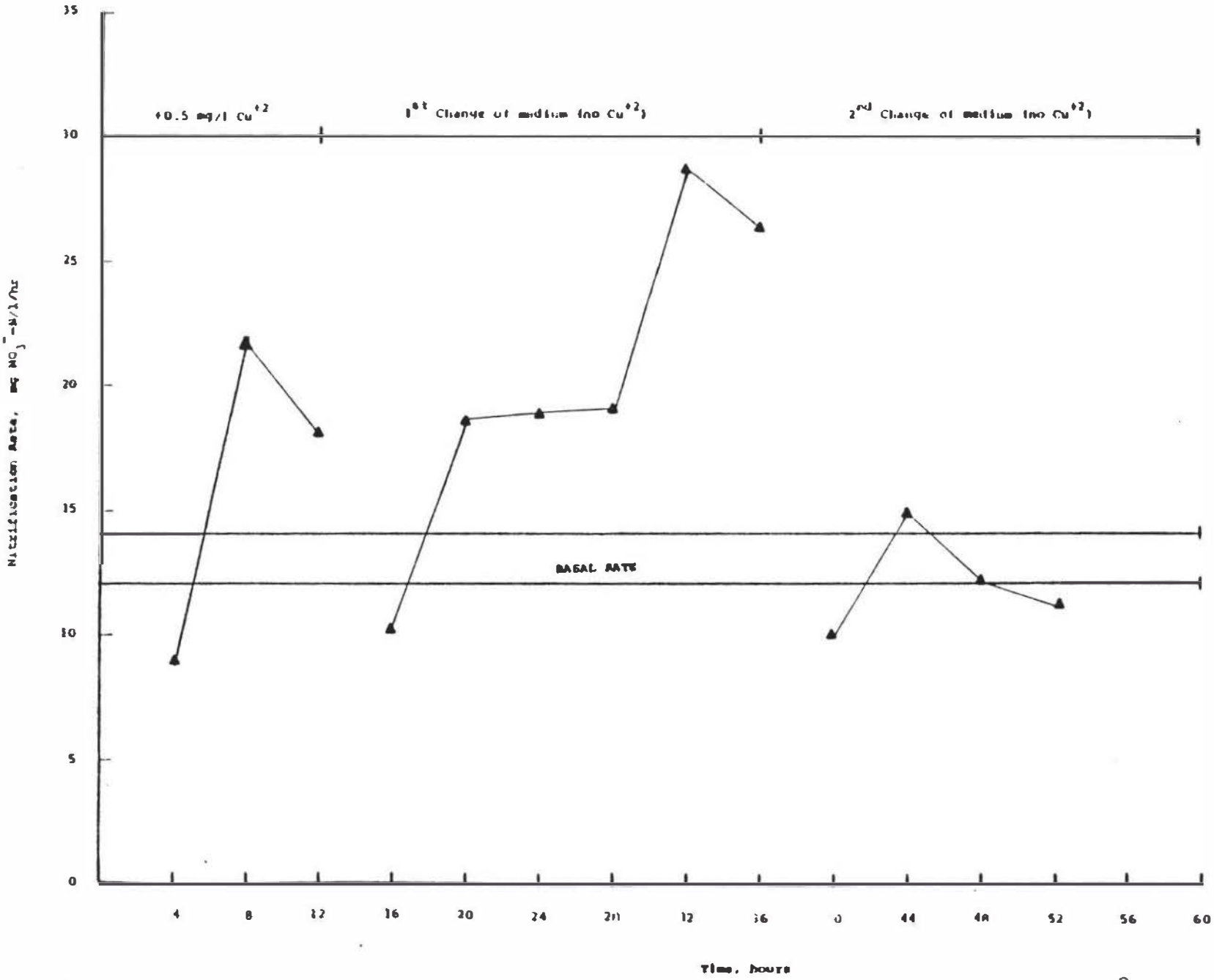

Figure 9. Nitrification rate vs. time at a copper concentration study of 0.50 mg Cu^{+2}/l.

