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THESIS

"The Microbiological Destruction of Thiocyanate
in Activated Sludge Plants Treating
Coke Oven Effluent."

Submitted for the degree of Ph.D.
at the
University of Aston in Birmingham

Thesis
628.356 HIA   190413

October 1975.

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ACKNOWLEDGEMENTS.

The author wishes to express his thanks to Dr. M. Hutchinson of the Water Research Centre and Dr. D. White of Leeds University for valuable advice on the detection of thiobacilli. Gratitude is also expressed to Mr. N.R. Hall for techniques used in chemical analysis and in the operation of laboratory scale biological treatment plants. The photomicrographs were obtained with kind co-operation of Mr. R. Edwards, Mr. D. Nairn and Mr. H. Neilson. Thanks are also expressed to Mrs. Janis Kelly who typed the manuscript.

This work was carried out in the Chemical Section, Research Centre, British Steel Corporation, Tubes Division, Corby. Gratitude is expressed to the British Steel Corporation for financing the project and for providing the time and facilities required.

TITLE OF THESIS.

"The Microbiological Destruction of Thiocyanate in Activated Sludge Plants treating Coke Oven Effluents."

SUMMARY

The treatment of effluents produced during the manufacture of metallurgical coke is normally carried out using the activated sludge process. The efficiency of activated sludges in purifying coke oven effluent depends largely on the maintenance of species of micro-organisms which destroy thiocyanate. The composition, production, toxicity and treatment of coke oven effluent at Corby steelworks are described. A review is presented which follows the progress made towards identifying and monitoring the species of bacteria which destroy thiocyanate in biological treatment plants purifying coke oven effluents.

In the present study a search for bacteria capable of destroying thiocyanate led to the isolation of a species of bacteria, identified as *Pseudomonas putida*, which destroyed thiocyanate in the presence of succinate; this species had not previously been reported to use thiocyanate. Washed cell suspensions of *P. putida* destroyed phenol and thiocyanate simultaneously and thiocyanate destruction was not suppressed by pyridine, aniline or catechol at the highest concentrations normally encountered in coke oven effluent. The isolate has been included, as N.C.I.B. 11198, in the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen. Three other isolates, identified as *Achromobacter sp.*, *Thiobacillus thioparus* and *T. denitrificans*, were also confirmed to destroy thiocyanate.

A technique has been developed for monitoring populations of different species of bacteria in activated sludges. Application of this technique to laboratory scale and full scale treatment plants at Corby showed that thiobacilli were usually not detected; thiobacilli were eliminated during the commissioning period of the full scale plant. However experiments using a laboratory scale plant indicated that during a period

of three weeks an increase in the numbers of thiobacilli might have contributed to an improvement in plant performance. Factors which might have facilitated the development of thiobacilli are discussed. Large numbers of fluorescent pseudomonads capable of using thiocyanate were sometimes detected in the laboratory scale plant. The possibility is considered that catechol or other organic compounds in the feed-liquor might have stimulated fluorescent pseudmonads.

Experiments using the laboratory scale plant confirmed that deteriorations in the efficiency of thiocyanate destruction were sometimes caused by bulking sludges, due to the excessive growth of fungal flocs. Increased dilution of the coke oven effluent was a successful remedy to this difficulty.

The optimum operating conditions recommended by the manufacturer of the full scale activated sludge plant at Corby are assessed and the role of bacterial monitoring in a programme of regular monitoring tests is discussed in relation to the operation of activated sludge plants treating coke oven effluents.

CONTENTS

Section Page

1. INTRODUCTION. 1

2. LITERATURE REVIEW. 15

3. EXPERIMENTS USING MIXED CULTURES.

3.1 Laboratory scale treatment plants. 32

3.1.1 Plant treating coke oven effluents. 32

3.1.1.1 Source of bacteria. 36

3.1.1.2 Acclimation of sludge to coke oven effluent. 36

3.1.1.3 Continuous treatment. 44

3.1.1.4 Effect of concentrated liquor. 53

3.1.1.5 Addition of catechol. 55

3.1.1.6 Addition of pyridine. 56

3.1.1.7 Effect of different dilutions of feed-liquor on the occurrence of bulking sludges. 58

3.1.2 Plant treating synthetic coke oven effluent. 70

3.2 Testing of batch cultures. 74

3.2.1 Additions of glycine and alanine. 74

3.2.2 Addition of formate. 79

3.3 Results 81

4. ISOLATION AND TESTING OF PURE CULTURES. 84

4.1 Enrichment of thiocyanate and thiosulphate destroying bacteria. 84

4.2 Media. 86

4.3 Procedure for isolation of thiocyanate-destroying bacteria, using inorganic media. 86

4.4 Methods of testing isolates. 87

Section | Page

4.4.1 Stock cultures. 88
4.4.2 Gram reaction and Flagella. 88
4.4.3 Biochemical tests and resistance to penicillin, cetrimide. 90
4.4.4 Determination of thiocyanate-destruction. 93
4.4.5 Tests using washed resting cell suspensions. 95

4.5 Results.
4.5.1 *Thiobacilli*, isolates 1 and 2. 97
4.5.2 *Achromobacter* sp, isolate 3. 105
4.5.3 Fluorescent pseudomonads, isolates 4 and 5. 107
4.5.4 *Micrococcus* sp, isolate 6. 117
4.5.5 Summary of results. 119

5. MONITORING OF BACTERIAL POPULATIONS IN LABORATORY SCALE AND FULL SCALE TREATMENT PLANTS.
5.1 Sampling. 121
5.2 Dispersion. 122
5.3 Dilution. 127
5.4 Preparation of media. 129
5.5 Application of samples to media. 129
5.6 Thiobacilli. 132
5.6.1 Selected features. 132
5.6.2 Development of method. 135
5.6.3 Final method adopted. 138

5.6.4 Results. 138
5.7 Fluorescent pseudomonads. 142
5.7.1 Selected features. 142
5.7.2 Development of method. 142
5.7.3 Final method adopted. 144

Section | Page

5.7.4 Supplementary tests. 145

5.7.5 Results. 146

5.8 Total pseudomonads.

5.8.1 Selected features. 147

5.8.2 Development of method. 147

5.8.3 Final method adopted. 147

5.8.4 Supplementary tests. 148

5.8.5 Results. 148

5.9 Bacteria which used thiocyanate in the presence of organic carbon-sources:succinate and phenol.

5.9.1 Selected features. 149

5.9.2 Development of methods. 149

5.9.3 Final method adopted. 151

5.9.4 Results. 151

5.10 Bacteria which used thiocyanate in the presence of aeration-tank supernatant liquid.

5.10.1 Selected features. 154

5.10.2 Development of method. 154

5.10.3 Final method adopted. 156

5.10.4 Supplementary tests. 156

5.10.5 Results. 158

5.11 Total heterotrophic bacteria.

5.11.1 Selected bacteria. 158

5.11.2 Development of method. 158

5.11.3 Final method adopted. 159

Section | Page

5.11.4 Results 159

5.12 Background growth. 160

5.12.1 Results. 163

6, DISCUSSION. 179

APPENDIX.1 and 2. 201

KEY TO TABLES 206

KEY TO FIGURES 208

REFERENCES 210

1. Introduction.

The production of coke by the carbonisation of coal yields large volumes of toxic and corrosive effluents. The continuous requirement by the blast furnaces for metallurgical coke is accompanied by the problems of purifying and disposing of the effluent from coke ovens. The use of raw untreated liquors from coking plants for the wet quenching of coke leaves impurities in the coke; for example a reduction in the quality of the coke occurs particularly if calcium chloride is not substantially removed before quenching. The evaporation of coke oven effluent during coke quenching causes pollution of the surrounding atmosphere. Furthermore, the discharge of coke oven effluent in excess of that required for quenching may cause severe pollution of the receiving waterways and may impair the efficiency of domestic sewage works.

Coke oven effluent is formed from the evaporation of moisture in the coal, the pyrolysis of volatile organic matter and from the formation of by-products such as ammonium sulphate and benzole. The composition of coke oven effluent depends on the source and quality of coal, the temperature and duration of carbonisation and the efficiency of removing ammonia from the ammoniacal liquor by steam distillation. Barker et al (1958) gave typical concentrations of the main toxic materials present in coke oven effluents; the figures are given in Table 1. At the outset of the present study the effluents from Corby coke ovens were analysed and the concentrations of the major constituents are given in Table 2 on page 38. Comparison between tables 1 and 2 shows that the effluent at Corby had a relatively wide range of concentrations of ammonia. Spent still liquor, which constitutes most of the volume of Corby coke oven effluent, had relatively high concentrations of thiocyanate, phenol and chloride. The production and treatment of coke oven effluent at Corby are summarised in Fig 1.

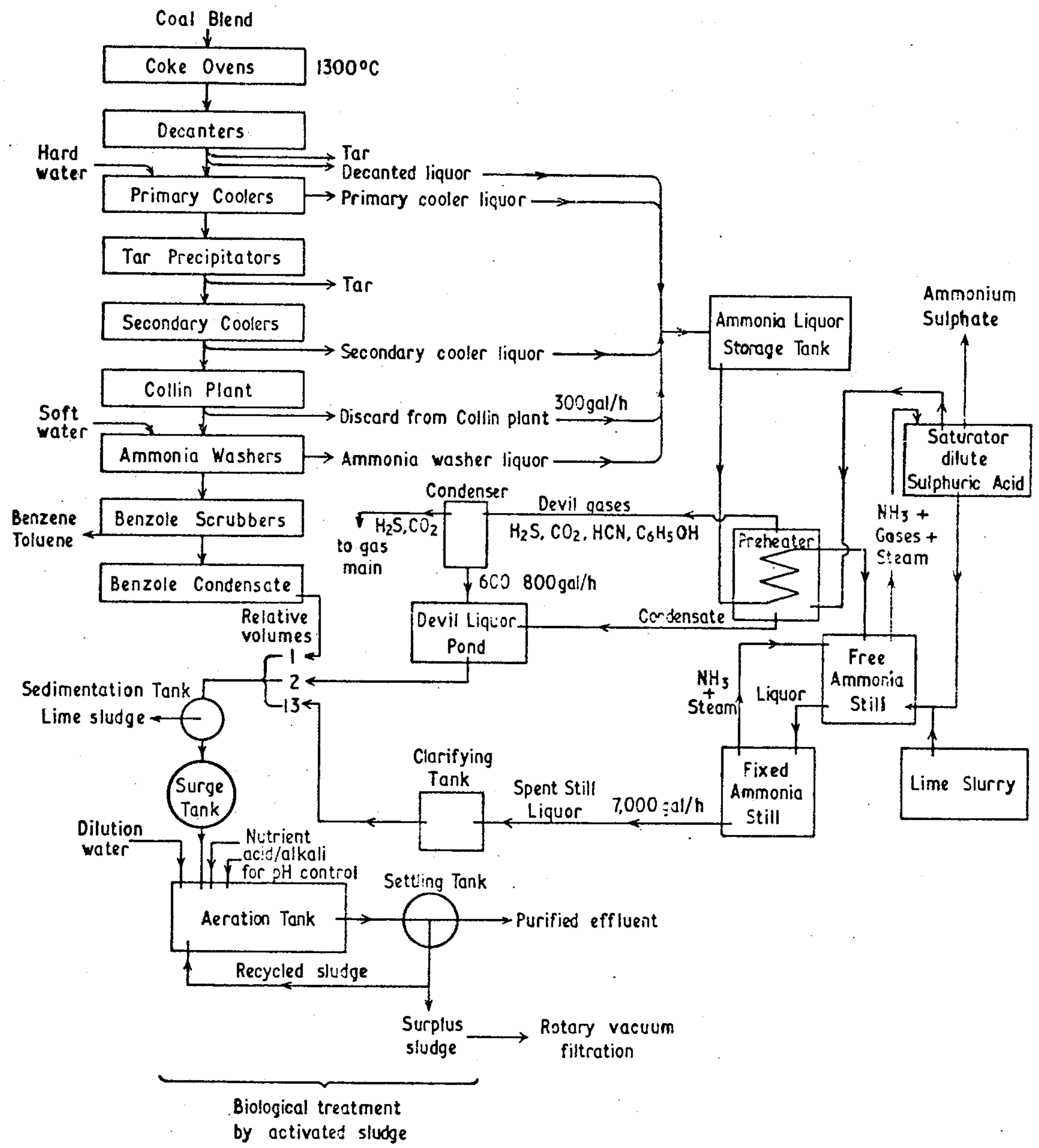
Table 1 Analysis of ammoniacal liquor from coke ovens.

Constituents / Liquors	Concentration, mg/l 1	2	3	4	5	6
PHENOLS:						
Monohydric -						
Phenol	470	1,360	1,420	1,290	770	980
o-Cresol	30	150	100	110	150	110
m-Cresol	80	370	280	290	220	220
p-Cresol	30	180	140	140	140	120
2:3-,2:4-,2:5,-) 2:6-Xylenols) o-Ethylphenol)	10	70	50	40	70	40
3:4-,3:5-Xylenols) m-mp-Ethylphenols)	10	60	30	20	30	20
Total monohydric	630	2,190	2,020	1,890	1,380	1,480
Dihydric -						
Catechol	-	10	30	30	30	20
3-Methylcatechol	-	10	30	30	30	20
4-Methylcatechol	-	20	20	30	30	20
Resorcinol	10	40	50	60	50	40
2-,5-Methylresorcinols	-	30	60	-	40	30
4-Methylresorcinol	-	-	10	-	20	-
Total Dihydric	10	110	200	150	200	130
Total Phenols	640	2,300	2,220	2,040	1,580	1,610
INORGANIC IONS:						
Ammonia "Free" NH_4^+	1,530	1,130	3,600	10,500	10,300	2,700
Ammonia "Fixed" NH_4^+	1,870	2,420	4,970	3,300	1,700	1,770
Ammonia Total NH_4^+	3,400	3,550	8,570	13,800	12,000	4,470
Sulphides S^{--}	50	0	420	830	3,750	80
Thiosulphate $S_2O_3^{--}$	1,450	610	400	490	240	39
Thiocyanate CNS^-	170	230	450	610	550	39
Chloride Cl^-	2,290	3,770	8,000	5,450	3,050	30
PERMANGANATE VALUE:	2,770	4,900	6,100	5,650	10,800	3,900

Key to Table 1:-

Liquors 1 = Richard Thomas and Baldwin, Ebbw Vale.
2 = British Benzol and Coal Distillation Limited, Trethomas
3 = Appleby-Frodingham Steel Company, Scunthorpe.
4 = Stewarts and Lloyds Limited, Corby.
5 = Birchenwood Gas and Coke Company Limited, Kidsgrove, Stoke.
6 = Colville's Limited, Clyde Iron Works, Tollcross, Glasgow.

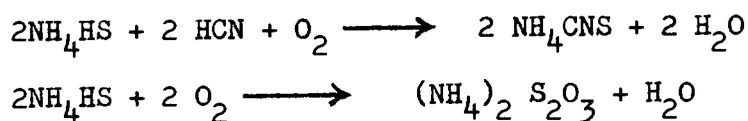
FIG 1 PRODUCTION AND PURIFICATION OF COKE OVEN EFFLUENTS AT CORBY STEELWORKS



The Collin process used at Corby for the wet desulphurisation of coke oven gas (Williams, 1954) produces an effluent which is added to the crude ammoniacal liquor fed to the ammonia stills. The discarded liquid from the Collin plant contains formate, ferrocyanide and thiocyanate. Since many other coking plants do not employ the Collin process, the presence of formate is a feature which distinguishes Corby coke oven effluent from many other carbonisation waste liquors.

Ammoniacal liquor is steam distilled to remove free and fixed ammonia. Effluent from the free ammonia stills enters the fixed ammonia stills where a slurry of calcium hydroxide is added. Here ammonium compounds react with milk of lime to release free ammonia. Spent liquor from the ammonia stills at Corby contains mainly mono- and dihydric phenols and substituted derivatives, pyridine and quinoline bases, thiocyanate, thiosulphate, chloride, formate and ammonia.

The gas leaving the retorts contains only small amounts of thiocyanate and thiosulphate. These pollutants are formed subsequently by the oxidation of sulphide and cyanide (Key, 1956):



$$2NH_4HS + 2\ HCN + O_2 \longrightarrow 2\ NH_4CNS + 2\ H_2O$$

$$2NH_4HS + 2\ O_2 \longrightarrow (NH_4)_2\ S_2O_3 + H_2O$$

The amounts of thiocyanate and thiosulphate formed by these oxidations depend on the amounts of sulphide and cyanide in the liquor, and on the oxygen content of the crude gas. The oxygen content is increased by the leakage of air into the retorts, especially when they are operated under slight vacuum. Since the oxidations occur relatively slowly, prolonged contact between the liquor and the gas results in increased concentrations of thiocyanate and thiosulphate.

Operation of the ammonia stills requires careful control to minimise the concentration of ammonia in the spent liquor. The

free gaseous ammonia is absorbed into dilute sulphuric acid to form ammonium sulphate. Regulation of the addition of lime slurry to the fixed ammonia stills is necessary to avoid an excessive amount of calcium hydroxide accumulating in the spent ammoniacal liquor. As a result of the liming process thiocyanate is present as calcium thiocyanate.

Devil liquor from the ammonia stills contains primarily phenol, thiocyanate, hydrogen sulphide, carbon dioxide and hydrogen cyanide. Benzole condensate is formed during steam distillation, which separates crude benzole from gas oil used for stripping aromatic hydrocarbons from the gas stream. The condensate contains thiocyanate, hydrogen sulphide, and hydrogen cyanide. Some of the cyanide present in devil liquor and benzole condensate is removed by evaporation from the hot liquors entering the balancing tank.

The temperature of the liquor leaving the ammonia stills is about 80°C and the pH is usually between 9.0 and 11.5. Bacteriological tests in the present study showed that the liquors from Corby coke ovens were sterile on entering the balancing tank before biological treatment. The relative volumes of spent still liquor : devil liquor:benzole condensate in Corby coke oven effluent are usually 13:2:1.

Three factors are concerned with the disposal of coke oven effluents specifically at Corby steelworks. Firstly the practice of wet quenching of coke using the treated or untreated effluent may have to be discontinued in order to reduce atmospheric pollution and to avoid the contamination of metallurgical coke. A greater volume of toxic liquid effluent would then require disposal. Secondly there is an insufficient volume of water in the streams surrounding Corby to permit adequate dilution of the effluent. This problem becomes particularly acute during periods of dry weather. Thirdly precautions must be taken to avoid the pollution

of drinking water. This possibility arises because the brooks receiving controlled discharges from Corby steelworks enter Willow Brook which in turn enters the River Nene. Water from the River Nene is pumped to the Empingham Reservoir, construction of which was begun in 1971 to meet the increasing demand for potable water in the East Midlands. These considerations emphasise the need for an awareness of the harmful effects on stream-life and potable water caused by constituents of coke oven effluent. The legal requirements to purify industrial wastes (Rivers Prevention of Pollution Act, 1951 and Water Resources Act 1963), must be satisfied and the standards applied to the quality of drinking water must be considered: (European Standards for Drinking Water, W.H.O. Geneva, 1970).

Thiocyanate was reported by Key (1956) to impart a relatively low toxicity to fish, compared with the more harmful effects of phenols, catechol and ammonia which are also present in carbonisation effluents. Key gave the toxic concentration of thiocyanate as 200 mg per litre, compared with 10 mg per litre for each of phenols, catechol and ammonia. However, the toxicity to fish of chlorinated effluents containing thiocyanate is increased by the formation of cyanogen chloride (Allen et al, 1946, and Hynes, 1966). A further objection to the discharge of coke oven effluents containing thiocyanate is that the oxidation of thiocyanate by sewage bacteria increases the concentration of ammonia in the sewage (Key, 1956). Bishofsberger (1971) stated that in order to achieve the biological oxidation of ammonia in coke oven effluent it was necessary to lower the concentration of thiocyanate to not more than 10 mg per litre. However Tomlinson et al (1966) found that nitrification by activated sludge treating domestic sewage was not affected by 300 mg potassium thiocyanate per litre.

Thiosulphate is readily oxidised by autotrophic and heterotrophic bacteria (Starkey, 1934, and Peck, 1962). Consequently it does not normally persist or accumulate in waters or soils (Lees, 1955). In studies of the toxicity to fish of chemicals in turbid waters, Wallen et al (1957) found that thiosulphate had a relatively low toxicity to Gambusia affinis. Nevertheless the complete oxidation of thiosulphate by bacteria requires a large amount of oxygen (Trudinger, 1967). The toxicity of other pollutants may be increased at lowered concentrations of dissolved oxygen (Herbert, et al 1955).

Several strains of bacteria found in streams and rivers are capable of oxidising phenol (Tabak et al, 1964, and Stanier, 1966). However phenol presents a serious pollution problem owing to its toxicity to fish (Wallen et al, 1957), and its inhibitory effect on nitrification in activated sludges (Tomlinson et al, 1966, and Stafford, 1974). Tomlinson also found that cresols and aniline inhibited nitrification, and Stafford confirmed that pyridine and 2- and 4- methyl pyridine had severe inhibitory effects. A further problem arises from the reaction of phenols with chlorine to form chlorophenols which impart tastes and odours to waters, (Southgate, 1960).

Ammonia is toxic to fish at the relatively low concentration of 1 mg per litre (Spicer, 1952). The toxicity of ammonia, which is due mainly to the undissociated base (Doudoroff et al, 1950), is increased at high pH values (Alabaster et al, 1954) and at low concentrations of dissolved oxygen (Downing et al, 1955).

Considerable toxicity to fish is caused by hydrogen sulphide (Doudoroff, 1950) and cyanide (Southgate et al, 1933, and Key, 1956). Lockett et al (1947) described the inhibitory effect of 1 mg of hydrogen cyanide per litre on the biological purification of

sewage by activated sludge.

Coke oven effluents have a very high oxygen demand which would exert a severe pollutional load on a stream. The high oxygen demand, expressed as the 4 hour permanganate value, is given in Table 1 on page 2 .

The safe range of pH values to fish was stated by Key (1956) to be pH 5.0 - 9.0. However alkalinities between pH 10 and 11 were sometimes recorded for Corby coke oven effluent (Table 2 , page 38). pH values greater than 10 are considered to be hazardous to fish life (Doudoroff, et al 1950).

The high temperature of spent ammoniacal liquor (80°C) could present a hazard to life in receiving waterways. Carpenter (1927) found that in general a rise of 10°C halved the survival times of fish in the presence of certain soluble toxic metal salts. The Public Health Act, Section 27 (1936) forbade the discharge to sewers of liquids having a temperature higher than 43°C.

The above account of the composition and harmful effects of coke oven effluents emphasises the need for strict control over their disposal. The problem of preventing pollution of the atmosphere, water-resources and agricultural land is increased by the large volumes of carbonisation effluents. At Corby steelworks the volume of toxic liquid effluents formed daily from the manufacture of coke is approximately 200,000 gallons.

Several methods have been employed for the disposal of coke oven effluents. Disposal by coke quenching destroys some of the toxic materials when the liquors come into initial contact with the coke at 1100°C - 1300°C. However during cooling, increased concentrations of pollutants remain in the gases emitted from the quenching towers.

Crude ammonial liquor has been used as a fertiliser on agricultural land (Schuster, 1931). As a top-dressing on grassland the liquor has produced increased yields. However ammoniacal

liquor contains relatively high concentrations of toxic substances such as phenol and thiocyanate. Liquors containing 0.4% of thiocyanate were toxic to many seeds and growing plants (Key, 1956).

Discharging onto colliery spoil-heaps permits the gradual removal of thiocyanate and phenol. This has been attributed largely to physical adsorbtion and absorbtion rather than to microbiological degradation, (Dobson, 1971). The dangers of this method of disposal are that the slag heaps may become unstable and continued dumping may cause the erosion of channels followed by contamination of the surrounding land and water supplies by untreated effluents. If the effluents are discharged into disused colliery mineshafts, underground water supplies may be contaminated and existing collieries may be affected.

Blackburn and Kershaw (1963) found that domestic sewage purification works employing bacteria beds are normally capable of accommodating up to 2% of coke oven effluent in the incoming sewage without the need for an increase in the capacity of the plant. However long periods, e.g. 8 days at 18°C (Key, 1956), may be required to induce the bacterial oxidation of thiocyanate in oxygenated water containing domestic sewage. Controlled discharges to sewers of coke oven effluents containing thiocyanate should therefore be carried out continuously, instead of intermittently, to permit the development of an active thiocyanate oxidising population of micro-organisms. Klein (1966) reported that crude ammoniacal liquor should not exceed 0.25% of the sewage flow. Small discharges of carbonisation effluents into sewers may therefore be acceptable. However pre-treatment to remove phenols and ammonia may be necessary. Restrictions imposed on discharges to sewers in England and Wales are controlled by the Public Health Acts 1936 and 1961 and the Public Health (Drainage of Trade Premises)

Act 1937.

Several chemical methods of treating coke oven effluents have been used. Ion exchange resins have been used successfully to remove thiocyanate and thiosulphate, followed by adsorbtion of phenols and colour by activated carbon, (Akeroyd, et al 1958).However the ion exchange resins and activated carbon required frequent regeneration and the cost of the treatment process was high. Van Stone (1972) reported that most contaminants in coke oven effluents could be removed by physical and chemical treatment. This involved flocculation and sedimentation to remove solids, followed by the adsorbtion of phenols by granular activated carbon and catalytic oxidation to remove free cyanide. The disadvantage of the system was that the treated effluent contained high concentrations of thiocyanate and ammonia.

The most widely used method of purifying coke oven effluent is by biological treatment. The four main types of biological treatment processes which have been used are: bacteria beds, packed towers using plastic media, rotating discs and activated sludge. Bacteria beds have the advantage of being resistant to shock loadings and are tolerant of combinations of sulphide and cyanide which suppress activated sludge (Cooper, 1975). However, relatively small loadings were possible using bacteria beds which often became blocked with tars and excessive accumulations of microbial film, (Cooper, 1975), which impaired the efficiency of transfer of materials between the micro-organisms and the waste being purified. The excessive accumulation of microbial mass in the bacteria beds might be caused by an inhibitory effect of coke oven effluent on populations of scouring organisms such as protozoa which feed on bacteria in the microbial film. Furthermore there is often insufficient space available to install the large filter areas

which are required to purify large volumes of coke oven effluent. Towers packed with plastic media achieved high rates of treatment (Cooper and Catchpole, 1966) but were less efficient in removing thiocyanate. The use of rotating discs has recently been investigated by the British Carbonisation Research Association (Cooper, 1975). Although this method of treatment is suitable only for small volumes of effluent, the consumption of energy was low and the final effluent had a low concentration of suspended solids. Carbonisation waste treatment by activated sludge is used more extensively than the three other processes described above.

The activated sludge process was originated by Arden and Lockett in 1913 and was reported by Arden et al (1914). Essentially the process involves the aeration of settled sewage with micro-organisms which actively purify the liquid waste. A large proportion of the microbial mass involved in purification is in a flocculated form. The "mixed liquor" contained in the aeration tank consists of suspended flocs of micro-organisms, or "activated sludge", together with the sewage undergoing biological purification. In the conventional process (Fair, et al, 1968) the waste to be purified is fed continuously into the aeration tank where it is utilised partly in the synthesis of increased microbial mass and partly in biological oxidations producing energy and waste products such as carbon dioxide, ammonia and sulphate. The overflow of mixed liquor from the aeration tank enters a settling tank where the flocs are allowed to settle. The purified supernatant liquid is discharged as the final effluent. There is a continuous feedback of cells from the settling tank to the aeration tank. This maintains a sufficient biomass to achieve purification of the waste. Excess activated sludge is removed from the system. Since part of the waste is incorporated into cells, a large proportion of the waste

might be discharged as suspended cell materials if separation of the cells was not practiced (Mitchell, 1972).

In view of the inhibitory effect of phenol on the oxidation of thiocyanate (Pankhurst, 1959), Abson and Todhunter (1958) recommended a two-stage aeration system. Phenols were removed in the first stage which permitted thiocyanate destruction to occur in the second stage, However, Cooper et al (1966) found that the simultaneous destruction of phenol and thiocyanate could be achieved using a single aeration tank, provided that inhibitory concentrations of phenol were prevented. Careful control of the process was therefore needed. For example, Bishofsberger (1971) reported that in order to achieve the complete destruction of thiocyanate in a single aeration vessel it was necessary to maintain the concentration of phenol at less than 25 mg per litre.

In laboratory experiments, Ashmore et al (1968) observed that when the influent concentration of ammonium thiocyanate reached 330-500 mg per litre, small increases in the concentration of thiocyanate caused large increases in the minimum aeration period. His work showed that high concentrations of thiocyanate contributed to the instability of the purification process. In pilot scale studies (BCRA Review Vol. 1 No. 1, 1974) fluctuations in the composition of the crude ammoniacal liquor resulted in a decrease in the efficiency of removal of thiocyanate in activated sludge units. Although activated sludge processes are capable of achieving the efficient destruction of thiocyanate, the accumulation of this toxic constituent of coke oven effluent often occurs during biological treatment. The complete destruction of thiocyanate was found to be the best single criterion of efficient biological purification of coke oven effluent (Ashmore et al., 1968).

Improvements in the biological oxidation of thiocyanate by activated sludge were found to be stimulated by intermittent additions of 1-10 mg per litre of certain organic compounds. The organic compounds, which were observed individually to bring about a beneficial effect, included: benzoic acid, para-aminobenzoic acid, para-hydroxyebenzoic acid, anthranilic acid and phthalic acid (Cooper and Catchpole 1966, b). The same authors found that a similar stimulating effect on thiocyanate oxidation could be brought about by additions of glucose or pyruvate (BCRA Report 64, 1971, and Coke Research Report 76, 1973). However the nature of the influence of these organic additives on the micro-organisms involved in thiocyanate destruction was uncertain.

The commonly used chemical monitoring tests for thiocyanate and phenol do not reveal the causes of decreased purification efficiency and often they fail to provide sufficient advanced warning of deteriorating conditions in the plant. Detailed studies of fluctuations in the proportions of different thiocyanate destroying micro-organisms during the biological treatment of coke oven effluent have rarely been undertaken. The information gained from microbiological monitoring tests may enable deteriorations in plant-performance to be anticipated at an earlier stage.

Owing to the instability of activated sludge systems purifying coke oven effluents, strict control of the operational conditions is required. Thiocyanate rapidly accumulates to toxic concentrations if the optimal conditions are not adhered to. The aims of the present work were to determine if micro-organisms other than those previously identified could utilise thiocyanate and to relate the occurrence of the different species to the efficiency of thiocyanate destruction during biological treatment.

The testing of laboratory scale plants and a full scale treatment plant during its commissioning period enabled an assessment to be made of the value of bacterial monitoring to plant-operation. Investigations using laboratory scale plants also provided the opportunity to assess the optimum conditions for the treatment of coke oven effluent at Corby.

2. Literature Review.

This section reviews the developments which have contributed to a more thorough understanding of the identity and importance of different species of micro-organisms in the destruction of thiocyanate in biological treatment plants.

Progress towards achieving efficient biological purification of carbonisation effluents has been achieved through advances in several related fields of study. These include aspects of the design and operation of biological treatment systems, improvements in the operation of coking plants, studies of the treatability of different coke oven liquors, improved methods for the chemical analysis of liquors, the physiology, biochemistry and ecology of micro-organisms involved in wastewater treatment and improved methods for the enumeration and identification of micro-organisms.

The bacterial degradation of thiocyanate was described by Beijerinck (1904), in studies of enrichment cultures of *Thiobacillus thioparus* and *T. denitrificans.* However the significance of thiocyanate destroying bacteria in the purification of carbonsation wastes had not been established at this time. The importance of bacterial activity in the purification of spent liquor from gas works in percolating filters was demonstrated later by Fowler et al (1911) and Happold (1925 and 1930). Although these studies showed that monohydric phenols were oxidised by bacteria, it was not until 1937 that a detailed study was made of the bacterial oxidation of thiocyanate in effluents from gas works. From sewage sludge which had been adapted to the purification of gas works liquor, Happold and Key (1937) isolated on nutrient agar a Gram negative rod shaped bacterium which could utilise ammonium thiocyanate. The organism was named *Bacterium thiocyanoxidans*. Difficulties were encountered in obtaining the organisms as a pure culture and Happold et al (1952), showed that the original

heterotrophic culture was probably mixed. However, in 1951 the same workers obtained a pure culture of a Gram negative, motile, obligately autotrophic rod which converted thiocyanate quantitatively into sulphate and ammonia. The organism was classified as a Thiobacillus in view of its ability to grow with carbon dioxide as the sole course of carbon and inorganic compounds of sulphur as the energy source. The organism was therefore given the name Thiobacillus thiocyanoxidans.

