EFFECTS OF CYANIDE ON TRICKLING FILTER MICROORGANISMS

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THESIS

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EFFECTS OF CYANIDE ON TRICKLING FILTER MICROORGANISMS

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TABLE OF CONTENTS

ABSTRACT

Sodium cyanide in concentrations of 0.3 ppm (as CN) was applied to a pilot plant trickling filter. This was later increased to 1 ppm. A second trickling filter, operated in parallel, was used as a control.

The 0.3 ppm concentration of CN did not seriously affect the BOD of the effluent, but a high nitrite concentration appeared in the effluent. The nitrites decreased after a week, falling below the control. Nitrates were also below those found in the control filter effluent.

A CN concentration of 1 ppm retarded nitrite and nitrate formation and increased the BOD of the effluent.

A shock load of 4 ppm CN also resulted in high nitrites.

Nitrite and nitrate formation in the filter effluent was retarded by 40 ppm CN, especially in samples from the control filter.

High cyanide concentrations (20 and 200 ppm) inhibited reduction of nitrate to nitrite by Psuedomonas aeruginosa (both unadapted and previously adapted to 200 ppm cyanide); 2 ppm was slightly inhibitory, but 0.2 ppm had no apparent effect. The high concentrations caused a slight initial increase (followed by a decrease) in nitrites formed by the adapted strain.

Laboratory toxicity studies using a glucose broth

and a synthetic broth indicated that choice of medium is an important factor in demonstrating toxic effects. Most organisms tested were tolerant to 20 and 200 ppm CN on glucose broth. On the synthetic broth, considerably less resistance was shown. Only one organism grew in contact with 200 ppm CN: Serratia marcescens; this occurred only in the presence of 1 ppm methylene blue.

A cyanide tolerant strain of Streptomyces albus was isolated from sewage.

The author believes that nitrite production is the result of emergency use of nitrate as an oxidizing agent by the exposed organisms. This action is also sensitive to cyanide, but the inhibition is much slower.

INTRODUCTION

Since dissolved cyanides are generally toxic to humans and to fish, it is in the public interests to prevent discharge of cyanides to natural streams. Gaseous hydrogen cyanide, which is evolved on hydrolysis of cyanide salts and other cyanide compounds, is also very dangerous.

Cyanide discharges arise from industrial wastes, such as spent plating liquors and gas works effluents, which, accidentally or deliberately, are dumped into streams or into municipal sewers. The damaged treatment plants fail to perform oxidation of the wastes and contain the cyanide in the effluent. Cyanides inhibit the biological oxidation of the sewage; thus the streams become overloaded with organics, which are both a nuisance and a potential public health hazard. These effects are probably more important than the toxicity of the cyanide itself.

In order to employ a biological cyanide disposal unit, or to operate a sewage treatment unit in the presence of cyanide contamination, the mechanisms involved must be more clearly understood.

While many cyanide wastes contain the cyanides as heavy metal complexes, only simple cyanides were studied in this investigation.

SURVEY OF PREVIOUS WORK

The cyanide ion has two outstanding chemical characteristics: it is easily oxidized and it forms very stable metallic complexes. Most of its biological activity can be explained on one or the other of these bases.

Cyanide inhibition of cell respiration led Warburg to discovery of the cytochrome systems. These cytochromes have in common a heme (tetrapyrrole) nucleus and a heavy metal ion as prosthetic group. Inhibition is due to the formation of a complex between the cyanide and the enzyme's metallic ion, thus reducing its effectiveness. The toxicity of cyanides to mammals is due to inactivation of the blood hemoglobin (21). Since the cytochromes are frequently essential in hydrogen transport, repression of these enzymes will seriously hamper cell metabolism.

Many other enzymes (i.e. the polyphenol oxidases) contain heavy metals as active groups and are thus likely to be cyanide-sensitive. The cytochrome oxidase system ("indophenol oxidase") is also inhibited by cyanides (45) (47).

Not all enzymes are inhibited by cyanide. Urease is activated (25); as is the plant proteolytic enzyme β-papain (24). Some enzymes, as the dehydrogenases (47), are neither inhibited nor stimulated by the cyanide ion.

Few general statements can be made concerning the effects of cyanide on bacteria, as these are too diverse physiologically to be covered in a few sentences. In order to discuss these effects in an orderly fashion, the bacteria will be considered in families.

Bacteria are generally classified in the class Schizomycetes of the sub-phylum Fungi. Most bacteria, as ordinarily conceived, belong to the order Eubacteriales. There are eleven families in this order. Of these, the following have representatives occurring in sewage and in biological treatment units (6)(48): Nitrobacteriaceae, Psuedomonadaceae, Rhizobiaceae, Micrococcaceae, Achromobacteriaceae, Enterobacteriaceae, Bacteriaceae and Bacillaceae.

The Nitrobacteriaceae are autotrophic. The chief genera found in trickling filters are Nitrosomonas and Nitrobacter. Both have been reported to be inhibited by cyanide in concentrations of 2.5×10^{-6} (37) and 5×10^{-6} molar (31) respectively. These are equivalent to 70 and 140 ppm CN. Rao and Rao have demonstrated that Nitrosomonas fails to adapt to ammonia oxidation in the presence of cyanide ion. These organisms are chiefly responsible for nitrite and nitrate formation on trickling filters and in soil. There are several reports of cyanide inhibition of soil nitrification, including Lees and Quastel (26) and Tam and Clark (44).

The Psuedomonadaceae are generally gram negative rods, and are frequently found in soils and water. One of the

most commonly encountered is Psuedomonas aeruginosa. Work reported on this organism is conflicting: Quiroga and Monteverde (36) report that five strains of Ps. aeruginosa produce HCN from amino acids. Mochtar (33) also noticed HCN production.

Barron and Friedeman (5), on the other hand, report that this organism is completely inhibited by HCN.

Acetobacter xylinum is reported by Cozic (13) to be cyanide-tolerant, but six other species are inhibited by 4×10^{-3} molar KCN (10,200 ppm CN).

No reports on the effects of cyanide on Rhizobiaceae were discovered in this survey.

Micrococcaceae are gram positive spheres. Because of their shape, they present less surface to their surroundings and therefore would be more likely to be highly tolerant of cyanide.

Burnet (11) reports that gram positive cocci are least sensitive to cyanide; Streptococcus tolerates 0.5% CN (5000 ppm).

Sarcina lutea is reported to be cyanide tolerant (18).

According to Braun (8), Staphylococci are more influenced by the cyanide than are the Streptococci under aerobic conditions. (This agrees with Burnet's data: "Staphylococcus aureus" is inhibited completely by 0.04% CN.)

The pathogenic cocci are reported by Sevag (39) to be stimulated by cyanides at 10,200 to 51,000 ppm concentrations.

Of the Achromobacteriaceae, only data concerning Alcaligenes fecalis are reported. Barron and Friedeman (5) report inhibition; Mochtar (33) reports HCN production.

The Enterobacteriaceae are highly fermentative gram negative rods, which include such pathogens as Salmonella typhosa and Shigella dysenteriae.

Escherichia coli, the bacteriologist's favorite, is reported by Burnet to resist 0.1% (1000 ppm) CN. However, Aubel, Rosenberg and Szulmajster (4) report that cell respiration is inhibited, except in the presence of pyruvate. Stickland (56) reported that 0.0001 M CN (2800 ppm) inhibits nitrate reduction. Dessy (14) reported that E. coli is killed by 10% NaCN in six hours.

Mochtar reported that Proteus produces HCN.

Burnet reported that Shigella does tolerate cyanide, while Salmonella typhosa does not. Braun and Kurman (9) reported that Shigella is less resistant under aerobic conditions.

Salmonella paratyphosa tolerates 0.01% but not 0.1%, according to Boné.

The Bacteriaceae are a "miscellaneous" group; no general trend was noted.

Bacillaceae are the spore-forming bacteria. Most investigations have been centered on Bacillus subtilis.

As found by Boné (7), their spores survive five days in 0.1% CN while the vegetative cells are killed. These vegetative cells survive in 0.01% CN. Similar inhibition

is found by Hartree (22), who found parallel results with 5,7-dichloro-8-hydroxyquinoline, also a heavy metal complexing agent.

Cyanides are not particularly toxic to bacteria. Southgate (41) reports more (total numbers) organisms in a sewage containing cyanide than in normal sewage.

Burnet stated that all cyanide-tolerant organisms he found possessed peroxidase (a heme-containing enzyme), except Shigella. (Since hydrogen peroxide easily oxidizes cyanides in vitro, this may imply a mechanism for detoxification.) He believed that the major effect of the cyanide was to lower the redox potential of the system.

Löffler and Rigler (28) believed that ability to liberate hydrogen sulfide from cystein is highly correlated with cyanide resistance. H_2S and HCN both attack metal ions; tolerance for one would render tolerance for the other more likely.

Molds have been reported resistant to cyanide. Dessy (14) reported that while E. coli is killed in six hours, "molds" resist 10% NaCN for over one day. Tam and Clark (44) reported that soil fungi and actinomycetes are little affected by calcium cyanide. Generally fungi are cyanide-tolerant. The chief interest seems to lie in demonstration of ability to use cyanide as a sole nitrogen source.

Some work has been done on the toxicity of cyanide to yeasts. Meisel (30) reported that $Hg(CN)_2$ is more toxic than KCN which, in turn, is more toxic than NaCN. He stated that

long exposure to low cyanide concentrations causes a loss of fat in yeast cells. This is not demonstrated in yeast cells exposed to high concentrations of cyanide for a short interval.

Winzler (45) found that cyanides inhibit yeast respiration by inhibiting cytochrome oxidase and combining with an (unspecified) enzyme system.

Some work has been done with green algae. Nitella and Chlorella are among the genera reported to be cyanide-tolerant (38)(15).

Some HCN is produced by higher plants, but its function is unknown.

The toxicity of cyanide to mammals and fish is well recognized.

An organism has been isolated from the effluent from a trickling filter adapted to thiocyanate containing wastes by Happold and Key (20). They named it "Bacterium thiocyanoxidans", but it is not well characterized. Meyerson and Skupenko (32) reported finding thiocyanate in streams containing cyanide. (This may be a clue to the mechanism of cyanide destruction or detoxification.)

The effects of cyanide on sewage and sewage treatment may be considered as the summation of the effects on the single organisms. However, the populations involved in the disposal process are not well characterized.

Ludzack, Moore, Krieger and Ruchhoft (29) have conducted a study on the effects of cyanide in sewage samples

in the biological oxygen demand (BOD) test. They concluded that cyanide causes a lag in, or inhibition of, metabolism, but does not sterilize the sewage. Five percent inhibition was caused by 0.3 ppm CN. They further conclude that the chief disposal mechanism in natural streams is volatilization.

There are two general methods of aerobic biological disposal: activated sludge process and the trickling filter method. Both employ a zoogleal mass of bacteria which oxidize the material; in the activated sludge process, the mass is not attached to any support.

The earliest reported work on the activated sludge process was that of Wooldridge and Standfast, in 1937 (48). They found that 10^{-4}M CN (200 ppm) or the vapor from solid KCN inhibited the bacterial action.

In 1946, Nolte and Bandt (34) set up a miniature plant using a modification known as the Magdeburg process and butyrates or m-cresol as the organic substrate. The protozoa were shocked by 5 ppm KCN but later recovered. At 62 ppm, after adaptation, the effluent was free from cyanide and butyrate. If normal activated sludge is supplied in the recycle, 330 ppm KCN will not interfere with oxidation of the butyrate. (Evidently multiplication of the organisms is more sensitive than their metabolism.) Similar results were noted when m-cresol was used as the substrate.

In 1948, Lockett and Griffiths (29) noted that 1 ppm HCN partially inhibited the oxidation process. HCN was blown off in the aeration tank.

Coburn, in 1949, (12) found that 5 ppm caused a partial inhibition of oxidation by the activated sludge, but 20 ppm caused complete inhibition. This latter inhibition was overcome on removal of the cyanide.

There is even less data on the trickling filter process.

Pettet and Thomas, in 1948, (35) noted that less than 1 ppm HCN had no effect on the BOD of the effluent. (This disagrees with Ludzack et al.) Increasing the cyanide to 2 ppm had little effect on BOD but nitrate formation was retarded. An increase to 4 ppm resulted in an increased BOD in the effluent, as also did 10 ppm. After the filter was in contact with these concentrations for a time, the abnormalities dissappeared and the cyanides were more-or-less completely destroyed.

When the cyanide was increased to 30 ppm, nitrification was completely destroyed. About two months were required for adaptation. Then, total nitrogen in the effluent was greater than in the control.

The Water Pollution Research Board (Great Britain) published some work on cyanide toxicity in their 1951 annual report (1). They stated that 1 ppm had little effect on the BOD of the effluent but that the permanganate oxygen demand had increased. Nitrification was initially inhibited.

In 1952, this group reported (2) that 50-100% of the cyanide fed to the filter could be included in the ammonia, nitrites and nitrates in the effluent. On adaptation, cyanide in copper, zinc and cadmium complexes were also

almost completely destroyed. This was not the case for the iron and nickel complexes.

These studies do not agree closely on details, but owing to the possibility of different flora on the filters and different materials in the sewage, this is not unexpected.

To generalize, the BOD value is less sensitive to cyanide than chemical oxidation values. Nitrate formation is inhibited. The cyanides are eventually destroyed on the filter, if continuous exposure is provided.

EXPERIMENTAL PROCEDURE

Two pilot-plant trickling filters, two feet in diameter and with a rock depth of six feet were set up in parallel, complete with separate settling tanks (70 gallons capacity), recirculation pumps and constant-head tanks. These units are located in a special building on the grounds of the East Lansing municipal sewage disposal plant. Each filter has a capacity of 600 gallons per day, when operated at a recycle ratio of six to one. Figure 1 is a schematic flow diagram of the system. Figures 2 to 4 are photographs of these units; Figure 3 is an overall view of the pilot-plant.

The sewage, free from industrial wastes, is obtained from the primary settling basin in the East Lansing plant. Control of flow rates is obtained by using orifices installed below constant-head tanks. A fan and ducts are installed to remove contaminated air from the room.

After the filters had matured, one was exposed to a continuous feed of 0.3 ppm (as CN) sodium cyanide. The feed unit was essentially a supply bottle inverted over a funnel, with a stopcock to regulate flow rates. (See Figure 2.) Cyanide concentrations in the sewage were varied by changing the concentration of cyanide in the feed solution. It was necessary to use distilled water in these solutions to avoid precipitation of the cyanides.

After four weeks, the cyanide was increased to 1 ppm.