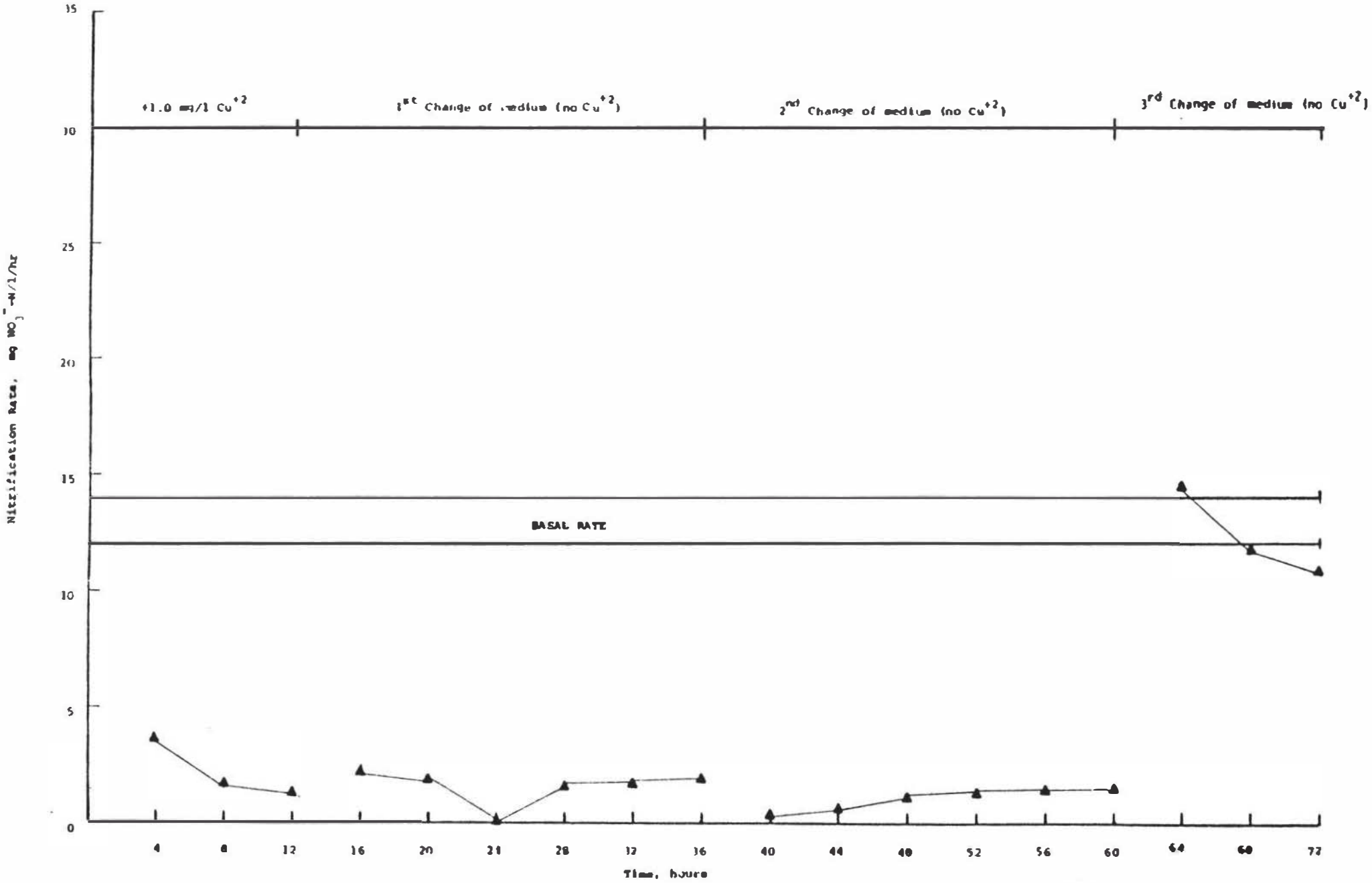

Figure 10. Nitrification rate vs. time at a copper concentration study of 1.0 mg Cu^{+2}/l.

cation decreased from that level. After replacement with copper-free medium, the nitrification process still was apparently inhibited until 64 hours after the initial poisoning period, when the rate returned to normal. A comparison of the rate of nitrification after 12 hours in the presence of different levels of copper (Fig. 11), showed that a gradual increase in rate occurred until the copper concentration reached 0.10 mg/l, then a drop occurred indicating inhibition at higher levels. The only level to show inhibition in the presence of copper and for a time, in its absence, was at copper concentration of 1.0 mg/l.