Happold (1954) again found difficulty in eliminating heterotrophs from isolates of T. thiocyanoxidans and he found that thiocyanate was rarely removed by the bacteria from agar media. He suggested that the cause might have been agar-toxicity. Silica gel media were therefore used in preference to agar. Although later work was to show that "T. thiocyanoxidans" should not be considered as a separate species, many of Happold's observations on the culturing of the organism are relevant to a detailed study of the bacterial destruction of thiocyanate: Happold observed that "T. thiocyanoxidans" caused no change in pH during thiocyanate oxidation and a marked clumping of the bacteria developed after removal of all the thiocyanate from liquid media. Ageing cultures formed deposits of elemental sulphur. "T. thiocyanoxidans" did not grow in the presence of organic substances unless thiocyanate was being oxidised. The organic substances tested were not found to stimulate growth or thiocyanate destruction, and at 1% w/v they were inhibitory. Phosphate was inhibitory at concentrations above 1260 mg/l.

Van der Walt et al (1955) obtained pure cultures of T. denitrificans which used thiocyanate anaerobically with nitrate as the oxidant. This evidence, together with Beijerinck's earlier observations, led De Kruyff et al (1957) to determine whether

thiocyanate oxidation was a valid criterion for the differentiation of species of thiobacilli. De Kruyff tested "*T. thiocyanoxidans*", *T. thioparus*, *T, denitrificans* and *T. thio-oxidans* for the ability to utilise thiocyanate aerobically and anaerobically in the presence of nitrate. "*T. thiocyanoxidans*", *T. thioparus* and *T. denitrificans* were found to have this ability, but *T. thio-oxidans* did not utilise thiocyanate. "*T. thiocyanoxidans*" was considered to be a variant of *T. thioparus*. Denitrification to form nitrogen gas from nitrate was a feature exhibited only by *T. denitrificans*. This organism was therefore considered to be a separate species.

Townsend and White (1960) found that the specimen of *T. thioparus*, N.C.I.B. 8370, used by De Kruyff grew on nutrient agar. This was contrary to Bergey's (1957) description of *T. thioparus* as a strict autotroph. The validity of De Kruyff's conclusions regarding the relatiohsip between "*T. thiocyanoxidans*", *T. thioparus* and *T. denitrificans* was therefore doubted.

Johnstone et al (1961) found that single cell-isolates of N.C.I.B. 8370 gave rise to cultures which varied in their ability to grow on nutrient agar and to utilise thiocyanate. These two features which showed variation were ones considered to be important in distinguishing between species of *Thiobacillus*. Lysis of some colonies of thiobacilli was observed. It was suggested that the lytic agent may have caused the inter-species changes.

The problems so far encountered in the identification of thiocyanate destroying bacteria appeared to have been associated with contaminating heterotrophs, inter-species changes and doubtful criteria for differentiating between the species of thiobacilli.

Despite these difficulties in identifying thiocyanate

destroying bacteria, it was becoming increasingly important to improve the performance of biological systems purifying carbonisation effluents. Studies were therefore made by Pankhurst (1959) and Abson and Todhunter (1958) to find the optimum conditions for the bacterial oxidation of thiocyanate, thiosulphate and phenol. These compounds are always present in carbonisation liquors. Investigations into the composition and treatability of effluents from coal carbonisation processes were also established in 1955 by the British Coke Research Association and the University of Leeds.

At this time it was assumed that thiobacilli oxidised thiocyanate in biological treatment plants. However, Hutchinson and White (1964) found that thiobacilli were absent in many activated sludge systems treating carbonisation effluents, but present in bacteria beds. The sensitivity of the thiobacilli to phenol and oxidised catechol was demonstrated and it was suggested that the film accumulated on filter media offered protection for the thiobacilli. However in the activated sludge system the bacteria were more exposed to inhibitory substances. Hutchinson (1964) isolated a heterotrophic bacterium which destroyed thiocyanate. He suggested that this or similar organisms might destroy thiocyanate in systems where thiobacilli were absent. It was emphasised that the effects of inhibitory compounds in the feed-liquor would depend on the group of bacteria present in the system.

The situation had arisen in which it was necessary to identify the group of bacteria which destroyed thiocyanate in each treatment system, yet efforts to do so were complicated by difficulties in isolating and identifying the thiobacilli. Furthermore the contribution of heterotrophs to the destruction of thiocyanate had not been established.

Significant progress towards overcoming many of these difficulties was made as a result of detailed taxonomic studies of the thiobacilli by Hutchinson et al (1965 and 1969). A numerical taxonomic study was made using Adansonian principles of classification; these principles have been summarised by Sokal and Sneath (1963). Equal significance was given to each feature, which was expressed as a numerical value. More than 100 strains were examined by multivariate analysis. Eight groups of strains were clearly differentiated. "T. thiocyanoxidans" was included within the T. thioparus group. Only two groups included strains which oxidised thiocyanate; these were T. thioparus and T. denitrificans. T. thioparus was considered to be a stable, distinct species which was strictly chemolithotrophic.

Hutchinson (1965) observed only rare instances of unusual strains associated with stock cultures used in the numerical taxonomic study, even after maintaining them for three years. He pointed out that the method of subculture might influence the stability of the cultures. Hutchinson used a dry method of subculture and suggested that this may have contributed to stability. Johnstone's (1961) apparent inter-species changes occurred when single cells were inoculated into 10 ml volumes of liquid media. Smith et al (1952) demonstrated that great variability can be induced by placing a small inoculum into a large volume of liquid. Genetic interchanges would be favoured more in a liquid environment than on solid media. The presence of a lytic agent was suggested to have caused certain instances of altered strains.

A diagnostic test scheme to aid the identification of species of thiobacilli was proposed, Hutchinson et al (1969), in which emphasis was placed on standardising the conditions

and duration of the tests. Two of the key tests of value in the detection and enumeration of thiobacilli were the deposition of elemental sulphur and the formation of acidity in 1% thiosulphate minerals medium.

The thiobacilli could now be identified with greater reliability. However the problems arising from altered strains and persistent contaminating heterotrophs had not entirely been overcome. The identification of an organism suspected to be a species of Thiobacillus, derived from an activated sludge system, still presented the problem of possible variability favoured by the liquid environment. The organism might require repeated subculturing on solid media before its properties were stabilised to allow a reliable identification to be made. An additional problem sometimes found was the lysis of colonies, (Johnstone, 1961 and Hutchinson, 1965). Lysis of thiobacilli during primary isolation onto solid media was also encountered by Mather (1971). The lytic agent was transmitted only via confluent growth and did not pass through a membrane filter with a pore size of 0.45 μ - 0.1 μ. The nature of the lytic agent was not known. When the present study was undertaken the possibility was therefore considered that organic substances released from lysed bacteria might make it more difficult to eliminate contaminating heterotrophs, even when using autotrophic media. Consequently, despite improved diagnostic test schemes, the primary isolation of thiobacilli sometimes presented problems of variable strains, lysis of colonies and persistent contamination by heterotrophs.

A genetic similarity between thiobacilli and certain pseudomonads was found by Jackson, Moriarty and Nicholas (1968). The feature considered to be of taxonomic value was the D.N.A.-

base composition of the cells. T. thioparus, T. denitrificans and "T. thiocyanoxidans" closely resembled Pseudomonas aeruginosa and P. fluorescens, according to the molar percentages of guanine and cytosine. "T. thiocyanoxidans" and P. stutzeri both had a minor 'satellite' D.N.A. component, but that of "T. thiocyanoxidans" could have been caused by contamination of the culture. However, as pointed out by Trudinger (1969) the base-composition does not indicate the base-sequence and hence the genetic information.

The genetic affinity between thiobacilli and pseudomonads is accompanied by biochemical similarities between the two groups. The oxidation of inorganic sulphur compounds, particularly thiosulphate, is achieved not only by thiobacilli but also by aerobic pseudomonads, Parker et al (1953), Trudinger (1967) and Kelly (1968). Autotrophic growth on thiosulphate is not a feature specific to the thiobacilli. In view of the wide range of substrates metabolised by many species of aerobic pseudomonads (Stanier, et al 1966) it was possible that some species might be capable of utilising thiocyanate. Further evidence in support of this possibility arose from the detection of large numbers of pseudomonads in biological systems purifying carbonisation effluents, Happold (1932) and Pankhurst (1959). However, up to this time pseudomonads were considered to be primarily responsible for the oxidation of phenols. Hutchinson (1965) had shown that heterotrophs were probably responsible for thiocyanate destruction in treatment systems where thiobacilli were absent but the identity of the heterotrophs was not established.

The utilisation of thiocyanate by a heterotroph resembling Pseudomonas stutzeri was reported by Stafford et al 1969). The organism was strictly heterotrophic and used thiocyanate only

in the presence of organic substances, e.g. succinate or phenol. Its ability to oxidise thiocyanate and phenol simultaneously indicated that it may play a major role in the removal of these pollutants in biological treatment plants. The heterotroph was capable of utilising thiocyanate and phenol when both of these substrates were in high concentrations of 200 mg thiocyanate per litre and 470 mg phenol per litre. In biological systems treating coke oven effluent, the ability to tolerate high phenol-concentrations might enable the organism to adapt more successfully than the thiobacilli which are sensitive to phenol. However thiocyanate oxidation was inhibited by 72 mg ammonia per litre. Spent ammoniacal liquor from coke ovens usually contains at least 100-200 mg ammonia per litre and the concentration is often considerably higher, e.g. 1000 mg per litre. The toxicity of the effluent usually suppresses nitrification so that very little of the ammonia is oxidised during biological treatment. Furthermore, ammonia is formed from the complete oxidation of thiocyanate. The role of P. stutzeri in the biological treatment of coke oven effluent was therefore uncertain.

The decomposition of thiocyanate by bacteria other than thiobacilli was described by Mather (1971). He isolated an Achromobacter sp and a Hyphomicrobium sp, both of which oxidised thiocyanate in mineral media. The Achromobacter sp was claimed to be primarily responsible for thiocyanate destruction in carbonisation effluent treatment plants. Thiocyanate-oxidation by the isolates occurred optimally in an ammonium thiocyanate minerals medium, lacking any added organic carbon source. Many organic substances were tested, including several amino acids and growth factors, but none of them stimulated thiocyanate-oxidation. Among the substances tested was para-aminobenzoic

acid (P.A.B.A.). P.A.B.A. had been found to cause improvements in the biological treatment of coke oven wastes, Cooper et al (1966). Mather did not observe any stimulation of thiocyanate-oxidation following additions of P.A.B.A. to pure cultures. However the proportion of thiobacilli in the activated sludge declined after P.A.B.A. was added to the feed liquor. None of Mather's isolates of thiocyanate-oxidising bacteria oxidised phenol and only a small proportion of the total bacteria in the laboratory system appeared to be capable of oxidising thiocyanate. However the activity of a lytic agent was proposed as a possible cause of failure to obtain thiocyanate destruction by many pure cultures. 0.1% nitrate as potassium nitrate was found to promote thiocyanate oxidation. The addition of nitrate was recommended to stimulate rapid recovery after thiocyanate had accumulated in treatment plants.

The monitoring of numbers of viable thiobacilli assisted Mather by enabling the effect of P.A.B.A. on this species to be observed in a mixed culture in the laboratory system, rather than in pure culture. The application of bacterial monitoring had revealed a possible means of influencing the types of bacteria in the treatment plant. The increased rates of treatment following additions of P.A.B.A. and other growth factors, described by Cooper et al (1966b) could have been caused by a change from a sludge dominated by thiobacilli to one dominated by heterotrophs. Such a change could be favourable in view of the sensitivity of the thiobacilli to phenol and oxidised catechol (Hutchinson, 1964). Greater growth yields could also have been stimulated by the additions of growth factors. An increased growth yield would contribute to a greater quantity of sludge available for the adsorption, absorption and oxidation of the waste.

Since Beijerinck's experiments in 1904, five different strains of bacteria had been found to decompose thiocyanate. Four of these strains destroyed thiocyanate autotrophically; these were Thiobacillus thioparus, T. denitrificans, Achromobacter sp and Hyphomicrobium sp. The remaining organism resembling Pseudomonas stutzeri was a strict heterotroph. Evidence from the bacterial monitoring of treatment plants by Hutchinson (1964) and Mather (1971) showed that thiobacilli were often absent from activated sludge plants. In these systems, the Achromobacter sp or heterotrophs such as P. stutzeri were implicated in the destruction of thiocyanate. An activated sludge dominated by heterotrophic thiocyanate destroying bacteria was considered to be less sensitive to inhibition than a sludge dominated by thiobacilli. Some form of monitoring procedure to determine the nature of the bacterial population might therefore aid plant-operation.

Happold and Key (1932) were able to correlate counts of viable bacteria with the efficiency of operation of bacteria beds at Coventry gas works. Using plate counts it was demonstrated that low viable counts of oxidase positive bacteria were associated with poor purification. Spent and crude liquors from gas works appeared to exert a distinct toxic action on oxidase negative bacteria. The oxidase negative bacteria formed a high proportion of the bacterial population in the domestic sewage which was mixed with spent ammonia still liquor before being dosed to the bacteria beds. During periods of efficient purification the numbers of viable oxidase positive bacteria were greatly increased during biological treatment, while the oxidase negative bacteria declined in numbers.

The introduction of a surface drop viable counting technique by Miles and Misra (1938) provided a rapid means of estimating the numbers of viable bacteria. The surface drop technique was

used by Pankhurst (1959) during pilot scale studies of the biological oxidation of spent gas liquor. The numbers of total heterotrophic bacteria on nutrient agar and bacteria capable of growing on a phenol agar were monitored. These viable counts could sometimes be correlated with plant performance. However, on nutrient agar the ratio of one colonial type to another often varied from week to week. Pankhurst found that these variations could not easily be related to the performance of the treatment plant.

The accuracy of surface drop bacterial counts was investigated by Badger and Pankhurst (1960). They found no significant error using the method, while counting bacteria from a pilot plant oxidising thiosulphate. Plates of thiosulphate agar were inoculated with 0.1 ml surface drops and incubated for 5-6 days. Since thiobacilli develop relatively rapidly on thiosulphate agar the surface drop method appeared to be suitable for the enumeration of viable thiobacilli. Hutchinson (1965) used the surface drop technique for counting thiobacilli on 1% thiosulphate minerals agar.

In view of the slow growth of thiobacilli to form characteristic intracolonial deposits of elemental sulphur from thiosulphate, colony-counts required a prolonged incubation at 28-30°C for up to 28 days. Consequently although surface drops of several dilutions could be applied rapidly to media, maximum viable counts were not available for two or three weeks. Considerable experience was also needed to distinguish between the intracolonial deposits formed by thiobacilli and those formed by certain non-thiobacilli, Hutchinson (1965). In the present work it was therefore difficult to apply the monitoring of thiobacilli to biological treatment plants where efficient

operation depends on the ability to take prompt remedial action in anticipation of, or immediately following an accumulation of thiocyanate. Nevertheless the bacteria could be identified conclusively as thiobacilli and their numbers could be estimated reliably using the surface drop cultural method.

The surface drop method was used by Harris and Sommers (1968) in a plate dilution frequency technique. This enabled the counting of several different micro-organisms or physiological groups in complex systems, such as activated sludge, to be made with a precision similar to dilution tube methods. The technique may be applied to the monitoring of activated sludge plants treating carbonisation effluents.

The method of dispersion of bacteria from activated sludge should not decrease the viability of the bacteria and precautions should be taken to prevent re-aggregation. Allen (1944) found that homogenisation using a domestic cream maker increased viable bacterial counts on plates of nutrient agar. This medium was found to give the highest counts of heterotrophic bacteria in activated sludge treating domestic sewage. Gayford and Richards (1970) homogenised activated sludge using a Silverson mixer. A combination of sodium pyrophosphate and Lubrol W was used successfully as a deflocculating agent. Surface counts were preferred to pour plates. The use of ultra-sonics for dispersing bacteria resembling thiobacilli derived from a laboratory activated sludge system treating ammonium thiocyanate, was described by Williams, Stafford, Callely and Hughes (1970). Using a flexural sonicator they obtained a twenty-fold increase in total heterotrophs and *Thiobacillus* - type organisms. Sonication released the cells from flocs without affecting their viability. A short period of two minutes sonication was satisfactory, but after

twenty minutes sonication the viable counts declined. Procedures for enumeration bacteria in activated sludge were evaluated by Pike et al (1972). The purpose of this evaluation was to propose a reliable routine technique for counting viable heterotrophic bacteria. Minimal lethal effects on the bacteria were obtained by dispersion for one minute in a Kerry ultrasonic bath, using sodium tripolyphosphate as the diluent. Optimal counts were obtained using spread plates of casitone + glycerol + yeast extract agar, incubated at 22°C for six days.

The application of viable bacterial counts to the study of activated sludge presents difficulties associated with non-viable cells and debris, both of which normally form a significant proportion of the flocs. In the study of bacterial populations in activated sludge, Sladka et al (1970) and Pike et al (1971) found that total microscopic counts of bacteria were considerably greater than viable aerobic counts. A high proportion of cells in activated sludge are in a starved condition and in the declining phase of growth (Pike et al (1971)). Substrate accelerated death (Postgate and Hunter, 1963) may occur when cells in this condition of stress are suspended in a nutrient-deficient diluent and subsequently transferred to a nutrient-rich medium. These physiological changes imposed on bacteria in the endogenons phase of growth may result in a loss of viability. Pike estimated that the percentage of viable cells in activated sludge varied between 0.12% and 0.2% of the total cells. If the much greater percentage of non-viable cells contributes significantly to the biochemical activity of the activated sludge, it may be difficult to relate viable bacterial counts to the performance of the biological treatment process. Stevenson (1928) showed that biochemical activity could increase as the viability of a batch

culture decreased in the stationary phase of growth. A similar situation may exist in the stationary and declining phases of growth commonly encountered in activated sludge systems.

Interpretation of viable bacterial counts is complicated further by the numerous selective pressures exerted on the different populations in the activated sludge system. Although the nature of the waste has a selective influence on bacterial populations, other important factors include toxic materials, nutrient-availability, pH value, dissolved oxygen concentration, organisms in different trophic levels, aeration time and sludge wastage rate. Following investigations carried out at the Water Pollution Research Laboratory (1971 and 1972) it was concluded that bacterial populations in aerobic biological treatment processes rarely achieve a steady state because of the complexity of interactions between different species.

Interactions between populations of bacteria known to oxidise constituents of coke oven effluent were demonstrated by Jones and Carrington (1972). A thiocyanate-degrading strain resembling _Thiobacillus thioparus_ was unaffected by 100 mg phenol per litre, but in mixed culture, the active growth of a strain of _Moraxella/ Acinetobacter_ on phenol completely inhibited growth on thiocyanate. It was considered that large numbers of species rather than individual species normally metabolise specific components of waste waters. A consequence of this was that changes in the species which predominate at different times may occur irrespective of the nature of the waste.

The differentiation between thiobacilli and heterotrophs capable of oxidising inorganic compounds of sulphur may be aided by observing the products and intermediates formed during thiosulphate-oxidation. It was established by Nathansohn (1902) that the main products of thiosulphate-oxidation were sulphate,

sulphur and tetrathionate. Since then the pathway of thiosulphate-oxidation has been studied extensively using different species of bacteria. The microbial metabolism of inorganic sulphur compounds has been reviewed by Parker and Prisk (1953), Vishniac and Santer (1957), Kelly (1968), Trudinger (1967 and 1969) and Peck (1968).

Peck (1962) considered that two physiological groups of heterotrophs existed, one produced polythionates, the other produced sulphate from polythionates. Mixed cultures of these heterotrophs could convert thiosulphate to sulphate. However it is possible that individual heterotrophs may be able to oxidise thiosulphate to sulphate, Wieringa (1966). Kelly (1968) pointed out that in contrast to the heterotrophs, all the thiobacilli could in optimum conditions probably carry out the complete oxidation of thiosulphate to sulphate. Peck (1968) considered that sulphite was a key intermediate in the metabolism of inorganic sulphur compounds by thiobacilli. Kelly (1966) observed the transient accumulation of trithionate during thiosulphate-oxidation by a strain of Thiobacillus. Jones (1975) considered that the formation of trithionate during the oxidation of thiosulphate was a characteristic of the thiobacilli. Chromatographic methods for identifying polythionates were described by Pollard, McOmie and Jones (1955). A method for detecting polythionates in cultures of thiobacilli was also described by Pratt (1958).

A bacterial monitoring technique using the biochemical properties of mixed cultures of activated sludge was proposed by Woodward, Stafford and Callely (1974). The criteria used to determine the dominant type of thiocyanate oxidising bacteria were the stochiometry of thiosulphate-oxidation and the extent of inhibition of oxygen uptake at high concentrations of thiocyanate. Respirometric experiments were carried out using

activated sludge obtained from a laboratory scale plant treating ammonium thiocyanate. The stochiometry of thiosulphate-oxidation was found to resemble that of Thiobacillus thiocyanoxidans, Happold (1954). Thiosulphate was oxidised completely to sulphate. The inhibitory effect on oxygen uptake caused by 100 mM thiocyanate per litre was also taken to indicate a close resemblance to a pure culture of thiobacilli. In contrast to T. thiocyanoxidans, the Pseudomonas stutzeri of Stafford et al (1969) did not appreciably oxidise thiosulphate and was not suppressed by 100 mM of thiocyanate. The results of the test were available a few hours after obtaining the samples of activated sludge. The technique could therefore be incorporated in a programme of frequent tests to monitor the performance of biological treatment plants.

In the technique described by Woodward et al (1974) the characteristic biochemical activity of two different species of thiocyanate oxidising organisms was used to determine which of these species was dominant in activated sludge. This did not account for the possible influence of other thiocyanate oxidising bacteria on the overall biochemical activity of the sludge. In such conditions "T. thiocyanoxidans" and P. stutzeri might both be absent. Bacteria which might influence the stochiometry of thiosulphate-oxidation by activated sludge include the facultatively autotrophic Achromobacter sp and Hyphomicrobium sp isolated by Mather (1971). At the outset of the present investigation it was considered that other micro-organisms, not yet found to oxidise thiocyanate, might show characteristic biochemical activity towards thiosulphate and thiocyanate. Bacterial monitoring using cultural techniques would permit the isolation and identification of newly recognised thiocyanate

oxidising bacteria. Determination of the biochemical properties of a wider range of thiocyanate oxidising bacteria would then be possible. This information might enable the activity of mixed cultures used in rapid biochemical monitoring tests to be interpreted with greater accuracy. Cultural techniques might also be used to confirm the results of respirometric tests using mixed cultures.

3. Experiments using mixed cultures.

3.1 Laboratory scale treatment plants.

Two laboratory scale activated sludge plants were assembled as shown in Fig 2. One was used to investigate the biological treatment of effluents from the coke ovens at Corby steelworks. The duration of this investigation was 105 weeks. A synthetic coke oven effluent was treated using the second laboratory scale plant, over a period of 90 weeks.

3.1.1 Plant treating coke oven effluents.

The aims were, firstly to determine the species of bacteria involved in thiocyanate destruction in the continuous purification of coke oven effluent by activated sludge. Secondly the changes in bacterial populations were recorded to find if this information could assist the operation of the treatment plant. These bacteriological tests are described separately in sections 4 and 5. Thirdly the effects of possible inhibitors were studied in continuous culture. This approach was more relevant to the continuous operation of treatment plants than tests using batch cultures. Fourthly, the laboratory scale plant enabled an assessment to be made of the operating conditions specified by the manufacturer of the full scale treatment-plant installed at Corby steelworks.

In order to assess the value of microbiological testing of the activated sludge, it was necessary to gain experience from the operation of laboratory and full scale plants relying on conventional daily tests. These tests included the determination of thiocyanate, phenol, ammonia, nitrite, percentage sludge volume, suspended solids concentration, sludge density index, sludge age, flow-rates, pH, temperature and aeration. The significance of these factors is well established and their

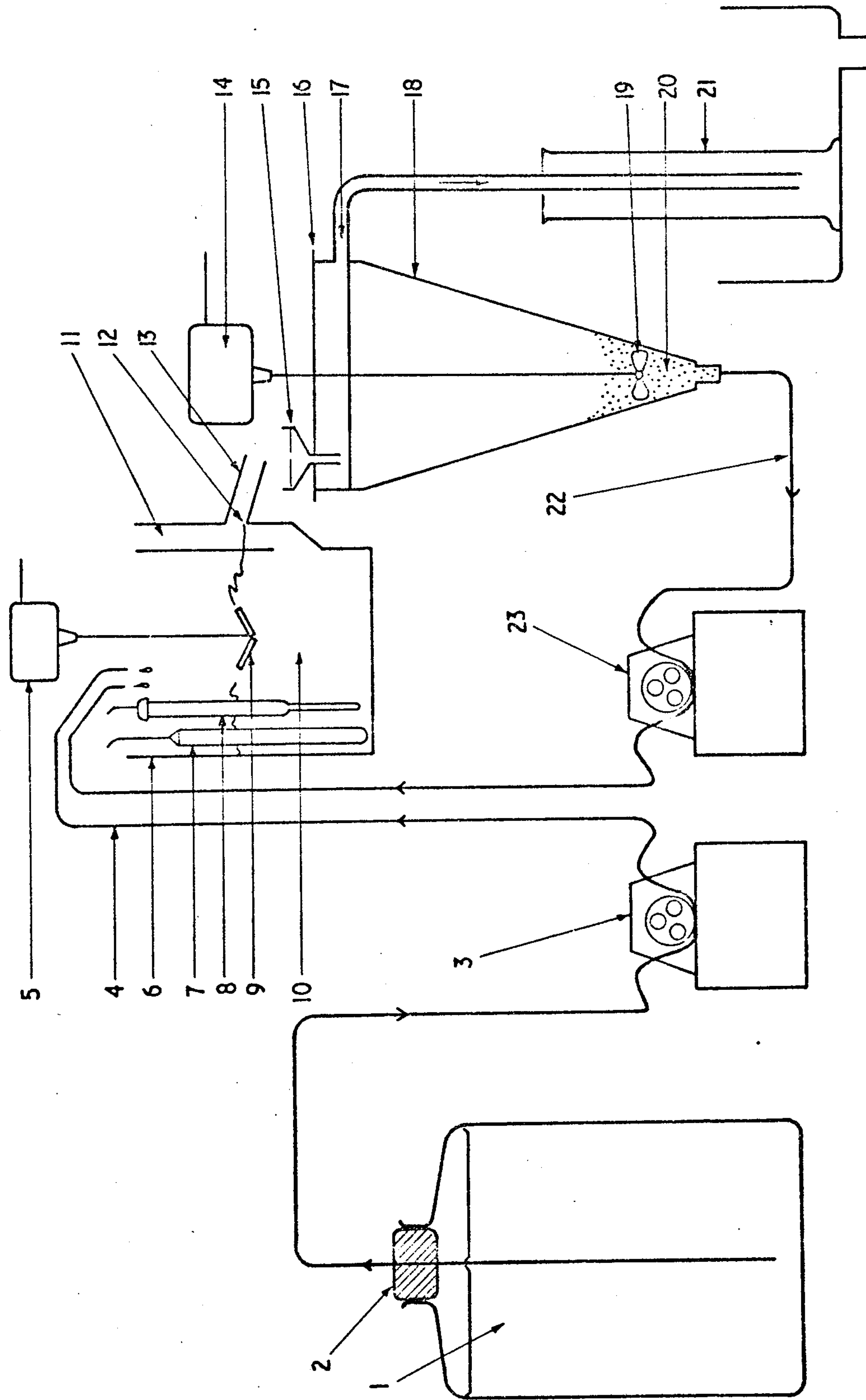
Figure 2.

Key to Fig 2.

1. Jerry can containing 25 litres of diluted coke oven effluent and 1 mg calgon/litre, pH 7.5 - 8.5
2. Non absorbent cotton wool plug.
3. Peristaltic pump for feed liquor.
4. Silicone rubber tube supplying feed liquor.
5. Stirrer motor for aeration tank.
6. Aeration tank, capacity 6 litres.
7. Heater.
8. Thermometer.
9. Aeration tank stirrer.
10. Completely mixed contents of aeration tank, 27°C, pH 6.7 - 7.5.
11. Baffle.
12. Overflow weir of aeration tank.
13. Delivery tube.
14. Stirrer motor for settling tank.
15. Funnel.
16. Lid.
17. Overflow weir of settling tank.
18. Settling tank, capacity 7 litres.
19. Settling tank stirrer.
20. Settled sludge.
21. Measuring cylinder containing final effluent and excess sludge.
22 Silicone rubber tube delivering recycled sludge.
23. Peristaltic pump for recycled sludge.

FIG 2

LABORATORY SCALE ACTIVATED SLUDGE PLANT TREATING COKE OVEN EFFLUENT



relation to successful plant operation has been discussed by several authors, including Eckenfelder and McCabe (1956), Hawkes (1963) and Fair et al (1968).

The degree to which plant performance could be controlled with the aid of conventional tests formed the basis on which the value of additional microbiological tests were assessed. For this reason, an account is given of the operation of the laboratory scale plants.

3.1.1.1 Source of bacteria.

Settled sewage sludge was obtained from the primary settling tanks at the Corby sewage purification works. The waste being treated at Corby sewage works was primarily domestic sewage. Coke oven effluent did not enter the sewage works. A sludge which had not been acclimatised to coke oven effluent was chosen to determine the induction period required to gain active thiocyanate destruction. A 300 ml sample of primary settled sewage sludge was diluted to 3 litres and aerated for three hours before being brought into contact with coke oven effluent.

3.1.1.2 Acclimation of sludge to coke oven effluent.

A considerable dilution of coke oven effluent was used initially for three reasons. Firstly coke oven effluent contained high concentrations of toxic substances not normally encountered by micro-organisms in domestic sewage. Secondly, concentrations of thiocyanate above 100 mg per litre were found to have an inhibitory effect on the rate of thiocyanate destruction by sludge which had not been acclimated to coke oven effluent. Thirdly a prolonged lag period of five to fourteen days was normally observed before the destruction of thiocyanate commenced. The beginning of thiocyanate destruction was detected readily when the initial concentration was low, e.g. 5-20 mg/l CNS.

A dilution of x100 was used to give an initial concentration of 5.3 mg/l CNS. The proportion of spent still liquor to 'devil' liquor, 13:2 (v/v), was the same as in the liquors to be fed continuously in the full scale treatment-plant, as shown in Fig 1 on page 3. It was not essential to include benzole-condensate in the feed-liquor. This was because the concentrations of pollutants including thiocyanate, phenols and ammonia in benzole condensate were relatively small. Analyses of the three major liquors from the coke ovens at Corby are given in Table 2. Before mixing with pre-aerated sewage sludge, the pH of the diluted liquors was lowered from 10.5 to 6.8 using HCl (S.L.R.). The phosphate nutrients solution (Table 3, No. 1) was added after pH adjustment to avoid the precipitation of calcium phosphate in alkaline liquor. This precaution was necessary in view of the large requirement for phosphate for the complete oxidation of thiosulphate (Vishniac et al, 1957, and Baalsrud, 1952). The pH of the coke oven liquors + nutrients mixture was adjusted to 6.8 using concentrated hydrochloric acid. One litre of the pre-aerated sludge was then added. The batch culture was stirred at 18°-22°C using a cone-aerator shown in Fig 3. This surface-aerator provided complete mixing and adequate aeration and agitation facilitating rapid biological oxidation.

During the period of acclimation to coke oven effluent it was necessary to maintain the sludge in batch-culture. If continuous culture had been attempted during the prolonged lag period preceding rapid thiocyanate destruction, the bacteria would have been washed out of the system. Thiocyanate destruction began after five days. In contrast, the destruction of phenol began almost immediately, (Table 4).

Table 2 Analysis of coke oven effluents at Corby steelworks.

Figures = mg/l
NT = not tested.
Methods in Appendix 2, p 205.

1. Spent Still Liquor.

Weeks	pH	P.V. 4h	CN	CNS	PhOH	S_2O_3	Cl	H_2S	Formate	Total NH_3	
1	8.5	4,030	0	600	1,700	301	10,224	0	262	4,675	
6	10.7	4,537	NT	675	1,750	307	10,472	NT	304	510	
8	9.1	3,890	NT	650	1,325	282	9,118	NT	759	1,530	
11	11.7	4,450	NT	675	1,725	416	9,053	NT	662	523	
13	NT	3,930	NT	545	1,425	365	10,220	NT	768	1,530	
31	10.1	5,403	NT	700	2,125	NT	10,650	NT	322	816	
Means	10.0	4,373	0	614	1,675	334	9,956	0	515	1,597	

2. Devil Liquor.

Weeks	pH	P.V. 4h	CN	CNS	PhOH	S_2O_3	Cl	H_2S	Formate	Total NH_3	Fixed NH_3
1	NT	8,000	73	135	3,950	230	142	10	0	374	65
6	8.7	6,878	18	255	3,000	301	1,225	138	NT	1,037	2,171
8	8.5	5,170	22	100	2,100	198	754	NT	NT	248	668
11	9.3	6,670	32	350	2,375	480	1,527	364	NT	4,862	3,077
13	9.6	7,260	143	395	2,650	288	1,408	944	NT	9,231	1,020
31	6.9	10,435	108	100	5,625	NT	NT	NT	NT	258	354
Means	8.6	7,402	66	223	3,283	299	1,011	144	0	2,668	1,226

3. Benzole Condensate.

Weeks	pH	P.V. 4h	CN	CNS	PhOH	S_2O_3	Cl	H_2S	Formate	Total NH_3	Fixed NH_3
1	NT	606	30	68	9.0	256	0	230	0	604	58
6	8.8	364	11	95	9.2	147	0	17	NT	153	68
8	9.2	647	48	60	5.0	115	NT	231	NT	529	44
11	9.1	426	19	59	8.8	166	NT	126	NT	673	48
13	9.1	372	22	92	4.8	122	NT	145	NT	629	60
31	9.2	559	9	140	16.0	NT	NT	NT	NT	NT	NT
Means	9.1	496	28	76	8.8	161	0	148	0	518	56

Table 3. Stock nutrient solutions for activated sludge.