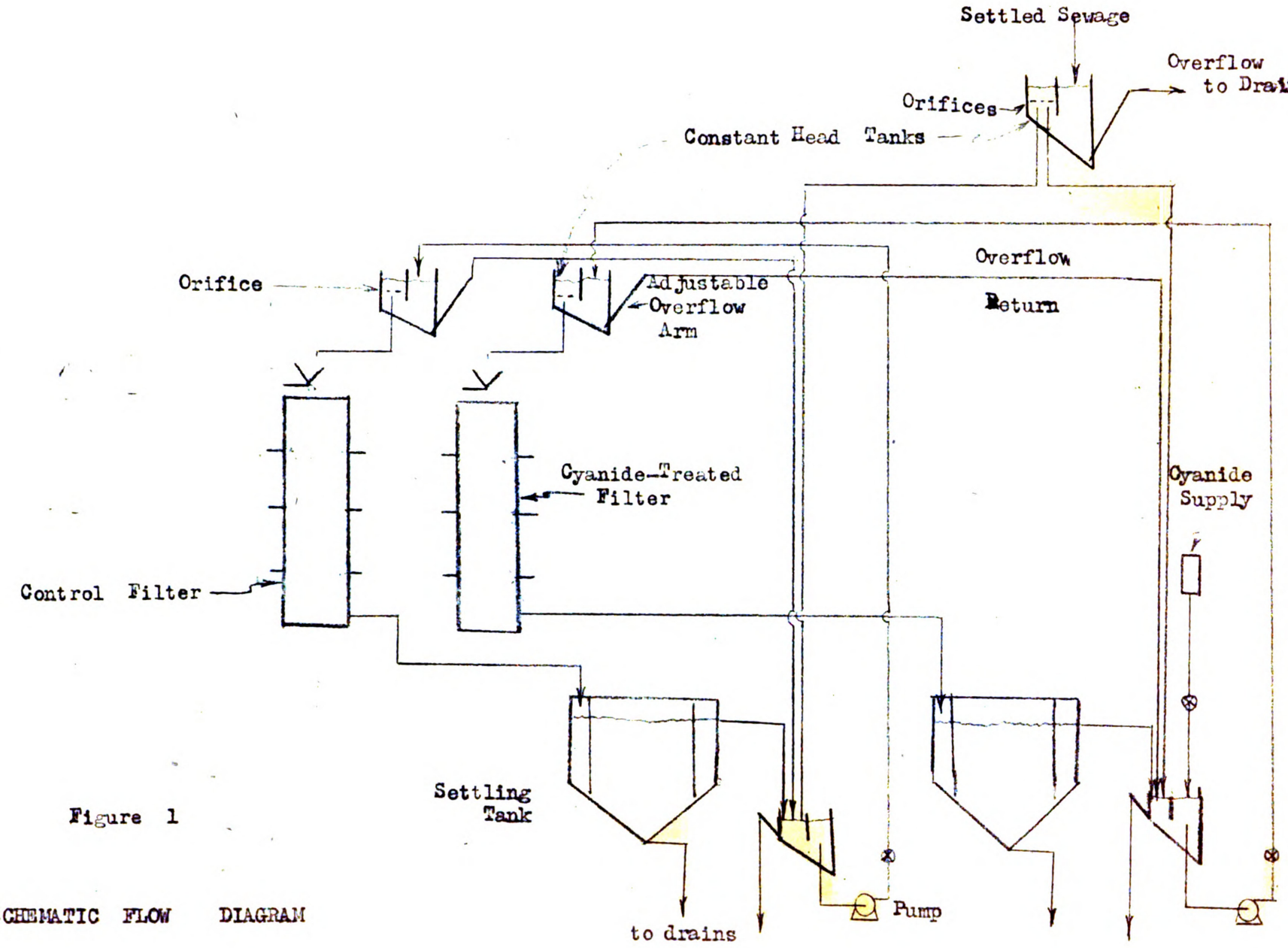


Figure 1

SCHEMATIC FLOW DIAGRAM

Figure 2

CYANIDE FEED AND SETTLING TANK

Figure 3

OVERALL VIEW

Figure 4

TRICKLING FILTERS

Samples were taken daily: at the bases of the filters ("effluent"); at the recirculation pump intake ("influent") and at the constant-head tank proportioning the raw sewage. (To retard clogging of orifices, a screen is inserted above this tank.) Daily duplicate determinations of biological oxygen demand (BOD) and single determinations of oxygen consumed by dichromate (DOC) by Shaw's method (40), nitrates (phenoldisulfonic acid method) (3), and nitrites were performed. Nitrates and nitrites were determined colorimetrically, employing a Coleman Model 9 Nephlo-Colorimeter. (See Appendix II for calibration curves.)

In addition, formaldehyde determinations by chromotropic acid (10) and by condensation with phenylhydrazine (43) and cyanide determinations by Prussian blue formation, by picric acid reduction, and by ferric thiocyanate formation were also made. Calibration curves for these analyses are included in Appendix II.

At the end of the pilot-plant investigation, the control filter was subjected to a shock load of 4 ppm cyanide by adding the necessary amount of cyanide solution directly to the settling tank. Nitrite production was followed in this test.

Toxicity studies, using pure cultures of organisms reported from sewage and trickling filters (6)(48), were made in the laboratory, both on a synthetic medium and on glucose broth. The organisms were obtained from various members of the Bacteriology Department. Organisms employed were:

Aerobacter aerogenes, Alcaligenes fecalis, Chromobacterium amethystinum, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Ps. fluorescens, Salmonella typhosa, Serratia marcescens, "Staphylococcus aureus" (Micrococcus pyrogenes var. aureus), and Streptococcus liquifaciens.

Isolation and identification of cyanide-tolerant organisms from sewage and trickling filter effluent was attempted. The procedures outlined in Bergey's manual (6) were followed.

The effect of cyanide on nitrite production in the filter effluent under aerobic conditions was also investigated in the laboratory. Fifty-milliliter portions of the effluents from each filter were exposed to 40 ppm CN in sterile 500-ml. Erlenmeyer flasks loosely stoppered with cotton. Samples were withdrawn aseptically; nitrites were determined colorimetrically as in routine analysis. The temperature was 22±1°C.

Similar experiments were performed on two strains of Ps. aeruginosa, one not previously exposed to cyanide, the other adapted to 200 ppm CN. Diluted 48 hr. cultures were added to 100 ml. of a sterile medium consisting of physiological saline plus 0.1% glucose and 0.1% potassium nitrate. These organisms were added aseptically to the flasks together with various concentrations of cyanide (0.2, 2, 20 and 200 ppm). Nitrite concentrations after various time intervals were determined. A temperature of 20°C was maintained during these tests in an air incubator.

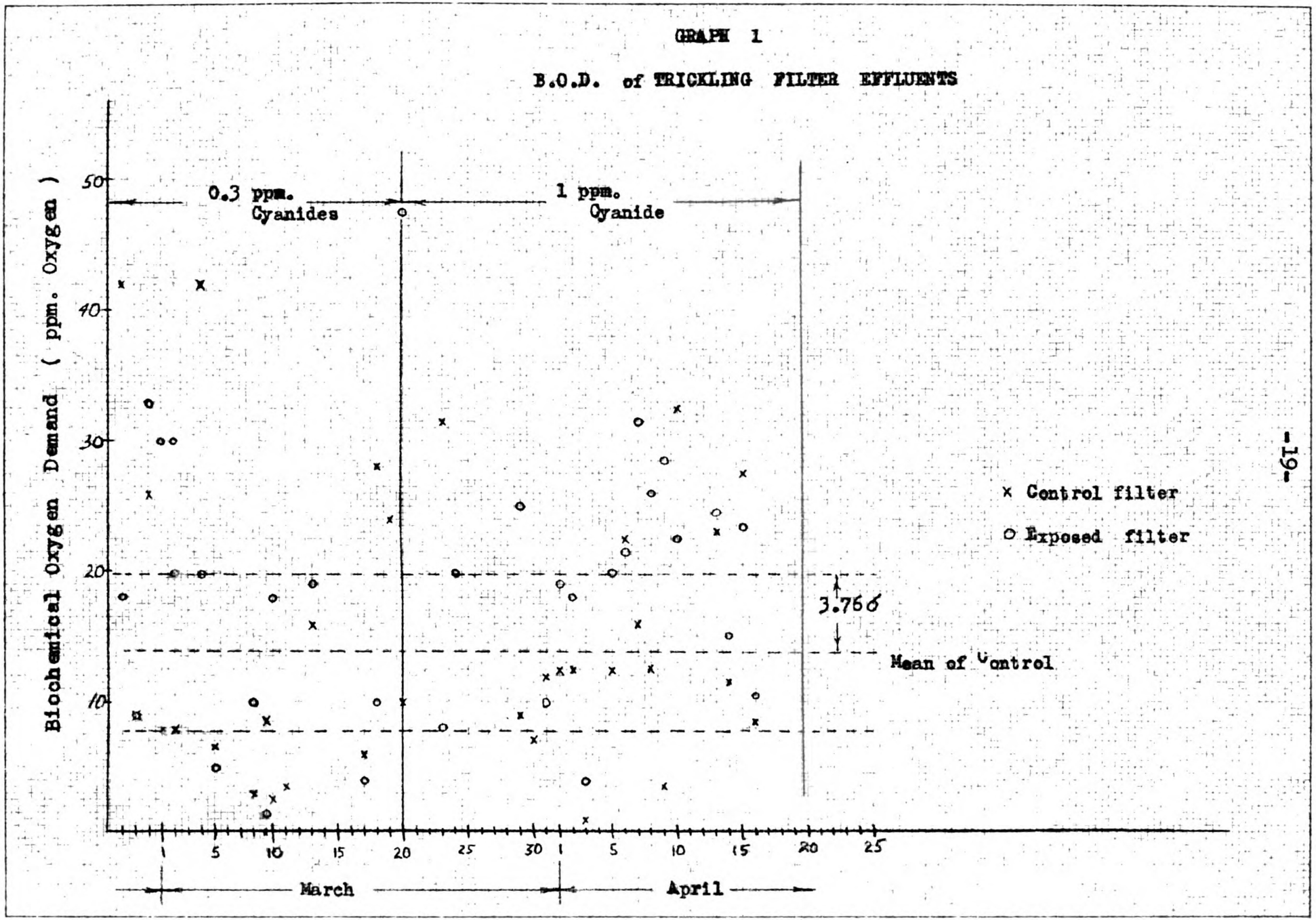
PRESENTATION AND ANALYSIS OF RESULTS

Graph 1 shows the effect of low concentrations of cyanide on the BOD of the trickling filter effluent. Cyanide in a concentration of 0.3 ppm was not particularly detrimental to the effluent quality, but addition of 1 ppm CN resulted in a merked tendency for higher BOD values. The mean BOD of the control filter effluent was 14 ppm oxygen, with a standard deviation of 1.6 ppm.

A similar plot of dichromate oxygen consumed showed a scatter which was difficult to interpret. The effluent from the control filter had a mean of 43 ppm oxygen, with a standard deviation of 29 ppm. A correlation between BOD and DOC is included in Appendix II.

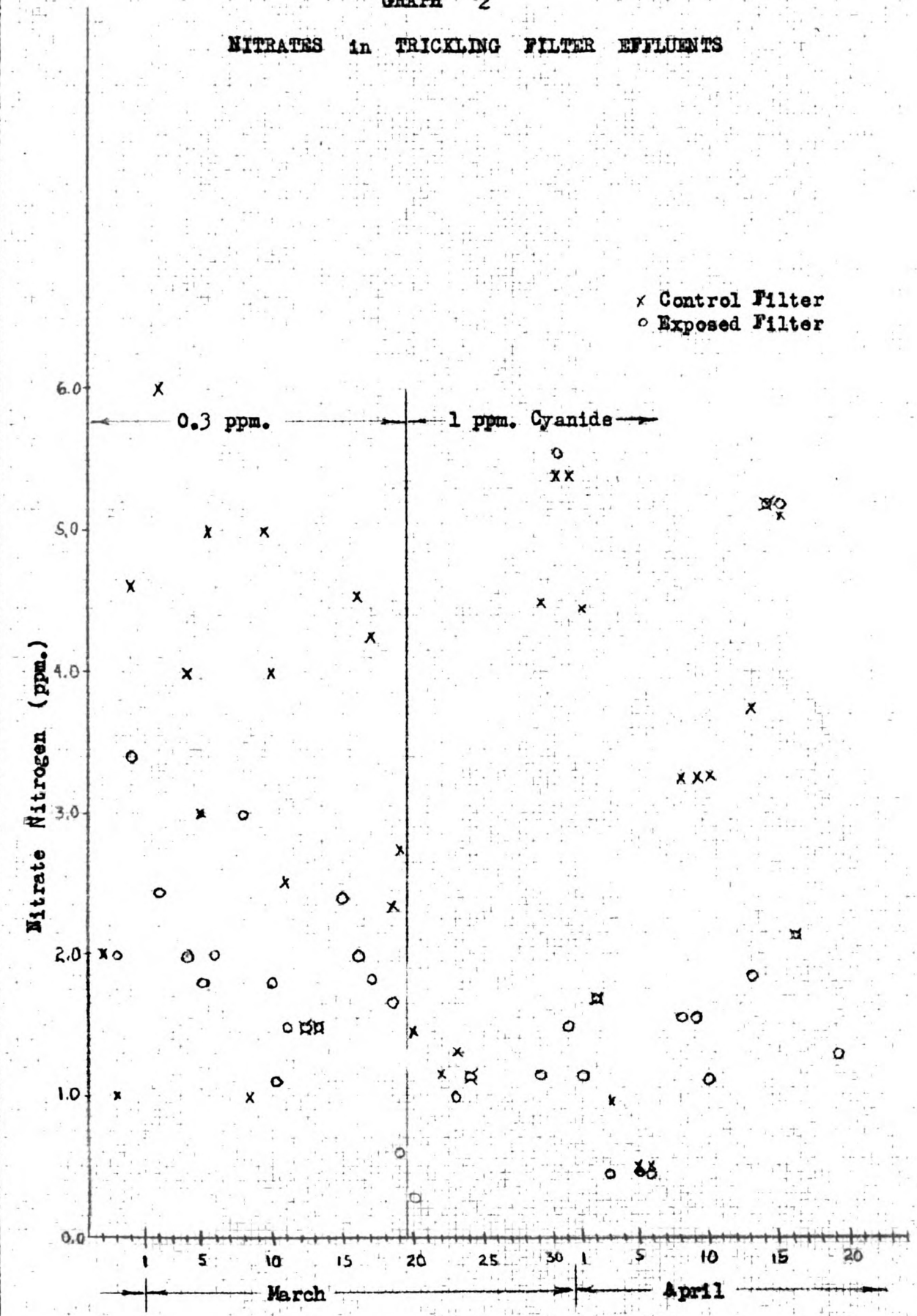
Graph 2 shows that 0.3 ppm cyanide partially inhibited nitrate formation, and that 1 ppm caused even more marked inhibition. Neither concentration inhibited nitrate formation completely. The mean nitrate concentration in effluent from the control filter was 3.1 ppm (as N). The standard deviation was 1.6 ppm.