In pure Nitrosomonas cultures isolated from activated sludge, Skinner and Walker (1961) found that copper concentrations greater than 1 mg Cu^{+2}/l inhibited ammonia oxidation and that 4 mg Cu^{+2}/l reduced ammonia oxidation by about 75%. Loveless and Painter (1968) found that in pure cultures of Nitrosomonas, ammonia oxidation was inhibited at copper levels greater that 0.05 mg Cu^{+2}/l (at pH 7.3). In contrast, Tomlinson and coworkers (1966) found activated sludge to be much more tolerant to copper, exhibiting 25% ammonia oxidation at 150 mg Cu^{+2}/l. As seen in Table 11, in the nitrifying biofilm (at a copper concentration of 1.0 mg/l) nitrification occurred at 10% the normal rate after the 12 hour direct contact with the biofilm. After 24 hours in fresh medium, the nitrification rate was completely inhibited. When comparisons with nitrification in pure cultures are made, the nitrifying biofilm seems to be at least as sensitive as the pure culture. On the other hand, activated sludge nitrifying organisms seem more tolerant of copper than the nitrifying biofilm.

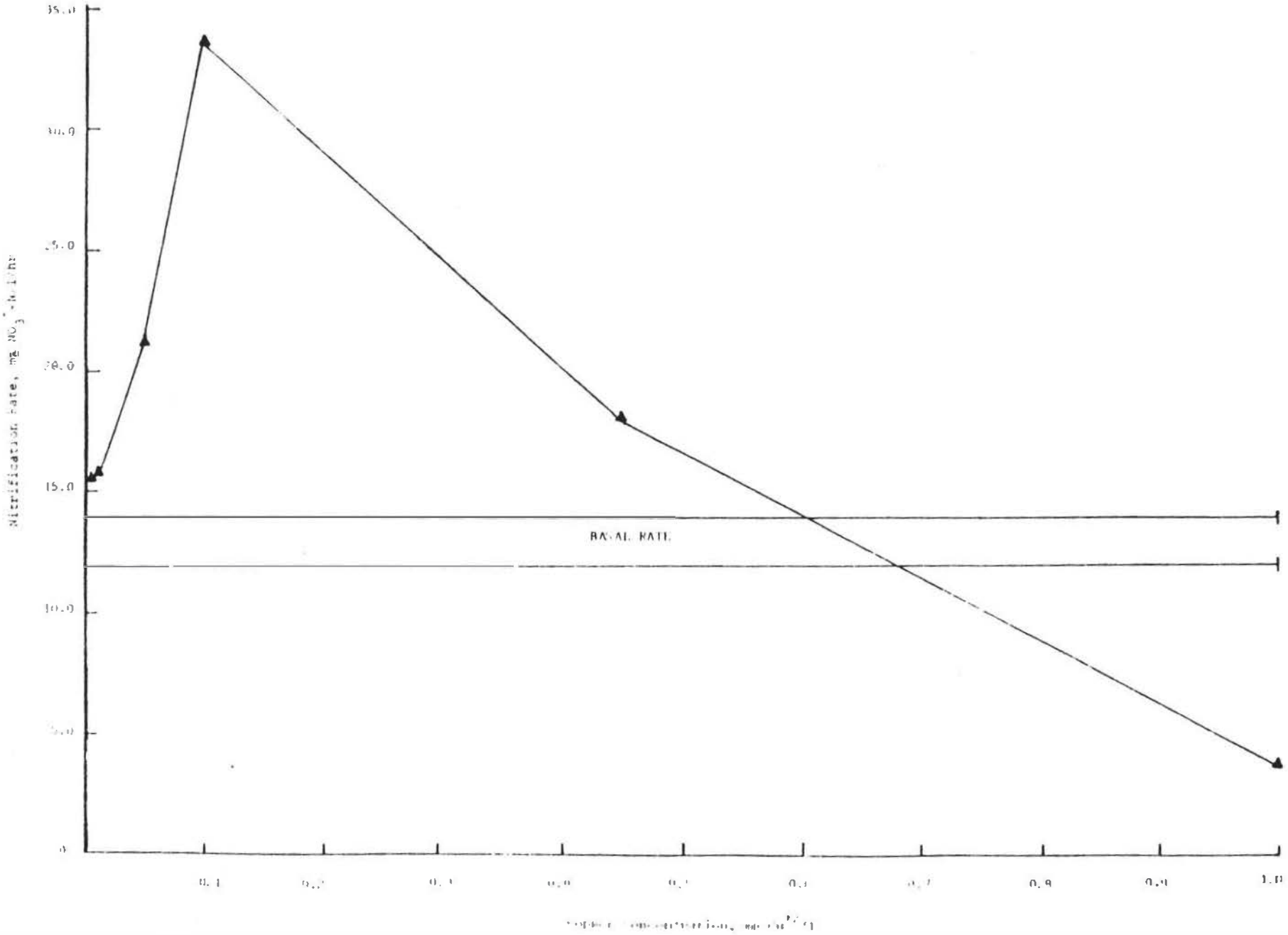

Figure 11. Nitrification rate vs. copper concentration after 12 hours direct contact.

Table 11. Variation in the nitrification rates at differing concentrations of copper.

mg Cu^{+2}/l	% Nitrification rate after 12 hours of copper contact	% Nitrification rate 24 hrs. after Cu^{+2} removal	Hours to return to normal
0	----	100% (basal rate)	---
0.005	119%	244%	app. 66
0.01	121%	133%	72
0.05	193%	190%	56
0.10	258%	161%	56+
0.50	138%	145%	48
1.00	10%	0%	68