No. 1:	$FeCl_3.6H_2O$	50g
	$MgSO_4.7H_2O$	200g
	H_3PO_4.(S.G.1.75)	100 ml
	HCl acid (S.L.R.)	25 ml
	Distilled water	2,375 ml

1ml added to 1 litre giving final concentrations in the feed liquor of : 8mg/l Mg
4mg/l Fe
20mg/l P

No. 2:	Calgon, sodium hexametaphosphate, $Na_2(Na_4P_{16}O_{18})$	66g
	Distilled water	1,000 ml

1 ml added to 1 litre giving a final concentration in the feed liquor of : 20mg/l

FIG 3 CONE - AERATOR FOR AERATION TANK OF LABORATORY SCALE ACTIVATED SLUDGE PLANT

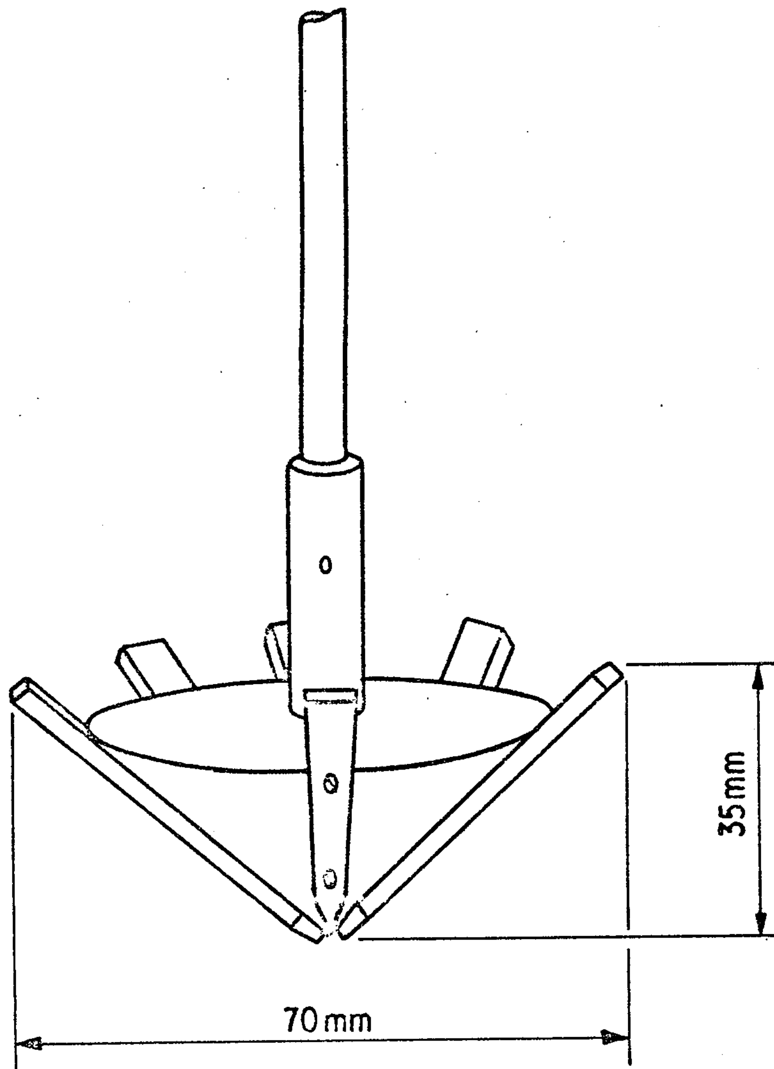


Table 4 Acclimation of sewage sludge to coke oven effluent in batch culture.

Culture volume = 4 litres.
Temperature = 18°-22°C.
Dissolved oxygen = not less than 4 mg/l.
pH = 6.5 - 7.5

Days	Time	CNS (ppm)	PhOH (ppm)	pH	Comments
1	16.00	5.3	10.5	6.8	40 ml of liquor added at 16.00 hours.
2	10.00	5.3	1.0	7.6	Adjusted pH to 6.6.
	15.30	5.3	0	7.5	Adjusted pH to 6.5.
5	09.30	5.0	0	6.5	
	13.30	5.0	0	6.5	
6	10.30	5.0	0	6.4	Induction period for CNS destruction = 6 days.
7	09.00	0	0	6.8	Added 80 ml of liquor. Adjusted pH.
	09.30	14.0	24.5	6.8	
8	10.40	11.6	13.2	6.9	
9	10.00	0	0	7.1	Added 80 ml of liquor. Adjusted pH.
	10.45	12.4	22.6	6.7	
	13.25			7.4	Adjusted pH to 6.6.
10	09.00	12.3	0	7.1	Slow destruction of CNS but rapid destruction of PhOH.
11	08.30	8.3	0	7.2	
	13.15	4.5	0	6.5	
	14.15	3.8	0	6.6	
	16.10	1.0	0	6.7	Added 100 ml of liquor. Adjusted pH.
	16.20	16.2	33	6.8	
14	09.30	0	0	7.1	Added 150 ml of liquor. Adjusted pH.
	09.20	35	72	6.9	
	11.30	33	26.5	6.9	
15	09.00	0	0	6.8	Added 150 ml of liquor. Adjusted pH.
	09.15	34	63	6.7	Sludge increasing in volume. Flocculation rapid. % S.V. ½ hr = 3.
16	08.30	0	0	7.2	Added 150 ml liquor. Adjusted pH.
	08.40	35	66	6.9	
17	08.45	0	0	6.7	% S.V.½ hr = 3.2, Good sludge-settlement. Supernatant liquid renewed when adding fresh 150 ml vols of liquor.
	10.00	35	66	6.7	
18	09.00	0	0	7.2	
	10.00	36	71	6.7	
19	09.00	0	0	7.2	% S.V.½ hr = 3.3. Transferred 3 litres of culture to laboratory scale plant. Retained 1 litre as a 'fill and draw' culture.

After 15 days the destruction of thiocyanate was well established. Since flocculation and sludge settlement occurred rapidly, the enrichment had developed sufficiently for it to be transferred to continuous culture in the laboratory scale activated sludge plant.

The stirrer in the aeration tank was immersed immediately below the surface of the liquid so that the blades projected 1 mm above the surface before stirring commenced. This setting enabled maximum aeration and agitation to be achieved throughout the aeration vessel when the stirrer was set at the optimum speed. The optimum speed of the stirrer caused complete mixing without excessive splashing and maintained a dissolved oxygen concentration of not less than 1·5 mg/l in the liquid surrounding the flocs. The effect of low levels of dissolved oxygen in the aeration vessel is shown in Figure 4. This test was done using a dissolved oxygen meter, when the system was continuously purifying coke oven effluent at a high rate of treatment. When the aerator was stopped the rapid drop in oxygen concentration from 1.6 to 1.0 mg per litre did not cause an accumulation of thiocyanate. However, thiocyanate accumulated rapidly when the dissolved oxygen concentration fell below 1.0 mg per litre. This test showed that the critical oxygen concentration for thiocyanate destruction was 0.8 mg per litre. A small stirrer was required at the base of the settling tank. This prevented the settled sludge from blocking the orifice leading into the tube for recycled sludge.

One litre of the enrichment was retained as a 'fill and draw' culture in which the supernatant liquid was renewed when each fresh addition of feed liquor was made. This prevented the accumulation of waste products, such as ammonia and sulphate,

FIG 4 ACCUMULATION OF THIOCYANATE IN THE AERATION TANK AT LOW CONCENTRATIONS OF DISSOLVED OXYGEN

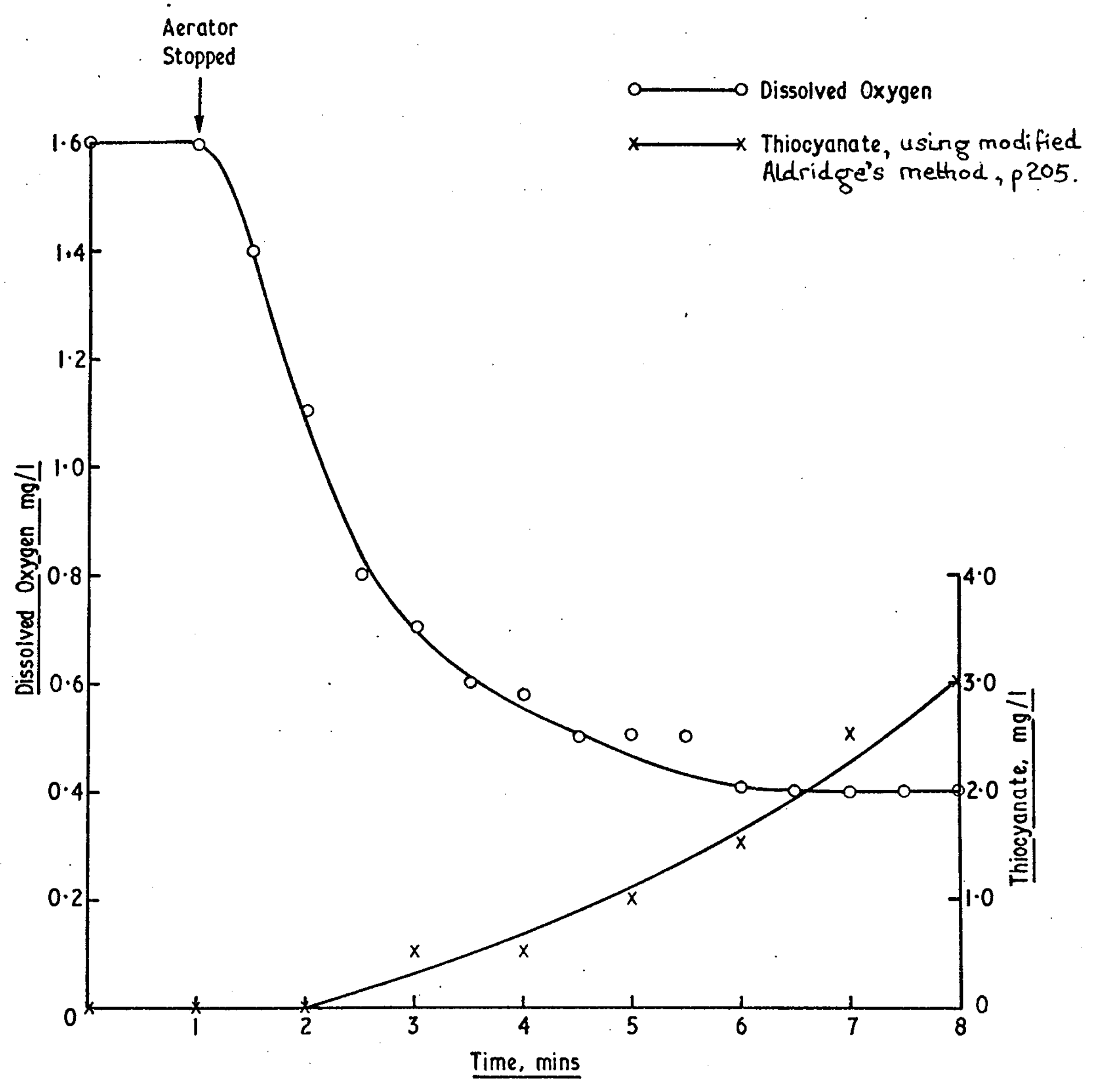
Laboratory scale plant purifying coke oven effluent (1:1 v/v dilution)

Temperature 27°C

pH 7·0

Aeration time 10h

Thiocyanate in feed liquor 220 mg/l



which might have suppressed thiocyanate destruction. The fill and draw culture was used as a reserve of acclimated sludge which could be added to the laboratory scale plant when required.

3.1.1.3 Continuous treatment.

The main conditions specified by the manufacturer, for the operation of the full-scale treatment plant are listed in Table 5 . The initial objective was to assess these operating conditions using the laboratory scale plant.

25 litres of the diluted feed-liquor, 1:1 v/v, was adjusted from pH 9.7 to pH 7.0 using concentrated hydrochloric acid. 25 ml of a stock solution of calgon, Fig 3 , No. 2, were then added to give a final concentration of 20 mg/l of calgon in the feed-liquor. In order to prevent excessive washing out of sludge from the system during the first six days, the feed-liquor was introduced intermittently instead of continuously. Controlled intermittent feeding enabled calculated concentrations of up to 50 mg/l thiocyanate to accumulate in the aeration tank. It was then possible to estimate the rate of thiocyanate destruction following each dosing of feed-liquor. After six days of intermittent dosing the activity of the sludge was sufficient to maintain continuous treatment of the feed-liquor, see Table 6 . Complete destruction of phenol usually occurred rapidly. The analysis for phenol is therefore omitted from Table 6.

Continuous treatment of the 1:1 (v/v) diluted effluent was initially maintained at a slow rate of treatment, 72 ml/h. If a greater dilution of the liquor had been used, continuous treatment might have been possible at an earlier stage. Attempts were not made to increase the rate of flow substantially at this stage because the amount of activated sludge available for biological oxidation was small, as shown by the small settled

Table 5. Conditions specified for operation of the full scale biological treatment plant at Corby.

1. Proportions of coke oven liquors v/v,:
spent still liquor : devil liquor : benzole condensate = 13:2:1.

2. Dilution of mixed coke oven liquors, with water, v/v = 1:1.

3. Nutrient addition as calgon, at a concentration in the feed liquor of 20 mg/l.

4. pH of feed liquor = 7.5 - 8.0

5. pH in aeration tank = 6.5 - 7.5

6. Temperature in aeration tank = 27° - 28°C.

7. Rate of treatment, expressed as retention time in hours of feed liquor in the aeration tank

$$\text{Aeration-time, A.T.} = \frac{\text{aeration tank capacity. gal.}}{\text{rate of flow of feed liquor, gal/h.}}$$

A.T. = 20h

Table 6

Key to Table 6

$\%SV\frac{1}{2}h$	= percentage of aeration tank sludge settled in $\frac{1}{2}$h.
SS	= mg/l suspended solids in aeration tank. 100 ml of aeration tank mixed liquor filtered through Whatman No. 1 paper. Solids collected on filter paper washed with 200 ml distilled water and dried for 1h at 110°C.
S.D.I.	= sludge density index = $\frac{\%\ SS \times 100}{\%SV\frac{1}{2}h}$
↑	= rate of flow increased.
OFF ↓	= flow stopped.
A.T. = Aeration time	= hrs retention time of feed liquor in the aeration tank. = $\frac{\text{aeration tank capacity, ml}}{\text{feed liquor flow, ml/h.}}$

Aeration tank capacity = 6,000 ml

Aeration tank temperature = 27°C.

Feed liquor = 1:1 (v/v) dilution of coke oven effluent, with 20 mg/l calgon.

Table 6 Development of continuous treatment of coke oven effluent in the laboratory scale plant.

Days	Time	CNS mg/l	pH	Feed-liquor ml/h	Sludge-return ml/h	%SV ½h	SS	S.D.I.	A.T.
1	08.30	< 5	6.4	72	220	1			83
	10.30	< 5	6.3	72	220				
2	09.00	< 5	6.7	72↑	220	1			
	13.00	< 5	6.7	96↑					
	15.30	< 5	6.7	144					
3	09.00	< 5	6.9	192↑	520	1			
	11.15	< 5	6.9	216					28
4	09.15	< 5	6.9	216↑	540	1.5			
	11.15	< 5	6.9	252↑					
	16.10	15	6.9	260↓ OFF					
5	09.20	< 5	6.9	ON AT 260	540	3			
	10.00	10	6.8	260↓ OFF					
	10.30	< 5	6.9	ON AT 180					
	16.00	< 5	7.2	180					
6	09.00	< 5	7.0	180↑	520	3			
	10.15	< 5	7.0	228					
7	09.30	< 5	7.0	228↑	520	3			
	16.00	< 5	7.0	252↑					
	16.30	< 5	7.0	300	660				20
8	09.15	< 5	7.0	300	660	3	495	1.7	
9	09.00	< 5	7.1	312	660	3	500	1.7	
10	09.00	< 5	7.1	300	660	3	490	1.6	
23	09.00	< 5	7.1	300	660	4			
60	09.00	< 5	7.1	312	660	10	1580	1.6	20
70	16.00	< 5	7.2	300	660	16	2560	1.3	
80	09.15	< 5	7.1	328	720	28	2800	1.0	18

sludge volume of only 1%. The flow of feed-liquor was stopped when more than 5 mg/l of thiocyanate accumulated in the aeration tank. Samples for the determination of thiocyanate were taken from the overflow weir of the aeration tank, Fig 2, page 35. The pumping of feed-liquor into the aeration tank was restarted as soon as the thiocyanate concentration fell below 5 mg/l. If thiocyanate continued to accumulate the flow of feed-liquor was again stopped until the thiocyanate was destroyed. The flow of feed-liquor was then restarted at a reduced rate. By adhering to this procedure, the rate of treatment was gradually increased to 300 ml/hr. This was equivalent to a retention time of 20h. The period required to increase the rate of treatment from 72 ml/h to 300 ml/h was seven days.

Although a 20h retention time had been achieved the amount of sludge developed after seven days of continuous treatment was small. It was considered that, at the 20h retention time the optimum suspended solids concentration in the aeration tank was between 5,000 and 10,000 mg/l. However the aeration tank so far contained only 495 mg/l suspended solids on day 8 (Table 6). A larger amount of sludge would have enabled the system to adapt to fluctuations in the composition of the coke oven effluent. Small changes in flow rates, temperature and dissolved oxygen concentration were also unavoidable. These fluctuations were minimised in the operation of the laboratory scale plant but may be more difficult to control in the full-scale plant. Changes in the composition of spent still liquor from the coke ovens at Corby are given in Table 7 .

A period of 63 days was required before the suspended solids concentration and percentage sludge volume approached the required values of 5,000 mg/l and 20% respectively. The cause

Table 7. Changes in composition of spent still liquor over a period of five weeks.

*Figures = mg/l

ND = not determined.

analysis \ days	1	8	19	27	34
pH	11.3	9.0	9.5	11.1	11.3
CNS	637	737	600	700	1012
S_2O_3	942	532	181	783	665
*P.V. 4h	3700	2828	1919	3366	4356
C.O.D.	5690	4999	3759	5440	6896
PhOH	1100	900	600	1050	1450
NH_3	612	1632	833	1598	1173
COD/PV4h	1.54	1.54	1.95	1.61	1.58
Coking Time, h	21	21	21	21	ND
Oven Temp °C	1210	1210	1210	1210	ND
Ovens pushed	187	ND	199	202	ND

of the slow increase in sludge may have been that the toxic coke oven effluent was insufficiently diluted at the start of continuous treatment. The concentration of the liquor may then have been high enough to have an inhibitory effect on the micro-organisms. A greater dilution of 1:4, liquor:water v/v, may therefore have permitted a more rapid development of sludge.

In order to keep the activated sludge in contact with the feed-liquor, the flow-rate of recycled sludge was approximately twice the flow-rate of feed-liquor. This prevented anaerobiosis in the settling tank. However an excessive rate of sludge-recycling would have reduced the retention time of the feed-liquor in the aeration tank, causing thiocyanate to accumulate.

Continuous treatment was maintained successfully for 60 days under the conditions listed in Table 5 on page 45. During this period 99% of the thiocyanate in the feed-liquor was normally destroyed, leaving between 1 and 5 mg/l in the final effluent from the aeration tank. The concentration of thiocyanate in the feed-liquor was 420-430 mg/l.

Failures of the pumps and stirrers occurred occasionally. These mechanical failures sometimes caused thiocyanate to accumulate. However, provided that the faults were remedied immediately, continuous treatment was usually restored quickly.

The following features of activated sludge in the aeration tank were important in assessing the efficiency of the treatment process. The suspended solids concentration enabled the amount of sludge available for biological treatment of the waste to be estimated. During the 60 day period, at a retention time of 20h, the suspended solids concentration was 2,200 - 4,000 mg/l. However, a concentration of 5,000 - 10,000 mg/l as recommended above, would probably have imparted

greater stability to the treatment process by accommodating the fluctuations in feed liquor composition. The percentage sludge-volume was also a valuable guide to the amount of sludge and its settling properties. A sludge-volume of 20-30% was satisfactory. Sludge-volumes greater than 30% were sometimes accompanied by low sludge-density indices. The sludge density index, S.D.I., varied between 0.8 and 3.0. When the S.D.I. fell below 1.0, sludge-settlement was poor. This condition was sometimes associated with a high proportion of fungal filaments in the flocs, causing the sludge to "bulk". Excessively high S.D.I. values, greater than 2, were sometimes associated with a large amount of calcium carbonate caused by excessive liming of the ammoniacal liquor.

Microscopic examination of the activated sludge was also a useful operational aid. In optimum conditions of treatment the flocs were predominantly bacterial with very few fungal filaments. If the proportion of fungal filaments became high, settlement was poor and bulking occurred, see Fig 5 on page 65. When the flocs had a fairly open texture, as shown in Fig 6, page 66, this permitted a rapid exchange of materials between the bacteria and the surrounding liquid.

Free-living ciliated protozoa were often present but their numbers were usually relatively small when the 1:1 v/v dilution of coke oven effluent was being treated. Greater numbers of free-living protozoa, e.g. Colpidium, were present when a more diluted effluent was being treated, e.g. 1:4 v/v. Peritrichous ciliates, such as Epistylis were normally not present.

After the period of 60 days during which continuous treatment had been successful, repeated failures of the pumps and stirrers occurred. For three weeks it was therefore

necessary to pump in the feed-liquor intermittently. Between successive dosings of feed-liquor there were periods of up to three days when the sludge was being alternately aerated and settled in the absence of the main substrates from the feed-liquor. These periods of starvation caused the sludge to enter further into the endogenous phase of growth. Consequently the proportion of dead material in the flocs increased and the quantity of sludge in the system declined. After 22 days during which continuous treatment could not be maintained due to mechanical failures, the percentage sludge volume had dropped from 24% to 10% and the dry weight concentration of suspended solids fell from 2400 mg/l to 1340 mg/l. When the pumps were repaired it was necessary to operate the system at a 30h - 40h aeration-time to allow sufficient sludge to be built up. When the percentage sludge volume and dry weight of suspended solids concentration had been restored to 20% and 2500 mg/l respectively, the aeration-time could then be lowered to 20h.

3.1.1.4 Effect of concentrated liquor.

Increased concentrations of thiocyanate, phenol and thiosulphate were caused by an unusually long coking time of 21 hours. This sometimes produced a smaller volume of concentrated coke oven effluent. The 1:1 v/v dilution of this concentrated liquor could not be treated successfully at an aeration-time of 20h. When attempts were made to retain a 20h aeration-time, thiocyanate began to accumulate immediately in the effluent from the aeration-tank. It also became difficult to prevent excessively low pH values of less than 6.0 in the aeration-tank. This problem occurred even when the pH of the feed-liquor was raised from 7.2 to 8.3, see Table 8 . Since nitrite was not detected in the aeration tank

Table 8 First series of tests while treating 1:1 v/v dilution of coke oven effluent : Means of daily tests on laboratory scale plant.

Samples	Tests	Nature of 1:1 v/v feed-liquor	
		Typical liquor	Strong liquor
Feed-liquor :	thiocyanate, mg/l	420	673
	phenol, mg/l	620	820
	thiosulphate, mg/l	210	323
	pH	7.2	8.3
	aeration-time, h	20	33
Effluent from aeration-tank	thiocyanate, mg/l	1	5
	phenol, mg/l	0	0
	thiosulphate	0	0
	pH	6.8	6.2
	% sludge volume, settled in ½h	21	25
	dry weight of suspended solids, mg/l	2,400	2,700
	temperature °C	27	27
Period of testing, days.		51	28

the low pH was attributed to the oxidation of thiosulphate which was present at a high concentration of 323 mg per litre in the feed-liquor. Continuous treatment of the concentrated liquor was possible when the aeration-time was increased to 33h and when the pH in the aeration tank was maintained at 6.4 - 7.5. However, despite these measures the removal of thiocyanate was incomplete; 5 mg CNS/litre usually remained undestroyed. When the pH was allowed to fall to 5.2 - 5.9, thiocyanate accumulated rapidly.

Throughout this series of tests while treating a 1:1 v/v dilution of coke oven effluent, the settleability of the sludge was poor. This was shown in the percentage sludge volume test, where the rates of flocculation and settlement were slow and the supernatant liquid remained turbid. Bulking of the sludge had therefore occurred. The extent of sludge-bulking was usually sufficient to prevent the percentage sludge volume from rising above 25%. Furthermore the dry weight concentration of suspended solids did not reach 3000 mg/l, see Table 8. This limited the biomass available for adsorption, absorbtion and oxidation. Owing to the limited amount of sludge, decreases in the aeration-time to below 20h usually caused excessive losses of sludge from the system and consequent accumulations of thiocyanate.

3.1.1.5 Addition of catechol.

Catechol is a constituent of coke oven effluent which, when partly oxidised, has been found to suppress the growth of bacteria capable of destroying thiocyanate (Hutchinson et al, 1964). When the laboratory system had been acclimatised to the concentrated liquor, catechol was included in the feed at a concentration of 50 mg/l, to find if it had an inhibitory effect on thiocyanate-

destruction. No detrimental effect occurred after seven days of continuous treatment at an aeration time of 33h. During this time ultra violet analysis of the aeration tank effluent confirmed that catechol had been destroyed in the treatment process. The concentration of catechol was then increased to 100 mg/l in the feed liquor. After a further period of 67 days continuous treatment, no detrimental effect of catechol was found. Both thiocyanate and catechol were almost completely destroyed provided that low pH values, below 6.2, were prevented. The addition of catechol was therefore discontinued.

Before any other investigations were carried out, the addition of catechol was stopped and the feed-liquor was replaced by a 1:1 v/v dilution of coke oven effluent lacking added catechol.

3.1.1.6 Addition of pyridine.

The resistance of pyridine to biological oxidation has been reported elsewhere (B.C.R.A. report No. 64, 1971). Pyridine was investigated in this study in order to gain more information on the nature of its inhibitory effect. Pyridine was included in the feed-liquor to the laboratory system at a concentration of 10 mg/l. This was the highest concentration likely to be encountered in the coke oven effluent, Barker et al (1958). After five days the settling property of the sludge deteriorated slightly. At the same time the colour of the final effluent from the settling tank changed from pale yellow to brown and was turbid due to the increased wash-out of micro-organisms which had not undergone settlement. The poorly settled, bulking sludge consisted of small loose-textured flocs which were 80 µ - 250 µ across instead of the optimal size of about 400 µ. Fungal filaments and free-living ciliated protozoa were not observed at this time.

Despite these signs of deterioration, a further 23 days were required before thiocyanate began to accumulate in the aeration tank. During the first week it was possible to maintain an aeration-time of 33h. It then became necessary to increase the aeration-time to 50h to prevent an accumulation of thiocyanate. The percentage sludge volume declined progressively from 19% to 10% throughout the 28 days when pyridine was added. On the 27th day the final effluent contained 10-30 mg CNS/litre, despite the prolonged aeration-time. On the 28th day the rate of destruction of thiocyanate declined rapidly and 140 mg CNS/ litre quickly accumulated in the aeration tank.

Four methods were used in attempts to restore activity. Firstly the aeration-time was increased to 60h. This permitted continuous treatment, but it was not possible to restore the aeration-time to 20h. Secondly the feed-liquor containing pyridine was added intermittently in volumes of 500 ml. Each addition gave an initial concentration of 30-40 mg CNS/litre throughout the system. A period of 6-8h was required to destroy the thiocyanate introduced in each batch-addition and the rate of thiocyanate destruction did not increase when successive batches of feed-liquor were added. After seven days a third attempt was made to restore activity. The feed-liquor was replaced by a diluted liquor, 1:4 v/v (liquor:water) which contained no added pyridine. However this did not permit an increase in the rate of thiocyanate destruction after one week. The fourth method was to add activated sludge which had been acclimated to coke oven effluent in the fill and draw culture. The acclimated sludge had not been in contact with pyridine at any time. 100 ml of acclimated sludge was added to the aeration tank, but the continued inhibitory effect of

pyridine again prevented the restoration of continuous treatment.

Due to the lack of a continual supply of feed-liquor which would have diluted inhibitory substances out of the system, further deterioration occurred and endogeny became more advanced. This caused a further decline in the percentage sludge volume from 10% to 3%. Consequently the system was almost completely inactive in thiocyanate destruction and oxygen consumption two weeks after continuous treatment had been prevented by the addition of pyridine.

In view of the inability to restore activity by adding acclimated sludge, a test was done to assess the toxicity of mixed liquor from the aeration-tank. Details of this test are given in Table 9. The sample of mixed liquor was taken from the aeration tank when the thiocyanate concentration had slowly declined to below 5 mg/l. Throughout the 96h test-period, thiocyanate destruction by the acclimated sludge was suppressed completely by mixed liquor from the aeration-tank. Following the unsuccessful attempts to restore activity, the laboratory scale plant was emptied and cleaned. The activated sludge was replaced by acclimated sludge from the fill and draw culture, in preparation for a different series of tests.

3.1.1.7 Effect of different dilutions of feed-liquor on the occurrence of bulking sludges.

The development of bulking sludges was investigated further in a second series of tests. The results are summarised in Table 10. The bulking of activated sludges has been attributed to several causes. These include a high C:N ratio in the feed-liquor, low pH values of the aeration tank mixed liquor (Eikelboom, 1975), a deficiency of phosphorus, Greenberg et al (1955), toxicity (Heukelenkian, 1941) and over-

Table 9 Inhibitory Effect of Aeration-Tank mixed liquor on thiocyanate-destruction by acclimated sludge.

		Cultures 1	Cultures 2
Contents of each 3 litre - culture	acclimated sludge from fill and draw culture	85 ml	85 ml
	mixed liquor from aeration tank.	0 ml	1500 ml
	tap water	1500 ml	0 ml
	Calgon	20 mg/l	20 mg/l
	spent still liquor + devil liquor 13:2 (v/v)	150 ml	150 ml
	initial pH	7.0	7.0

Additions	Hours	Cultures 1 CNS mg/l	Cultures 1 pH	Cultures 2 CNS mg/l	Cultures 2 pH
	0	50	7.0		7.0
	0.3	50	7.0		7.0
	1.0	46	7.0		7.0
	1.5	41	7.1		7.0
	2.0	34	7.1		7.0
	3.0	10	7.3	50	7.0
	4.0	0	7.6		7.0
150 ml spent still + devil liquors added to 1	18.0	50	7.0		6.5
	48.0	0	7.7		6.4
150 ml spent still + devil liquors added to 1	72.0	50	7.0		7.0
	96.0	0	7.8		6.4

Table 10 Second series of tests while treating 1:1 v/v dilution of coke oven effluent.