Graph 3 shows the concentration of nitrites in the filter effluents. Immediately after addition of 0.3 ppm CN, very high nitrites were noted. After a week, the nitrite concentration decreased below that of the control. After the cyanide was increased to 1 ppm, the low nitrite


GRAPH 1
B.O.D. of TRICKLING FILTER EFFLUENTS
Biochemical Oxygen Demand (ppm. Oxygen)
50
40
30
20
10
0.3 ppm. Cyanides
1 ppm. Cyanide
× Control filter
○ Exposed filter
3.76σ
Mean of Control
1
5
10
15
20
25
30
1
5
10
15
20
25
March
April

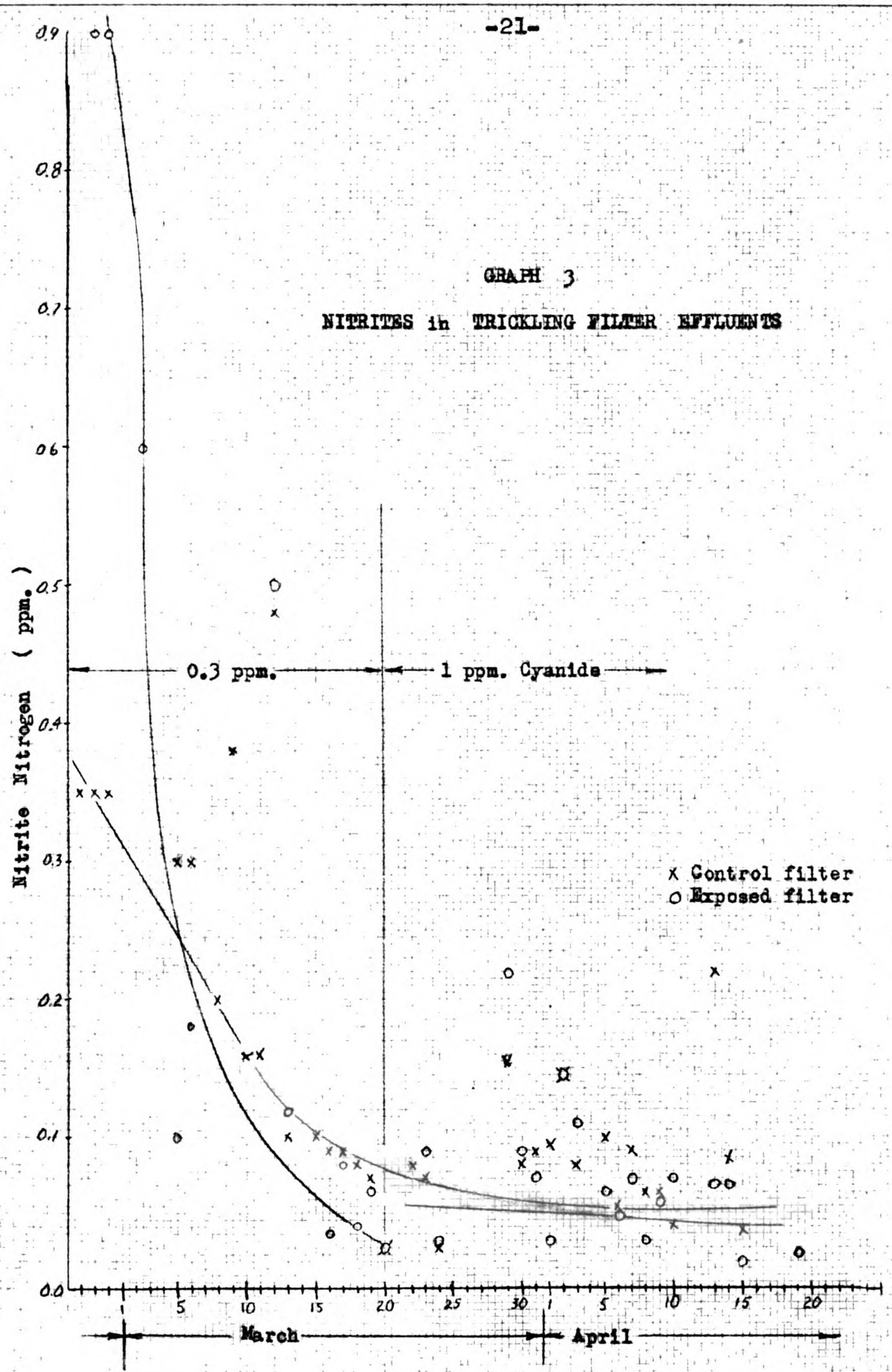
GRAPH 2

NITRATES in TRICKLING FILTER EFFLUENTS



GRAPH 3

NITRITES in TRICKLING FILTER EFFLUENTS



concentrations persisted. The steady decrease in nitrites on the control is not explained. Nitrites on the control averaged 0.16 ppm (as N), the standard deviation was 0.12 ppm.

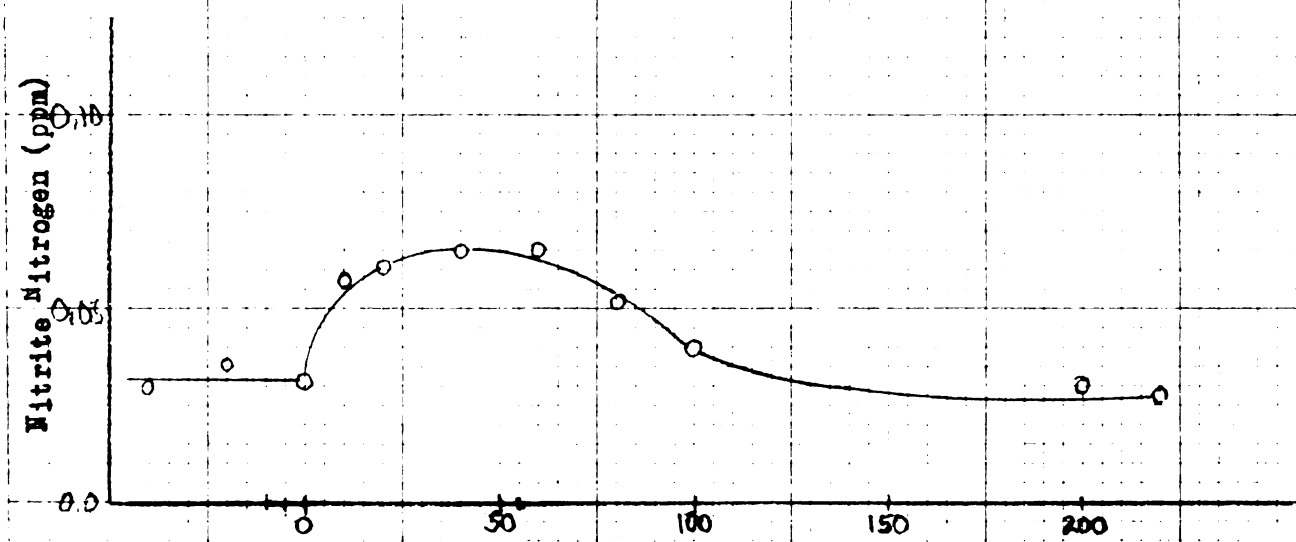
Graph 4 shows the effects of a shock load of 4 ppm CN on a filter not previously exposed to cyanides. An initial peak of 0.07 ppm NO_2-N was observed an hour after the cyanide was applied. In three hours this decreased to the current average value for the filter (0.03 ppm NO_2-N). A second delayed rise to 0.28 ppm N was noted on the second day, which returned to normal in about two weeks.

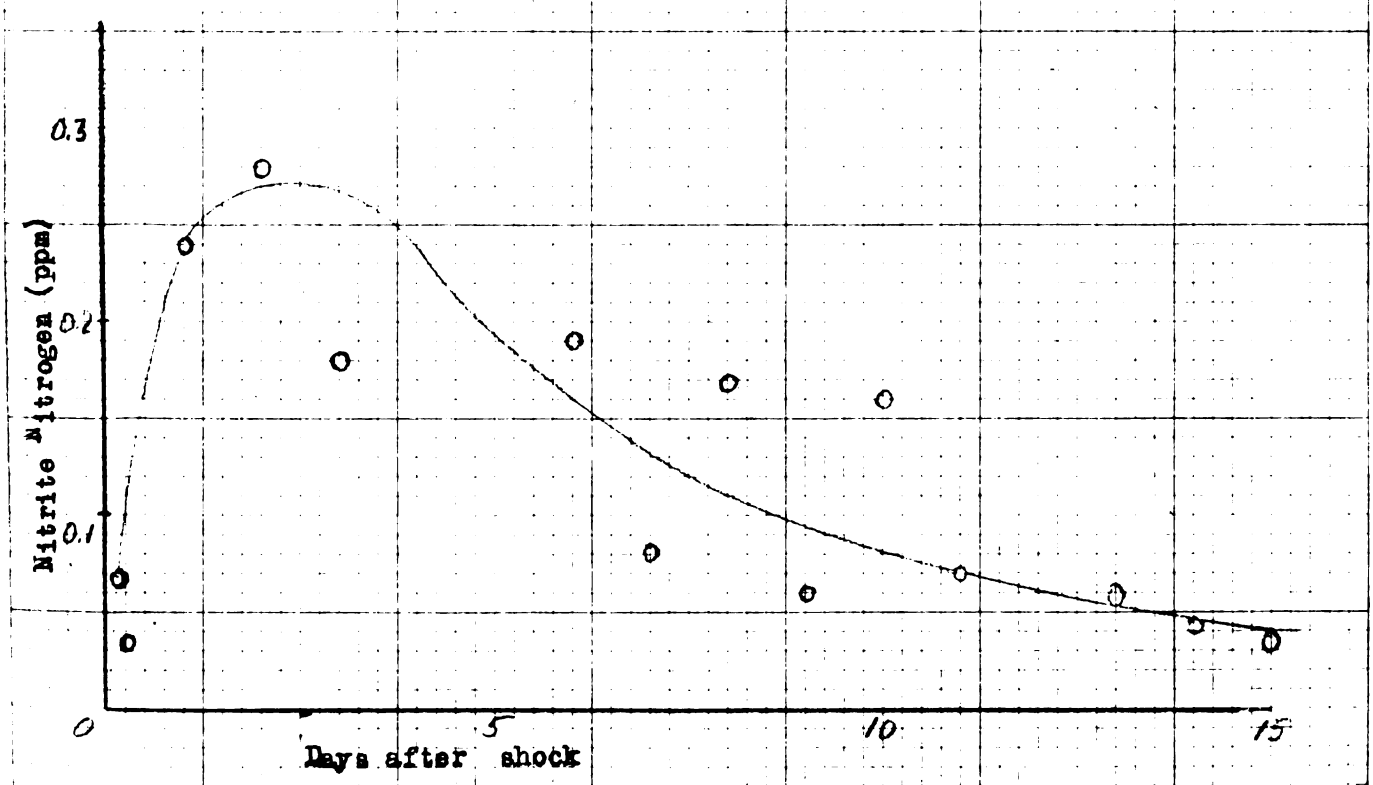
The effect of 40 ppm cyanide on nitrite formation in filter effluent (under aerobic conditiions in the laboratory) is shown on Graph 5. Cyanide initially stimulated nitrite formation in the effluent from the filter exposed to 0.3 ppm CN, but inhibited it in the previously unexposed effluent. Both controls behaved similarly. Nitrate formation occurred in the controls; relatively little in the cyanide containing samples.

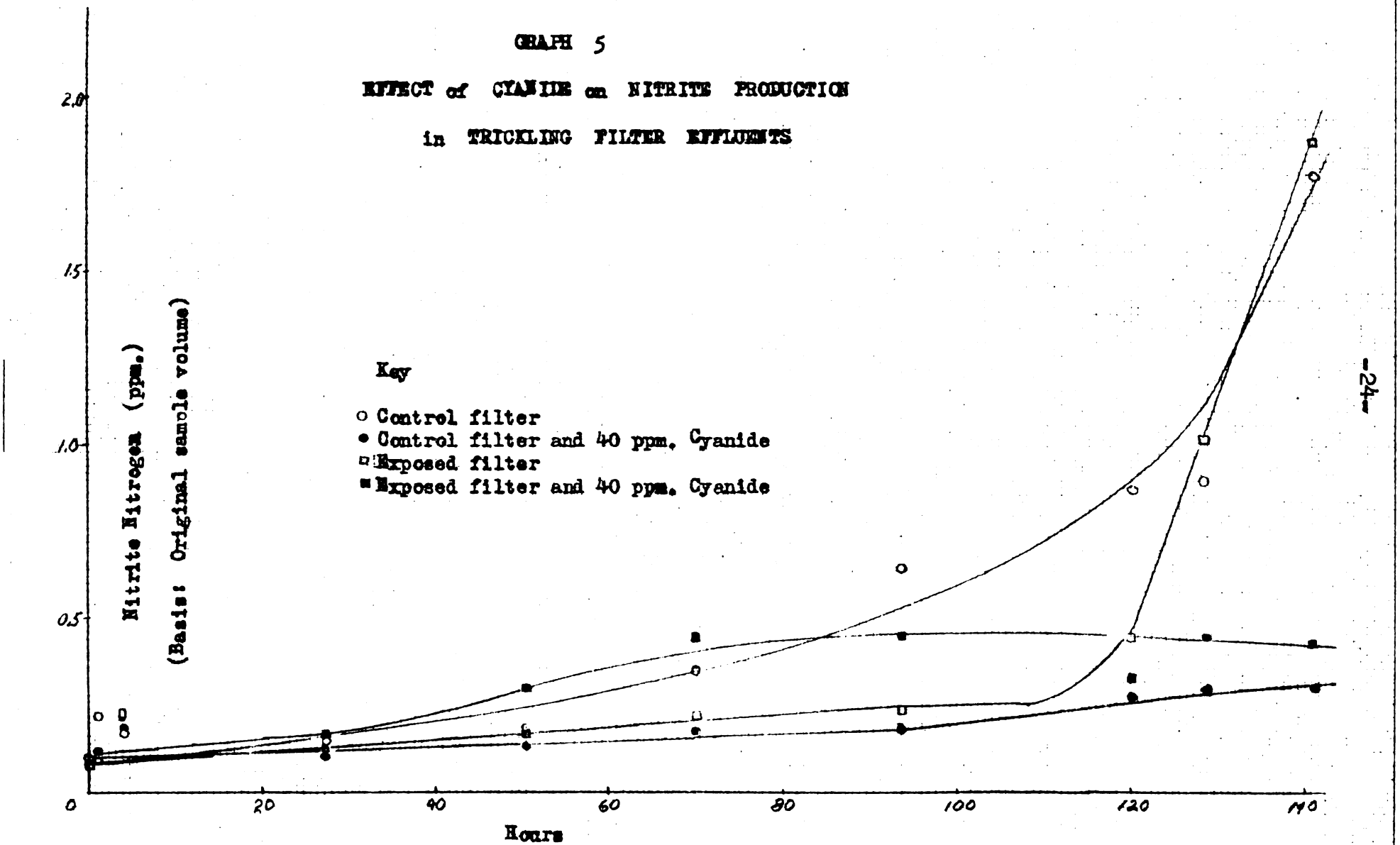
Graphs 6 and 7 show the effects of various cyanide concentrations (0 to 200 ppm) on nitrate reduction by a pure culture of Psuedomonas aeruginosa. Cyanide at 0.2 ppm had no effect (or was possibly slightly stimulative). Cyanide at 2 ppm was slightly inhibitory; at 20 ppm the inhibition was marked. A cyanide concentration of 200 ppm had a slight stimulation on the strain adapted to 200 ppm, but had a decisive inhibition of the unadapted strain.