This is probably due to the fact (as suggested in part by Tomlinson et al.,1966) that activated sludge contains more organic material to complex the metal contaminants than does the biofilm. Another possible factor contributing to the differences exhibited by the three nitrifying systems (pure culture - sludge - biofilm) may be that different strains of Nitrosomonas (and/or Nitrobacter) can vary in their sensitivity to copper (Tomlinson et al.,1966).

The nitrifying biofilm exhibited 90% ammonia oxidation inhibition in the presence of 1.00 mg Cu^{+2} /l (Table 11) and is, therefore, more sensitive to copper than nitrifying activated sludges which exhibited only 75% inhibition at 150 mg Cu^{+2} /l(Tomlinson et al., 1966). This suggests that plants using nitrifying biofilms for removal of ammonia might be more effected by shock (or chronic) doses of copper than those using activated nitrifying sludges.

LITERATURE CITED

1. Aleem, M.I.H. The physiology and chemo-autotrophic metabolism of Nitrobacter agilis. Thesis, Cornell University, N.Y. 1959.

2. Aleem, M.I.H.; Alexander, M. Cell-free nitrification by Nitrobacter. J. Bacteriol. 76: 510. 1958.

3. Aleem, M.I.H.; Alexander, M. Nutrition and physiology of Nitrobacter agilis. Appl. Microbiol. 8: 80. 1960.

4. Beg, S.A.; Siddiqi, R.H.; Ilias, S. Inhibition of nitrification arsenic, chromium, and fluoride. Jour. Water Poll. Control Fed. 54: 482. 1982.

5. Boon, B.; Laudelout, H. Kinetics of nitrate oxidation by Nitrobacter winogradski. Biochem. J. 85: 440. 1962.

6. Borchardt, J.A. Nitrification in the activated sludge process. In The Activated Sludge Process. Div. of San. and Water Resources eng., Univ. of Michigan, Ann Arbor. 1966.