			6	69	103	114
Days after start of 2nd series of tests			6	69	103	114
Purification efficiency, % destruction of CNS			89	89	> 99	82
Characteristics of activated sludge flocs	Size-range μ		80-400	100-300	400-800	80-250
	bulked or well settled		bulked	bulked	well settled	bulked
Composition of flocs, relative amounts,	fila-ments	bacterial	low	high	low	high
		fungal	high	high	low	low
	non-filamentous, globular form		low	low	high	low
Free-Living ciliates, relative numbers			very few	very few	numerous	absent
Thiocyanate in feed liquor, mg/l			425	415	420	420
Thiocyanate in final effluent, mg/l			45	45	1	75
Suspended solids concentration in aeration-tank mixed liquor, mg/l			320	510	4,040	350
% sludge volume settled in ½h.			1	9	23	7
Sludge density index			3.2	0.6	1.8	0.5
Aeration-time, h			60	20	20	20
Sludge age*, days			9.3	2.3	3.3	2.3
Remedy: Increase in dilution of feed-liquor, followed by progressive decrease in dilution. Liquor:Water v/v.			1:10 for 15dys 1:4 for 28dys	1:8 for 17dys 1:3 for 7dys 1:2 for 3dys		1:4 for 10dys 1:2 for 4dys
Period required to regain efficient purification and rapid sludge-settlement while treating liquor at increased dilutions.			9dys	7dys		5dys
Period treating 1:1 v/v dilution of feed-liquor before CNS accumulated.			6dys	20dys		20dys

$$\text{* Sludge age, days} = \frac{V \times Sm}{Q \times S_s}$$

where V = aeration tank volume, litres.
Sm = concentration of suspended solids in aeration tank mixed liquor, mg per litre.
Q = rate of flow of feed-liquor, litres per day.
S_s = concentration of suspended solids in final effluent, mg per litre.

loading the system with organic waste. The micro-organisms found elsewhere to be associated with bulked sludges include filamentons bacteria ; Sphaerotilus, Beggiatoa, Thiothrix (Pipes 1969) and fungi : Geotrichum (Pipes et al 1963, Schofield 1971) and Zoophagus (Cooke et al 1958, Pipes 1965). Hawkes (1965) found that the amounts of the fungus Sepedonium sp accumulating in bacteria beds were related to the sewage-strength. The fungi isolated from bulked activated sludges in the present investigation did not resemble any of the fungi cited above. The probable causes of sludge-bulking in the present study were firstly, the high organic loading primarily as phenol, and secondly, toxic constituents, e.g. phenol-derivatives and pyridine bases.

A relatively concentrated feed-liquor, at a dilution of 1:1 v/v was treated to re-affirm the occurrence of sludge-bulking. Attempts were then made to eliminate bulking and restore sludge settleability. This was done by increasing the dilution of the feed-liquor, in order to lower the organic loading on the system and to reduce the concentrations of toxic materials. In order to investigate the effects of different dilutions, it was necessary to avoid the effects of changes in the composition of the effluent from the coke ovens. Therefore, throughout the second series of tests, the different dilutions of feed-liquor were all obtained using the same sample of coke oven effluent.

Six days after the beginning of continuous treatment of the 1:1 v/v dilution of coke oven effluent, the sludge showed poor settling properties, (Table 10). Bulking caused a loss of sludge from the system which resulted in low values for suspended solids, percentage sludge-settlement and sludge-age. Consequently, thiocyanate accumulation even at an increased aeration-time of 60h. The remedy of increasing the dilution of feed-liquor to

1:10 v/v resulted after 9 days in an improvement in sludge-characteristics, and almost complete thiocyanate-destruction. The dilution of feed-liquor was then changed to 1:4 v/v and finally to 1:1 v/v after 43 days. During this period the volume of settleable sludge increased. When a less diluted liquor was introduced, the aeration-time was increased correspondingly so that there was no initial rise in the loading applied to the system. Provided that thiocyanate did not accumulate, the aeration-time was then gradually decreased to 20h, over a period of several days until the next decrease in dilution was made. On day 103 treatment of the 1:1 v/v diluted liquor had been restored. The tests carried out at this time gave results which were typical of those obtained when the efficiency of thiocyanate-destruction was high.

Microscopic examination showed that on days 6 and 69 a large proportion of the bulked sludges consisted of fungal filaments. An explanation of the large amount of fungal growth was sought. In view of the high concentration of phenol, 600-1000 mg/l in the 1:1 v/v dilution of feed-liquor, phenol was a possible source of organic carbon for fungal growth. A test was therefore done to find if the fungi present in the system were capable of utilising phenol. Pure cultures of fungi were isolated from the aeration tank mixed-liquor on days 6 and 69. This was done by streaking onto plates of malt agar (B.B.L. 11400). Six isolated colonies of the fungi grown for 5 days at 28°C were each inoculated into 10 ml of a sterile mineral salts basal medium (Bushnell Haas broth, Difco 0578-02) supplemented with phenol at a concentration of 470 mg/l. After 8 days at 28°C all the cultures gave good growth and complete destruction of phenol, showing that the fungi were capable of utilising

phenol as the sole source of organic carbon.

Sludge-ages are given in Table 10 as this test is widely accepted as a useful aid to the operation of activated sludge plants, Gould (1953) Hawkes (1963) and Fair (1968). The "sludge age" may be defined as the average total time of detention of the flocs in the system (Hawkes 1963). Values for the biochemical oxygen demand, or B.O.D., were determined using spent liquor from the ammonia stills during initial estimations of sludge ages according to the following equation given by Hawkes (1963):

$$\text{Sludge age, days} = \frac{\text{lb dry weight of activated sludge in the system}}{\text{lb weight of B.O.D. entering the system per day.}}$$

In the above equation the weight of B.O.D. entering the system per day has been inserted in place of the weight of suspended solids entering the system per day. This change was made because, after sedimentation, the coke oven effluent was primarily a soluble waste containing very little suspended solid material. However the B.O.D. values were not considered to be accurate in view of the probable inhibitory effects on biochemical oxidation caused by constituents of coke oven effluent. The use of an acclimated "seed" of micro-organisms from the final effluent of the laboratory scale plant may have overcome this problem to some extent. Nevertheless, the B.O.D. value of 2,430 mg per litre obtained for Corby spent still liquor was low in relation to the 4h permanganate value of 2,900 mg per litre for the same sample. This indicated that biochemical oxidation had been suppressed in the conditions specified for the B.O.D. determination (Beavis D, 1967). Consequently the use of B.O.D. values in determining sludge ages was discontinued. The equation finally adopted was that given by Klein (1966), see Table 10 on page 60.

In the present study, sludge-ages between 3 and 5 days were satisfactory, but sludge-ages below or above this range were often associated with bulking sludges.

The second series of tests showed that bulking sludges developed on three successive occasions during the treatment of a 1:1 v/v dilution of coke oven liquor. On all three occasions it was possible to regain efficient purification and rapid sludge-settlement by increasing the dilution of feed-liquor. However, bulking of the sludge developed again within three weeks of returning to a 1:1 v/v dilution.

A typical fungal floc obtained from the laboratory scale plant is shown in Fig 5. Flocs of this nature had poor settling properties. Bacterial flocs which settled rapidly are shown in Figures 6 and 7. Fig 8 shows dense rapidly settling bacterial flocs which developed three days after increasing the dilution of feed liquor from 1:1 v/v to 1:4 v/v (liquor:water).

Considerable improvements in plant performance occurred when a rapid increase in thiobacilli developed from 10^2 to 1.6×10^8 per ml. Details of bacteriological and conventional tests carried out at this time are given on page 166 Table 29, column (iii). Changes in the composition of the feed-liquor which might have provided conditions suitable for the increase in bacterial activity included: the low concentration of inhibitory coloured materials, an increased availability of phosphate and ferrous iron in acidified liquor and possibly the presence of formate in the feed-liquor. The significance of these factors is considered in sections 5.6.4 and 6.

These alterations in the conditions of treatment were followed within five days by changes in the microscopic appearance

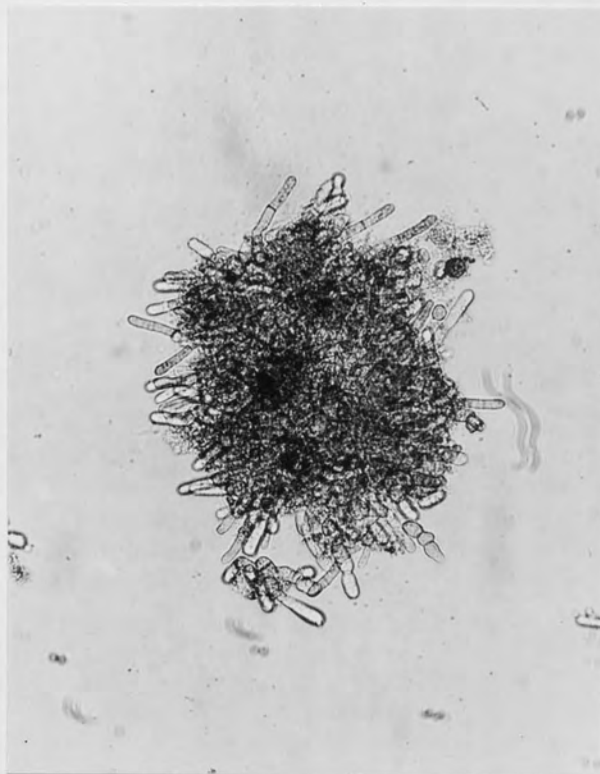


Figure 5.

"Bulking" of activated sludge:
A typical fungal floc from the aeration tank of the laboratory system treating 1 : 1v/v dilution of coke oven effluent.
Magnification X200.

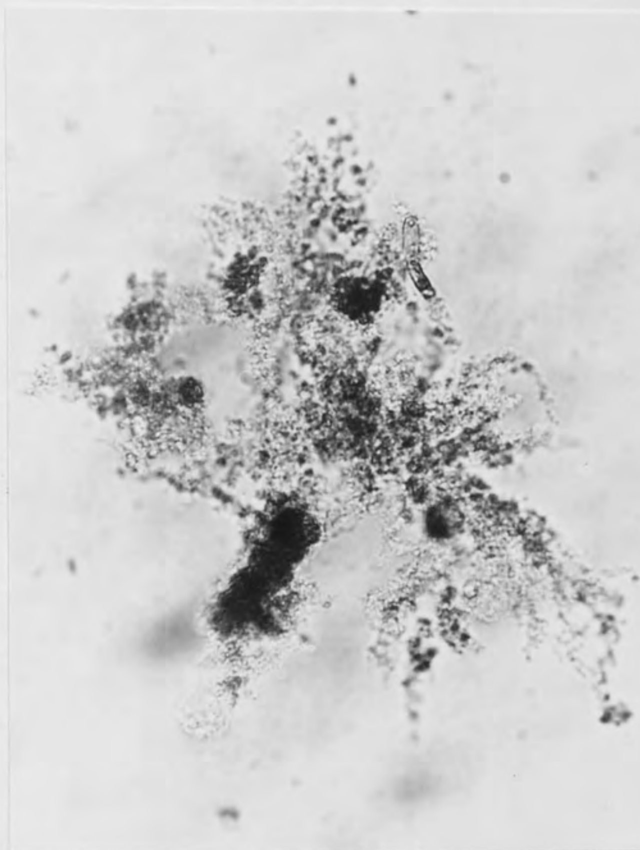


Figure 6.

A typical open textured, rapidly settling bacterial floc, obtained from the laboratory scale plant during efficient purification of coke oven effluent; 1 : 1v/v dilution.
Magnification X200.

Figure 7.

Activated sludge : Open-textured bacterial flocs which showed rapid settlement. Obtained from laboratory scale plant during efficient purification of coke oven effluent 1:1^{v}/v dilution.

Magnification x100

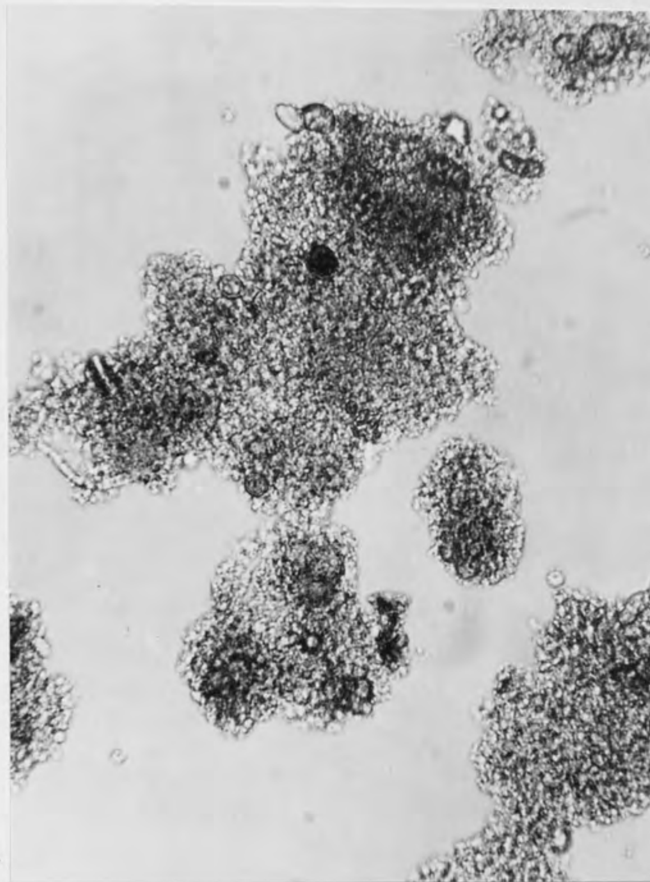


Figure 8 .

Dense rapidly settling bacterial flocs, from the laboratory scale aeration tank, during efficient purification of 1 : 4v/v (liquor : water) dilution of coke oven effluent.
Magnification X400.

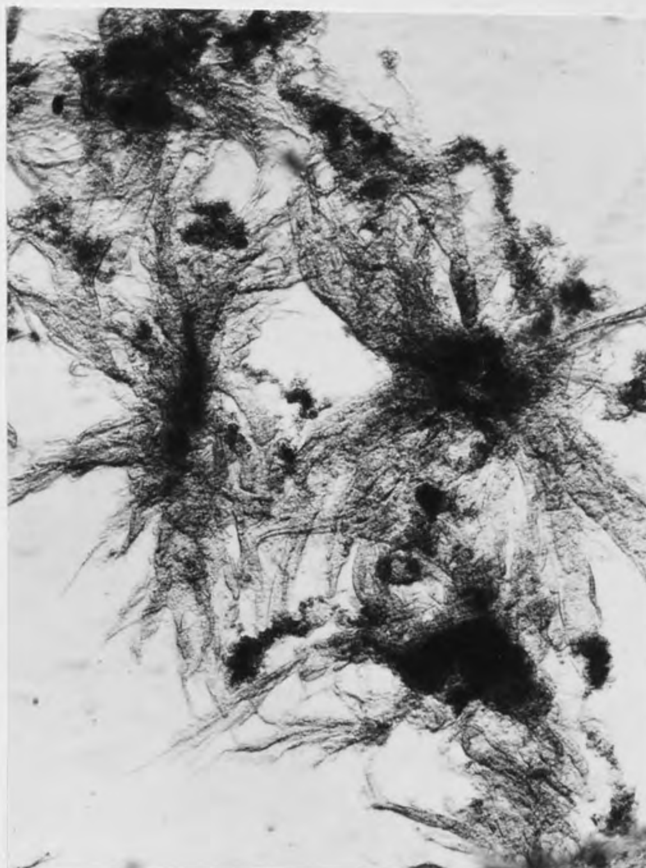


Figure 9.

Tasselled bacterial flocs. A further increase in the amount of filamentous bacteria caused a deterioration in sludge-settlement. Origin: Laboratory scale aeration tank purifying 1 : 1v/v dilution of coke oven effluent.

and settleability of the activated sludge. The development of filamentous bacteria gave the flocs a tasselled apperance, shown in Fig 9 on page 69. When in this condition, the activated sludge remained active in the removal of thiocyanate, but the rate of treatment was limited by a slow rate of sludge-settlement.

3.1.2 Plant treating synthetic coke oven effluents.

The purification of synthetic coke oven effluents was investigated to find the effects of individual liquor-constituents on the minimum aeration-time, achieved without an accumulation of thiocyanate in the system. Throughout these investigations the concentration of thiocyanate in the feed-liquor was 1000 mg/l, as ammonium thiocyanate. The liquor-constituents investigated were sodium thiosulphate, ammonium chloride, phenol and pyridine. The source of micro-organisms was a 300 ml sample of sludge obtained from the primary settling tank at Corby sewage works. The sludge was acclimated to a solution of ammonium thiocyanate containing a mineral nutrients solution (Table 3, page 39, No.1). The initial concentration of thiocyanate ws 50 mg/l. The procedure for progressively increasing activity during batch-culture was the same as described in section 3.1.1.2. During the period when activated sludge was being developed in the fill and draw culture, it was observed that approximately one gram of thiocyanate was destroyed per gram dry weight of activated sludge produced. Continuous treatment was established after six weeks using a feed-liquor containing 1000 mg CNS/l, as ammonium thiocyanate. The minimum aeration-time was established by gradually increasing the rate of continuous treatment over a period of six weeks until it was no longer possible to prevent an accumulation of thiocyanate in the final effluent.

The different liquor-constituents were added successively to the feed-liquor over a total period of 42 weeks. The minimum aeration-time was determined for each formulation of synthetic coke oven effluent. At each stage, four weeks were allowed for acclimation, followed by a period of two weeks during which the minimum aeration-time was determined. The range of minimum aeration-times required for the treatment of a 1:1 v/v dilution of raw coke oven effluent is included in Table 11 to enable comparisons to be made. Since thiosulphate had a negligible effect on the minimum aeration-time, it was omitted from the feed-liquor containing phenol. The effect of pyridine on the purification of an ammonium thiocyanate solution was found separately.

Thiosulphate at 200 mg per litre had a negligible suppressing effect on the rate of treatment. 8000 mg chloride per litre caused a slight suppression which resulted in an increase in the minimum aeration time from 5.4 to 6.8h. Sludge bulking often became a major problem during active phenol destruction. Pyridine had a toxic effect which was shown after 24 hours by the accumulation of sulphur in the flocs. The deposition of sulphur revealed the incomplete oxidation of thiosulphate and thiocyanate by the thiobacilli. Severe inhibition of activity followed after a further 24h. All the synthetic liquors could normally be purified at faster rates than coke oven effluent.

The suppressing effect of low pH values on thiocyanate destruction is shown in Fig 10. At pH values below 6.0 the rate of thiocyanate destruction was significantly lower than at pH 6.4. However, after 100h at pH 5.1, the batch culture regained its original high rate of thiocyanate destruction, 24h after returning to pH 6.4.

Table 11.

Effect of Different Liquor-Constituents on the Minimum Aeration-Time.

<table>
<tr><th>Feed-Liquor</th><th colspan="2">Formulation (*)</th><th>Minimum aeration-time h</th></tr>
<tr><td rowspan="6">Synthetic</td><td colspan="2">1000 mg CNS/l, as NH_4CNS</td><td>4.3</td></tr>
<tr><td colspan="2">1000 mg CNS/l, as NH_4CNS + 200 mg S/l as $Na_2S_2O_3.5H_2O$</td><td>5.5</td></tr>
<tr><td rowspan="3">1000 mg CNS/l as NH_4CNS + 200 mg S/l as $Na_2S_2O_3.5H_2O$ +</td><td>1000 mg Cl/l as NH_4Cl</td><td>5.4</td></tr>
<tr><td>4000 mg Cl/l as NH_4Cl</td><td>5.4</td></tr>
<tr><td>8000 mg Cl/l as NH_4Cl</td><td>6.8</td></tr>
<tr><td colspan="2">1000 mg CNS/l as NH_4CNS + 8000 mg Cl/l as NH_4Cl + 2000 mg phenol/l.</td><td>11-60 (x)</td></tr>
<tr><td></td><td colspan="2">1000 mg CNS/l as NH_4CNS + 50 mg pyridine/l</td><td>> 60</td></tr>
<tr><td>Raw</td><td colspan="2">1:1 v/v dilution of raw coke oven effluent ; spent still liquor : devil liquor, 13:2 v/v.</td><td>18-32 (x)</td></tr>
</table>

Temperature maintained at 27°C. pH in aeration-tank adjusted to 6.7 - 7.3

(*) All formulations included a minerals nutrient solution, Fig 3 , solution No. 1., page 39.

(x) Minimum aeration-times were increased by bulking sludges.

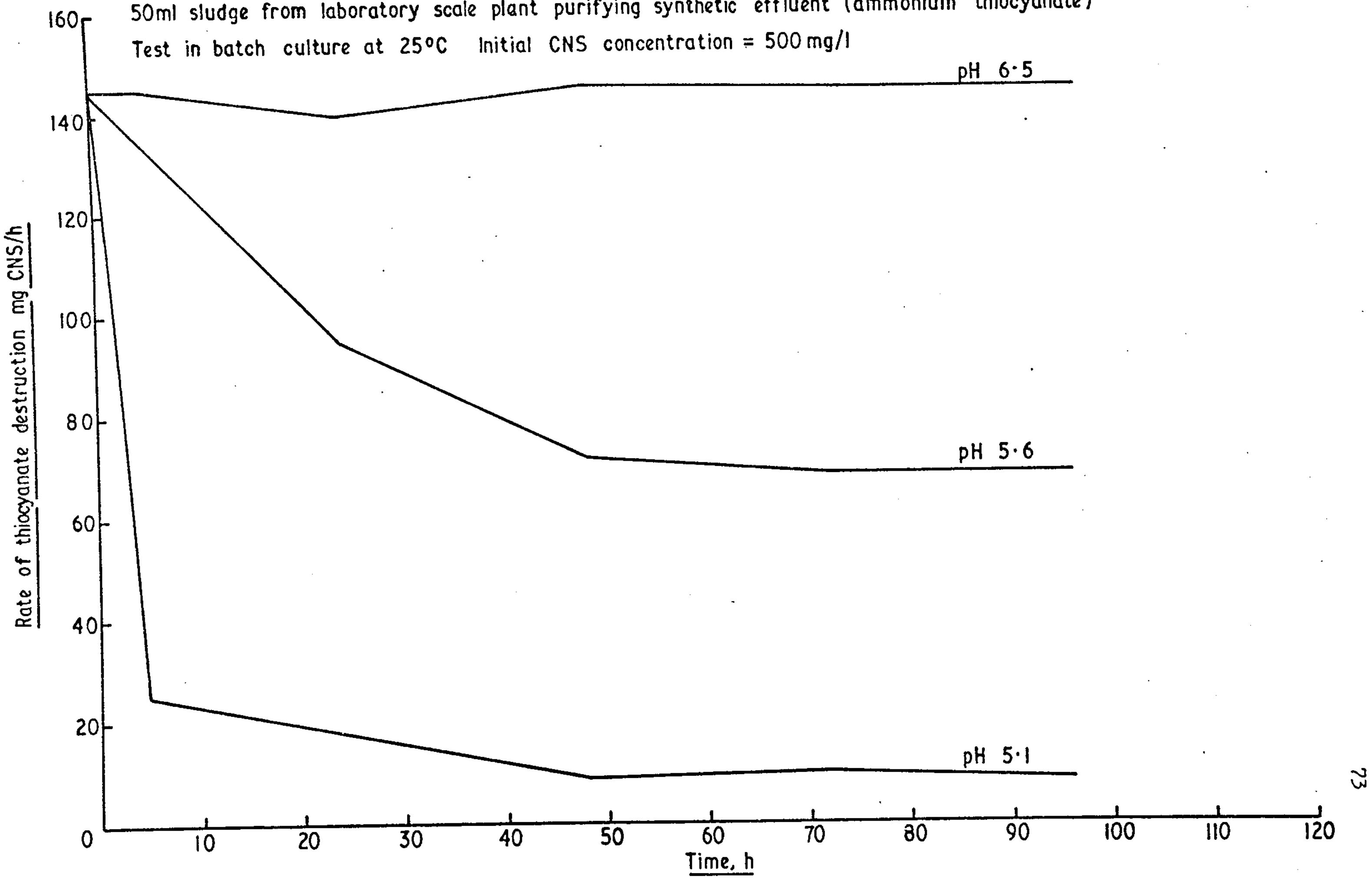


FIG 10 DESTRUCTION OF THIOCYANATE AT LOW pH VALUES
50ml sludge from laboratory scale plant purifying synthetic effluent (ammonium thiocyanate)
Test in batch culture at 25°C Initial CNS concentration = 500 mg/l
pH 6·5
pH 5·6
pH 5·1
Rate of thiocyanate destruction mg CNS/h
0
20
40
60
80
100
120
140
160
10
30
50
70
90
110
Time, h

Occasionally it was necessary to stop the flow of feed liquor due to mechanical failures. Similar difficulties were likely to occur on the full scale plant. The effect of a three day period of starvation was therefore investigated using a batch culture of activated sludge from the laboratory scale plant, Fig 11. Five days were required to regain rapid oxygen consumption and active thiocyanate destruction. A dissolved oxygen meter was used in this test.

3.2 Testing of batch cultures.

3.2.1 Additions of glycine and alanine.

The addition of small concentrations of glycine and alanine, 5 mg/l in the feed-liquor, have been reported to stimulate the biological treatment of coke oven effluent. The observed effect of additions of these amino-acids was the attainment of shorter minimum aeration-time, (B.C.R.A. report 64, 1971). In the present study, short-term exploratory tests were done in batch-culture, to find if glycine or alanine stimulated the destruction of thiocyanate and phenol.

Activated sludge was obtained from the laboratory scale plant treating a synthetic coke oven effluent containing ammonium thiocyanate, 1000 mg CNS/l, phenol, 2000 mg/l, and ammonium chloride, 8000 mg Cl/l, see section 3.1.2. As shown in Table 12, no increase in the rate of thiocyanate-destruction was observed in the presence of 5 mg glycine/l.

The test using glycine was repeated using a synthetic coke oven effluent in place of the solution of potassium thiocyanate. The synthetic effluent consisted of a 1:4 v/v dilution (liquor: tap water) of the synthetic effluent which was fed into the laboratory scale system. A fresh sample of activated sludge from the laboratory scale plant was used. On three successive tests, 5 mg glycine per litre again had no significant effect on thiocyanate

FIG 11. DISSOLVED OXYGEN-UPTAKE AND THIOCYANATE-DESTRUCTION DURING RECOVERY FROM A THREE-DAY PERIOD OUT OF CONTACT WITH THIOCYANATE

Sludge from laboratory scale plant purifying synthetic effluent (ammonium thiocyanate)
Test in batch culture at 25°C and pH 7·5

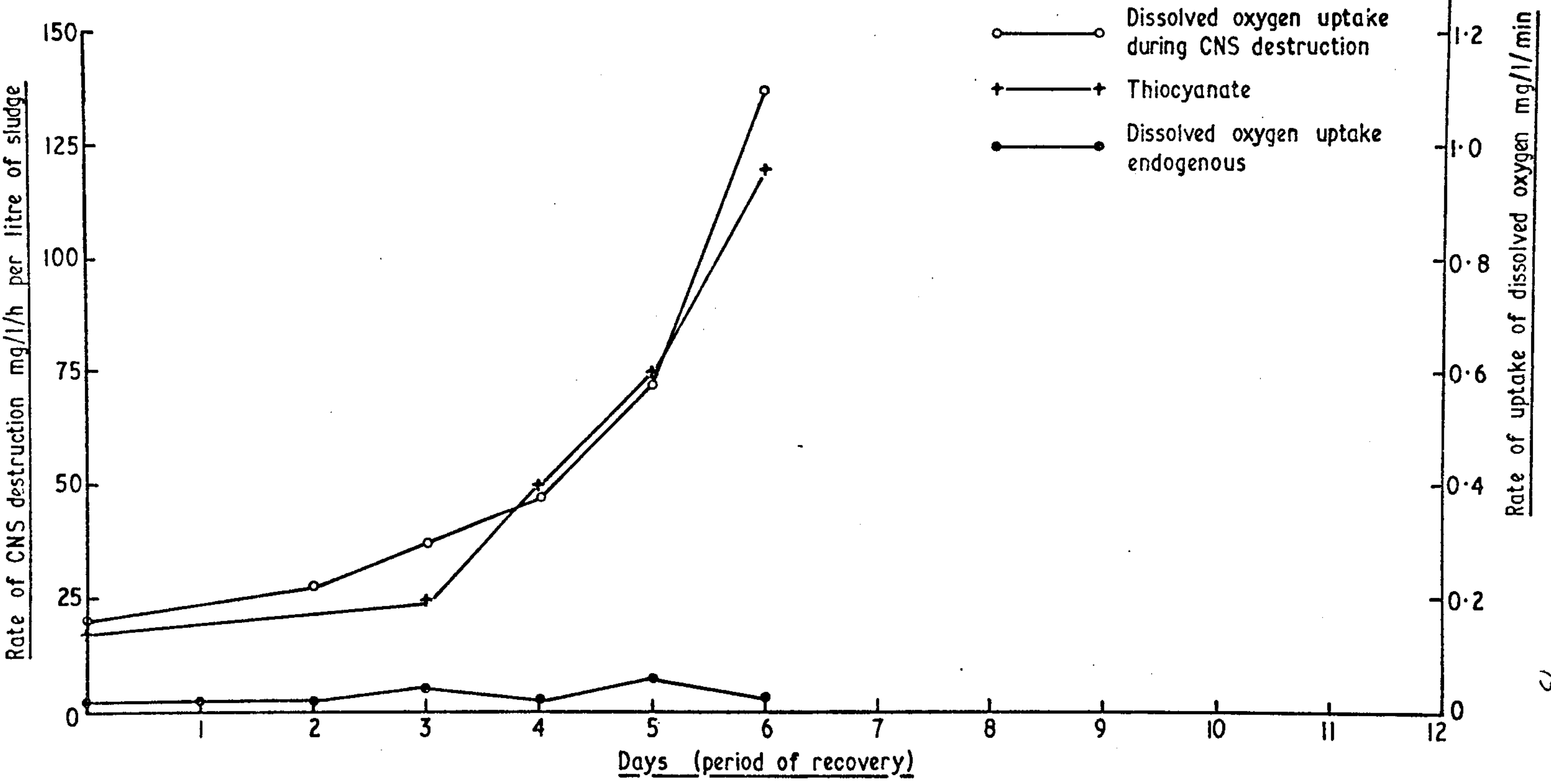


Table 12 Rates of destruction of thiocyanate and phenol by batch cultures, in the presence of glycine and alanine.

* Each figure = rate of destruction of substrate, calculated from 10 tests during a 6h period. Rates were found to be linear after a 1-5 min. lag for CNS destruction. No lag was observed for phenol destruction.

Temperature maintained at 20°C.

pH maintained at 6.5.

Initial CNS concentration = 250 mg/l.

Initial phenol concentration = 500 mg/l.

Thiocyanate and phenol were destroyed simultaneously.

	Rates of destruction of substrates mg/l/h	
Substrates	No glycine added	5 mg glycine/l added
NH_4CNS	25* 25 25	25 28 28
Phenol	100 98 113	102 105 113

	Rates of destruction of substrates mg/l/h	
Substrates	No alanine added	5 mg alanine/l added
NH_4CNS	19 26 22	19 26 26
Phenol	92 41 112	110 73 113

destruction. A fourth test, using 10 mg glycine/l was also ineffective.

The effect of alanine was tested under the same conditions. Again a fresh sample of 250 ml activated sludge from the plant treating synthetic effluent was used. As shown in Table 12, the first test indicated a slightly increased rate of phenol-destruction, but no effect on thiocyanate-destruction was observed. When the supernatant liquid was replaced by a second batch of synthetic effluent, the rate of phenol-destruction was almost doubled in the presence of alanine. However, the rate of thiocyanate-destruction remained unchanged. Replacement of the supernatant liquid by a third batch of synthetic effluent resulted in increases in the rates of destruction of both thiocyanate and phenol, in the presence of 5 mg alanine/l.

Following the short term exploratory tests described above, glycine and alanine were tested separately, in continuous culture, using the laboratory system treating synthetic coke oven effluent described in section 3.1.2. In separate investigations, each of the two amino-acids was included in the synthetic feed-liquor at 5 mg/l. No effect on the minimum aeration-time was found during a test-period of six weeks. When 25 mg alanine/l was included in the feed-liquor, a serious sludge "bulking" problem resulted. The bulked sludge which was found to contain an excessive amount of fungal filaments,is shown in Fig 12. The higher influent C:N ratio might have promoted fungal growth (Hawkes 1963). The short term tests in batch-culture indicated that alanine may stimulate the destruction of thiocyanate and phenol. However in continuous culture during a period of six weeks, neither glycine nor alanine enabled a lowering of the minimum aeration-time to be achieved. Raising the concentration of alanine from 5 mg/l to 25 mg/l

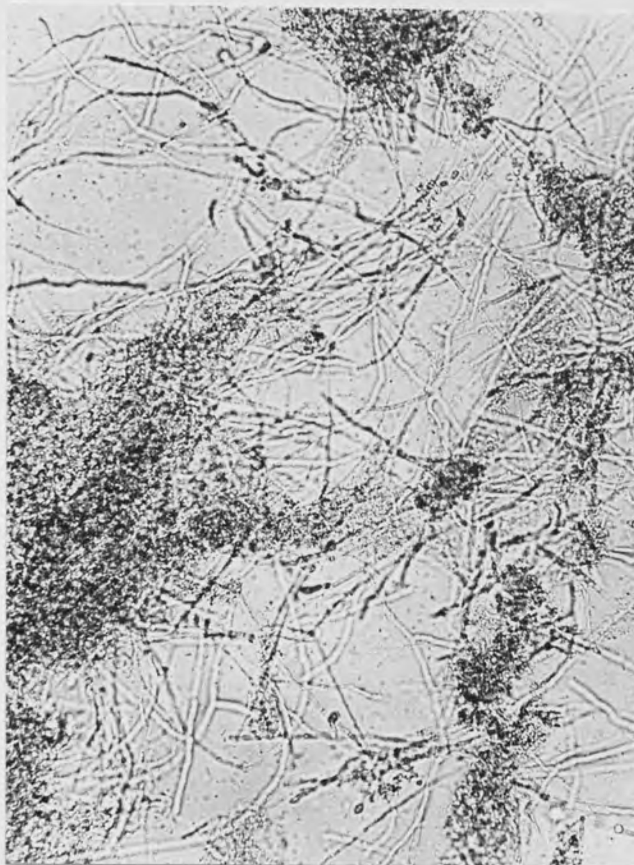


Figure 12.