GRAPH 4

EFFECT of SHOCK LOAD of 4ppm. CYANIDE on FILTER EFFLUENT

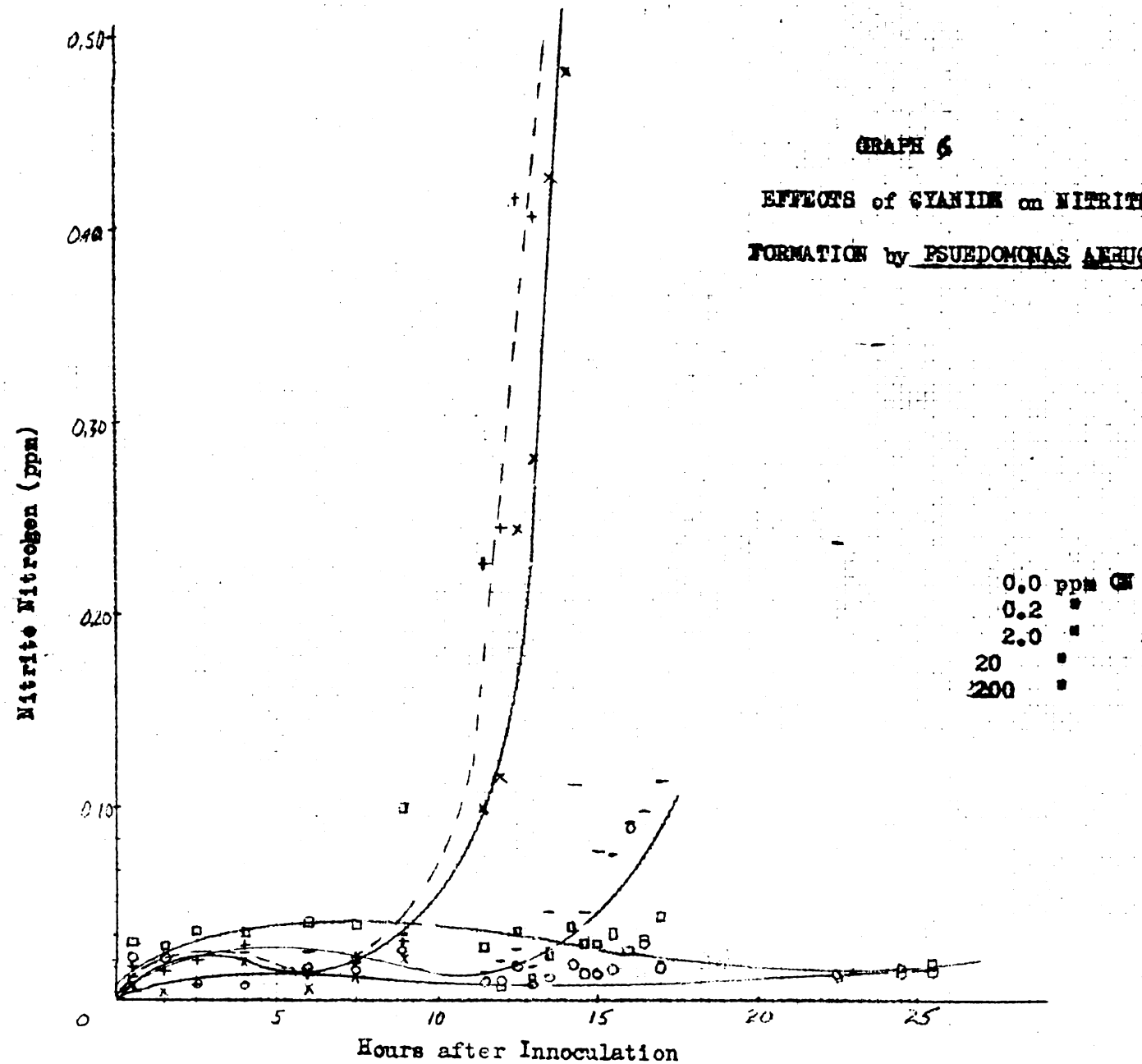





GRAPH 5
EFFECT of CYANIDE on NITRITE PRODUCTION
in TRICKLING FILTER EFFLUENTS
Key
Control filter
Control filter and 40 ppm. Cyanide
Exposed filter
Exposed filter and 40 ppm. Cyanide
Nitrite Nitrogen (ppm.)
(Basis: Original sample volume)
Hours
0
0.5
1.0
1.5
2.0
20
40
60
80
100
120
140

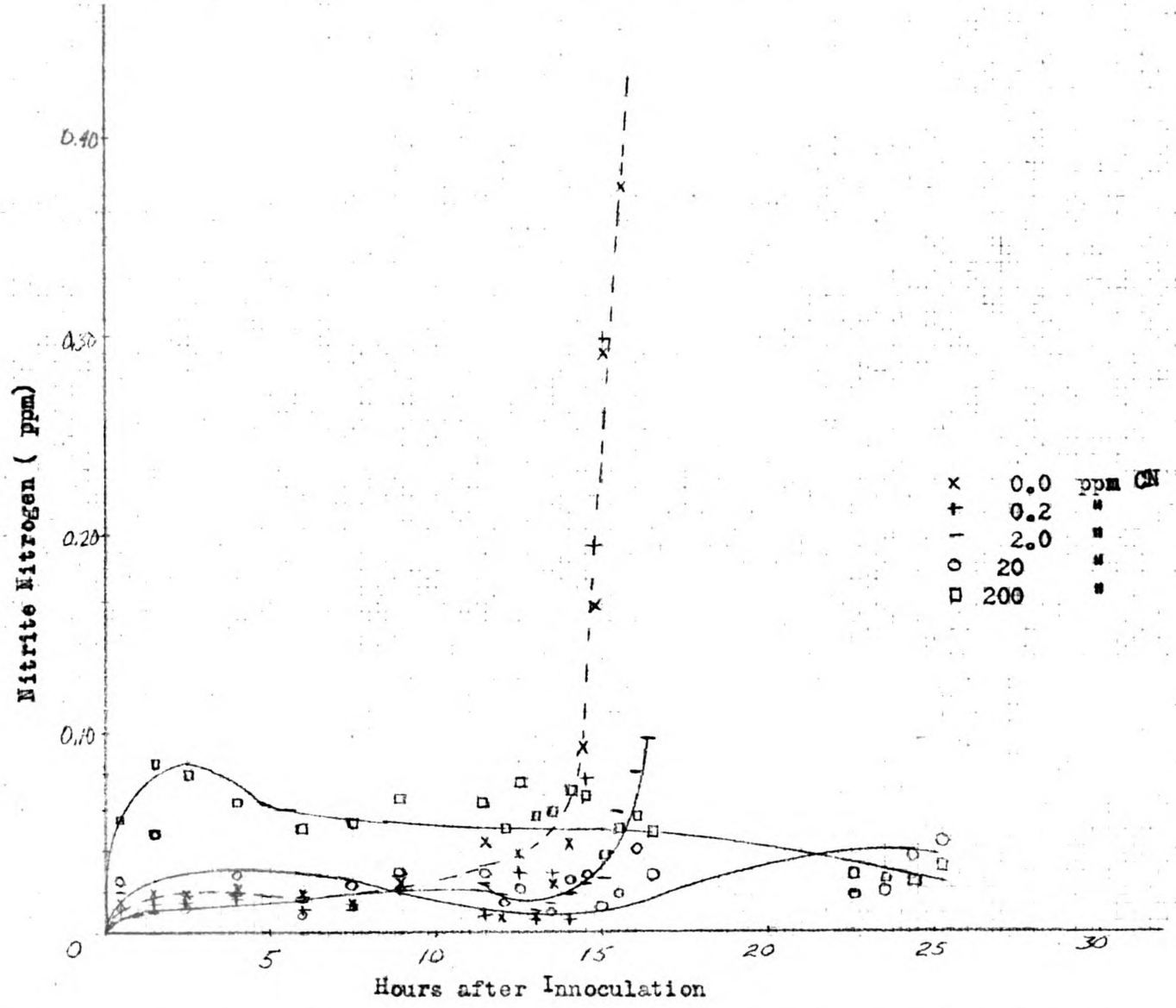
GRAPH 6

EFFECTS of CYANIDE on NITRITE FORMATION by PSUEDOMONAS AERUGINOSA



GRAPH 7

EFFECTS of CYANIDE on NITRITE FORMATION by CYANIDE-ADAPTED PSUEDOMONAS AERUGINOSA



with respect to nitrate reduction. Evidently adaptation to cyanide did not involve a significant change in nitrogen reduction mechanism.

Tables 1 and 2 show the results of toxicity studies. All organisms tested were more resistant to cyanide on glucose broth than on the synthetic broth. Pigment formation was retarded in several instances.

Enterobacteriaceae and close relatives: A. aerogenes, E. coli, Proteus vulgaris, and Serratia marcescens were very tolerant; fortunately Salmonella typhosa was the most sensitive.

The most cyanide-sensitive organisms were Alcaligenes (an Achromobacteriacea) and Chromobacterium amethystinum (a Rhizobiacea), especially on the synthetic medium.

Ps. aeruginosa and Ps. fluorescens were moderately tolerant of cyanides.

Of the gram positive cocci, Staphylococcus aureus and Streptococcus liquifaciens were both tolerant; Staphylococcus was more sensitive. This agrees with Braun's findings.

High concentrations of cyanide (200 ppm) were very toxic in synthetic broth. Only Serratia grew after 24 days; then, only in the presence of 1 ppm methylene blue in the medium.

Some of the organisms (i.e. Proteus) were revived on synthetic broth after contact with cyanide by addition of 1 ppm methylene blue to the subculture.

All tolerant organisms were catalase positive after

Table 1

Toxicity Studies on Glucose Broth

Organism	Days:	0 ppm CN				20 ppm CN					200 ppm CN			
		1	2	3	4	1	2	3	4	7	1	2	3	4
Aerobacter aerogenes		++	++	++	++	++	++	++	++	++	+	++	++	++
Alcaligenes fecalis		(+)	+	+	+	-	-	(+)	+	+	-	-	-	-
Chromobacterium sp.		+	+*	+	+	-	+	+	+*	+	-	-	-	-
Escherichia coli		++	++	++	++	+	++	++	++	++	-	+	++	++
Proteus vulgaris		+	++	++	+++	+	++	++	++	++	-	++	++	++
Psuedomonas aeruginosa		+	++*	++	++	+	++*	++	++	++	-	-	(+)	+*
Psuedomonas fluorescens		+	++	++	++	+	++	++	++	++	+	++	++	++
Salmonella typhosa		+	+	+	++	-	+	++	++	++	-	-	-	-
Serratia marcescens		++	++*	++	++	++	++*	++	++	++	-	-	-	+
Staphylococcus aureus		-	-	(+)	+	-	-	-	+	+	-	(+)	+	+
Streptococcus liquifaciens		+	++	++	++	++	++	++	++	++	-	+	++	++

Key: - no growth
+ growth
* characteristic pigment appears

Table 2

Toxicity Studies on Synthetic Broth

20 ppm CN

Organism	Days:	1	2	3	4	5	10	11
Aerobacter aerogenes		(+)	(+)	-	-	-	-	-
Chromobacterium sp.		-	-	-	-	(+)	++	++
Proteus vulgaris		-	-	-	-	-	+	++
Psuedomonas aeruginosa		-	(+)	++	++	++	++	++
Psuedomonas fluorescens		-	-	-	(+)	+	++	++
Serratia marcescens		-	-	+	++	++	++	++

200 ppm CN

Aerobacter aerogenes	-	-	-	-	-	-	-
Chromobacterium sp.	(+)	(+)	-	-	-	-	-
Psuedomonas aeruginosa	-	-	-	-	-	-	-
Psuedomonas fluorescens	-	-	-	-	-	-	-
Serratia marcescens	-	-	-	-	-	-	-

(Organisms grown for two sucessive transfers on Synthetic broth before innoculation.)

Composition of Synthetic Broth:

Sucrose	30 gm.
K_2HPO_4	1 gm.
$MgSO_4$	0.32 gm.
$CaCl_2$	0.08 gm.
$(NH_4)_2SO_4$	1 gm.
Water	1 liter

growth in the presence of cyanide. The cyanide did not noticably affect the level of growth attained but affected only the period of lag.

A cyanide-tolerant organism was isolated from raw sewage containing 200 ppm CN, and was identified as Streptomyces albus. Table 8 in Appendix I describes its characteristics. It is very cyanide-tolerant, surviving three succesive transfers on a synthetic medium containing cyanide as sole source of nitrogen. This Strep. albus is also catalase positive, but does not release hydrogen sulfide on Kligler's Iron Agar.

The high nitrite concentrations can have two likely origins: oxidation of ammonia or reduction of nitrate. Since the chief ammonia oxidizer (Nitrosomonas) is reported (37) both to be completely inhibited by cyanide and to be unable to adapt to tolerate cyanide and since the Psuedomonas experiments show that low cyanide concentrations do not inhibit nitrate reduction, reduction of nitrate is the more likely major source of nitrites in the effluents from filters exposed to low concentrations of cyanide. Nitrates may be used as emergency oxidizing agents, if the cyanide attack on the cytochromes impaired the use of oxygen as the oxidizing agent. This nitrate reduction can stop at nitrite or procede all the way to ammonia or nitrogen. The secondary decrease in nitrite may represent either a gradual inhibition of this mechanism or development of a more efficient reduction system. Since nitrate concentrations were not used during

this secondary repression, this second possibility should not be ignored. In any case, there is insufficient nitrate to oxidize a significant fraction of the sewage.