7. Buchanan, R.E.; Gibbons, N.E. eds. Bergey's Manual of Determinative Bacteriology, Eighth Ed. Williams and Wilkins Co., Baltimore. 1974. 1268 pp.

8. Buswell, A.M.; Shiota, T.; Lawrence, N.; Meter, I.V. Laboratory studies on the kinetics of the growth of Nitrosomonas with relation to the nitrification phase of the BOD test. Appl. Microbiol. 2: 21. 1954.

9. Campbell, N.E.R.; Aleem, M.I.H. The effect of 3-chloro-6-(trichloromethyl)pyridine on the chemoautotrophic metabolism of nitrifying bacteria. II. Nitrite oxidation by Nitrobacter. Antonie van Leeuwenhook 31: 137. 1965.

10. Clark, C.; Schmidt, E.L. Uptake and utilization of amino acids by resting cells of Nitrosomonas europaea. J. Bacteriol. 93: 1309. 1967.

11. Downing, A.L. et al. Effect of inhibitors on nitrification in the activated sludge process. Jour. Inst. Sew. Purif. 87: 547. 1964.

12. Engel, M.S.; Alexander, M. Growth and autotrophic metabolism of Nitrosomonas europaea. J. Bacteriol. 76: 217. 1958.

13. Erikson, R.H.; Hooper, A.B. Preliminary characterization of a variant CO-binding heme protein from Nitrosomonas. Biochem. Biophys. Acta. 175: 231. 1972.

14. Hackette, S.L.; Syke, G.E.; Burton, C.; Segel, I.H. Characterization of an ammonia transport system in filamentous fungi with methyl ammonium - C14 as the substrate. J. Biol. Chem. 245: 4241. 1970.

15. Harold, F.M. Antimicrobial agents and membrane function, p. 45-104. In A.H. Rose and F.J. Wilkinson (ed.), Advances in microbial physiology, vol. 4. Academic Press, Inc. New York. 1970.

16. Hochster, R.M.; Quastel, J.H. eds. Metabolic Inhibitors, vol. II. Academic Press, New York, N.Y. pp. 615-629. 1963.

17. Hockenbury, M.R.; Grady, C.P. Jr. Inhibition of nitrification. Effect of selected organic compounds. Jour. Water Poll. Control Fed. 49: 768. 1977.

18. Hofman, R.; Lees H. The biochemistry of the nitrifying organisms 2. The free-energy efficiency of Nitrosomonas. Biochem. J. 52: 140. 1952.

19. Hooper, Alan B.; Terry, Kathleen, R. Specific inhibitors of ammonia oxidation in Nitrosomonas. J. Bacteriol. 115: 480. 1973.

20. Jensen, H.L. Effect of organic compounds on Nitrosomonas. Nature 165: 974. 1960.

21. Kholdebarin, B.; Oertli, J.J. Effect of pH and ammonia on the rate of nitrification of surface water. Jour. Water Poll. Control Fed. 49: 1688.

22. Kiesow, L. On the mechanism of chemosynthesis. Biol. Abstr. 42: 1185. 1962.

23. Laudelout, H.; Van Tichelen, L. Kinetics of the nitrite oxidation by Nitrobacter winogradski. J. Bacteriol. 79:39. 1960.

24. Lees, H. Inhibitors of nitrification. In Metabolic Inhibitors, Ch. 37. Hochester and Quastel Eds. 1963.

25. Lees, H. The biochemistry of the nitrifying organisms. 1. The ammonia oxidizing systems of Nitrosomonas. Biochem. J. 52: 134. 1952.

26. Lees, H. The biochemistry of the nitrifying bacteria. In Symposium on Autotrophic Microorganisms, pp. 84-98. Cambridge Univ. Press, London. 1954.

27. Lees, H. The biochemistry of autotrophic bacteria. Butterworth's Scientific Publications, London. 1955. 112 pp.

28. Lees, H.; Quastel, J.H. Biochemistry of nitrification in soils, III. Nitrification of various organic nitrogen compounds. Biochem. Jour. 40: 824. 1946.