Deterioration in the setting property of activated sludge caused by the addition of 25mg alanine per litre. Excessive development of fungal filaments causing "bulking".
Origin: Laboratory scale system purifying synthetic effluent of ammonium thiocyanate.
Magnification X200.

caused an increase in the minimum aeration-time, caused by poor-settlement of the bulked sludge.

3.2.2 Addition of formate.

At Corby Works, formate enters coke oven effluent as a product of the hydrolysis of cyanide, during the wet desulphurisation of coke oven gas in the Collin process. The concentration of formic acid in Corby coke oven effluent as a result of this process may exceed 1000 mg/l. Most other coking plants do not employ the Collin process. High concentrations of formic acid are therefore common only in the coke oven effluent at Corby. Hall (Corby Works, 1968) suggested that the high rates of treatment he had achieved using laboratory scale plants treating Corby coke oven effluent may have been due to a stimulating effect of formate. In order to investigate this possibility, preliminary short term tests were done to find the effects of adding formate to batch cultures treating a synthetic coke oven effluent, see Table 13.

Ammonium formate was added at a concentration of 400 mg/l in a two litre batch culture of activated sludge. A control culture, lacking ammonium formate, was also set up. The conditions of the test were the same as in the testing of glycine and alanine. The six successive additions of ammonium formate had no effect on the rate of destruction of thiocyanate, but phenol-destruction was more complete on three successive occasions when formate was added. Chemical analysis showed that formate was destroyed by the culture. The pH rose significantly, from 6.8 to 7.8, only in the culture which destroyed formate.

A rise in pH during biological treatment was also observed occasionally in the laboratory scale plant purifying coke oven effluent. Here it was sometimes necessary to adjust the pH of the feed liquor to 4.0 to maintain a pH of 6.4 - 7.5 in the

Table 13. Rates of destruction of thiocyanate and phenol by batch cultures, in the presence of ammonium formate.

* Each figure = rate of destruction of substrate, calculated from 10 tests during a 6h period. Rates were found to be linear after a 1-5 min. lag for CNS destruction. No lag was observed for phenol destruction.

Temperature maintained at 20°C.

Initial CNS concentration 230-270 mg/l.

Initial PhOH concentration 445-480 mg/l.

Thiocyanate and phenol were destroyed simultaneously.

	Rates of destruction of substrate, mg/l/h	
Substrates	No formate added	400 mg $HCOONH_4$/l added
NH_4CNS	31* 36 29 30 16 23	31 36 29 30 23 23
Phenol	178 160 153 61 58 60	178 160 153 71 79 72
Mean Changes in pH during destruction of substrates.	7.1 → 7.4	6.8 → 7.8

aeration tank. When the liquor was acidified using hydrochloric acid, mineral acidity was partly replaced by acidity due to formic acid formed from calcium formate. Alkalinity would then have resulted from the removal of formic acid during biological oxidation. In contrast to this, the calcium formate present in an alkaline feed liquor might have been oxidised to calcium carbonate, causing no change in the pH value.

3.3 Results.

The outcome of the laboratory scale experiments on the biological treatment of Corby coke oven effluent is summarised as follows:

The inhibitory nature of the waste caused a prolonged lag in the development of the optimum biomass in the system. Strict adherance to the principle of avoiding an accumulation of thiocyanate was essential to improve process-stability.

Dissolved oxygen concentrations above 1.0 mg per litre permitted the maximum rate of thiocyanate destruction. However when activity was high, a decrease in the rate of aeration caused the oxygen concentration to fall rapidly to a critical level of 0.8 mg per litre. Thiocyanate then accumulated rapidly.

The optimum dry weight concentration of suspended solids in the aeration tank was found to be 4,000 - 5,000 mg per litre, in the absence of deposits of calcium carbonate. This figure was lower than the expected value of 5,000 - 10,000 mg per litre.

The treatment of a 1:1 v/v dilution of coke oven effluent resulted in a gradual deterioration in plant-performance shown by the development of an excessive amount of fungal flocs. This caused bulking of the activated sludge. Dilution to 1:2 v/v (liquor:water) was a successful remedy to the problem of sludge-bulking.

In the absence of sludge-bulking, a daily sludge wastage rate of 5% to 10% was satisfactory, provided that the optimum dry weight concentration of suspended solids had been achieved.

The optimum range of sludge-ages was found to be between 3 and 5 days, when the system was operating successfully at an aeration time of 20h and the daily sludge wastage rate was carefully controlled.

In addition to the need for greater dilution, it was necessary to exercise strict control of the pH in the aeration vessel to within the range 6.4 to 7.5. This was necessary in order to maintain the rapid destruction of thiocyanate, to minimise the amount of calcium carbonate deposited in the flocs and to minimise the amount of phosphate nutrient lost from alkaline solution by precipitation as calcium phosphate. A high influent concentration of thiosulphate caused excessive acidity to develop in the aeration tank, where the pH fell to below the optimum range of 6.4 - 7.5 for thiocyanate destruction. Conversely, the oxidation of high concentrations of formic acid in acidified feed-liquor appeared to be the cause of excessive alkalinity. Consequently it was necessary to provide the facility for frequent dosing of either acid or alkali.

The additions of 5 mg per litre of glycine and alanine did not result in an improvement in purification efficiency. Alanine at 25 mg per litre caused excessive filamentous fungal growths to develop, resulting in sludge-bulking. Neither catechol or chloride had an inhibitory effect on the purification process, at the concentrations normally present in the waste.

The toxic effect of pyridine was observed by a deterioration in the settleability of the activated sludge and an increase in intensity of brown colouration in the final

effluent, before thiocyanate began to accumulate.

Increased rates of treatment were achieved when the acidified feed-liquor contained greater concentrations of inorganic phosphate, ferrous iron and formate.

The conventional tests outlined at the beginning of this section did not reveal the gradual accumulation of inhibitory substances. Microscopic examination of the activated sludge proved to be a useful indication of the efficiency of purification. Efficient treatment was accompanied by the presence of moderate numbers of free-living ciliates, e.g. *Colpidium*. These were eliminated when concentrated liquors were treated and when thiocyanate accumulated in the system. Microscopy provided an advanced warning of sludge bulking, enabling remedial action to be taken at an earlier stage. Counts of viable bacteria were called for to determine the extent of inhibition which occurred when thiocyanate accumulated.

4. Isolation and testing of pure cultures.

The experimental work using pure cultures was concerned with the enrichment, isolation, identification and testing of strains of bacteria which destroy thiocyanate.

4.1 Enrichment of thiosulphate and thiocyanate destroying bacteria.

Bacteria were obtained from a 50 ml sample of sludge derived from one of the primary settling tanks at Corby sewage purification works. This works was treating mainly domestic sewage.

The enrichment medium used was that described by Vishniac et al (1957) for the growth of Thiobacillus thioparus which was active in carbon dioxide fixation and the oxidation of sulphur compounds. The sludge sample was aerated at room temperature (18°C) in a 10 gallon carboy containing 30 litres of thiosulphate minerals medium, of the following composition : $Na_2S_2O_3.5H_2O$, 300g., K_2HPO_4, 120g., KH_2PO_4, 120g., $MgSO_4.7H_2O$, 24g., NH_4Cl, 12g., stock trace metals solution 300 ml., tap water 30 litres. The trace metals stock solution consisted of : EDTA, 50g., $ZnSO_4.7H_2O$, 22g., $CaCl_2$, 5.54g., $MnCl_2.4H_2O$, 5.06g., $FeSO_4.7H_2O$, 4.99g., $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.1g., $CuSO_4.5H_2O$, 1.57g., distilled water, 1000 ml. The trace metals solution was similar to that used by Postgate (1966) specifically for the enrichment of thiobacilli. The pH of the trace metals solution was adjusted to 7.0 with 10% KOH. A slight precipitate could not be avoided while preparing this solution. After mixing, 10 ml of the fresh trace metals solution, including a small amount of precipitate was added to each litre of culture medium.

Initially the culture was aerated with a mixture of air (10 vols) and carbon dioxide (1 vol), blown through separate sintered glass spargers. After 36 hours the culture acquired a

slight white turbidity due to the increased numbers of dispersed bacteria and the formation of a suspension of elemental sulphur from the incomplete oxidation of thiosulphate. When thiosulphate-oxidation became rapid, the pH dropped to 4.0 and required periodic readjustment to 7.0 with sodium bicarbonate. The supply of carbon dioxide was stopped when the drop in pH became rapid. After 48 hours, 4 litres of the thiosulphate grown enrichment were transferred to a 4 litre beaker and supplied with 1ppm of the inorganic nutrients solution (No. 1, Table 3, page 39), together with 25 mg CNS/litre as NH_4CNS. The 4 litre culture was adjusted to pH 6.7 with 10% NaOH solution, and aerated with compressed air supplied through a sintered glass sparger. The induction-period needed before thiocyanate destruction began was 68 hours. The bacteria flocculated much more than during thiosulphate destruction, leaving a clear supernatant. The rate of thiocyanate oxidation increased following the addition of two further batches of 0.13 g NH_4CNS. Rapid flocculation allowed the 4 litre enrichment of thiocyanate oxidising bacteria to be maintained as a "fill and draw", or semi-continuous culture. This was done by periodically stopping aeration after the complete destruction of thiocyanate and allowing settlement of the flocculated bacteria. The supernatant liquid was then replaced by a fresh solution of ammonium thiocyanate and nutrients. The pH was then adjusted to 6.7. Very little change in pH occurred during the oxidation of thiocyanate. High rates of thiocyanate destruction, 1.0 g CNS per hour, were achieved in the 4 litres enrichment culture. Three litres of this culture were transferred to a 13 litre laboratory scale activated sludge plant, and maintained, in continuous culture, treating a solution of ammonium thiocyanate in tap water. The solution fed into the aeration tank consisted of NH_4CNS (1400ppm CNS) and 1ppm of the inorganic nutrients

solution. The pH of the feed was adjusted to 8.0. Using this method an enrichment of thiobacilli was developed continuously purifying a solution of ammonium thiocyanate. Isolate 5, page 115, was obtained from a laboratory scale plant treating coke oven effluent.

The continuous enrichment culture was used to obtain pure cultures of thiocyanate-destroying thiobacilli.

4.2 Media.

Details of all the media used for the isolation of pure cultures described in this section and for the monitoring of bacterial populations in Section 5, are given in the Appendix on page 201. Of these media, the ones selected for the isolation of pure cultures were as follows:

Description of medium	No. of medium in Appendix. (page 201).
1% thiosulphate minerals medium.	1
6% thiosulphate minerals medium.	1
0.02% ammonium thiocyanate minerals medium.	3
Thiocyanate + succinate medium.	7
Phenol medium.	8
Nutrient agar.	14
Coke oven effluent medium.	15

4.3 Procedure for isolating thiocyanate destroying bacteria using inorganic media.

Two series of subcultures were made,using bacteria derived from the 13 litre continuous enrichment culture treating ammonium thiocyanate. Each series was obtained as follows: 10 ml of flocculated bacteria from the aeration tank was dispersed in a pyrex glass conical tube, using a PTFE coated glass rod. The dispersed bacteria were diluted in sterile quarter strength Ringer solution. Bacteria were streaked from the 10^{-4} dilution,

onto thiosulphate minerals agar, medium 1. This medium was chosen for the first subculture because thiosulphate grown colonies were usually larger and more clearly isolated that colonies grown on thiocyanate minerals agar, medium 3. The plates were incubated at 30°C for 5 days. A series of subcultures was then made, at five day intervals, alternately to solid and liquid medium 3. Fifteen subcultures were made in each series. At each stage the cultures were tested for the ability to grow on nutrient agar and to deposit elemental sulphur on medium 1. Additional diagnostic tests were carried out on isolates from the 9th and 15th subcultures.

4.4 Methods of testing isolates.

In view of work by Stafford et al (1969) and Mather (1971), it was considered that some of the heterotrophic isolates obtained in the present study might belong to the genera *Pseudomonas* or *Achromobacter*. The origin of the bacteria from domestic sewage containing river-water and drainage from soil suggested that the initial tests should also enable the family *Enterobacteriaceae* and species of *Aeromonas* to be recognised. In the choice of diagnostic tests, advice was therefore gained from Dr Collins (1972) at the Freshwater Biological Association. Seven test recommended by Dr Collins were used in the present investigation. These tests included motilty, flagella, fluorescence, penicillin sensitivity, oxidase reaction, oxidation or fermentation of glucose and arginine utilisation.

Additional tests used to distinguish between species of *Pseudomonas* were selected from those described by Stanier et al (1966). These tests included the formation of phenazine pigments, gelatin liquefaction, denitrification and growth at 4° and 41°C. Resistance to 0.3% - 0.1% cetrimide was also used as this

ability has been attributed to certain pseudomonads, particularly Pseudomonas aeruginosa (Lowbury, 1965).

4.4.1 Stock cultures.

Isolates were compared with stock cultures of bacteria possessing known characteristics. The stock cultures used were: Thiobacillus thioparus (N.C.I.B. 8370), Pseudomonas stutzeri (N.C.I.B. 10331) and Pseudomonads fluorescens. Stock cultures were subcultured to fresh media at intervals of two weeks, and were incubated at 30°C.

4.4.2 Gram reaction and flagella.

Some isolates, 3a and 3b, showed Gram-variability after 24h growth, so results were recorded where possible between 18h and 24h growth.

Flagella.

The method of Rhodes (1958) showed flagella in some of the isolates, but the technique finally adopted was to examine the bacteria by electron microscopy.

Only young cultures, not more than 24h old, were used to demonstrate the flagella because on ageing the flagella become more easily detached from the cells. Where slime-layers were formed, this occurred more in ageing cultures. The presence of a slime-layer prevented the penetration of staining reagents so that the outline of the cells and flagella was not clear. When a slime-layer was present, it often included deposits from the medium. The obscuring effect was increased by rapid expansion and distortion of the slime-layer when the sample was brought under vacuum. Before preparing the bacteria for electron microscopy, it was therefore useful to test for the presence of a slime-layer or capsule by negatively staining with India ink and observing under the light microscope at X 1200. Crystalline deposits were also formed under vacuum when quarter strength

Ringer solution was used to suspend the bacteria. This problem was avoided by suspending the cells in sterile distilled water.

Correct preparation of the carbon + collodion support-film for the bacteria was important. Collodion was used to overcome the brittleness of the thin carbon film. A layer of collodion in amyl acetate was placed on the surface of distilled water in a beaker. Copper grids, 2 mm, 200 mesh, were washed in distilled water and placed, mat-surface down, onto the surface of the layer of collodion. A strip of paper was then placed carefully on top of the grids. The paper strip was then lifted off with the grids and collodion adhering to it. The paper + grids + collodion was inverted so that the paper supported the grids which in turn were covered by the layer of collodion. The preparation was then coated with carbon under high vacuum, beneath an electric arc passing between two carbon rods. A current of 50 amps was used. A layer of carbon, 100Å thick, allowed the passage of electrons, and was sufficiently strong to form an unbroken supporting layer for the bacteria.

When cultures in liquid media were used, the cells were washed three times, centrifuging at 4000 rpm for 5 minutes. The cells were resuspended in sterile distilled water. When cultures on solid media were used, cleaner preparations were obtained when sterile membrane filters were used to separate the bacteria from the agar during incubation.

A sample of the freshly grown culture was mounted in a 0.05 ml drop of sterile distilled water on a clean glass slide. Sufficient bacteria were added to give a visible turbidity. Three drops of a 4% solution of uranyl acetate in distilled water were added to the suspension which was left for five minutes in a moist container to prevent drying. Uranyl acetate served as an electron-dense negative stain and aided fixation

of the specimen.

Several drops of the stained suspension of bacteria were applied to each carbon + collodion coated grid. The aim was to load the grid with a uniform distribution of 50-100 cells per grid-square. A 2% solution of egg albumen was sometimes used to cause the bacteria to adhere more strongly to the layer of carbon.

Factors influencing the quality of the preparation included: age of culture, thickness of carbon film, intensity of negative staining, density and distribution of cells on the grid. Consequently, in order to obtain a satisfactory specimen illustrating the number and position of insertion of flagella, several preparations were usually required; see Fig. 13 , page 91 .

4.4.3 Biochemical tests and resistance to penicillin, cetrimide.

Oxidation/fermentation of glucose.

Carried out according to the method of Hugh and Leifson (1953). The sensitivity of the test was improved, when testing isolate 5, by replacing bromothymol blue by bromocresol purple.

Arginine metabolism.

According to Thornley (1960).

Oxidase.

According to Kovacs (1956).

Penicillin sensitivity.

Penicillin discs (Oxoid) 2.5 u and 5.0 u were placed on nutrient agar which had been inoculated with a pure culture of the bacteria. Plates were incubated at 25°C for 5 days and the zone of inhibition was noted.

Resistance to cetrimide.

Resistance to 0.03% - 0.1% cetrimide was used as a supplementary test in the identification of pseudomonads; details are given in section 5.8.

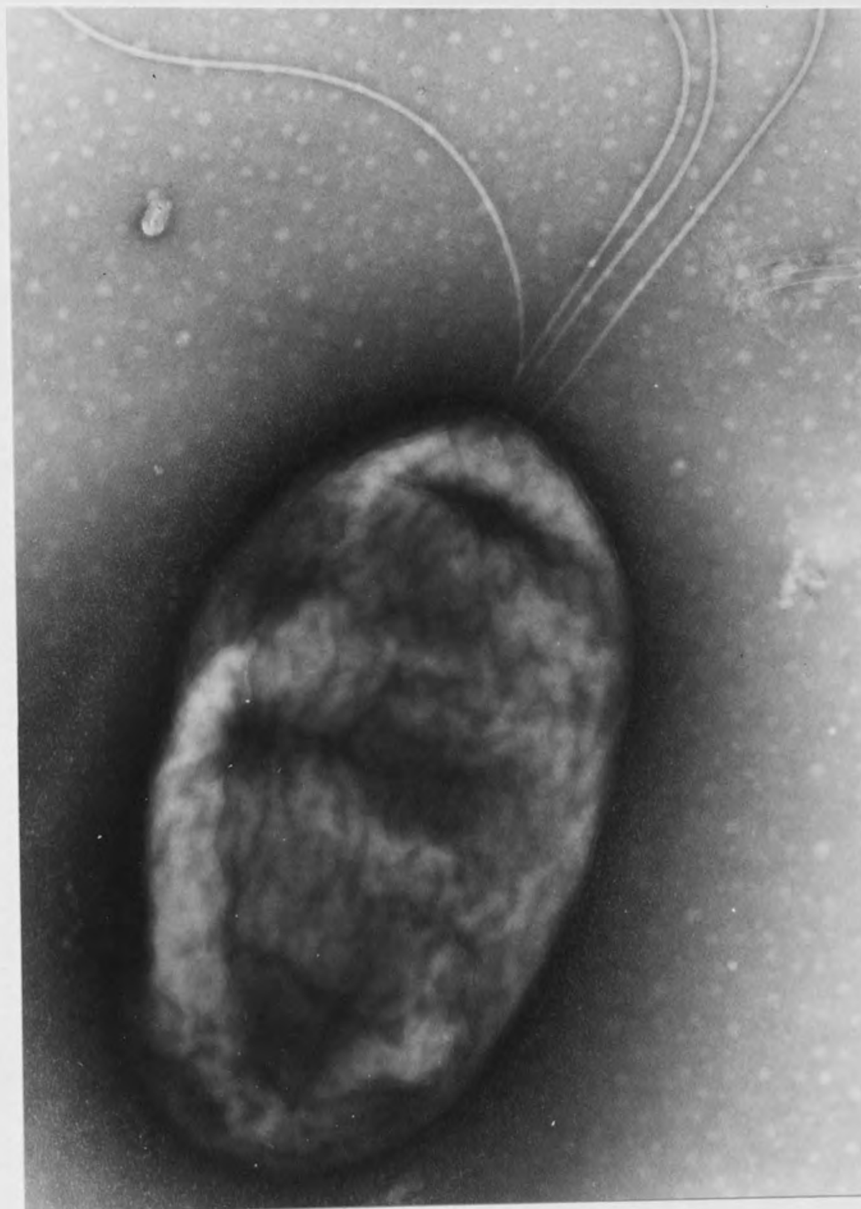


Figure 13.

Pseudomonas putida, isolate 4, showing four flagella inserted at one pole of the organism.
Electron micrograph: X 12,000.
Negatively stained with 4% uranyl acetate.

Formation of pigments.

Fluorescein:

Isolates were streaked onto King's medium B, (King et al, 1954) and incubated at 22°C for up to 7 days. Fluorescence was observed under ultra violet light at 350 mμ.

Phenazine pigments:

Isolates were streaked onto King's medium A and incubated at 22°C for up to 14 days.

Denitrification.

The bacteria which were tested had been isolated from an aerobic environment in the biological treatment plant, where the ability to denitrify might have been diminished or lost. In order to select for denitrifying ability the technique described by Stanier et al (1966) was used. This technique involved semi-aerobic culture for 24h at 30°C, followed by strictly anaerobic culture for 5 days at 30°C. The medium used in both stages of the test was yeast extract supplemented with 1% potassium nitrate and 0.1% Ionagar (Oxoid). A positive result was shown by the formation of turbidity and nitrogen gas which was trapped beneath an overlay of Ionagar.

Anaerobic reduction of nitrate in the presence of thiosulphate.

Medium: 300 ml peptone water containing $Na_2S_2O_3.5H_2O$, 0.1 g, KNO_3, 0.6 g. Distributed to bottles, completely filled, screw-capped, each containing an inverted Durham tube. The medium was tyndallised. After inoculation the bottles were incubated at 25°C for 14 days. Tests were then made for nitrite and nitrogen gas.

Gelatin Liquefaction.

Tubes of 10 ml nutrient gelatin were stab-inoculated and incubated at 25°C for 3 weeks. The tubes were cooled to 8°C before observing for liquefaction.

Deposition of elemental sulphur.

Intracolonial deposits of elemental sulphur were observed microscopically at X 100, in transmitted light, on plates of inorganic thiosulphate agar (medium 1, page 201) incubated for up to 3 weeks at 28°C. Sulphur was also detected by extraction into carbon disulphide, followed by evaporation of the extract. Yellow crystals of elemental sulphur were deposited. Further tests to aid the identification of species of Thiobacillus were selected from the scheme described by Hutchinson et al (1969).

4.4.4 Determination of thiocyanate destruction.

Liquid medium 7 was modified to determine conclusively if isolated bacteria could oxidise thiocyanate. The modification was to include 25ppm thiocyanate in the liquid media, instead of 152ppm CNS. This was to avoid the possible inhibitory effect of high concentrations of thiocyanate, to allow for a lag period of slow thiocyanate destruction by pure cultures, and to enable small changes in thiocyanate concentration to be measured more accurately. The liquid cultures were aerated using compressed air which was sterilised by passage through platinised asbestos packed into a silica tube in a furnace at 1000°C. This removed any possible traces of volatile organic compounds which might stimulate heterotrophic growth in the liquid media. The air was then passed through a membrane filter assembly (Sartorius Ltd). The apparatus is shown in Fig 14. Precautions were also taken to eliminate contaminating organic substances from the pure cultures inoculated into the sterile liquid media. This was done by inoculating a pure culture, grown for 18 hours at 28°C on a solid medium, using a platinum wire point, instead of a wire loop. The volume of liquid medium inoculated was 200 ml. This provided a volume sufficient to dilute to a negligible concentration any traces of organic substances which might still

Fig.14.

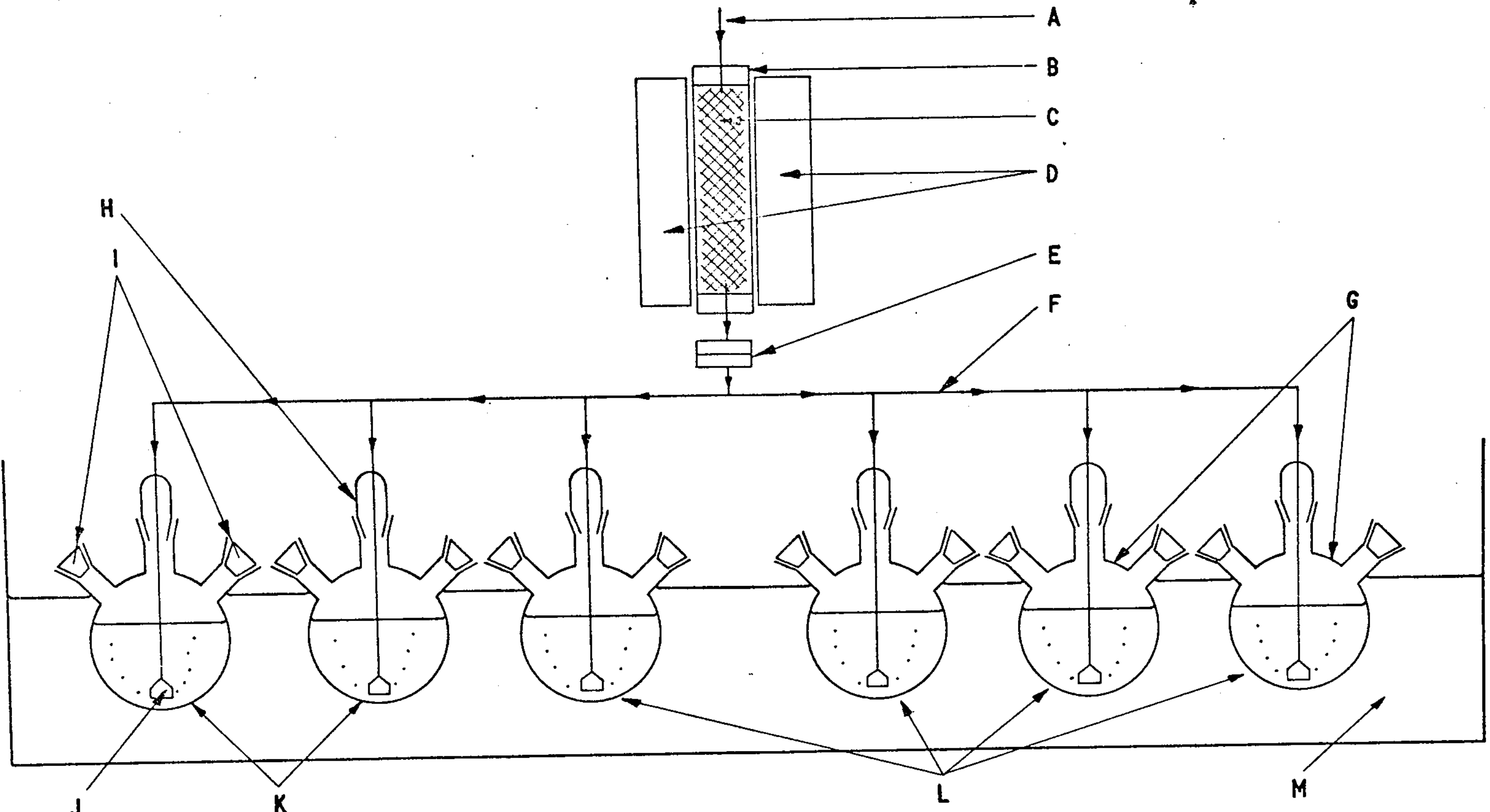


DIAGRAM OF BATCH CULTURES USED TO TEST FOR THIOCYANATE OXIDATION BY PURE CULTURES

A: Compressed air, B: Silica tube, C: Platinised asbestos, D: Furnace at 1,000 °C, E: Membrane filter assembly (Sartorius Ltd), F: Pyrex glass manifold, G: Pyrex glass culture flasks with two sampling ports, H: Quickfit cone + socket joint, I: Silicone sponge plugs, J: Sintered glass sparger, K: Uninoculated sterility testing liquid medium, L: Inoculated liquid culture media containing thiocyanate, M: Water-bath at 30°C.

have contaminated the medium and allowed samples to be taken frequently for accurate determinations of thiocyanate-concentration. 5 ml samples of the liquid cultures were filtered and the supernatant was acidified with 1 ml HCl acid (S.L.R.) 0.5 ml iron alum was then added and the intensity of red colour, due to ferric thiocyanate was determined spectrophotometrically, at 458 mμ. The cultures were incubated in 250 ml round bottomed flasks, each with three cone and socket 'Quickfit' ports. Sterility-testing liquid medium, Oxoid nutrient broth No. 2, and liquid medium 3, were aerated in parallel with the inoculated culture media. All the flasks were incubated at 28°C, in a water bath, see Fig 14.

4.4.5 Tests using washed resting cell suspensions.

The aim of these tests was to assess enzyme-activity without the influences of products of metabolism, dead or lysed cells, constituents of the growth medium or growth of the culture. If washed resting cell suspensions were capable of destroying thiocyanate to an appreciable extent, e.g. 50% (+), in a short period of two hours, this was evidence that the cells possessed an active enzyme or enzyme-system for using thiocyanate.

A pure culture of the isolate under test was activated by growth in a liquid medium containing thiocyanate. The medium was potassium thiocyanate + disodium succinate, medium 7. In this medium thiocyanate served as the sole source of nitrogen. Tests were also carried out to find if isolates could destroy thiocyanate as the sole source of carbon and nitrogen. This was done by omitting the disodium succinate from the medium. When the culture was in log growth after 18h - 24h at 28°C in a shaking bath, the cells were quickly washed by centrifuging and resuspended in the basal mineral salts liquid medium lacking thiocyanate. Cells in this state had maximum potential enzyme

activity but were maintained temporarily in a resting, non-growing condition. Thiocyanate was added to activate the enzyme. Enzyme-activity was assessed in terms of the rate at which thiocyanate was destroyed over a period of up to two hours.

Basal mineral salts liquid medium, medium 7 lacking thiocyanate and succinate, was prepared and dispensed to capped flasks in precise volumes of 50 ml in 250 ml flasks. This avoided any great depth of medium which would permit deoxygenation during log. growth. Stock solutions of potassium thiocyanate, 400 mg CNS/litre, and disodium succinate, 6.7 g/litre, were sterilised separately.

Young 18h - 24h cultures were subcultured 18h - 24h before the start of the test to 250 ml flasks containing liquid medium 7, and grown for 18h - 24h at 28°C. If a culture became very turbid during this period, indicating that it may have passed beyond the log phase of growth, it was re-activated by aseptically adding 50 ml liquid medium 7. The culture was then aseptically divided approximately equally between the two flasks and returned for a further 2h - 3h to regain a moderate turbidity.

After 18h - 24h the activated culture was distributed equally betweeen four centrifuge tubes. The cells were washed by centrifuging at 10,000 rpm for 10 minutes in a M.S.E. super-speed refrigerated centrifuge at 4° - 8°C. If the temperature had been allowed to rise during centrifuging, cell-lysis might have occurred. After centrifuging, the supernatant liquid was decanted and discarded. Each bouton of cells was resuspended in a small quantity of basal mineral salts medium.

The contents of all four centrifuge tubes were transferred to one, and the volume was made to 25 ml with basal medium. The cells were spun down as before and resuspended in 10 ml of basal medium.

The 10 ml of washed resting cell suspension was divided into four equal parts of 2.5 ml. Each 2.5 ml volume was pipetted into a separate 100 ml conical flask. Equal volumes of basal medium were added and the flasks were capped and placed in the shaking bath to equilibrate for 5 minutes at 28°C. 0.5 ml of the stock potassium thiocyanate solution was pipetted to each flask giving a concentration of 50 mg CNS/litre. One flask was removed immediately and placed for 5 minutes into boiling water to provide a killed cell control. The three other flasks were removed at 15 minute intervals during a two hour test period.

The cells were killed by boiling as in the control, and thiocyanate was determined spectrophotometrically in the supernatant liquid obtained by centrifuging the cells at 10,000 rpm for 15 minutes.

4.5 Results.

4.5.1 Thiobacilli : isolates 1 and 2.

During the isolation sequence in inorganic media, it was found that growth on thiocyanate minerals agar, medium 1, page was usually slow and resulted in very small 0.2 mm to 0.8 mm diameter watery, circular colonies. The minute colonies were difficult to isolate and sometimes it was necessary to use a binocular microscope (X 40), in order to transfer them individually to fresh medium. Elemental sulphur was not often deposited on medium 3, even after 2-3 weeks. Only a slight turbidity was produced in liquid inorganic CNS medium (3).