The Psuedomonas experiments show that cyanide does inhibit nitrate reduction, if the cyanide level exceeds 2 ppm. At lower concentrations of cyanide, gradual inhibition may take place.

The shock effects may be similarly explained. Initial stimulation of nitrites, and rapid repression may represent the desperation attempt of the trickling filter organisms to offset an impaired cytochrome system and then gradual inhibition of the nitrate reductase system. As the cyanides are washed out, the reductase system may recover more rapidly (or the attack is more readily reversed) than the cytochrome system. Thus, a second increase in nitrites is not unlikely. The extreme duration of this second period of increased nitrites indicates that 4 ppm CN is very toxic to the filter.

Contact of the microorganisms with somewhat higher cyanide concentrations than previously encountered resulted in an initial increase in nitrites often followed by a decrease. This was demonstrated on the filter itself (both under continuous exposure and under a shock load), batchwise contact with the filter effluents and in nitrate reduction by Psuedomonas aeruginosa. All are involved with nitrate reduction. The extreme duration of nitrite persistence on the shocked filter eliminates cyanide itself as the possible precursor of the nitrites.

SUMMARY

Sodium cyanide in concentrations of marginal toxicity (0.3 and 1 ppm CN) was applied to a pilot-plant trickling filter. At the end of these runs, the control filter was subjected to a shock load of 4 ppm CN.

BOD of the effluent was not affected by 0.3 ppm CN, but an initial increase and later decrease in nitrites and an initial decrease in nitrates were noted.

A concentration of 1 ppm cyanide retarded nitrites and nitrates in the effluent and also caused increased BOD values. The shock load also produced the high nitrites and low nitrates.

Cyanide in a concentration of 40 ppm retarded nitrite and nitrate formation in the effluent from the filters.

Psuedomonas aeruginosa reduced nitrate to nitrite in the presence of 0.2 ppm CN, without inhibition. Cyanide at 2 ppm is slightly inhibitory; 20 and 200 ppm are decidedly inhibitory.

Laboratory toxicity studies were attempted on organisms reported from trickling filters or sewage, using both glucose broth and a synthetic broth. Most organisms were tolerant of 20 and 200 ppm CN on glucose broth. On synthetic broth, 200 ppm was very toxic: Serratia marcescens was the only organism to grow, and this only in the presence of

1 ppm methylene blue. Cyanide affected the length of the lag period, but did not appreciably alter the level of growth attained.

The author believes that the nitrites originate from emergency reduction of nitrate; this oxidative alternative may also be inhibited by cyanide, but at a much slower rate.

CONCLUSIONS

(1) Cyanide in a concentration of 0.3 ppm interferes with the nitrogen metabolism of a trickling filter operating on municipal sewage, but is not appreciably detrimental to the BOD of the filter effluent.

(2) Cyanide in a concentration of 1 ppm is markedly detrimental to the quality (BOD) of the effluent. Nitrate formation is partially inhibited.

(3) Cyanide at 40 ppm concentrations inhibits nitrification in the filter effluents.

(4) Shock loads of cyanide applied to an unadapted filter result in increased nitrites in the effluent.

(5) Cyanide toxicity to microorganisms is a function of the medium supporting these organisms.

(6) Cyanide toxicity involves increased lag periods, rather than decreased population levels attained. Cyanide exerts a bacteriostatic action.

(7) Most of the sewage organisms tested are found to tolerate 20 and even 200 ppm CN on glucose broth.

(8) Cyanide in concentrations over 2 ppm retards the use of nitrates as auxiliary oxidizing agents by Ps. aeruginosa. At lower concentrations, it is only slightly inhibitory.

(9) The increased nitrites probably have their origin in reduction of nitrates.

SUGGESTIONS FOR FURTHER WORK

(1) Investigation of conductimetric titration with nickel and "versene" as a possible analytical method for cyanide.

(2) Investigation of the effects of methylene blue on a trickling filter inhibited by a shock load of cyanide. For some organisms, methylene blue can act as a substitute for the cytochrome system.

(3) Investigation of formaldehyde as a possible detoxifier for cyanides. Reaction kinetics might be determined by following the cyanohydrin condensation reaction in a conductivity cell.

(4) Isolation of the denitrifying organisms from the trickling filters. The general organism present, Zooglea ramigera, is reported by Bergey's (6) not to be a denitrifier.

(5) Attempted adaptation of Nitrobacter and Nitrosomonas to low concentrations of cyanide. Metabolic activity may be followed as nitrate or nitrite.

(6) Investigation as to the correlation between ammonia production and nitrite depression.

(7) Systematic investigation of the significant components in media for the demonstration of cyanide toxicity. This might yield some insight into the mechanisms involved.

(8) Investigation of possible intermediates in cyanide

destruction. For reproducible results, the use of pure cultures of well-known organisms is suggested.

(a) Formate might be measured quantitatively by reduction with acid and magnesium, with colorimetric determination of the formaldehyde formed.

(b) Formic dehydrogenase might be demonstrated in the organisms by decolorizing methylene blue in a Thurnburg vacuum tube.

(c) Thiocyanate may be determined colorimetrically as the iron complex.

(d) Nitrite may be determined by the usual method employed in this work.

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BA: Biological Abstracts

CA: Chemical Abstracts

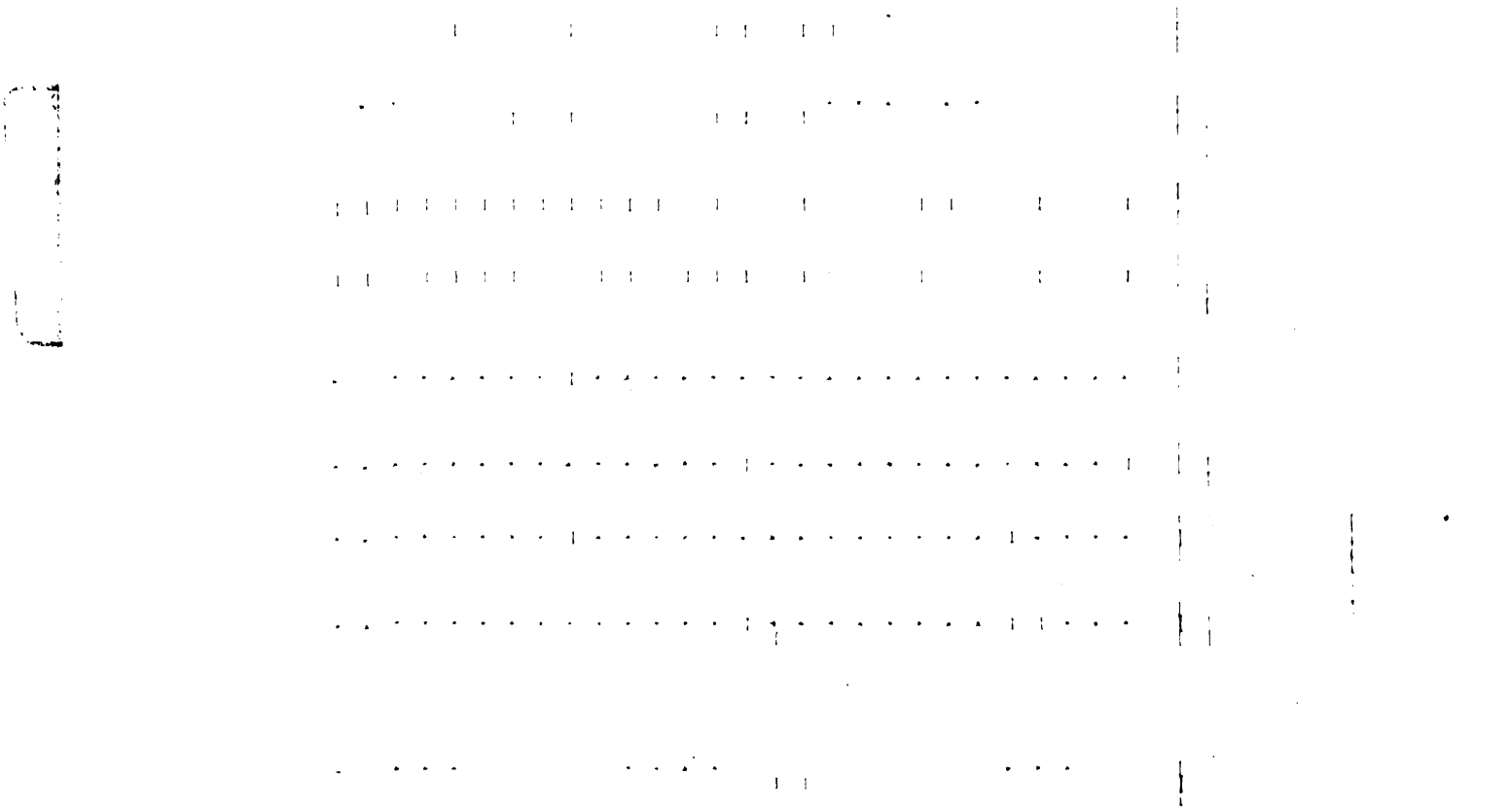
APPENDIX I

EXPERIMENTAL DATA

Table 3

Trickling Filter Effluents

	Date	Temperature (°C)	Nitrites (ppm N)		Nitrates (ppm N)		DOC (ppm O)		BOD (ppm O)	
		1	1	2	1	2	1	2	1	2
0.3 ppm CN	Feb. 26	13	0.35	1.2	-	2.0	-	-	42	18
	27	13	0.35	0.9	2.0	1.0	10	26	9	9
	28	12.5	0.35	0.9	4.6	3.4	58	19	26	33
	March 2	17.5	-	0.6	6.0	2.4	-	-	8	30
	4	19.5	-	-	4.0	2.0	24	27	42	20
	5	11	0.3	0.1	3.0	1.8	9	68	6.5	5
	6	16	0.3	0.18	5.0	2.0	9	-	7.5	10
	8	19	0.2	1.0	1.0	0.3	-	-	3	10
	9	19	0.38	1.5	5.0	1.8	7	48	8.5	1.5
	10	19	0.16	2.0	4.0	1.1	88	90	2.5	18
	11	12	0.16	0.42	2.5	1.5	21	38	3.5	-
	12	-	0.48	0.5	1.5	1.5	-	-	-	-
	13	-	0.12	0.1	1.5	1.5	64	58	16	19
	15	16	-	0.1	-	2.4	-	200	-	-
	16	12.5	0.09	0.04	4.6	2.0	-	-	-	-
	17	12.5	0.09	0.08	4.3	1.8	-	36	6	4
	18	12.5	0.08	0.05	2.3	1.6	67	-	28	10
	19	13.5	0.07	0.06	2.8	0.6	-	-	24	69
1 ppm CN	20	15	0.03	0.03	1.5	0.3	-	-	10	48
	22	13	0.07	-	1.2	-	85	-	-	-
	23	17	0.06	0.08	1.4	1.0	55	-	32	8
	24	13	0.03	0.04	1.2	1.2	-	-	-	20
	29	14	0.16	0.22	4.6	1.2	-	-	9	25
	30	15.5	0.08	0.09	5.5	5.7	-	-	7	-
	31	12.5	0.09	0.07	5.5	1.5	-	-	12	10
	April 1	12.5	0.1	0.03	4.5	1.0	60	-	12.5	19
	2	14	0.14	0.14	1.7	1.7	-	-	12.5	18
	3	10.5	0.08	0.11	1.0	0.6	-	-	1	4



	Date	Temperature (°C)	Nitrites (ppm N)		Nitrates (ppm N)		DOC (ppm C)		BOD (ppm O)	
		1	1	2	1	2	1	2	1	2
1 ppm CN	April 5	13	0.10	0.06	0.6	0.5	-	-	12.5	20
	6	16	0.05	0.04	0.6	0.5	38	44	23	22
	7	16	0.09	0.07	-	-	94	86	16	32
	8	12	0.06	0.03	3.2	1.6	-	-	13	26
	9	14	0.06	0.05	3.2	1.6	-	-	4	28
	10	16	0.04	0.07	3.2	1.2	-	-	32	22
	13	15	0.22	0.07	3.7	2.8	28	16	23	24.5
	14	16	0.08	0.06	5.2	5.2	18	72	11.5	16.5
	15	16	0.04	0.02	5.0	5.2	-	-	27	23.5
	16	13.5	-	-	2.2	2.2	-	-	8.5	10.5
	19	-	-	0.025	-	1.3	-	-	-	-
Mean			0.155		3.08		43.2		13.8	
Standard deviation			0.125		1.63		29.2		1.6	