29. Lewis, R.F. Isolation and growth of Nitrosomonas in pure culture. Dissertation, Rutgers State University, New Jersey. 1959.

30. Loveless, J.E.; Painter, H.A. The influence of metal ion concentrations and pH values on the growth of a Nitrosomonas strain isolated from activated sludge. J. Gen. Microbiol. 52:1. 1968.

31. Meiklejohn, J. Some aspects of the physiology of the nitrifying bacteria. In Symposium on Autotrophic Microorganisms. pp.68-83. Cambridge Univ. Press, London. 1954.

32. Meyerhof, O. Untersuchungen über den Atmungsvorgang nitrifizierender Bakterien. I. Die Atmung des Nitratbildners. Pflugers Arch. ges. Physiol. 164: 353. 1916.

33. Nicholas, D.J.D.; Wilson, P.W.; Heinen, W.; Palmer, G.;Beinerts, H. Use of electron paramagnetic resonance spectroscopy in investigations of functional metal components in microorganisms. Nature, London 196: 433. 1962.

34. Nicholls, P.; Schonbaum, G.R. Catalases. pg. 147-225, In P.D. Boyer, H. Lardy, and K. Myrback (eds.), The enzymes, vol. 8. Academic Press Inc. N.Y. 1963.

35. Oginsky, E.L.; Umbreit, W.W. An introduction to bacterial physiology. W.H. Freeman and Co., San Francisco. 1959. 443 pp.

36. Painter, H.A. A review of literature on inorganic nitrogen metabolism in microorganisms. Water Research 4: 393. 1970.

37. Pokallus, R.S. Toxicity of nitrite to Nitrosomonas europaea. Doctoral thesis. Rutgers State Univs., New Jersey. 1963.

38. Process Design Manual for Nitrogen Control. Technology Transfer, U.S. Environmental Protection Agency. 1975.

39. Randall, C.W.; Buth, D. Nitrite build-up in activated sludge resulting from temperature effects. Jour. Water Poll. Control Fed. 56: 1039. 1984.

40. Randall, C.W.; Buth, D. Nitrite build-up in activated sludge resulting from combined terperature and toxicity effects.. Jour. Water Poll. Control Fed. 56: 1045 - 1049. 1984.

41. Schoberl, P.; Engel, H. Das. Verhalten der nitrifizierenden Bakterien gegenüber gelöstem Sauerstoff. Arch. Mikrobiol. 48: 393. 1964.

42. Skinner, F.A.; Walker, N. Growth of Nitrosomonas europaea in batch and continuous culture. Arch. Mikrobiol. 38: 339. 1961.

43. Stojanovic, B.J.; Alexander, M. Effect of inorganic nitrogen on nitrification. Soil Sci. 86: 208. 1958.

44. Tomlinson, T.G.; Boon, A.G.; Trotman, C.N.A. Inhibition of nitrification in the activated sludge process of sewage disposal. Jour. Appl. Bacteriol. 29: 266. 1966.

45. Ulken, A. Die Herkunft des Nitrits in der Elbe. Arch. Hydrobiol. 59: 486. 1963.

46. Van Droogenbroeck, R.; Laudelout, H. Phosphate requirements of the nitrifying bacteria. Antonie van Leeuwenhoek 33: 287. 1967.

47. Wild, H.E. Jr.; Sawyer, C.N.; McMahon, R.C. Factors affecting nitrification kinetics. Jour. Water Poll. Control Fed. 43: 1845. 1971.

48. Wood, L.B.; Hurley, J.E.; Matthews, P.J. Some observations on the biochemistry and inhibition of nitrification. Water Research 15: 543. 1981.

49. Young, J.C. Chemical methods for nitrification control. Jour. Water Poll. Control Fed. 45: 637. 1973.

50. Zavarzin, G.A. On the inducer of the second phase of nitrification, I. Concerning the participation of respiratory pigments in nitrification. Mikrobiologiia 27: 401. 1958.