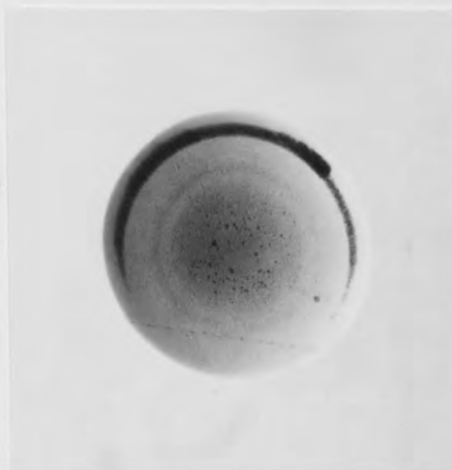
When grown on thiosulphate minerals agar, medium 1, two types of colony usually developed, within 5 days at 30°C. One form was similar in appearance to the colonies grown on medium 3, remaining small (up to 0.5 mm diameter) and showing no deposition of elemental sulphur. The other growth form produced

larger colonies which deposited elemental sulphur within the colonies; a feature of the thiobacilli. Considerable experience was required in order to distinguish, using transmitted light microscopy (X 100-150), between black crystalline deposits of elemental sulphur shown in Figs. 15, 16 and 17, and slightly paler brown deposits shown in Fig. 18, page 102. Hutchinson et al (1969) concluded that similar brown deposits consisted of sodium sulphate. In the present investigation the colonies showing the brown deposit also gave (i) moderate or rapid growth on nutrient agar and (ii) some of them also fluoresced on King's medium B. These two tests for heterotrophy and pigment-formation assisted the procedure for the enrichment of the strictly autotrophic thiobacilli by enabling the presence of facultatively autotrophic sodium sulphate depositors to be recognised. A third test found to be of value in the present study in confirming the persistence of bacteria capable of heterotrophic growth was the change in final pH which was caused when a pure culture was grown in minerals 1% thiosulphate liquid medium 1. The non-sulphur depositing facultative autotrophs caused a rise in pH from 6.8 to 8.0 - 9.0. In contrast to this, the sulphur depositing strict autotrophs caused the pH to fall, after 28 days at 28°C, to pH 4.0 - 5.5.

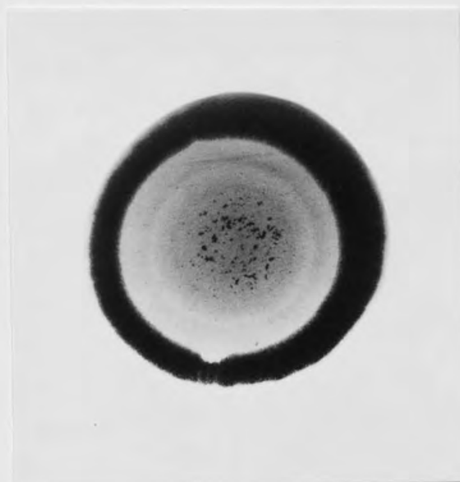
The use of the inorganic enrichment medium containing thiocyanate as the only source of added carbon, other than dissolved carbon dioxide and bicarbonate, did not eliminate non-thiocyanate destroying bacteria which were capable of heterotrophic growth on nutrient agar. This demonstrated that tolerance of thiocyanate did not necessarily imply thiocyanate destruction. However, two facultatively autotrophic isolates, 4 and 5, were found to destroy thiocyanate.

The extent of sulphur deposition by isolated colonies on media 1 and 3 sometimes declined after successive subculturing

(i) 3 days



(ii) 5 days



(iii) 10 days

Figure 15 .

Thiobacillus sp: Isolated colonies on 1% thiosulphate minerals agar, (i), (ii) and (iii). Progressive stages in the deposition of elemental sulphur from thiosulphate. Incubation at 28°C.
Magnification X100. Transmitted light.
Origin: Laboratory scale plant treating coke oven effluent.

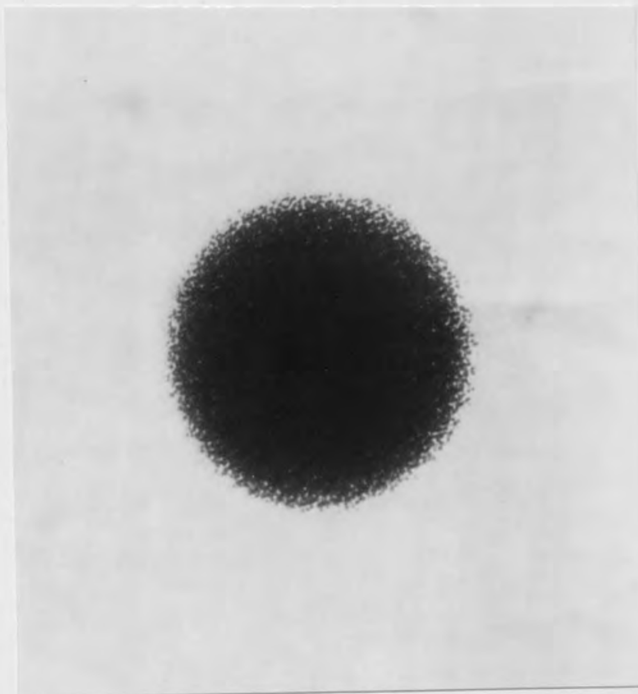


Figure 16 .

Thiobacillus sp: Isolated colony on 0.02% ammonium thiocyanate minerals agar. Black, granular intracolonial deposits of elemental sulphur formed from thiocyanate; a characteristic of T.denitrificans and T.thioparus, isolates 1 and 2. Age of culture, 10 days at 28°C. Magnification X150. Transmitted light.
Origin: Laboratory scale plant treating coke oven effluent.

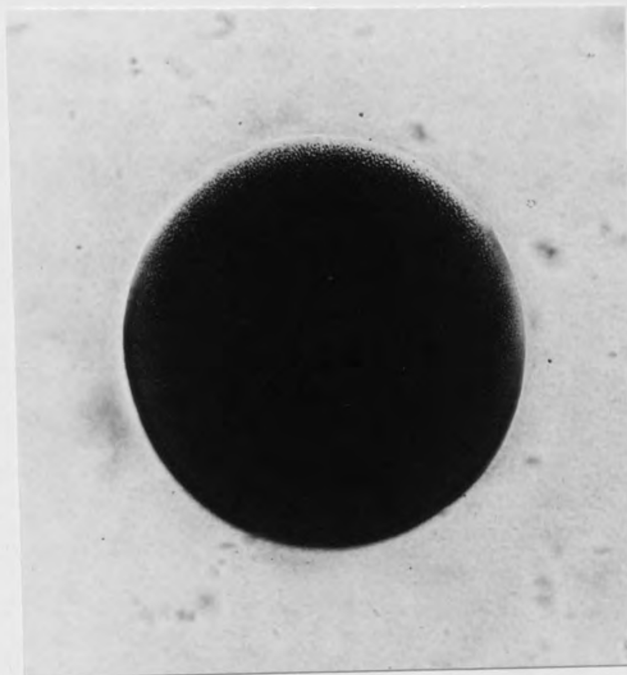


Figure 17 .

Thiobacillus sp: Isolated colony on 1% thiosulphate minerals agar, showing characteristic smooth margin of colony and dense granular intracolonial deposition of elemented sulphur. Age 28 days at 28°C. Magnification X100. Transmitted light. Origin: Laboratory scale plant treating coke oven effluent.

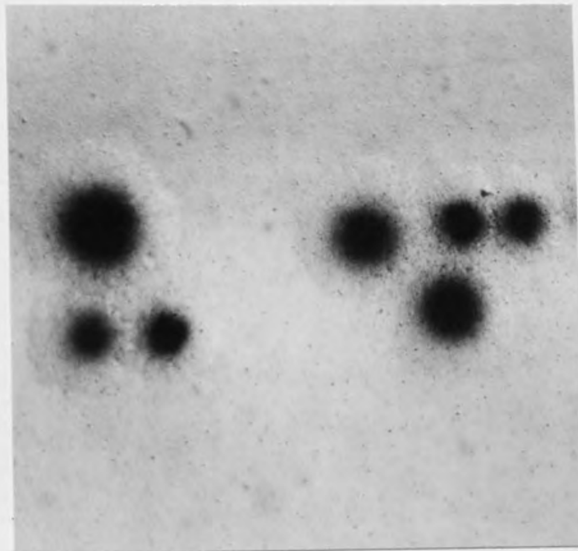


Figure 18 .
Facultatively autotrophic Pseudomonas sp. Colonies on 1% thiosulphate minerals agar, showing diffuse dark brown intracolonial deposits. Age 5 days at 28°C.
Magnification X50. Transmitted light.
Origin: Laboratory scale plant treating coke oven effluent.

had been carried out. This may have been due to an excessively long period of 2-3 weeks between subcultures. Furthermore, the ability of the sulphur depositing bacteria to grow in inorganic thiocyanate media was variable. One isolated colony, isolate 1, showed the pattern of results given in Table 14 , page 104.

Isolate No. 1:

The following features aided the identification of isolate 1 as belonging to the genus Thiobacillus: Growth on minerals 1% thiosulphate medium to produce acidity and to deposit elemental sulphur. Failure to grow on nutrient agar indicated that the isolate was strictly autotrophic. The demonstration of strict autotrophy eliminated the possibility that the isolate might have been one of the facultatively autotrophic species of thiobacilli, e.g. T. novellus, or T. intermedius (Hutchinson, 1969). The ability of isolate 1 to oxidise thiocyanate in medium 3 identified it as either T. thioparus or T. denitrificans. In view of the ability of isolate 1 to reduce nitrate to form nitrogen gas in anaerobic culture identified the isolate as Thiobacillus denitrificans.

Isolate No. 2:

Isolate No. 2 resembled isolate No. 1 in the tests described above and given in Table 14, but isolate 2 did not denitrify to produce nitrogen gas. However in this test, isolate 2 caused an accumulation of nitrite at the expense of nitrate. Isolate No. 2 was therefore tentatively identified as Thiobacillus thioparus.

An exploratory test was carried out to determine the effect of formic acid on thiocyanate destruction by N.C.I.B. 8370, Thiobacillus thioparus. Formic acid was used because fluctuating concentrations of this compound enter Corby coke oven effluent. A concentration of 400 mg formic acid per litre was used in the

Table 14. Testing of Thiobacillus spp., isolates 1 and 2.

Tests	Isolate 1	Isolate 2	N.C.I.B. 8370
+ Destruction of thiocyanate in medium 3*	+	+	+
+ Growth in thiosulphate medium 1*: (i) final pH (ii) intracolonial sulphur deposition on the solid medium	5.0 +	4.3 +	4.2 +
Growth on nutrient agar	-	-	-
+ Anaerobic growth in thiosulphate + nitrate broth	+	-	-
Gram stain	-	-	-
Motility	+	+	+
Cell size	Rods, 0.6 μ x 1.0 - 1.6 μ	Rods, 0.6 μ x 0.8 - 1.8 μ	Rods, 0.7 μ x 1.0 - 1.6 μ

* Details of media given in Appendix, page 201.
+ Tests continued for 28 days at 28°C.

test because rapid increases of this order had sometimes been recorded (Hall, Corby Works, 1975). Laboratory scale tests using batch mixed cultures of activated sludge showed, in the present study (page 79), that formic acid was destroyed rapidly as it entered the culture. Nevertheless knowledge of the possible effects on T. thioparus caused by shock doses of formic acid was considered to be valuable.

A freshly grown, 5 days at 28°C, unflocculated culture of N.C.I.B. 8370 was prepared in thiosulphate minerals medium 1. A 10µl volume of this culture was dispensed aseptically to each of forty one-ounce loosely screw capped bottles, each containing 5 ml of thiocyanate minerals medium 3. Surface viable counts on solid medium 1 showed that approximately 120 viable cells were inoculated using each 10 µl drop, dispensed from a micrometer syringe. The initial concentration of thiocyanate was adjusted to 50 mg per litre to allow small changes in the concentration to be detected readily. 400mg of formic acid per litre was included in twenty of the bottles of medium 3. The inoculated bottles were incubated, unshaken, at 28°C for 30 days. Tests on each culture were carried out at intervals of five days to determine the surface viable count and the concentration of thiocyanate. The results are summarised in Table 15. The initial concentration of 400mg formic acid per litre suppressed thiocyanate destruction and caused lower viable counts to be recorded on medium 1.

N.C.I.B. 8370 was found to grow well in liquid CNS + succinate medium 7 after 4 days at 28°C; the viable count increased from 10^2 to 10^7 per ml. However CNS destruction was not detected during this period.

4.5.2. Achromobacter, isolate 3.

Two cultures were used, isolates 3a and 3b. Both formed pale yellow growth on nutrient agar, after being transferred as isolated colonies from the 9th and 14th subcultures to thiocyanate minerals medium 3. The two cultures were treated

Table 15. Testing of N.C.I.B. 8370: The effects of 400 mg formic acid per litre on viable colony counts and thiocyanate destruction.

Incubation at 28°C.

Days	* Medium 3		Medium 3 + 400 mg formic acid per litre	
	Mean CNS concentration, mg per litre	Surface viable count, per ml on medium 1 *	Mean CNS concentration, mg per litre	Surface viable count, per ml on medium 1 *
0	50	24	50	24
5	15	8.5×10^4	40	10^2
10	10	10^4	30	10^2
15	5	3×10^4	30	$< 10^2$
20	0	7×10^3	30	$< 10^2$
25	0	8×10^4	25	$< 10^2$
30	0	1.6×10^4	25	$< 10^2$

* Details of media given in Appendix on page 201.

separately, as follows: An isolated nutrient agar grown colony was transferred to 5 ml of sterile nutrient broth and grown for 24 hours at 30°C. Twelve tests were then made using the nutrient broth cultures and on a specimen of Pseudomonas fluorescens. Each test was carried out using six replicas. The results were consistent and are summarised in Table 16. The pattern of results for isolates 3a and 3b were characteristic of the Achromobacter group of species. Isolates 3a and 3b showed only slight growth on the coke oven effluent medium. The isolates failed to destroy thiocyanate autotrophically in medium 3, but showed slight thiocyanate destruction in the presence of succinate, in test 4.4.4.

4.5.3 Fluorescent Pseudomonads : isolates 4 and 5.

Isolate 4:

A 10 ml sample from the 4 litre batch NH_4 CNS enrichment was dispersed as described in section 4.3. A 0.1 ml sample of dispersed bacteria was inoculated to each of four pour plates of King's medium B. After 48 hours at 22°C, 12% of the colonies had produced a diffusible yellow-green pigment which fluoresced in ultra violet light as 350 mμ. The remaining colonies did not fluoresce. A fluorescent organism was isolated by streaking onto a second series of plates of King's medium B. A single, isolated fluorescent colony was then subcultured several times to different media, to determine firstly that a pure culture of the organism had been isolated and secondly to confirm the organism's ability to grow on the different media. The pattern of growth of isolate 4 is tabulated in Table 17, on page 109.

Freshly grown, 18h at 30°C, nutrient broth cultures of isolate 4 were tested in eight separate tests to confirm its ability to destroy thiocyanate. Results of these tests are summarised in Table 18, page 110. Details of one of the eight tests are given in Table 19 ,

Table 16. Testing of isolates 3a and 3b, Achromobacter sp.

Tests	Isolate 3a	Isolate 3b	*P. fluorescens*
Gram stain	Negative straight rods	Negative straight rods	Negative straight rods
Motility	+	+	+
Cell size	0.5 μ x 1-1.8 μ	0.5 μ x 1-1.8 μ	0.5 μ x 1-1.9 μ
Flagella	Pentrichous	Peritrichous	Polar
Oxidase	+	+	+
Oxidation/ Fermentation of glucose.	Oxidative only	Oxidative only	Oxidative only.
Utilisation of arginine	-	-	+
Fluorescence	-	-	+
Growth on 1% S_2O_3 agar, medium 1.	+	+	-
Growth on 6% S_2O_3 agar.	+	+	-
Growth on 0.02% NH_4CNS agar, medium 3	+	+	-
Penicillin	Sensitive	Sensitive	Resistant

Table 17. Growth of isolate 4, Pseudomonas putida, on six different media.

Incubation at 28°C for 14 days.

Agar media	Number of plates inoculated	Number of plates on which the isolate grew	Nature of Growth	Deposition of Elemental Sylphur
1% S_2O_3	8	8	Watery or dull white, 1-2 mm. Rapid growth.	-
6% S_2O_3	3	3	Watery or dull white, 2 mm became brown after 21 days. Rapid growth.	-
0.02% NH_4CNS	20	20	Watery or dull white 1-2 mm.	-
Thiocyanate + succinate	12	12	Rapid growth, 2-3 mm. Diffusible yellow-green fluorescent pigment.	-
0.047% phenol	4	4	Pinpoint watery colonies 1 mm.	-
Coke oven effluent	3	1	Pinpoint watery colonies 1 mm.	-

Table 18. Tests for thiocyanate destruction by isolate 4.

Initial concentration of CNS = 25 mg/l in all flasks.

Incubation at 28°C.

Test No.	Substrate	Thiocyanate destroyed		Period of test days.	
		in CNS + succinate medium	in inorganic CNS medium	Total	For induction of CNS destruction
1	NH_4CNS	+	-	7	3
2	NH_4CNS	-	-	11	Failed
3	KCNS	-	-	5	Failed
4	NH_4CNS	-	-	4	Failed
5	NH_4CNS	-	-	7	Failed
6	NH_4CNS	+	-	13	6
7	NH_4CNS	+	-	22	20
8	KCNS	+	-	8	6

Table 19. Test for thiocyanate destruction by isolate 4 in inorganic and organic media.

	Nutrient broth Control. Sterile	Liquid inorganic CNS (medium 3) Control. Sterile			Liquid Inorganic CNS medium 3						Liquid CNS + succinate medium 7					
					Replica 1			Replica 2			Replica 1			Replica 2		
Hours	Turbidity	Turbidity	CNS	pH	Turbidity	CNS	pH	Turbidity	CNS	pH	Turbidity	CNS	pH	Turbidity	CNS	pH
0	-	-	20	6.7	-	20	6.7	-	20	6.7	-	20	6.7	-	20	6.7
2	-	-	20	6.7	-	20	6.7	-	20	6.7	-	20	6.7	-	20	6.7
24	-	-	20	6.7	-	20	6.7	-	20	6.7	Slight	10	6.7	-	18	6.7
67	-	-	20	6.7	Slight	20	6.7	Slight	20	6.7	Opaque White	0	6.7	Opaque White	12	6.7
91	-	-	20	6.7	Slight	20	6.7	Slight	20	6.7	Added 30ppm CNS			Opaque White	10	6.7
115	-	-	20	6.7	Slight	20	6.7	Slight	20	6.7	Opaque White	0	7.4	Opaque White	0	7.2
122	-	-	20	6.7	Slight	20	6.7	Slight	20	6.7	Added 30ppm CNS			Added 30ppm CNS		
124	-	-	20	6.7	Slight	20	6.7	Slight	20	6.7	Opaque White	20	7.5	Opaque White	26	7.3
139	-	-	20	6.7	Slight	20	6.7	Slight	20	6.7	Opaque White Adjusted pH to 6.5	20	9.0	Opaque White Adjusted pH to 6.5	20	8.5
165	-	-	20	6.7	Slight	20	6.7	Slight	20	6.7		20	8.5		20	8.0

page 111. Isolate 4 was found conclusively to destroy thiocyanate in four of the eight tests, but substantial growth and thiocyanate destruction occurred only in the media containing succinate. It is possible that thiocyanate may have been destroyed in tests, 2, 3, 4 and 5, in Table 18, if a longer induction period had been allowed. Growth consistently developed in the inorganic thiocyanate medium to produce a slight turbidity, although no detectable drop in the concentration of thiocyanate occurred. Surface drop viable counts on solid medium 7 showed that a cell density of approximately 10^7 per ml was normally required before thiocynate destruction was detected. Strict control of the pH was necessary. In the thiocyanate + succinate medium the pH rose from 6.7 to 9.0 during thiocyanate destruction. Above pH 7.5 the rate of destruction of thiocyanate was suppressed.

Diagnostic tests were carried out using a nutrient broth culture of isolate 4 grown for 24h at 30°C. Isolate 4 was found to have the characteristics tabulated in Table 20, page 113. The results showed that isolate 4 bore a close resemblance to Pseudomonas putida, as described by Stanier et al (1966). Electron microscopy confirmed the presence of up to four polar flagella, shown in Fig. 13 , page 91.

Additional tests were carried out on isolate 4 which had been re-isolated on King's medium B as a single colony, derived from one of the flasks used to test for thiocyanate destruction. The additional tests which are given in Table 21, on page 114, confirmed the purity of the culture and the ability of isolate 4 to grow on media containing thiocyanate or thiosulphate. Growth was rapid and usually fluorescent on thiocyanate + succinate agar, medium 7. On inorganic thiocyanate agar, medium 3, growth was slow and always non-fluorescent growth occurred on both 1% thiosulphate agar, medium 1, and on 6% thiosulphate agar.

Table 20. Testing of fluorescent pseudomonads, : isolates 4 and 5.

Tests	Isolate 4	Isolate 5
Gram stain	-	-
Motility	+	+
Cell size	Rods 0.4 µ x 0.8 - 1.5 µ	Rods. 0.6 µ x 1.0 - 2.0 µ
Flagella	Polar. Between 2 and 4 inserted at one end of cell.	Polar. Between 2 and 4 inserted at one end of cell.
Oxidase	+	+
Oxidation/fermentation of glucose	Oxidative only.	Oxidative only.
Acid formed from: glucose lactose	 + +	 + +
Utilisation of arginine	+	+
Penicillin	Resistant	Resistant
1% cetrimide	Resistant	Resistant
Fluorescein	+	+
Phenazine	-	-
Gelatin liquefaction	-	-
Growth at: 4°C 17°C 41°C	- + -	- + -
Denitrification	-	-
Thiocyanate destruction: in medium 3* in medium 7*	 - Variable (±)	 - +

* Details of media given in Appendix on page 201.

Table 21 . Characteristic growth of isolate 4 on five different media.

Incubation at 28°C.

Agar media	Growth
0.02% NH_4CNS	Slow, non-fluorescent.
CNS + succinate	Rapid, fluorescent.
1% S_2O_3	Moderate, non-fluorescent, brown intracolonial deposit, no elemental sulphur
6% S_2O_3	Moderate, non-fluorescent, brown intracolonial deposit, no elemental sulphur.
King's B	Rapid, fluorescent.

Table 22 . Growth of isolate 5 in seven different liquid media.

Cultures tested daily for 14 days.
Incubation at 28°C in 10 ml static cultures.

Liquid Media	Results
1% S_2O_3, medium 1	Rapid growth with flocculation. S_2O_3 destroyed with increase in pH from 6.7 to 9.0.
6% S_2O_3	As above.
0.02% NH_4CNS, medium 3	Slow growth, slight visible turbidity, no flocculation, no detectable CNS destruction.
CNS + succinate*, medium 7.	Rapid growth, with flocculation, complete destruction of CNS
CNS + glucose	Rapid growth with flocculation, complete destruction of CNS.
CNS + 470 mg phenol/l	Rapid growth with flocculation, simultaneous destruction of CNS and phenol
470 mg phenol/l, as sole carbon source. No CNS present.	Rapid growth, with flocculation, complete destruction of phenol.

* In this medium the following substances, when tested separately, had no detectable suppressing effect on CNS destruction:

50 mg NH_3/litre as NH_4Cl

450 mg Cl/litre as $CaCl_2$

Isolate 5:

Fluorescent pseudomonads were detected in large numbers, greater than 10^7 per ml of activated sludge, in the laboratory scale plant, described in section 3.1.1., a 4 litre fill and draw culture and in the fullscale biological treatment plant at Corby. All three systems were treating coke oven effluent. The monitoring of fluorescent pseudomonads in treatment plants is described in section 5.7. Isolate 5, a fluorescent pseudomonad derived from the fill and draw culture, section 3.1.1, was found to grow rapidly on thiocyanate + succinate agar, medium 7. The rate of growth of isolate 5 on this medium was consistently greater than that of isolate 4. Furthermore, isolate 5 was found to destroy thiocyanate rapidly in the presence of succinate, in six consecutive tests. Again the test method was that described in section 4.4.4. Further tests showed that isolate 5 destroyed thiocyanate when succinate was replaced by glucose, or phenol at a concentration of 470 mg/l.. Thiocyanate and phenol were destroyed simultaneously. Isolate 5 gave a similar pattern of results as isolate 4, i.e. it closely resembled _Pseudomonas putida_. However, whereas the ability of isolate 4 to destroy thiocyanate was variable, thiocyanate destruction was a consistent feature of isolate 5.

The possibility that isolate 5 might contribute to thiocyanate destruction in the biological treatment plant was considered to be likely in view of its ability to destroy thiocyanate and phenol simultaneously. Following this observation, the influence of other constituents of coke oven effluent on thiocyanate destruction by isolate 5 was investigated. Growth tests using seven different liquid media are summarised in Table 22. Isolate 5 also destroyed thiocyanate in 100 ml flasks containing 50 ml of sterile activated sludge and 100 mg phenol per litre.

Isolate 5 grew autotrophically on 1% or 6% thiosulphate causing a rise in the pH of the medium. However, negligible growth occurred in the basal minerals medium containing ammonium thiocyanate. Rapid growth occurred in all the organic media. In medium 7 thiocyanate was destroyed in the presence of ammonia at 50 mg/l, and chloride at 450 mg/l as calcium chloride.

Following these exploratory tests, washed resting cell suspension of isolate 5 were prepared to confirm the presence of an active enzyme for thiocyanate destruction. Tests using washed cells were also used to obtain more precise information on the effects of individual coke oven effluent constituents. The constituents tested were phenol, catechol, ammonia, aniline and pyridine. A refrigerated centrifuge, see section 4.4.5, was not available at Corby. Therefore the effects of additives on thiocyanate destruction by washed cells were investigated by Mr A Antzoulatos, under the supervision of Mrs I Williams in the Department of Biological Sciences, University of Aston in Birmingham. Details of this investigation are given in Mr Antzoulatos' M.Sc. project report, December 1974. The concentrations of additives included in the washed cell suspensions were the highest concentrations likely to be encountered in the biological treatment of Corby coke oven effluent. Effects on the growth of isolate 5 were estimated using Miles and Misra surface drop viable counts on thiocyanate + succinate agar, medium 7.

Phenol at concentrations up to 400 mg/l had no apparent effect on the growth rate of isolate 5, but thiocyanate destruction wasdelayed. Phenol and thiocyanate were destroyed simultaneously. Catechol at 100 mg/l stimulated growth and thiocyanate destruction. Catechol and thiocyanate were destroyed simultaneously. Ammonia at 300 mg/l caused substantial suppression of thiocyanate

destruction, but complete inhibition was not observed. Ammonia and thiocyanate were destroyed simultaneously. Aniline and pyridine, each at 10 mg/l, had no effect on growth or thiocyanate destruction when tested separately. Isolate 5 was found to utilise phenol and catechol, but aniline and pyridine were not utilised.

4.5.4 Micrococcus, isolate 6.

During the procedure for isolating autotrophic thiocyanate destroying bacteria, section 4.3, it was difficult to eliminate organisms which could grow on heterotrophic media such as nutrient agar. One of the heterotrophs, isolate 6, formed bright yellow, circular, rough edged colonies, 1-2 mm in diameter on thiocyanate + succinate agar, medium 7. Isolate 6 was subcultured several times to the different media listed in Table 23 . Isolate 6 consistently grew on the inorganic and organic media containing thiocyanate and on the inorganic thiosulphate media. No growth was recorded on the coke oven effluent medium.

Isolate 6 had the following characteristics which aided its identification:

1. Gram positive cocci, 2 μ in diameter.
2. Cells grouped in cubical packets of eight cells, occasionally cells also grouped in pairs.
3. Aerobic.
4. Catalase-positive.
5. Penicillin-sensitive.
6. Heterotrophic; colonies on nutrient agar were yellow, circular rough edged and 1-2 mm in diameter.
7. The isolate was inert in Hugh and Liefson's test for the oxidation/fermentation of glucose.

Additional tests showed that isolate 6 destroyed thiosulphate

Table 23 . Growth of isolate 6 on six different media.

Incubation at 28°C

Agar Media	Number of plates inoculated	Number of plates on which the isolate grew	Nature of Growth
1% S_2O_3	6	6	Slow, 1 mm colonies.
6% S_2O_3	6	6	Slow, 1 mm colonies.
0.02% NH_4CNS	6	4	Slow, 0.5 - 1.0 mm colonies.
Thiocyanate + succinate	6	5	Rapid, yellow 2 mm colonies.
Nutrient agar	6	6	Rapid, yellow 2-3 mm colonies.
Coke oven effluent	6	0	No growth.

in medium 1, and showed a slightly greater amount of growth on thiocyanate + succinate agar than on the same medium lacking thiocyanate. However, thiocyanate was not destroyed in liquid inorganic or organic media using the test described in section 4.4.4.

4.5.5 Summary of results.

A search for bacteria capable of destroying thiocyanate in coke oven effluent treatment plants gave six isolates which grew on solid media with thiocyanate as the only carbon compound. The six isolates included two species of Thiobacillus, Micrococcus sp, Achromobacter sp, and two isolates of fluorescent pseudomonads.

When grown in pure, unshaken batch cultures, N.C.I.B. 8370 T. thioparus, showed variability in the extent of thiocyanate oxidation, although complete destruction of thiocyanate by all replicas was achieved after prolonged incubation for 28 days at 28°C. Viability and thiocyanate destruction by N.C.I.B. 8370 were both suppressed in batch culture by 400 mg of formic acid per litre.

The fluorescent pseudomonads were confirmed to destroy thiocyanate in the presence of succinate; however thiocyanate was not destroyed in media lacking an organic source of carbon. Washed cell-suspensions of one of the fluorescent bacteria, isolate 5, destroyed thiocyanate rapidly in the presence of succinate. The effects of individual constituents of coke oven effluent on thiocyanate-destruction by this isolate were determined. Isolate 5 destroyed thiocyanate and phenol simultaneously.

An inorganic thiocyanate medium supported slow growth of the fluorescent pseudomonads and the Micrococcus sp., even though thiocyanate was not destroyed. The possibility was investigated that growth was supported by volatile organic compounds in the

air or traces of organic substances in the inoculum. The inorganic thiocyanate medium still supported slow growth without thiocyanate-destruction after precautions had been taken to remove these sources of contaminating organic compounds. Chemical tests were therefore essential to confirm that thiocyanate destruction had occurred. In the presence of succinate, thiocyanate destruction was not normally detected until a cell density of approximately 10^7 per ml had developed.

5. Monitoring of bacterial populations in treatment plants.

The role of the different thiocyanate destroying bacteria in biological treatment plants were not known at the beginning of this study. In view of the results of investigations by Hutchinson et al (1964) and Mather (1971) it was suspected that thiobacilli may have a minor role in thiocyanate-destruction particularly when undiluted coke oven effluents were being treated. The monitoring of populations of bacteria in the present study was done in order to show which genera were dominant in the laboratory scale and full scale systems. Bacterial counts were carried out on the laboratory scale plant at intervals of approximately ten days over a period of 393 days. A full scale biological treatment plant was also monitored during the first six months of its operation.

5.1 Sampling.

The following precautions were taken to obtain representative samples of activated sludge. Firstly, samples were always taken from the overflow weir of the aeration tank (12, Fig 2, page 35), in which complete mixing was continuously maintained. Secondly, the overflow weir was kept free from blockages. Thirdly, the tube (Fig 2 , 13) delivering sludge from the overflow weir into the settling tank was kept free from blockages. Fourthly, samples were tested immediately after collection to prevent changes in the numbers and proportions of bacterial species taking place.

The percentage of aeration tank-sludge settled after 30 minutes is a guide to the quantity of micro-organisms available for biological oxidation. However a further contribution to purification is made by micro-organisms remaining in suspension in the supernatant liquid. It was accepted that at high dilution-rates a high proportion of these suspended micro-organisms would be washed out of the system. Nevertheless, in order to

relate bacterial monitoring to overall plant-performance, both flocculated and dispersed bacteria were sampled.

Samples were collected in sterile 150 ml Pyrex glass stoppered bottles. The volume taken was 100 ml, leaving an air-space above the sample to prevent deoxygenation from occurring before the sludge was dispersed. 1% potassium dihydrogen orthophosphate was included to buffer against pH changes. This precaution was usually not critical because the samples were normally tested immediately after collection. When it was not possible to test samples immediately they were stored at $4^{o}C$. Bacterial counts were carried out as soon as possible after sampling.