1 Control filter
2 Exposed filter

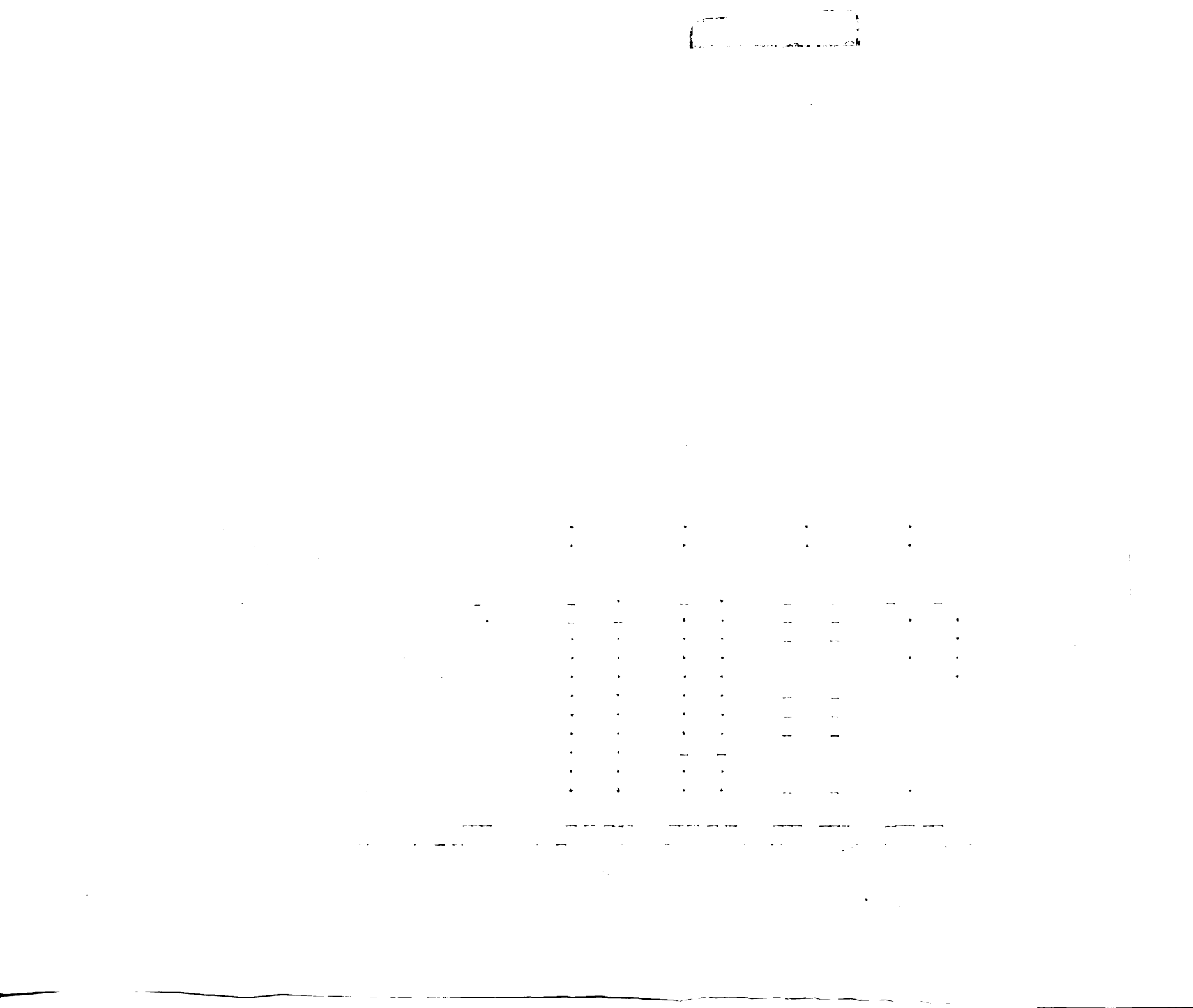


Table 4

Trickling Filter Effluent After Shock Load of 4 ppm Cyanide

Elapsed time (minutes)	Nitrites (ppm N)
-40	0.03
-20	0.036
0	0.033
10	0.058
20	0.062
40	0.066
60	0.066
80	0.051
100	0.040
200	0.030
220	0.028
(days)	(ppm N)
1	0.24
2	0.28
3	0.17
6	0.19
7	0.08
8	0.17
9	0.06
10	0.16
11	0.07
13	0.06
14	0.044
15	0.036

Table 5

Batchwise Nitrite Formation in Filter Effluents

A: No Previous Exposure to Cyanide

Elapsed Time (hours)	Sample Volume (ml.)	Total Volume Removed (ml.)	Evaporation Loss (ml.)	Nitrites 1 (ppm N)	Nitrites 2 (ppm N)	Corrected Nitrites 1 (ppm N)	Corrected Nitrites 2 (ppm N)	
0	(1)	(1)	0	0.12	0.12	0.12	0.12	
1	0.5	0.5	0.05	0.22	0.14	0.22	0.14	
3	1	1.5	0.2	0.16	0.18	0.159	0.179	
27	2	4	1.5	0.18	0.15	0.171	0.146	
51	2	6	2.9	0.20	0.15	0.186	0.14	
70	2	8	4.0	0.37	0.20	0.345	0.181	
94	2	10	5.3	0.75	0.22	0.65	0.189	
120	2	12	6.8	0.96	0.35	0.81	0.288	
129	1	13	7.2	1.1	0.38	0.90	0.304	
142	2	15	8	2.3	0.40	1.77	0.31	
0				1.5	1.5	1.5	1.5	Nitrates
142				12	5.6	9.2	4.3	"

B: Previously Exposed to 0.3 ppm Cyanide

0	(1)	(1)	0	0.08	0.08	0.08	0.08	
1	0.5	0.5	0.05	0.09	0.09	0.09	0.09	
3	1	1.5	0.2	0.22	0.22	0.217	0.217	
27	2	4	1.5	0.16	0.19	0.155	0.183	
51	2	6	2.9	0.19	0.32	0.177	0.30	
70	2	8	4.0	0.25	0.44	0.226	0.445	
94	2	10	5.3	0.29	0.51	0.251	0.444	
120	2	12	6.8	0.52	0.40	0.43	0.328	
129	1	13	7.2	1.3	0.58	1.04	0.468	
142	1	14	8	2.5	0.58	1.9	0.451	
0				1.5	1.5	1.5	1.5	Nitrates
142				11	6	8.4	4.6	"

1: Control 2: 40 ppm Cyanide

Table 6

Effects of Cyanide on Nitrite Formation

by Unadapted Psuedomonas aeruginosa

Time elapsed (hours)	Cyanide Concentrations				
	0 ppm	0.2 ppm	2 ppm	20 ppm	200 ppm
	Nitrites (ppm NO :				
0	0.0	0.0	0.0	0.0	0.0
0.5	0.008	0.016	0.014	0.022	0.036
1.5	0.006	0.0	0.014	0.022	0.032
2.5	0.0	0.020	0.008	0.008	0.042
4.0	0.022	0.027	0.025	0.029	0.037
6.0	0.007	0.012	0.029	0.016	0.046
7.5	0.012	0.023	0.027	0.017	0.046
9.0	0.020	0.030	0.034	0.027	0.10
11.5	0.09	0.023	0.017	0.014	0.028
12.0	0.125	0.25	0.022	0.014	0.012
12.5	0.25	0.42	0.034	0.022	0.042
13.0	0.28	0.41	0.022	0.008	0.010
13.5	0.43	0.56	0.056	0.012	0.025
14.0	0.48	0.61	0.115	0.022	0.034
14.5	0.54	0.74	0.056	0.018	0.034
15.0	0.74	0.89	0.070	0.018	00036
15.5	0.90	1.10	0.067	0.022	0.044
16.0	0.92	1.18	0.089	0.082	0.031
16.5	1.05	1.34	0.095	0.034	0.036
17.0	1.20	1.55	0.11	0.022	0.046
22.5			0.24	0.022	0.019
23.5			0.34	0.020	0.020
24.5			0.45	0.018	0.015
25.5			0.52	0.034	0.036

Innoculum: 7.44×10^7 cells

Temperature: 20°C

Medium: Physiological saline with 0.1% KNO_3 and 0.1% glucose

Table 7

Effects of Cyanide on Nitrite Production by Previously Adapted Psuedomonas aeruginosa

Time Elapsed (hours)	Cyanide Concentrations				
	0 ppm	0.2 ppm	2 ppm	20 ppm	200 ppm
	Nitrites (ppm N)				
0	0.0	0.0	0.0	0.0	0.0
0.5	0.016	0.012	0.020	0.025	0.056
1.5	0.020	0.014	0.010	0.050	0.086
2.5	0.020	0.016	0.020	0.014	0.080
4.0	0.022	0.018	0.018	0.023	0.062
6.0	0.022	0.012	0.016	0.013	0.050
7.5	0.015	0.015	0.014	0.023	0.053
9.0	0.022	0.024	0.028	0.029	0.068
11.5	0.042	0.010	0.024	0.018	0.064
12.0	0.006	0.0	0.014	0.010	0.052
12.5	0.036	0.030	0.030	0.021	0.070
13.0	0.010	0.007	0.008	0.008	0.054
13.5	0.027	0.033	0.016	0.012	0.056
14.0	0.055	0.080	0.020	0.016	0.059
14.5	0.10	0.082	0.023	0.028	0.073
15.0	0.16	0.20	0.029	0.018	0.038
15.5	0.29	0.30	0.060	0.025	0.050
16.0	0.37	0.39	0.062	0.025	0.026
16.5	0.45	0.61	0.080	0.042	0.059
17.0	0.60	0.79	0.10	0.030	0.048
22.5				0.022	0.033
23.5				0.025	0.031
24.5			0.70	0.040	0.029
25.5				0.050	0.035

Innoculum: 6.75×10^7 cells

Temperature: 20°C

Table 8

Characteristics of Cyanide-Tolerant Organism Isolated From Sewage

Morphological

Mycelium: Conidia formed in chains

Much branching, small elongated cells

Colonies: White on Nutrient agar, potato plug, calcium malate agar, Kligler's iron agar, starch agar.

Flakey white on surface of nutrient broth

Physiological

Aerobic

Reduces nitrates to nitrites

Peptonizes litmus milk

Actively proteolytic, liquifies gelatin

Catalase positive

Survives three sucessive transfers on synthetic medium (agar), with 200 ppm CN as the sole nitrogen source.

Isolated on synthetic agar in the presence of 200 ppm CN.

Characteristics fit best those of Streptomyces albus, Bergey's (6) page 934.

APPENDIX II

ANALYTICAL METHODS

ANALYTICAL METHODS

Routine analyses of BOD, nitrites and nitrates were performed according to the procedures outlined in Standard Methods (3). The nitrite procedure was slightly modified to give greater accuracy at low concentrations: the sample is diluted to 20 ml. instead of 50 ml. before colorimetric comparison.

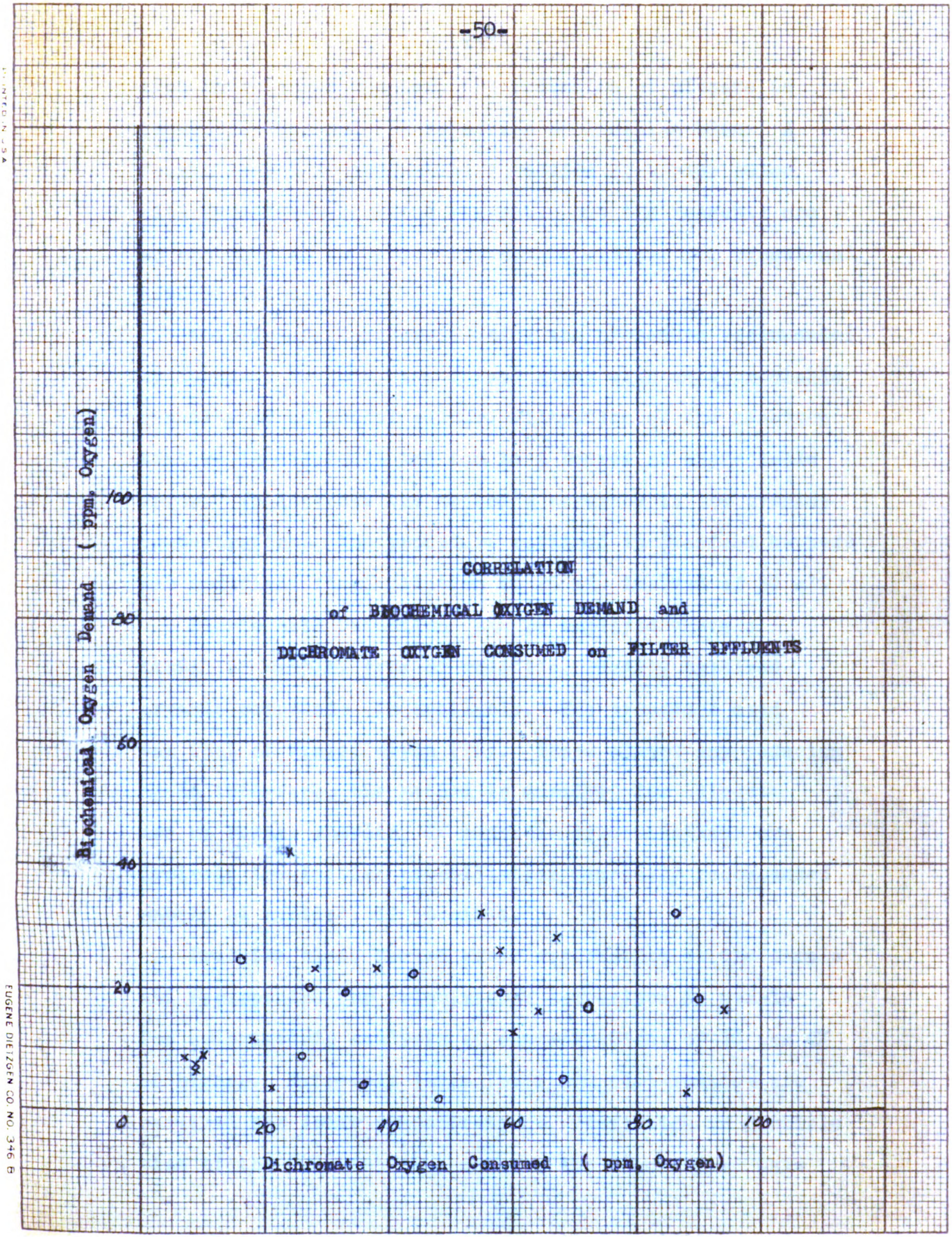
Shaw's method for DOC was chosen because of the relative speed and simplicity of the procedure. The analytical results showed too much scatter to be useful. A graph showing the correlation between BOD and DOC is included. The correlation is poor. There is no significant difference between the results from the treated and the control filters. This can probably be attributed to the poor precision of the determination.

It is also difficult to obtain a suitable analytical procedure for cyanide.

There are two common general procedures for cyanide determination: titration and colorimetry.

Titrimetric procedures include (1) titration with silver nitrate and various indicators (Liebig method), and (2) titration with divalent nickel.

Colorimetric procedures include (1) formation of the



iron thiocyanate complex, (2) reduction of picrate to iso-purpurate, and (3) formation of Prussian blue. A fourth method is available: formation of a blue color with pyridine-pyrazalone reagent, but the reagents are so unstable as to render the method useless for occasional determinations.

Thiocyanate complexes, using the procedure recommended by Standard Methods, are formed along with an appreciable quantity of colloidal sulfur. Filtration of the reaction mixture is difficult and can be a source of appreciable error. To increase the method's accuracy in low cyanide concentrations, the procedure outlined in Standard Methods was modified: final dilution to 25 ml. instead of 50 ml.

Picric acid reduction can be employed where a distilled sample, free of volatile, readily oxidized organic compounds is available. The procedure used was that of Finkelstein (16). Since almost any reducing agent interfere, the method is unsuited for direct use on sewage samples. If numerous determinations are desired, the initial distillation generally recommended should be avoided, if possible.

Prussian blue, formed after evaporation of the sample and resuspended for colorimetric comparison is usable for colorimetric analysis at concentrations of 10 ppm CN or higher. Extremely large (over 100 ml.) samples are required for lower concentrations. Variation in particle size of the Prussian blue reduces the accuracy of this

of this method. For this investigation, the procedure of Friel and Wiest (17) was employed, diluting the sample to 5 ml. before comparison.

Titration with silver nitrate is not feasible in the presence of chlorides, commonly present in sewage. Simple acidification and distillation is not adequate, unless the chlorides are previously removed. Removal can be accomplished but is tedious.