5.2 Dispersion.

In order to obtain the highest viable counts, different methods were assessed to disperse the bacteria from the activated sludge flocs. The only method which was available in the laboratory at the beginning of this study was dispersion using a Kenwood mixer. This did not cause adequate deflocculation even at the highest speed. The Bell Cream Maker had been successfully used by Allen (1944) and James (1964). The most recent recommended methods used ultra sonic dispersion, Gayford et al (1970), Williams et al (1970). An exploratory test was done to find the effects of dispersion using a Bell Cream Maker and ultrasonics, on viable counts of fluorescent and non-fluorescent bacteria detected on King's medium B. The results given in Table 24 showed that the Bell Cream Maker caused a drop in viable counts compared with the undispersed sample. Ultrasonics increased the counts, the greatest count being obtained after 3 minutes sonication. It was possible that greater counts may have been obtained if a longer period of sonication had been used. Before the test, it was expected that a more prolonged sonication time might have disrupted the cells, giving a lower

Table 24 . Effects of different methods of sludge-dispersion on bacterial counts using King's medium B.

Treatment		Fluorescent pseudomonads	Non-fluorescent bacteria	Total bacteria
Ultra-sonics	10sec	1.6×10^6	2.0×10^7	2.2×10^7
	1 min.	2.2×10^6	4.0×10^7	4.2×10^7
	3 min.	3.0×10^6	7.4×10^7	10^8
Bell Cream Maker	5 min.	5.3×10^5	4.5×10^6	5.0×10^6
Untreated		1.1×10^6	9.8×10^6	1.1×10^7

The figures given are the means of duplicate counts on each sample using pour-plates. Viable counts were made after 3 days at 28°C.

count. In view of the results, longer sonication-times were tried in further tests.

10 ml of activated sludge from the laboratory scale plant treating coke oven effluent was placed in a boiling tube and sonicated for 30 minutes. During this period 1 ml volumes were removed at 5 minute-intervals using separate sterile pipettes. Each 1 ml volume was decimally diluted in quarter strength Ringer solution. The greatest dilution used was 10^6. 20 µl of each dilution was applied in triplicate, using the surface drop method (Section 5.5), to well-dried plates of five different media, see Table 25. Media 1 and 3 were chosen to show the effects of sonication on the growth of species of bacteria which respectively used thiosulphate and thiocyanate autotrophically. Medium 4 was used in order to complete the study of the effects of sonication-time on viable counts of fluorescent pseudomonads. Total heterotrophic bacteria were estimated using Plate Count agar (Oxoid CM 325). Detailed results, given in Table 25 and Fig. 19, showed that approximately tenfold increases in viable counts were obtained on most of the media after 5 minutes sonication. After 10 minutes fluorescent pseudomonads declined sharply while counts on the thiocyanate and thiosulphate media remained high. After 25 minutes sonication fluorescent pseudomonads were not detected and the counts on thiocyanate and thiosulphate media declined. The total counts on Plate Count agar were high between 5 minutes and 25 minutes. After 20 minutes sonication the temperature of the sample had risen from 28°C to 35°C and at 30 minutes the temperature was 40°C. Enzyme denaturation was considered to be likely at about 35°C or more. After 10 minutes the counts on all media were high. The bacteria which, in this sample, appeared most sensitive to ultrasonics were the fluorescent pseudomonads

Table 25 . Effect of different sonication times on surface drop bacterial counts.

Keys to types detected:

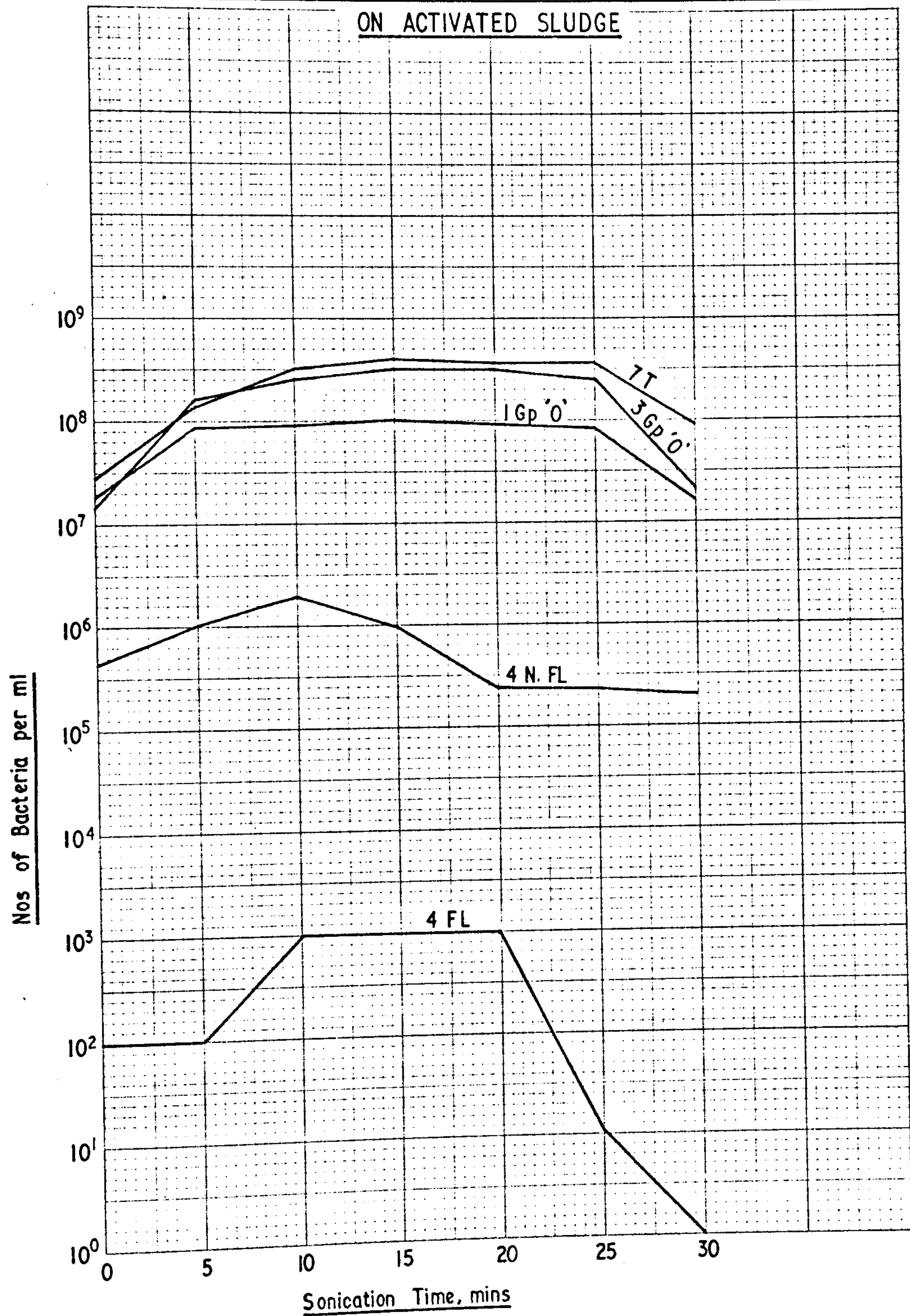
Details of media given in Appendix, page 201.

3.Gp '0'	=	non-thiobacilli, or 'trautwein '-types
7.T	=	total bacteria grown on KCNS + succinate
4.FL	=	fluorescent pseudomonads
4.N.FL	=	non-fluorescent bacteria on King's B
4.T	=	total bacteria on King's B
13.T	=	total bacteria on plate count agar.

Medium	Types Detected	* Sonication times, mins.						
		0	5	10	15	20	25	30
1	Gp'0'	2.7×10^7	9.3×10^7	9.4×10^7	10^8	9.7×10^7	9.3×10^7	3×10^7
3	Gp'0'	1.5×10^7	2×10^8	4×10^8	5×10^8	5×10^8	4×10^8	3×10^7
7	7.T	5.3×10^7	1.8×10^8	5×10^8	6×10^8	5.5×10^8	5.5×10^8	9.4×10^7
4	FL	10^2	10^2	10^3	10^3	10^1	ND	ND
	N.FL	6.4×10^5	10^6	3×10^6	10^6	3.9×10^5	3.7×10^5	3×10^5
	T.4	6.4×10^5	10^6	3×10^6	10^6	3.9×10^5	3.7×10^5	3×10^5
13	T.13	4.5×10^7	3.9×10^8	3.9×10^8	4.2×10^8	5×10^8	3.8×10^8	3×10^8

* Using an 'Ultra-cleen' Model 100., L.& R. Manufacturing Co., Kearny, N. Jersey.

FIG 19



detected on King's medium B.

Unfortunately, thiobacilli were not detected in the sludge sampled for use in this test. Knowledge of the sensitivity of thiobacilli in activated sludge to ultrasonics would have been valuable. Later tests using activated sludge obtained from Simon Hartley Limited showed that 10 minutes sonication was satisfactory for dispersing thiobacilli.

Based on the results of the test described above, an optimum sonication-time of 10 minutes was selected.

5.3 Dilution.

Initially dilutions were made using quarter strength Ringer solution. However it was possible that reaggregation of the dispersed bacteria occurred in this diluent. The diluent was therefore changed to sodium tripolyphosphate (5 mg/l) in deionised water autoclaved for 15 minutes at 121°C. This was in accordance with the procedure recommended by Pike et al (1972). Surface drop bacterial counts given in Table 26 showed that counts obtained using quarter strength Ringer solution were similar to those obtained using sodium tripolyphosphate. Nevertheless it was advisable to ensure against the possibility of reflocculation by retaining sodium tripolyphosphate as the diluent. Any delay either in preparing dilutions or in applying them to the media would have provided an opportunity for reaggregation to occur. The freshly dispersed samples of activated sludge were decimally diluted from 10^1 to 10^6.

Autoclaving sometimes caused a drop in pH of the 5% sodium tripolyphosphate solution from pH 6.8 to pH 4.7, possibly due to hydrolysis. The low pH caused a decrease in the counts of viable bacteria. It was therefore necessary to readjust the pH of the diluent to pH 6.8. This was done using sterile 10% caustic soda.

Table 26 . Comparisons between six different media for the enumeration of fluorescent pseudomonads and total bacteria.

Media	Types	Diluent	
		Sodium Tripolyphosphate	¼ strength Ringer soln.
4	FL	1.6×10^6	1.1×10^7
	T	9.5×10^7	1.5×10^8
5	FL	6.3×10^5	3.5×10^5
	T	3.8×10^6	2.3×10^6
6	FL	1.5×10^7	1.2×10^7
	T	9×10^7	9.8×10^7
7	FL	5.5×10^5	8.8×10^5
	T	6×10^7	6.5×10^7
13	FL	ND	ND
	T	9.2×10^7	8.8×10^7
14	FL	ND	ND
	T	1.3×10^8	1.4×10^8

Key to Table 26 .

Media:
- 4 = King's medium B
- 5 = 0.1% cetrimide agar.
- 6 = casitone + glycerol + yeast extract agar.
- 7 = KCNS + succinate agar.
- 13 = plate count agar.
- 14 = nutrient agar.

Types:
- FL = fluorescent pseudomonads
- T = total bacteria

Figures = Nos of bacteria per ml of activated sludge.

Means of duplicate counts.

ND = Not detected.

5.4 Preparation of media before inoculation.

The media were poured to the correct thickness and then dried adequately before inoculation. If the thickness of agar was inadequate, crystallisation of medium-constituents or cracking of the agar occurred during drying or inoculation of the plates. An agar-thickness of 1 cm was usually sufficient in pertri-dishes or repli-dishes. Dry plates were needed so that the surface-drops were absorbed into the agar within 10 minutes. This prevented the coalescence of surface drops and bacterial multiplication in unabsorbed liquid. If the agar surface remained moist the characteristic colony-morphology of thiobacilli and Pseudomonas stutzeri was not easily recognised. The plates were dried at 37°C for 24 hours, then at 60°C for 1 hour immediately before inoculation.

5.5 Application of samples to media.

An inoculum of 20 µl was applied to the surface of the agars. This volume was usually found to be absorbed into the agar over an area approximately 1 cm in diameter. Up to about one hundred colonies could sometimes be counted accurately throughout this area provided that colony-growth was not rapid and spreading was prevented. The growth of thiobacilli on adequately dried agars was well suited to surface drop colony counts. This was because colonial growth was slow, forming small 1 mm diameter, well defined colonies. 50 to 100 thiobacilli per 20 µl drop could usually be counted accurately after 6-28 days at 28°C. In contrast, the growth of pseudomonads on unmodified King's medium B and on the KCNS + succinate agar was often very rapid. Not more than 50 pseudomonad colonies per 20 µl drop could normally be counted. Consequently pseudomonads were usually counted after 2 days at 28°C before colonies became confluent. 12 x 20 µl drops could be applied to each 85 mm

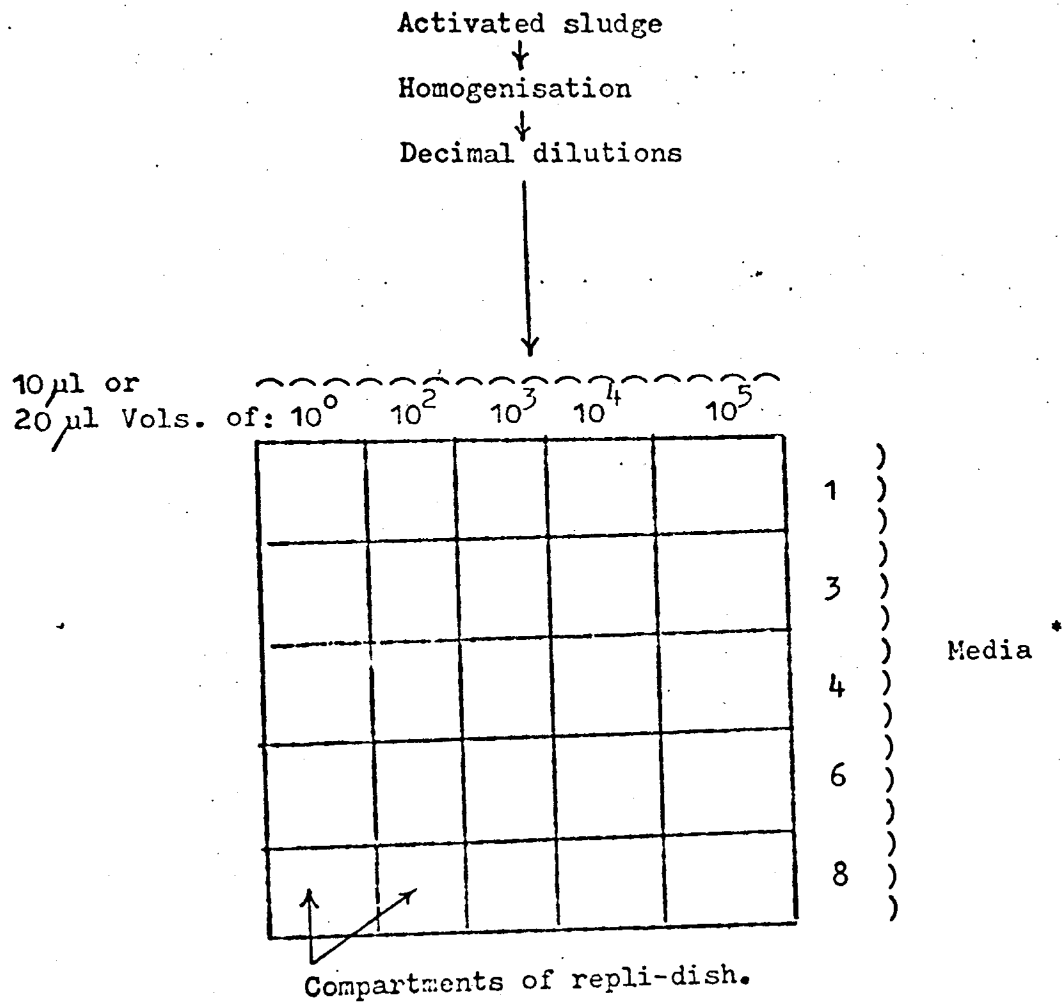
diameter petridish without excessive crowding. Surface drop bacterial counts could therefore be done in triplicate for each dilution without using a large number of petridishes. The amount of medium used for each test was minimised by the surface drop method. 10 µl drops were also satisfactory but greater accuracy in colony counting resulted from the use of 20 µl drops.

If the media had been adequately dried immediately before the surface drops were applied, absorption of the drops was usually complete after 10 minutes. The plates were then incubated at 28°C in an inverted position to prevent the condensation of moisture on the agar surfaces.

It was convenient to apply the diluted samples to repli-dishes (Sterilin Ltd) containing five different media, as shown in Fig 20. This saved space in the incubator which would otherwise have been occupied by several petri-dishes each containing a different medium. Furthermore, bacterial counts on the different media could be compared more readily when all the media were in a single repli-dish. A single 20 µl drop was applied to 3 ml of agar in each compartment of a repli-dish. After complete absorption of the drops the repli-dishes were inverted and incubated at 28°C.

Accurate dispensing of the 10 µl or 20 µl drops was of great importance in determining the accuracy and reproducibility of the surface-drop technique. At the beginning of the bacterial monitoring study, Pasteur pipettes were used, with 1 mm bore silicone rubber tubing of a standard internal diameter, enabling drops of a constant volume to be delivered. A 5 cm length of the silicone rubber tubing was fitted tightly as a sleeve over the narrow distal end of a Pasteur pipette. A 1 cm length of silicone tubing was left extending beyond the tip of the pipette. The modified pipette was plugged with silicone sponge (Hakuto Co. Ltd) and fitted into a Pyrex glass test tube using a silicone

Fig.20 The Use of a repli-dish containing different selective media for the monitoring of bacterial populations in activated sludge.



	No.	Groups of bacteria detected
*Media =	1	Total thiobacilli
	3	Thiocyanate destroying thiobacilli
	4	Fluorescent pseudomonads
	6	Total heterotrophs
	8	Pseudomonas stutzeri

Details of the media are given in Appendix on page 201.

sponge plug and autoclaved at 121°C for 15 minutes. Aluminium foil was placed over the wide end of the pipette to keep the silicone sponge dry during sterilisation. The larger silicone sponge plug enabled the pipette to be transferred quickly to and from the sterile test tube, minimising the possibility of contamination. The tip of the pipette was held not more than 1 cm above the agar to avoid splashing or excessive spreading of the drop.

Although the modified Pasteur pipettes were cheap and convenient to use, they were found to have disadvantages in operation. Firstly the volume of drops was found to vary between 20 µl and 30 µl. This was because it was difficult to control the rate of delivery of the drops. The most convenient way was to allow the level of the diluted sample to fall between two marks 1 cm apart on the pipette. The rate of delivery of the sample was found to be constant between these marks provided that the silicone plug in the pipette was a constant size and the pipette was always held vertically.

In view of the disadvantages of the above method a microlitre syringe was used. The syringe, type A-RN obtained from Scientific Glass Engineering PTY, Ltd. delivered 10 µl and was calibrated in 0.2 µl divisions. Its components were made of Pyrex glass, stainless steel and P.T.F.E. It was therefore autoclavable and none of the parts were biodegradable or toxic.

Before the microlitre syringe was used it was considered that fragments of undispersed flocs might block the narrow bore of the needle. However this problem was not encountered. The only disadvantage was that both hands were required to draw up the plunger fully to obtain a 10 µl sample. This slowed the inoculation procedure and increase the possibility of contamination introduced from the laboratory atmosphere. Otherwise the S.G.E.

microlitre syringe was satisfactory.

The most rapid and reliable method of applying microlitre volumes of the samples onto agar surfaces was with an adjustable digital microlitre pipette with disposable tips (Gilson France S.A.). The model used was the P200, recommended for 0-200 µl volumes and accurate to ± 1 µl. In addition to providing greater accuracy, the plunger could be used with one hand at all times. Two precautions were needed to prevent contamination of the sample from air-borne micro-organisms. Firstly the wide ends of the disposable polypropylene tips were plugged with non-absorbent cotton wool. Secondly, when each 10 µl or 20 µl sample had been dispensed aseptically, the plunger was kept fully depressed until the polypropylene tip had been returned inside the bottle of diluted sample. The Gilson P200 microlitre syringe was used in the technique finally adopted.

The accuracy of the surface drop colony counting method was tested using a freshly grown culture of a fluorescent pseudomonad capable of destroying thiocyanate (isolate 5, page 115). The results of the test are given in Table 27. Similar counts were obtained from different surface drops derived from the same dilution. The results showed that the technique gave sufficiently accurate and reproducable results.

5.6 Thiobacilli.

The stability of the purification process depends on the sensitivity of the dominant bacteria to inhibitory constituents of coke oven effluent. Since the thiobacilli are particularly sensitive, monitoring their numbers in activated sludge was considered to be important.

5.6.1 Selected Features.

The most important diagnostic features of thiobacilli used in bacterial monitoring were the deposition of elemental sulphur

Table 27 . Test for accuracy of surface drop colony counts.

Test-organism : Isolate 5 grown in King's B broth for 24 h at 28°C. Decimal dilutions prepared from 10^1 to 10^7.

Inoculum: 10 µl drops of the above dilutions applied using a Gilson microlitre syringe.

Agar: King's medium B dried for 24 h at 37°C, then for 1 h at 60°C.

Result:

Dilution	No. of colonies per drop.	Totals	Means	Colony count per ml.
10^4	Uncountable due to confluent growth.			
10^5	18, 18, 16, 17, 17, 15	101	17	1.7×10^8
10^6	1, 2, 1, 1, 2, 2	9	1.5	1.5×10^8

Range of counts per ml using 10^5 dilution = 1.5×10^8 - 1.8×10^8.

from thiosulphate on 1% thiosulphate minerals media and on thiocyanate minerals media, and the development of acidity in liquid 1% thiosulphate minerals medium after 28 days incubation at 28°C.

5.6.2 Development of methods.

In the preliminary stage of bacterial monitoring, rapid tests for the presence of large numbers of thiobacilli in the laboratory scale plant treating synthetic coke oven effluent were carried out. Initially this was done at weekly intervals by streaking 1/50 ml volumes of undispersed sludge from the aeration tank onto 1% thiosulphate minerals medium, medium 1 section 5.13. Incubation was for 7 days at 28°C to obtain prominant sulphur depositing colonies. This test did not indicate the numbers of thiobacilli present. Small numbers of thiobacilli, closely adhering to the flocs, could easily have been overlooked. The observation that T. thioparus flocculated markedly while destroying thiocyanate, see section 4.1, illustrated the need for deflocculation and dilution if the highest viable counts were to be obtained.

Initially, for the quantitative estimation of thiobacilli, 0.1 ml volumes of dispersed, decimally diluted sludge were spread over the entire surface of 1% thiosulphate minerals agar plates. Difficulties arose during incubation due to the spreading of colonies across the moist surface of the medium. The sulphur depositing thiobacilli tended to be masked by spreading growth. Even when thiobacilli developed as isolated colonies, the presence of surface-moisture caused the colonies to spread so that the characteristic circular colonies with smooth margins, see Fig 17, page 101, were not easily identifiable. Sulphur deposition did not develop well in colonies grown on moist agar surfaces. Following these observations, the volume of liquid sample applied

to the agar was reduced and care was taken to ensure that the plates were adequately dried at 60°C for 1 hour before inoculation. The surface drop method of bacterial counting satisfied the need for only small volumes of liquid samples applied to the agar.

Three media were used for the detection of thiobacilli. These three media, detailed in the Appendix, page 201, were 1% thiosulphate minerals agar at pH 6.7, (medium 1), 1% thiosulphate minerals agar at pH 4.1, (medium 2), and 0.02% ammonium thiocyanate minerals agar at pH 6.7, (medium 3). Medium 1 gave the most rapid result for total thiobacilli. On this medium, after incubation for 4 days at 28°C the characteristic deposition of sulphur within the colonies was usually noticeable microscopically, (X 100), using transmitted light. For confirmation it was adviseable to prolong incubation for 6 to 28 days before counting the colonies of thiobacilli. This was to enable sulphur-deposition to develop fully. This precaution was taken because after only 2-4 days incubation, colonies of certain non-sulphur depositing non-thiobacilli appeared very similar to the sulphur depositing thiobacilli. The similarity was in the appearance of granular deposits formed within the colonies. The deposits in the non-thiobacilli were found not to be sulphur, see Fig 18 page 102. The difference in appearance between the two forms of intracolonial deposits became clear only after incubation for at least 6 days. After this period, sulphur appeared opaque, dense and black in transmitted light, whereas the other deposit remained brown or dark brown. The non-thiobacilli were often found to be fluorescent pseudomonads after subculturing to King's medium B (medium 4). The thiobacilli did not grow on King's medium B.

The rapid growth of non-thiobacilli on thiosulphate minerals agar, at pH 6.7 often masked the growth of some Thiobacillus colonies. For this reason medium 2 at pH 4.1 was used in order to reduce the growth of non-thiobacilli and to select the acid-tolerant thiobacilli. It was considered that T. intermedius might have contributed to the formation of acid from thiosulphate-oxidation in the laboratory scale treatment plant. T. intermedius had been found by Hutchinson (1965) to show clearly defined globular deposits of sulphur on medium 2. The detection of this species was a further reason for experimenting with medium 2. However, surface drop counts showed that T. intermedius was not detected on medium 2. Furthermore the growth of thiobacilli was suppressed by the low pH. For these reasons the use of medium 2 was discontinued in the monitoring of thiobacilli.

Medium 3 was used specifically to detect thiobacilli which used thiocyanate. Since the efficiency of the purification process depends considerably more on thiocyanate-destruction than on thiosulphate destruction, counts of thiobacilli on medium 3 were considered to be of greater significance than counts on medium 1. However, sulphur-deposition by thiobacilli grown on the thiocyanate medium 3 developed at a much slower rate than on the thiosulphate medium 1. Using medium 1 the highest counts of thiobacilli could be obtained after 6 days at 28°C. With the experience gained from carrying out several counts on medium 1 it was possible also to predict whether the Thiobacillus-count was likely to be high or low, after only 4 days at 28°C. It was necessary to incubate plates of medium 3 for 14 to 28 days at 28°C to obtain the highest counts of thiobacilli which formed sulphur from thiocyanate. In view of these observations, it was decided that the monitoring method should include the use of medium 1 to obtain a rapid estimation of the total numbers of thiobacilli, and medium 3

to obtain counts of thiocyanate-destroying thiobacilli. Sulphur deposition from thiocyanate in medium 3 was a feature specific to T. thioparus and T. denitrificans. Monitoring of the total numbers of these two species was therefore possible using medium 3.

5.6.3 Final method adopted.

After dilution of the dispersed sludge, 20 µl volumes of each dilution were immediately applied to the surface of well dried plates of 1% thiosulphate minerals agar medium 1, and to 0.02% ammonium thiocyanate minerals agar, medium 3. The inoculated thiosulphate plates (1) were inverted after absorbtion of the 20 µl drops and incubated at 28°C for six days. After 4 days the plates were examined for colonies developing intra-colonial deposits of sulphur. For this a magnification of X 100 in transmitted light was sufficient. The final count was recorded after 6 days at 28°C. The thiocyanate plates (3) were inverted as before and incubated at 28°C. Sulphur depositing colonies were observed after 7, 14 and 28 days. As an aid to identifying the thiobacilli, reference was frequently made to photographs, Fig 16,page 100 and Fig 21,page 141. Stock-cultures of isolates identified as thiobacilli, grown on the same media were also referred to.

5.6.4 Results.

During the first twenty three weeks of bacterial monitoring, the recognition of thiobacilli in platings from mixed populations in the aeration tank was difficult. The identification of colonial types was obscured by the spreading growth of non-thiobacilli (Gp'O' types,Table 30,page 167,) and by the apparent lysis of some colonies. Nevertheless, clearly defined sulphur depositing colonies were not detected for several weeks. Thiobacilli were probably absent during this period.

On day 59 an increase was made in the concentration of thiosulphate in the feed liquor to the laboratory system. A rise in the thiosulphate concentration from 270 mg/l to 410 mg/l was followed within 24 hours by a sharp drop in pH in the aeration tank, from 6.6 to 5.2. The continuation of a pH value of 5.2 - 5.9 in the aeration tank during the following day resulted in an accumulation of 270 mg thiocyanate per litre. Corresponding drops in bacterial counts were recorded on day 60 in Table 30, page 168 and Fig 23, page 175. The autotrophic media, 1 and 3, showed greater drops in viable bacterial counts than the heterotrophic medium 13, see Fig 23. Counts on media 1 and 3 fell from approximately 10^7 per ml to about 10^5 per ml.

Analysis of the aeration tank supernatant liquid at the time of the accumulation of thiocyanate on day 60 showed the presence of 270 mg/l thiocyanate, 25 mg/l phenol and 82 mg/l thiosulphate. A one hundred fold drop in total viable count was recorded on the autotrophic thiosulphate medium. It was unusual to detect residual thiosulphate in the aeration tank. This, together with the suppression of thiocyanate destruction and the greatly reduced viable counts, confirmed the detrimental effect on viable bacteria in the system. Rapid remedial action was taken to prevent a further decline in viable counts. The flow of feed liquor was stopped, and the pH was kept between 6.5 and 7.0. This allowed the thiocyanate to be destroyed slowly at the optimum pH and prevented the wash out of bacteria from the system. After complete destruction of the thiocyanate, the flow of feed liquor was restarted at a increased aeration time of 50 hours instead of 20 hours. Successful treatment was restored when the viable counts on media 1 and 3 had been restored to 10^7 - 10^8 per ml.

On day 198 moderate numbers of thiobacilli, 5×10^6 per ml, were detected on thiosulphate agar, medium 1, but none were

detected on the thiocyanate agar, medium 3, until day 218. From day 244 to day 254 more thiobacilli were detected on thiocyanate agar than on thiosulphate agar. At no time were the numbers of thiobacilli greater than 5×10^6 per ml using either medium 1 or 2. On day 230 an accumulation of 55 mg thiocyanate per litre developed and continued for five days but the numbers of viable thiobacilli did not appear to be affected by this.

Towards the end of this study, a combination of factors appeared to contribute to the optimum conditions required for the rapid development of thiobacilli capable of oxidising thiocyanate. In order to prevent excessive alkalinity developing in the aeration tank, the feed-liquor was adjusted to the unusually low pH of 4.0. At this pH greater concentrations of phosphate and ferrous iron were retained in solution than at the normal feed-liquor pH of 7.5. These factors, together with the absence of coloured inhibitory materials and the absence of fungal flocs, might have contributed to the rapid increase in numbers of thiobacilli. The improvements in plant performance at this time are given on page 166, Table 29, column (iii). Tests carried out on two other occasions (i) and (ii) are included for comparison. The increased counts of thiobacilli are also shown in Fig 24, page 176.

In the full scale biological treatment plant the occurrence of thiobacilli since startup operations is shown in Table 31, page 174 and Fig 25, page 177. Large numbers of thiobacilli were detected in the activated sludge used to seed the full scale plant. Fig 21 shows the predominance of thiobacilli in surface drops applied to medium 1. The thiobacilli were quickly eliminated in the presence of coke oven effluent fed into the system. Thiocyanate concentrations recorded between days 1 and 69 in the aeration tank of the main plant, varied between 100 and 320 mg per litre.

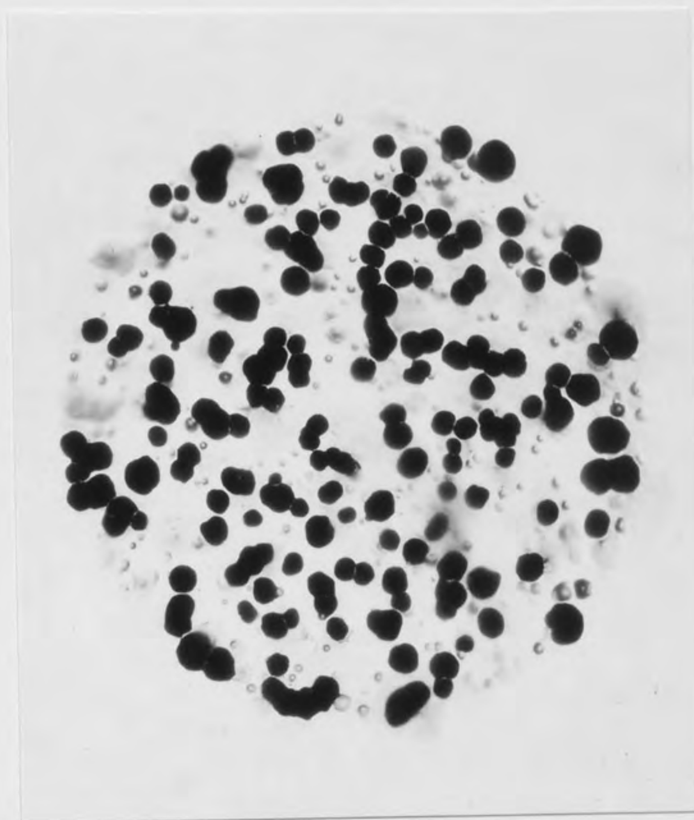


Figure 21 .