Titration of cyanide with nickel, using dimethylglyoxime as an indicator was unsucessful, due to the sluggishness of the nickel-glyoxime reaction. Further diffuculty was encountered in that cyanide reduced the sensitivity of the dimethylglyoxime, possibly by forming uncolored condensation products.

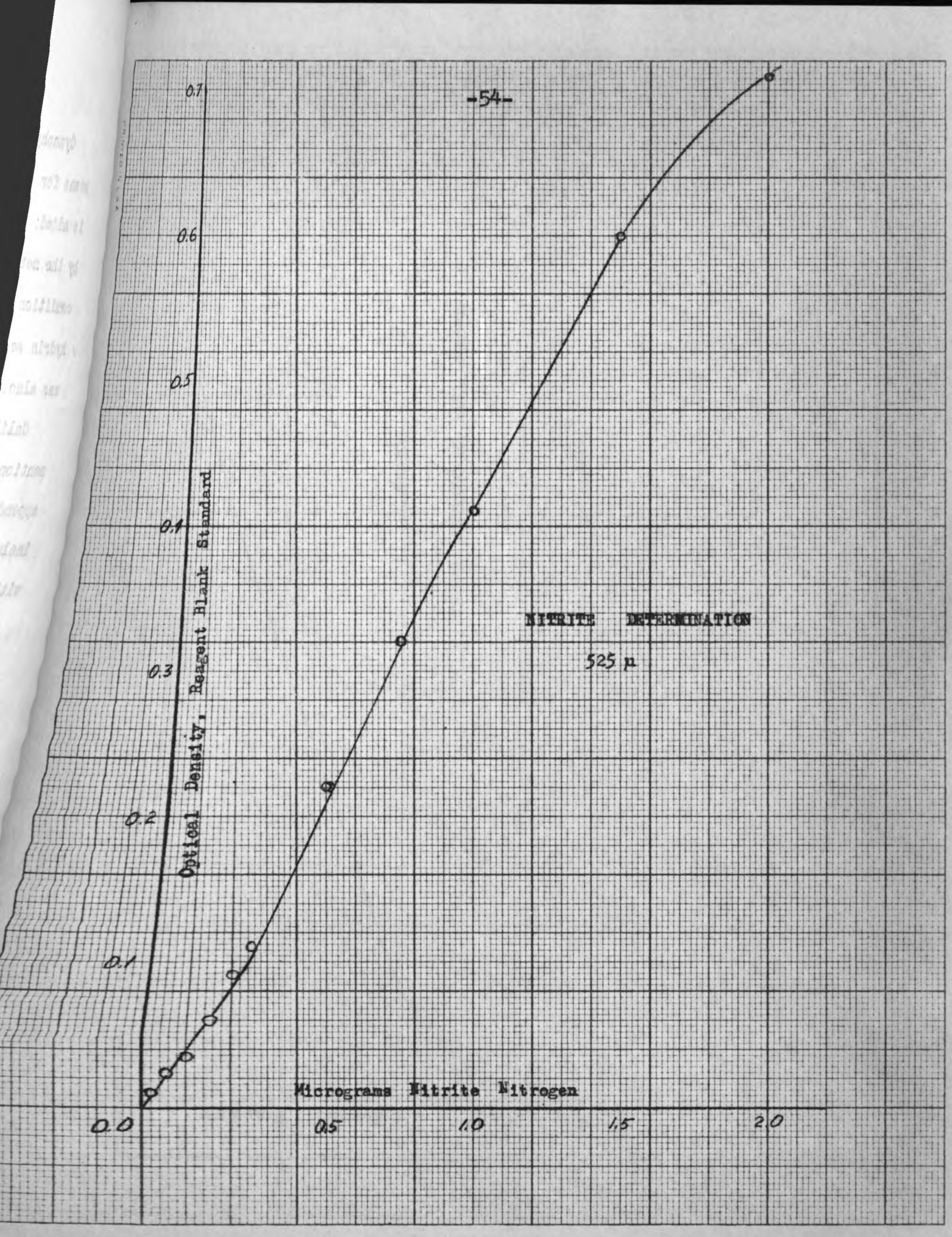
Addition of excess nickel and back titration with "versene" (diaminoethylene-tetra-acetic acid) is feasible if a suitable indicator can be found. Mureoxide (ammonium purpurate) is suitable (23), but is relatively expensive for routine analysis.

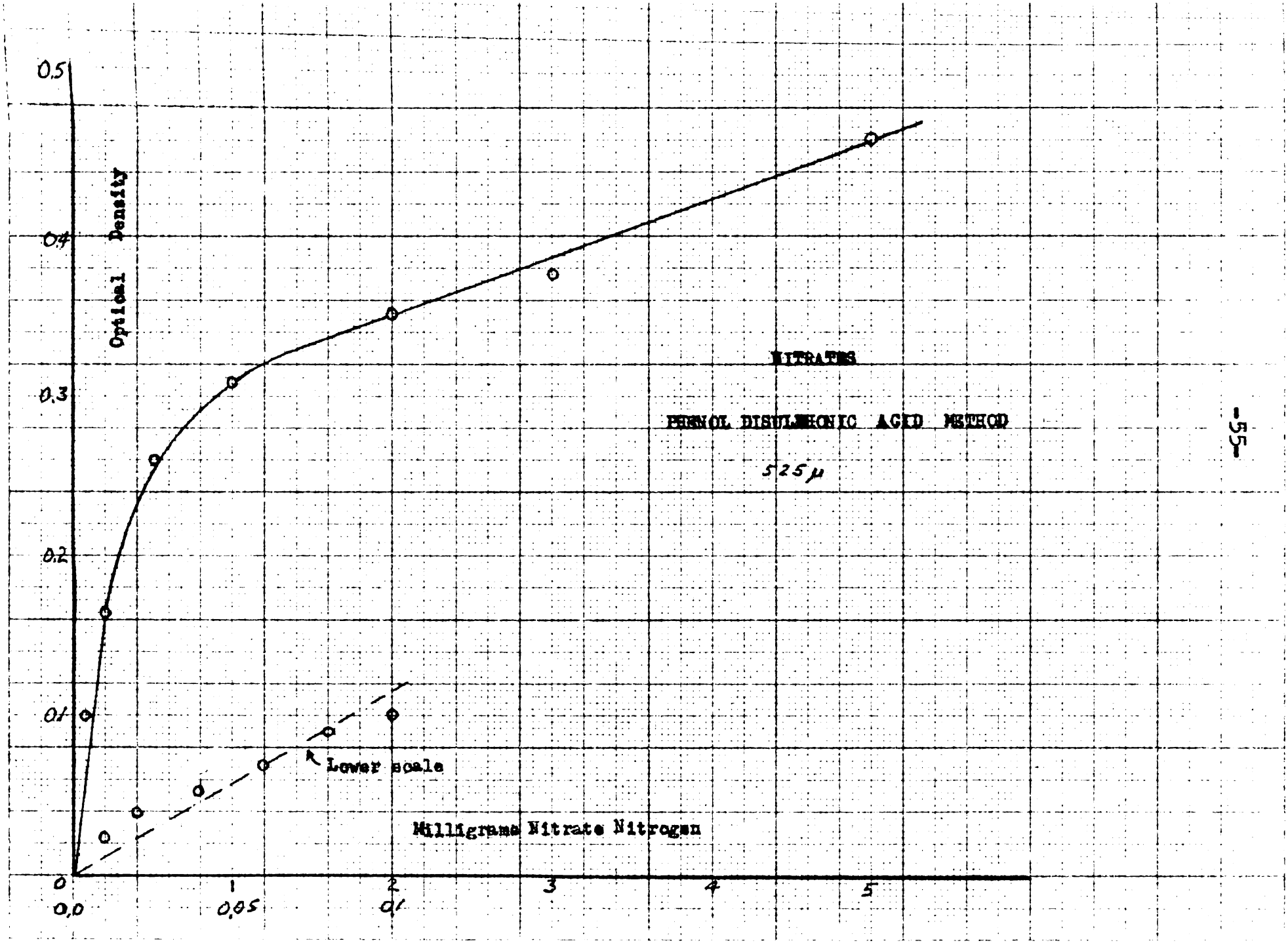
Titration of the cyanide directly by nickel in the presence of excess ammonia was attempted. There are several disadvantages: the end point is weak and the fumes are irritating to the analyst. The cyanide complex is more stable than the nickel ammine. Ammonia might be used as an external indicator, but this also is cumbersome.

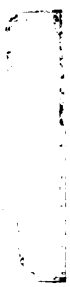
Conductimetric titration with nickel or with nickel and versene may be suitable.

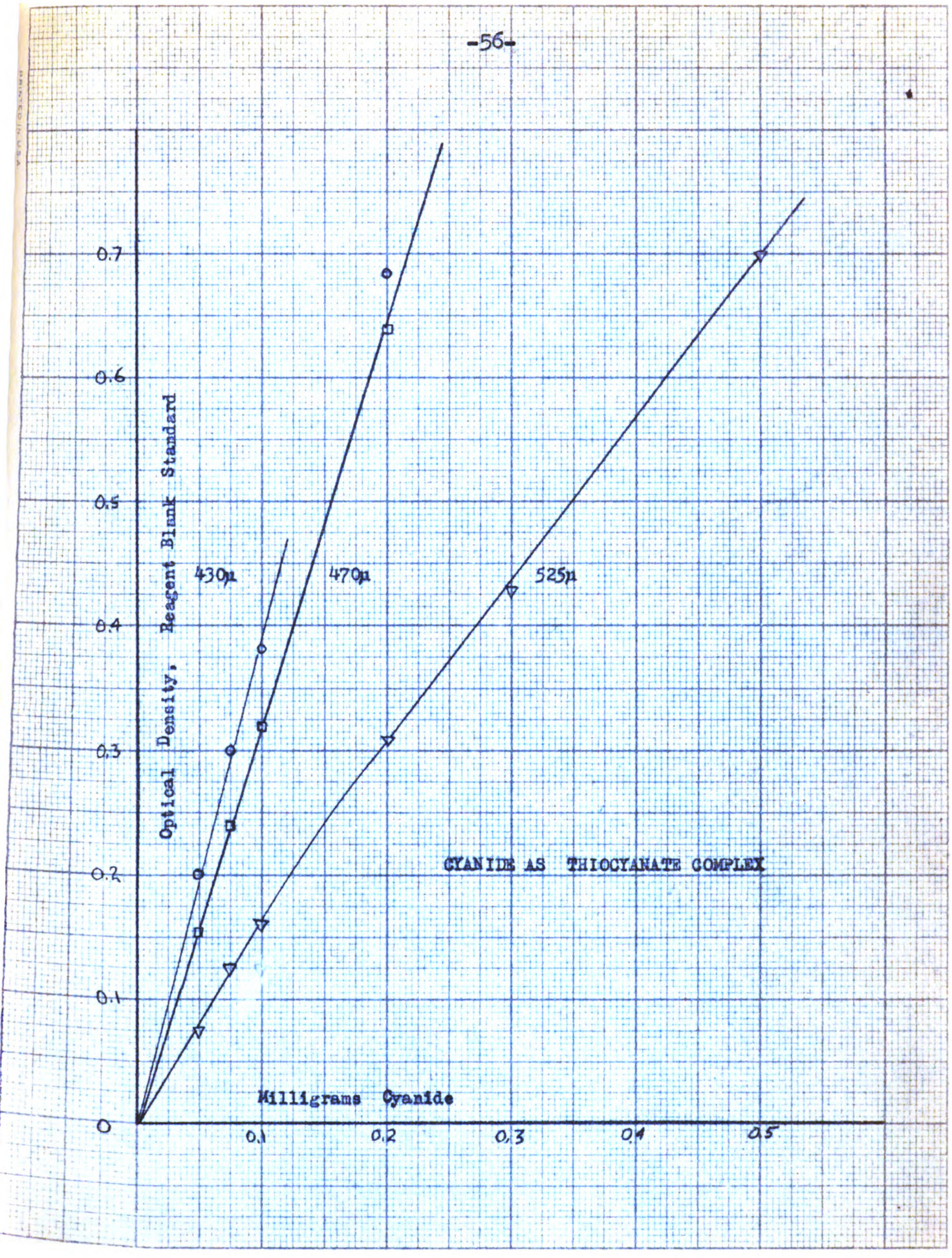
Cyanohydrin formation was also considered as a means for estimation of cyanide. Excess formaldehyde is added; the excess free formaldehyde is determined by the method of Tanenbaum and Bricker (43). Under conditions for formaldehyde determination, the cyanohydrin seemed to be quantitatively decomposed. This was almost instantaneous.

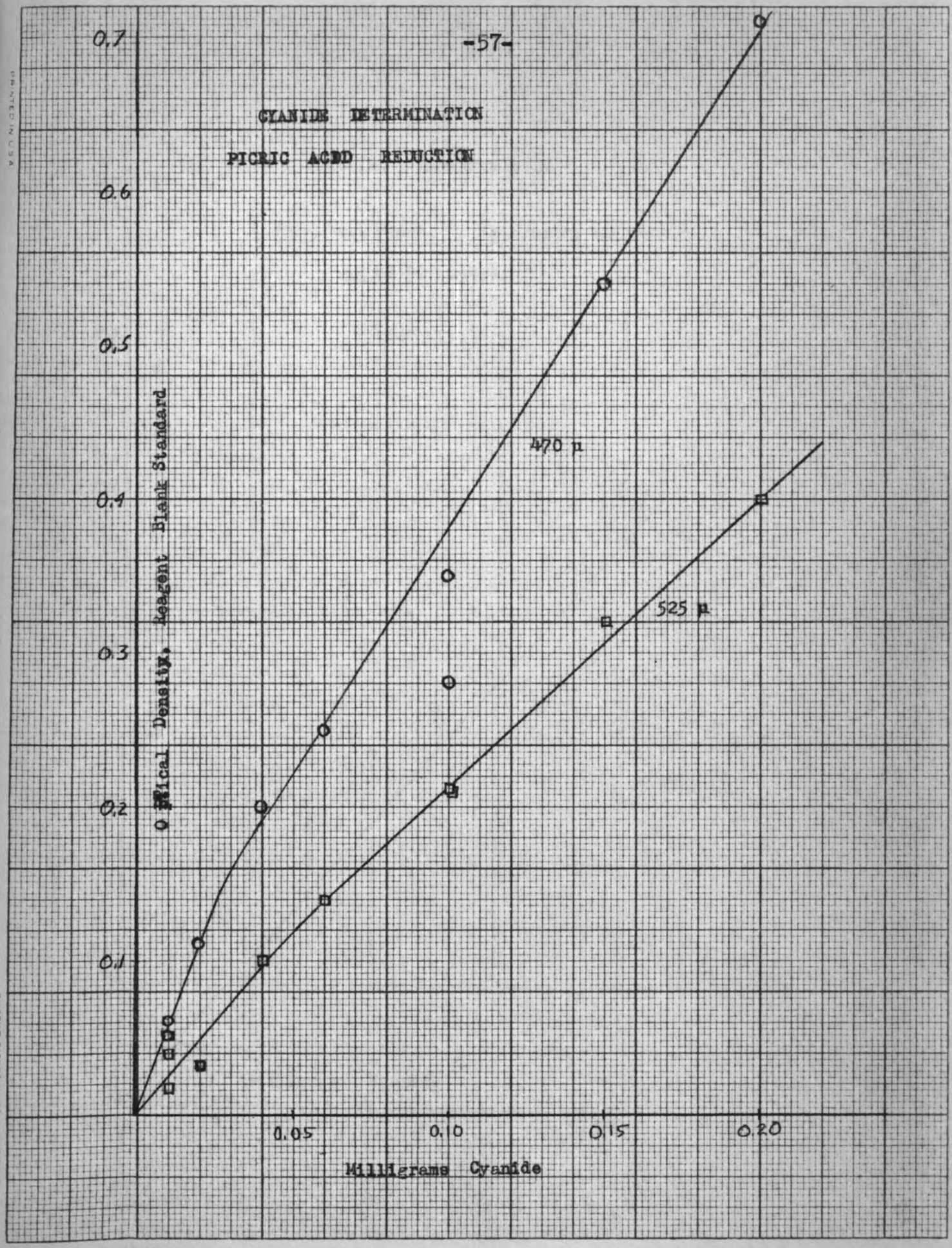
Calibration curves for all the colorimetric methods mentioned were prepared. These are included in this appendix. A second formaldehyde determination is included: formation of a colored condensation product with chromotropic acid (10).

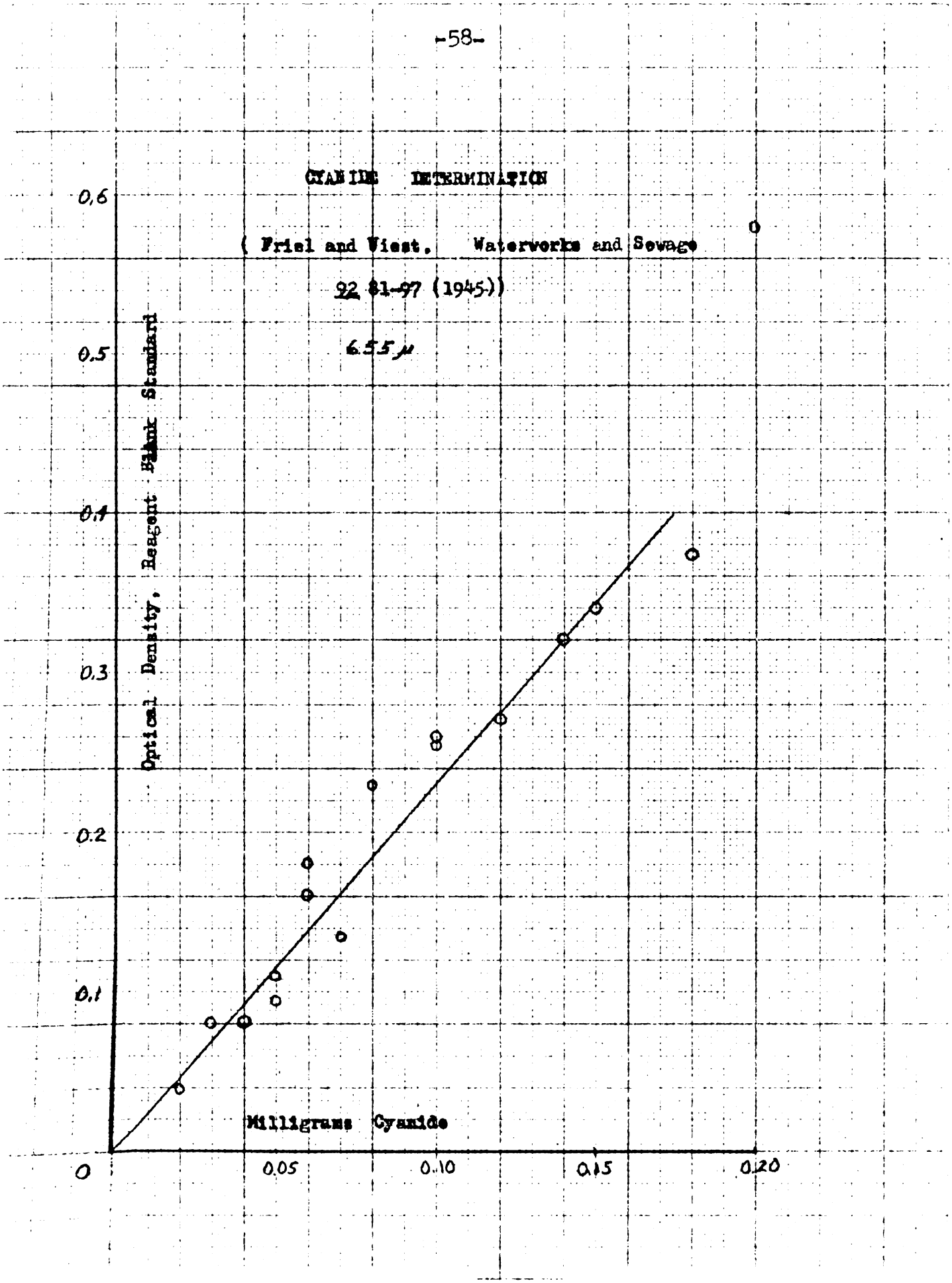

NITRITE DETERMINATION
525 μ
Optical Density, Reagent Blank Standard
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Micrograms Nitrite Nitrogen
0.5
1.0
1.5
2.0

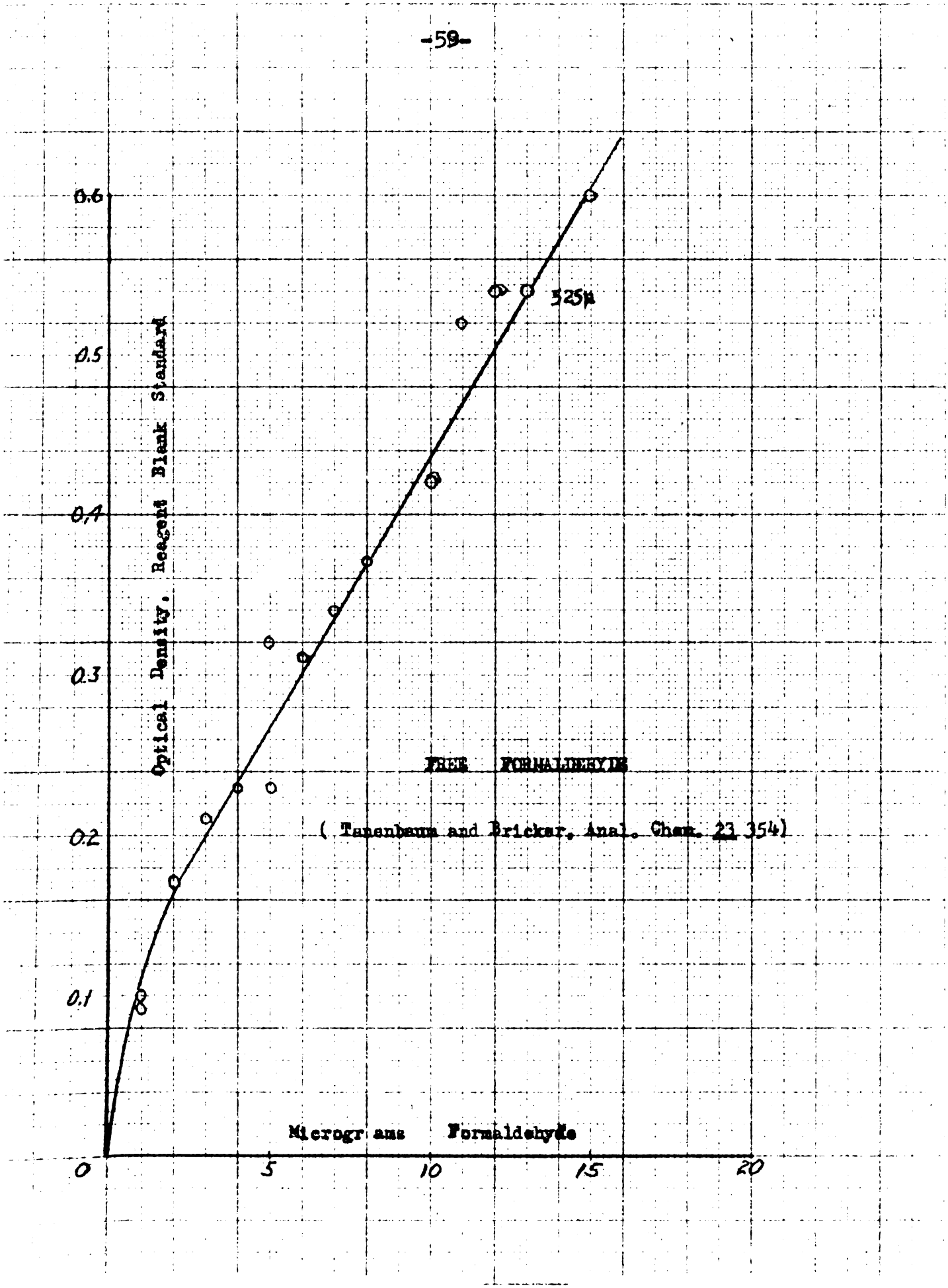

0.5
0.4
0.3
0.2
0.1
Optical Density
NITRATES
PHENOL DISULFONIC ACID METHOD
525 μ
Lower scale
Milligrams Nitrate Nitrogen
0
1
2
3
4
5
0.0
0.05
0.1




0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Optical Density, Reagent Blank Standard
430µ
470µ
525µ
CYANIDE AS THIOCYANATE COMPLEX
Milligrams Cyanide
0.1
0.2
0.3
0.4
0.5

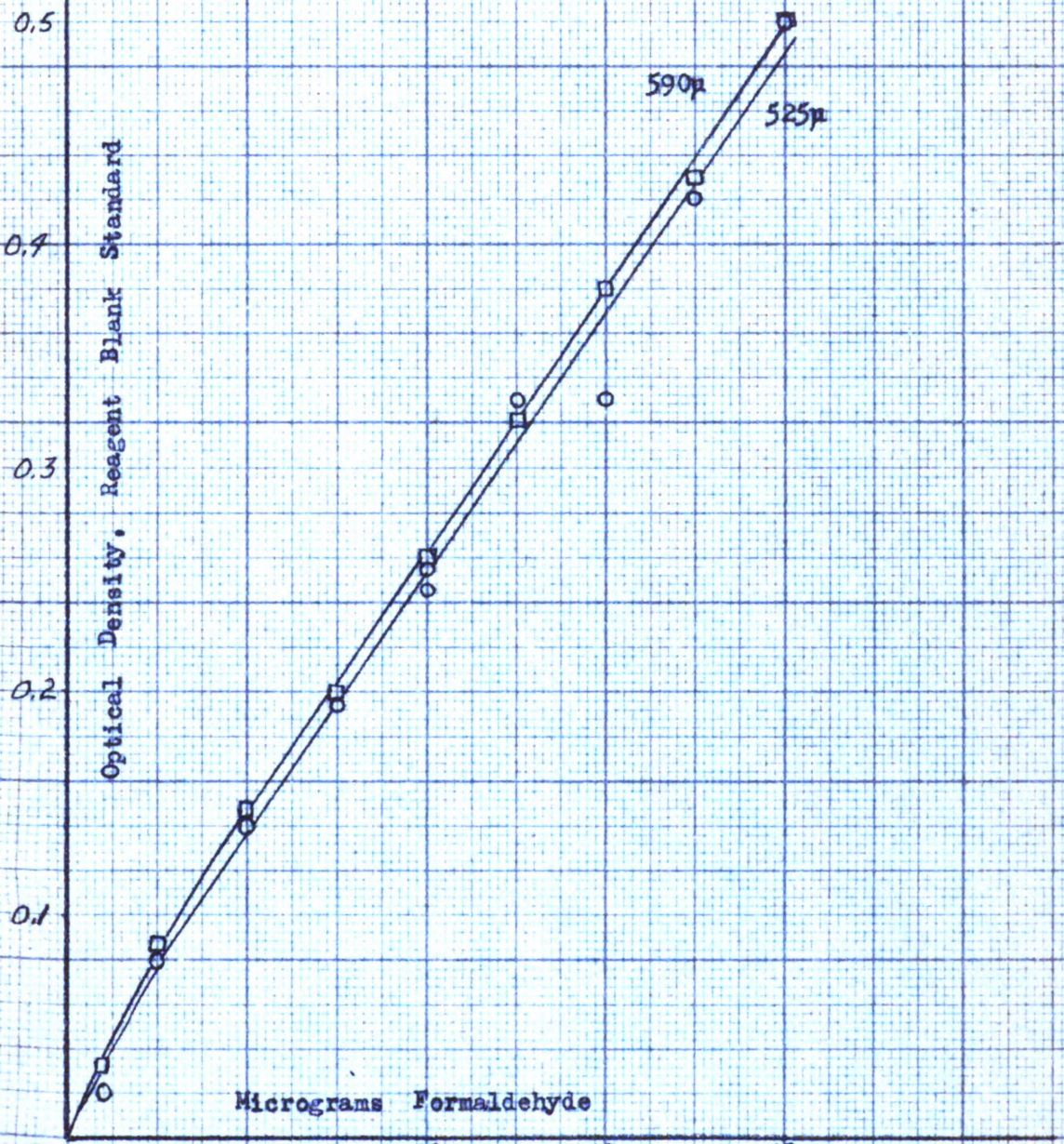



CYANIDE DETERMINATION
(Friel and Viest, Waterworks and Sewage
92, 81-97 (1945))
655 μ
Optical Density, Reagent Blank Standard
0.6
0.5
0.4
0.3
0.2
0.1
0
0.05
0.10
0.15
0.20
Milligrams Cyanide


0.6
0.5
0.4
0.3
0.2
0.1
0
Optical Density, Reagent Blank Standard
525μ
FREE FORMALDEHYDE
(Tanenbaum and Bricker, Anal. Chem. 23 354)
Micrograms Formaldehyde
5
10
15
20

FORMALDEHYDE *- CHROMOTROPIC ACID METHOD

(Bricker and Johnson, Ind. Eng. Chem., Anal. Ed. 17, 400 (1945))



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