Monitoring of thiobacilli in activated sludge:
Surface drop viable colony count using 1% thiosulphate minerals agar. 10 µl drop of 10^{-3} dilution of homogenised activated sludge. Origin: Activated sludge used to seed the full scale plant treating Corby Coke Oven effluent. Magnification X10. Transmitted light. Colonies of thiobacilli showing back deposits of elemental sulphur. Age of culture, 10 days at 28°C.

The thiobacilli were rapidly replaced by pseudomonads, see Fig 25 on page 177.

A period of twelve weeks was required before efficient purification was achieved in the full scale plant. This delay was caused by fluctuations in coke oven liquor concentration entering the system owing to difficulties in adjusting the rate of flow of feed liquor. This was considered to be an important factor preventing the reappearance of thiobacilli. Low temperatures of 12° - 15°C in the aeration tank also slowed the rate of increase in the amount of activated sludge in the system. When efficient purification had been established, thiobacilli remained undetected in the full scale aeration tank for the duration of the present investigation, i.e. for a further six weeks.

5.7 Fluorescent pseudomonads.

5.7.1 Selected features.

The formation on King's medium B of a yellow or greenish yellow diffusible pigment which fluoresced under ultra violet light at 350 mμ was used as the main diagnostic feature in the identification of fluorescent pseudomonads. Additional tests included a rapid positive reaction in the oxidase test, the presence of short Gram negative rods, and resistance to up to 0.1% cetrimide included in the solidified media. As described in sections 5.4 and 5.6 fluorescent pseudomonads were often detected using media other than King's B.

5.7.2 Development of methods.

The streaking of 1/50 ml volumes of undispersed activated sludge onto King's medium B, at weekly intervals followed by incubation for 4 days at 28°C showed that fluorescent pseudomonads were often present in large numbers. This justified the development of a surface-drop method for more accurate monitoring.

Although intense fluorescence developed quickly from the surface drops and maximum counts were usually obtained after 4 days at 28°C, some problems were encountered. Owing to the rapid and sometimes spreading growth of fluorescent pseudomonads on King's B, together with diffusion of the fluorescent pigment, the margins of the colonies were sometimes indistinct and confluent growth occurred. This made accurate colony-counting difficult after the optimum period of incubation needed to obtain the highest count. This problem was aggravated by the small area covered by each surface-drop; approximately 2.5cm^2 for a 20 μl drop on agar dried at 60°C for 1 hour.

Some non-fluorescent colonies often contributed to the unwanted spreading growth. A method of minimising the spreading of colonies was therefore required, provided that it did not reduce the colony-count or suppress fluorescence. A number of substances were considered for inclusion in King's medium B, with the aim of eliminating non-pseudomonads and preventing spreading. It was considered that the most useful substance would be one which permitted the growth of pseudomonads but caused substantial suppression of non-pseudomonads. Such a selective agent would enable the growth medium to be used for the enumeration of both fluorescent pseudomonads and total pseudomonads including those which were non-fluorescent, see section 3.6.8. Consequently the selective agents considered were cetrimide or cetyl trimethyl ammonium bromide, (Whitby et al, 1972), chloroxylenol and hexachlorophane, (Cruikshank, 1973), erythromycin, aureomycin, penicillin and nalidixic acid (Gilardi, 1972). Selection of pseudomonads by incubation at an elevated temperature of 35° to 40°C was also considered but incubation at the optimum temperature of the treatment plant was preferred, i.e. at 28°C. The antibiotics, erythromycin,

aureomycin, penicillin and nalidixic acid were not used because some suppression of viable counts of the pseudomonads was considered to be likely. Chloroxylenol was not used because its use in selective media for pseudomonads did not appear to be sufficiently documented elsewhere.

Cetrimide was chosen for inclusion in King's medium B because it had been used successfully elsewhere. Initially 0.03% cetrimide was used, as recommended by Lowbury and Collins (1955) and Lowbury (1965). However a rather high proportion, 20% of the colonies grown as King's B + 0.03% cetrimide, were found to be oxidase negative. Although some pseudomonads are reported to be oxidase negative, most of them are rapidly oxidase positive. It was therefore probable that many of the oxidase negative non-fluorescent colonies detected were not pseudomonads. The remaining 80% of the colonies grown on 0.03% cetrimide King's B agar were rapidly oxidase positive and most were fluorescent. Spreading was reduced by 0.03% cetrimide but, according to the oxidase test, the medium may not have been sufficiently selective to eliminate non-pseudomonads. Furthermore it was necessary to incubate the plates for 6 days instead of 4 days at 28°C to obtain the highest colony-counts.

The concentration of cetrimide was increased to 0.1% as in medium 5, given in the Appendix on page 202. At this concentration in King's medium B, a higher percentage, 90%, of the colonies were rapidly oxidase positive, but the counts of fluorescent pseudomonads were significantly less than with unmodified King's B. Again, 6 days incubation at 28°C were needed to obtain the maximum count, owing to the suppressing influence of cetrimide on the growth of pseudomonads.

5.7.3 Final method adopted.

In view of the lower bacterial counts and delayed growth

obtained by using cetrimide media, the definitive medium used in the enumeration of fluorescent pseudomonads was unmodified King's medium B.

5.7.4 Supplementary tests.

Colonies on King's medium B which were suspected to fluoresce but which did not do so conclusively were subcultured to a second plate of King's B for confirmation.

Coke oven effluent provides a highly specialised nutrient environment for the bacteria. It was therefore suspected that counting of pseudomonads by direct inoculation onto King's B might not give the highest counts. Counts made in this way were therefore compared with those obtained when a more general medium, nutrient agar, was used as the initial isolation medium. Colonies grown on nutrient agar were subcultured onto King's B to detect fluorescence. Incubation of plates for 3 days at 28°C following inoculation onto King's B gave counts of fluorescent pseudomonads as high as those obtained when nutrient agar was inoculated prior to King's B.

Fluorescence was often detected on C.G.Y. agar, medium 6., King's B + 0.1% cetrimide agar, medium 5., KCNS + disodium succinate agar, medium 7., and KCNS + phenol agar, medium 8. The proportions of the total number of fluorescent pseudomonads capable of growth on thiocyanate, thiocyanate + succinate, and thiocyanate + phenol could be estimated by comparing the counts of fluorescent colonies on these media. However care was needed when making these comparisons because pseudomonads capable of fluorescing on King's B did not always fluoresce on the thiocyanate media. Nevertheless, rapid growth of fluorescent pseudomonads at 28°C to form colonies 2-3 mm in diameter was usually accompanied by the formation of fluorescein. The intensity of fluorescence was variable, but as long as growth was rapid, fluorescence usually

developed even on the media containing thiocyanate.

5.7.5 Results.

In the laboratory scale plant, fluorescent pseudomonads did not appear to contribute significantly to purification during the first 100 days. This was shown by low viable fluorescent counts on King's B. The numbers of organisms per ml usually fluctuated between 10^2 and 10^5 per ml and they were absent on some occasions. Fluorescent pseudomonads capable of using thiocyanate on medium 7 were also in small numbers at this time, see Table 30 . However Fig 26,page 178 shows that between days 128 and 208, when catechol was added to the feed-liquor, there was a large progressive increase in fluorescent pseudomonads in the aeration tank. A corresponding rise occurred in numbers of fluorescent pseudomonads detected on medium 7 containing thiocyanate as the sole source of nitrogen. On day 254 high counts were obtained on both King's B (medium 4) and thiocyanate + succinate medium 7. 20 days later, fluorescent pseudomonads were not detected on medium 7, but their numbers remained high on medium 4.

The accumulation of thiocyanate which was caused by a sharp decline in pH resulted on day 60 in a drop in the total viable count on King's medium B from 1.3×10^6 to 4.4×10^5.

In the full scale treatment plant, acclimation of the sludge was shown by a rise in numbers of viable fluorescent pseudomonads from 1.5×10^4 per ml to 1.6×10^7 per ml. The first 18 days of full scale plant operation was also accompanied by the development of fluorescent pseudomonads capable of using thiocyanate on medium 7. After the first 18 days the population varied between 10^6 and 10^7 per ml.

5.8 Total pseudomonads.

5.8.1 Selected diagnostic tests.

Five tests were used to identify bacteria as pseudomonads. These tests were (i) the production of a non-fluorescent green or greenish blue phenazine pigment on King's medium A or on Pseudocel agar (media 9 and 10 respectively, page 203), (ii) the production of a fluorescent yellow or greenish yellow diffusible flourescein pigment on King's B, (iii) a rapid positive reaction in the oxidase test, (iv) resistance to up to 0.1% cetrimide and, (v) the growth of green or greenish blue circular colonies on C.L.E.D., medium 11.

5.8.2 Development of methods.

As described in section 5.7.2 media containing cetrimide suppressed pseudomonads. Nevertheless cetrimide has been widely used elsewhere in media for pseudomonads, particularly *P. aeruginosa*. Although this species was not identified in this study of activated sludge, some degree of resistance to cetrimide was a feature common to all of the *Pseudomonas* species isolated in section 4.1. Provided that the concentration of cetrimide was kept constant at 0.1% in King's B, colony counts using this medium could be used to indicate trends in the size of *Pseudomonas* populations. However the counts were taken to be below the real numbers per ml.

Replica plating showed that a high proportion, approximately 85%, of the total number of colonies recovered on nutrient agar and plate count agar grew on King's B 0.1% cetrimide and were rapidly oxidase positive. This was strong evidence that they were pseudomonads.

5.8.3 Final method.

Changes in the numbers of pseudomonads were monitored using King's medium B with the addition of 0.1% cetrimide to cause substantial inhibition of non-pseudomonads.

5.8.4 Supplementary tests.

The oxidase test was carried out on all colonies which grew to a diameter of at least 1 mm after 6 days at 28°C. The most convenient method was to use Taxo N discs (BBL) which were moistened with sterile distilled water, brought into contact with the colonies and incubated at 30°C for 30 minutes. Most of the pseudomonads gave a positive oxidase reaction within 10 seconds. A useful confirmatory test for pseudomonads was to subculture colonies onto unmodified King's B and observe fluorescence after 2-5 days at 28°C.

5.8.5 Results.

Total pseudomonads were monitored between days 198 and 320, Table 30 page 170, using the laboratory scale plant. Total viable counts of pseudomonads on medium 5 were normally between 10^6 and 10^7 per ml when the system was operating successfully. Non-fluorescent pseudomonads were in greater numbers than fluorescent pseudomonads for 29 days. After this period fluorescent pseudomonads dominated for a further 29 days.

The activated sludge used to seed the full scale treatment plant initially gave very low counts of pseudomonads on medium 5, see Table 31 page 174 and Fig 25, page 177. After 18 days the fluorescent, non-fluorescent and total viable counts on medium 5 had risen significantly. After this increase the counts remained between 10^5 and 10^7 per ml. When efficient continuous purification became established, the population of pseudomonads increased to 10^7 - 10^9 per ml.

5.9 Bacteria which used thiocyanate in the presence of the organic carbon sources; succinate and phenol.

5.9.1 Selected features.

The main feature chosen was the amount of colonial growth which developed on the selective media. Precautions were therefore taken to exclude contaminating sources of nitrogen and carbon from the media.

The sole source of added nitrogen was thiocyanate and the sole sources of added carbon were thiocyanate and either succinate in medium 7 or phenol in medium 8. Under these conditions, growth on the agar of colonies at least 1 mm in diameter was taken to indicate that thiocyanate had been used by the bacteria. Substantial use of thiocyanate was implied by the rapid growth of colonies after 6 days at 28°C to form colonies 2-3 mm in diameter. In the case of fluorescent pseudomonads, rapid growth together with the formation of fluorescein was evidence that thiocyanate had been rapidly utilised.

5.9.2 Development of methods.

Two media were used to detect bacteria capable of destroying thiocyanate in the presence of organic compounds as the sole sources of carbon. This was necessary in view of the absence of autotrophic thiobacilli from some plants treating carbonisation effluents, Hutchinson et al (1964). A heterotrophic bacterium isolated by Stafford et al (1969) used thiocyanate in the presence of succinate or phenol. Consequently, the first medium used to monitor heterotrophic thiocyanate destroying bacteria in this study contained succinate and potassium thiocyanate. Succinate was chosen for five reasons. Firstly, the British Carbonisation Research Association had found that pyruvate or substances which were readily oxidised to pyruvate sometimes stimulated the biological purification of coke oven effluents. (B.C.R.A., 1971).

Pyruvate enters the tricarboxylic acid cycle in which succinate is an important intermediate. Secondly, succinate stimulated thiocyanate destruction by Pseudomonas stutzeri, (Stafford et al, 1969) Thirdly, Lees (1955) reported that increased yields of Thiobacillus thioparus were obtained when succinate was added to the mineral medium containing thiosulphate. It was therefore suspected that greater yields of heterotrophic thiocyanate destroying bacteria might also be obtained using succinate. Fourthly, organic acids including succinate are intermediates in the bacterial oxidation of phenol which is a major constituent of coke oven effluent. Phenol is normally oxidised completely to carbon dioxide and water but traces of organic acids may be formed as secretions from the micro-organisms present in the activated sludge. The fifth reason for using succinate was that this organic compound had been found by Stanier et al (1966) to be readily utilised by most pseudomonads, particularly the fluorescent pseudomonads.

The KCNS + succinate medium first used in this study was that of Stafford et al (1969), modified by the addition of manganese. This medium had a thiocyanate concentration of 225 mg/l. Later it was expected that although colonies grew on this medium, the thiocyanate concentration may have been high enough to suppress the more sensitive thiocyanate destroying bacteria. The thiocyanate concentration was therefore lowered to 100 mg/l. However the amount of growth measured by the diameters of colonies was less than when 225 mg/l of thiocyanate was used. At 225 mg/l CNS the largest colonies reached 2-3 mm in diameter after 6 days at 28°C, but at 100 mg/l CNS, 1-2 mm was usually the greatest diameter except with occasional spreading colonies. Background growth sometimes accounted for colonies up to 1 mm in diameter. These approached the size-range of colonies on the KCNS + succinate agar. In order to aid the distinction between growth due to

thiocyanate-destruction and background growth the thiocyanate concentration was restored to 225 mg/l.

Phenol is the main source of organic carbon in coke oven effluent. It was therefore likely that phenol would be utilised by heterotrophic thiocyanate destroying bacteria acclimated to coke oven effluent. Bacterial counts on a medium containing both thiocyanate and phenol were therefore expected to have a bearing on the performance of the purification process, which relies on the efficient destruction of these two major constituents. *Pseudomonas stutzeri* destroyed thiocyanate and phenol simultaneously and its colonial morphology could be recognised on a phenol + KCNS medium (Stafford et al 1969). It was therefore expected that *P. stutzeri* could be detected and its numbers monitored, by using a KCNS medium containing phenol. Using high concentrations of 225 mg/l of CNS and 470 mg/l of phenol it was possible that the medium might be sufficiently selective to permit the growth of only *P. stutzeri*.

5.9.3 Final method.

The KCNS + succinate medium with 225 mg/l CNS was retained because fluorescent thiocyanate destroying pseudomonads were frequently detected on this medium, without the need to subculture the colonies to King's B for confirmation of their identity. Bacteria capable of growth on high concentrations of thiocyanate and phenol were detected using medium 8 (**Appendix, page** 203).

5.9.4 Results.

The use of a thiocyanate + succinate medium (7) facilitated theisolation of heterotrophic bacteria which were not previously known to destroy thiocyanate.

Bacterial monitoring using medium 7 gave three main colonial types commonly present in activated sludge from the laboratory system treating coke oven effluent. Two grew rapidly and were found to destroy thiocyanate in pure culture; The third colonial forms, recorded as type 'B' in Table 30, was sometimes in large numbers derived from the activated sludge. However in pure culture, type 'B' failed to destroy thiocyanate in liquid media. Fig 22 shows all three colonial forms. Two fluorescent isolates were studied in detail as isolates 4 and 5 in section 4.7.

On day 60, the low pH of 5.2 - 5.9 and high residual thiocyanate concentration of 270 mg per litre were accompanied by an unusually low viable count of 8.5×10^6 per ml on medium 7. After successful treatment had been restored the counts on medium 7 were restored to $10^8 - 10^9$ per ml.

Between days 168 and 254 the numbers of fluorescent pseudomonads detected on medium 7 increased to 8.8×10^6 per ml, see Table 30, page 170 and Fig 26. The count on day 254 showed that the number of fluorescent pseudomonads capable of using thiocyanate was almost as high as the total number of fluorescent pseudomonads detected using King's medium B, (Fig 26, on page 178). However it was necessary to interpret this observation in the light of the normal fluctuations which occurred in bacterial numbers, and in relation to the composition of the feed-liquor.

The medium containing high concentrations of thiocyanate and phenol was used to detect bacteria capable of tolerating fluctuations in the loading of these substrates on the system. The organism expected to fulfil this role was Pseudomonas stutzeri, which could be identified by its characteristic colonial form. However, between days 254 and 320 in the laboratory system, most of the bacteria recovered from medium 8 fluoresced when transferred to King's medium B. P. stutzeri is non-fluorescent. On several

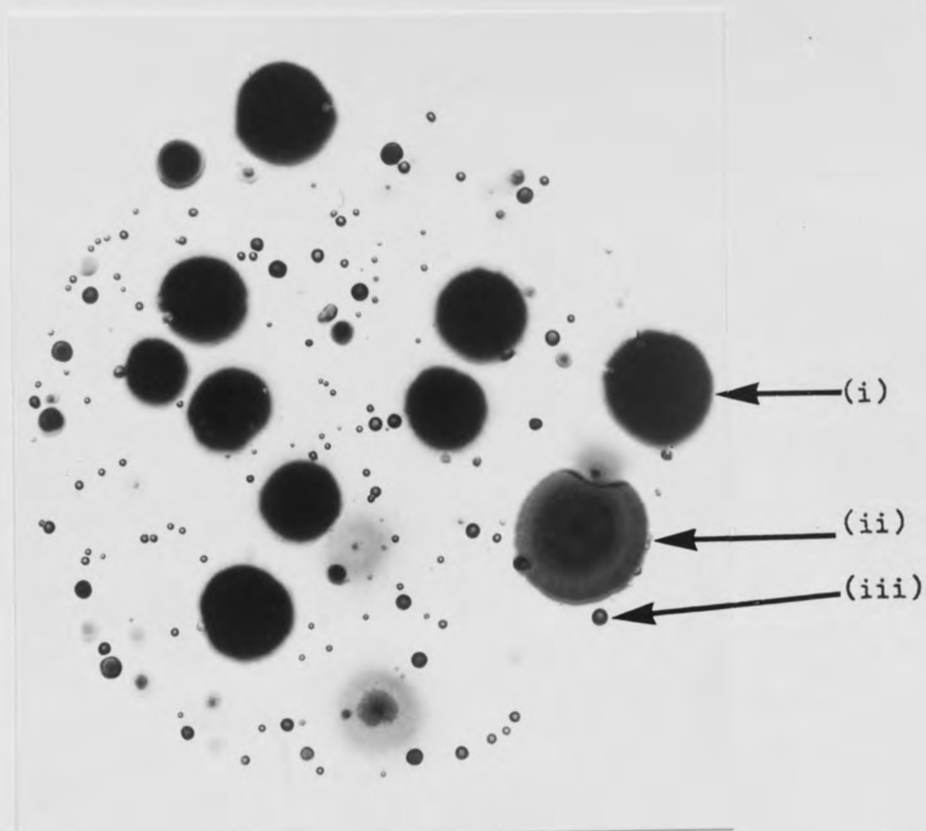


Figure 22.

Surface drop viable count using thiocyanate + succinate agar.
(i) = rapidly growing fluorescent colony, confirmed to destroy thiocyanate.

(ii) = rapidly growing non-fluorescent colony.
(iii) = slow growth of non-fluorescent colonial form, recorded as type 'b' in Table 30 .
Origin: Aeration tank of laboratory scale plant, treating coke oven effluent.
Dilution of sample = 10^{-3}.
Volume of surface drop = 10 μl.
Age of culture: 5 days at 28°C.
Magnification: X10 transmitted light.

occasions when the laboratory system was operating efficiently, very low counts were recorded on medium 8. This contrasted with higher counts on the same medium inoculated from the full scale treatment plant, in which phenol destruction was rapid but thiocyanate destruction was slow during start up operations.

5.10 Bacteria which used thiocyanate in the presence of aeration tank supernatant liquid.

5.10.1 Selected features.

When KCNS at 225 mg/l CNS was the main source of carbon added to the growth-medium, colonies at least 1 mm in diameter after 6 days at 28°C were recorded as bacteria capable of using thiocyanate as a source of carbon, in the presence of materials in the aeration tank supernatant liquid. Adverse conditions were suspected when the bacterial count declined significantly or when only minute colonies developed. A tenfold drop in numbers was considered to be significant, e.g. from 10^7 to 10^6 per ml. A predominance of very small 'pinpoint' colonies, not greater than 0.2 mm in diameter after 6 days at 28°C was taken to indicate the presence of an inhibitory substance or substances in the aeration tank liquid included in the medium.

5.10.2 Development of methods.

The artificial media described so far were designed to promote maximum growth of the bacteria to obtain the highest counts. This approach did not account for the conditions present in the liquid environment of the bacteria in the treatment plant. It was hoped that by including sterilised aeration tank supernatant liquid in a thiocyanate medium, the numbers of viable bacteria detected and their amount of growth may indicate whether conditions in the aeration tank were optional for thiocyanate destruction.

A test was done to find the amount of growth supported by a medium containing aeration tank supernatant liquid but no thiocyanate; medium 12. This medium was inoculated with dispersed, diluted activated sludge from the aeration tank of the laboratory scale plant treating coke oven effluent. The sludge was sampled when the plant was operating successfully at a retention time of 20 hours treating a 1:1 dilution of the raw effluent. Thiocyanate had not been added to the medium and no residual thiocyanate was present in the aeration tank at the time of sampling. However it was realised that the aeration tank supernatant liquid added to the minerals medium probably contained several sources of carbon and nitrogen derived from the effluent, and the activated sludge. In particular nitrogen was available as ammonia because nitrification did not occur significantly in the presence of toxic coke oven effluent constituents. Up to 1000 mg/l N as NH_3 was therefore present in the medium. As expected a fairly high background count was obtained on the aeration tank supernatant agar.

When 225 mg/l thiocyanate as KCNS was included in the medium the colony count was not greatly increased, but the size of colonies developed after 6 days at 28°C was greater, i.e. 10-20 mm instead of 0.4 - 1.0 mm diameter. The basal medium lacking thiocyanate therefore supported enough growth to invalidate colony counts on the thiocyanate containing medium, except in the case of the larger 1.0 - 2.0 mm colonies.

The value of the aeration tank supernatant agar was therefore doubted until pyridine was included in the liquor fed to the laboratory scale plant. When attempts were made to restore continuous treatment be feeding a diluted liquor with no added pyridine, the rate of thiocyanate destruction remained very low. The persistance of an inhibitory substance or substances

in the liquid in the aeration tank was therefore suspected. In order to confirm this suspicion, bacteria dispersed from the activated sludge at the time of suppressed thiocyanate destruction were inoculated onto two different batches of sterile aeration tank supernatant agar. One batch was prepared when the treatment plant was operating efficiently, before any inhibitors had been added. This agar was retained as a standard with medium free from inhibitors. The second batch of agar was prepared using the aeration tank supernatant liquid which was suspected to be inhibitory. The size of colonies grown on the standard medium was significantly greater than on the suspected inhibitory medium. In view of this observation, the estimation of bacterial growth by the surface drop method using the thiocyanate medium containing aeration tank supernatant liquid was considered to be valuable in confirming the presence of inhibitory substances.

5.10.3 Final method.

A fresh sample of bacteria from the aeration tank mixed liquid was dispersed, diluted and inoculated onto a thiocyanate agar supplemented with freshly sampled aeration tank supernatant liquid, medium 12. The same sample of bacteria was also inoculated onto a standard thiocyanate + aeration tank supernatant agar. The extent of growth developed on both media was recorded after 6 days at 28°C. The presence of an inhibitory substance in the freshly sampled aeration tank supernatant liquid was suspected if individual colony-growth was significantly greater than on the standard thiocyanate + aeration tank supernatant agar.

5.10.4 Supplementary tests.

If the nature of an inhibitor is known, its presence may be

confirmed by chemical analysis. However some of the substances normally present in the final effluent from the treatment plant interfered with the analysis. For example, pyridine had a maximum ultra violet absorbance at 256 mμ and two smaller peaks at 250 and 262 mμ. But the final effluent had absorbance throughout the ultra violet spectrum, masking the possible presence of small amounts of inhibitory substances such as pyridine. Several possible inhibitors including pyridine were tested to find their ultra violet absorbtion spectra, individually and in the liquor fed to the laboratory scale treatment plant. The inhibitors tested were thiocyanate, phenol, catechol, cresols, quinol and pyridine. The wavelengths of maximum ultra-violet absorbance shown by these compounds were as follows:

Maximum U.V. absorbance, mμ	Compounds.
220	Thiocyanate
270 and 276	Phenol
215 and 274	Catechol
215 and 272	Cresols, mixed isomers
223 and 288	Quinol
256 and two small peaks at 250 and 262	Pyridine

Although all of the above compounds showed characteristic U.V. absorptions in pure solutions, they could not be identified easily by this method when included in the feed liquor at the concentrations normally encountered in coke oven effluent. It followed from this that although ultra violet spectrophotometry may provide a rapid means of detecting several organic substances in river-waters, the method could not be applied accurately to the final effluent from the activated sludge plant treating coke oven effluent. More specific chemical tests for the inhibitors were needed.

The difficulties involved in applying specific chemical tests for inhibitors emphasised the requirement for the bacteriological tests described in this section.

The presence of an inhibitory liquid environment in contact with the bacteria was confirmed in batch mixed culture experiments, on page 59 .

5.10.5 Results.

Fig 23, page 175 showed that when thiocyanate accumulated to 270 mg per litre in the laboratory aeration tank on day 58, the suppressing effect on viable bacteria on day 60 was most clearly demonstrated by the drop in bacterial count using the medium containing liquid from the aeration tank.

5.11 Total heterotrophic bacteria.

5.11.1 Selected features.

All colonies which developed to least 1 mm in diameter on the heterotrophic media after 6 days at 28°C were counted.

5.11.2 Development of method.

Initially, in order to obtain the maximum count of heterotrophic bacteria, plate count agar medium 13 was used. This medium was chosen because it had been accepted in standard methods for the bacteriological analysis of drinking waters and river waters. Furthermore, it was found in this study to form a very clear agar, free from precipitates, enabling colony counts to be made readily.

In view of the considerable metabolic versatility of many species of bacteria in activated sludge treating coke oven effluents, it was considered that a more general purpose heterotrophic medium may be more suitable. In the Oxoid Manual, 1972, page 196, reference was made to the successful use of nutrient agar, medium 14, in the growth of the following species which have been isolated from activated sludge: Escherichia, Flavobacterium and Pseudomonas (Unz et al, 1970) and Thiobacillus. Presumably the 'thiobacilli' referred to included only T. novellus,

T. intermedius and possibly also the so called 'trautwein types'. All the remaining Thiobacillus species are normally considered to be strictly autotrophic although in this study the possibility of interspecies changes among thiobacilli was not excluded. The colony-counts obtained using nutrient agar were similar to those obtained using plate count agar. In view of the more general use of nutrient agar for heterotrophs, this medium was subsequently used instead of plate count agar.

Recent studies by Pike et al (1972) on the isolation of bacteria from activated sludges recommended the use of a casitone + glycerol + yeast extract agar (C.G.Y.) medium 6. In the later stages of the present bacterial monitoring C.G.Y. agar was assessed by comparing it with nutrient agar, plate count agar and King's medium B. The C.G.Y. counts were similar to those obtained using nutrient agar and plate count agar. However, C.G.Y. agar was found to have the advantage of usually inducing fluorescence by pseudomonads to an extent which at least equalled that of King's medium B.

5.11.3 Final method adopted.

The use of C.G.Y. agar enabled total heterotrophic bacteria and fluorescent pseudomonads to be enumerated on the same medium.

5.11.4 Results.

Total counts of viable heterotrophic bacteria were normally between 10^6 and 10^9 per ml in the laboratory system and between 10^7 and 10^9 in the full scale plant, during efficient purification. Although there were periods of two or three weeks when viable counts on the autotrophic media were greater than on heterotrophic media, this situation was frequently reversed without any observable effect on purification. Often the counts of autotrophs on medium 1 were similar to counts on the heterotrophic media 6, 13 and 14.

In view of the similarity between counts on autotrophic and heterotrophic media, the possibility of facultative autotrophy was investigated. Activated sludge from the laboratory system was dispersed as before and decimally diluted to 10^6. 10 µl drops of each dilution were applied to plate count agar, medium 13. All of the ten colonies which developed from a drop of the 10^5 dilution were subcultured to five different media, two of which were autotrophic, see Table 28. Background count agar, medium 15 was also inoculated to account for any residual growth which might have occurred. Eight of the ten isolates which grew on the heterotrophic media also grew on thiosulphate minerals agar. No background growth was recorded. Only one isolate showed slight growth on thiocyanate minerals agar but five grew slowly on thiocyanate + succinate medium. All of the ten isolates were Gram negative rods and the isolates which grew on medium 5 were oxidase positive. Nine of the ten organisms recovered on plate count agar were presumptively identified as pseudomonads on the basis of resistance to 0.1% cetrimide and a rapid positive oxidase reaction. The results of this test indicated that a high proportion of the total viable heterotrophs were pseudomonads, some of which were capable of growing autotrophically using thiosulphate.

5.12 Background growth.

Difficulties had been encountered in eliminating traces of organic materials which could support the growth of bacteria in both liquid and solidified media. The most likely explanations of this growth were considered to be contamination by volatile organic substances derived from the laboratory atmosphere and impurities in the medium-constituents including the purified agars used. When enumerating bacteria from activated sludge it

Table 28. Growth of ten isolates from the laboratory scale plant on heterotrophic and autotrophic media.

Isolates recovered from surface drop on plate count agar.	Growth of isolates on different media.					
	Heterotrophic media			Autotrophic media		
	5	4	7	1	3	15
i	+++	++++	+	++	-	-
ii	+++	++++	+	++	-	-
iii	+	++	+	++	-	-
iv	++	++	+	++	-	-
v	++	+++	-	++	-	-
vi	+++	++++	-	++	-	-
vii	++	+++	-	-	-	-
viii	++	++++	+	++	+	-
ix	+	++	-	+	-	-
x	-	+++	-	-	-	-

Key to Table 28

+ -	estimates of growth density on solid media.
5	0.1% cetrimide agar.
4	King's medium B.
1	1% thiosulphate minerals agar.
3	0.02% ammonium thiocyanate agar.
7	thiocyanate + succinate agar.
15	background count agar.

was also likely that, at dilutions from 10^0 to 10^3, substances in the inoculum applied to the agar also supported bacterial growth. It was considered that a source of organic carbon or nitrogen would be required at a concentration of at least 1 mg/l in order to support the growth of enough bacteria to interfere with bacterial counts. At dilutions of 10^4 and 10^5 the concentration of ammonia in the activated sludge had been lowered from not more than 1000 mg/l to 0.1 or 0.01 mg/l. The amount of growth supported by these low concentrations of ammonia would be negligible and substantial growth of colonies greater than 1 mm in diameter was then attributed to use of the nitrogen source, usually thiocyanate, added to the agar. It was therefore advisable to count colonies only at the greater dilutions of 10^4 and 10^5.

Further precautions were taken to minimise contamination by organic substances. These precautions included the washing of all glassware with 5% Decon 90 (Hopkin and Williams Ltd), followed by thorough rinsing with deionised water. Although Decon 90 effectively removed organic matter, it was biodegradable. Traces of this detergent could therefore have contributed to microbial growth. It was therefore necessary to remove all traces of Decon 90 with deionised water. Deionised water was used as the solvent for quarter strength Ringer and sodium tripolyphosphate solutions used as diluents for the activated sludge. Deionised water was also used in the preparation of all growth media.

A further check to determine the extent of background growth involved the inoculation of a mineral salts basal medium, lacking any major carbon or nitrogen source, solidified with a purified agar. Either Ionagar No. 2 (Oxoid L 12) or Bacto purified agar (Difco 0140.01) were used in medium 16. On this medium, the growth of colonies at least 1.0 mm in diameter, derived from the 10^4 or 10^5 dilutions, constituted background growth which was

therefore subtracted from the colony counts on the other media.

Precautions were taken to avoid contamination of the plates by volatile substances produced by microbial growth during incubation. The plates were incubated separately in sealed sterile plastic bags. Even so it was possible that traces of biodegradable materials could have been derived from the plastic. In this connection, polypropylene or aluminium caps were used in preference to non-absorbent cotton wool for stock bottles of media.

In the sterilisation of media, the autoclave was cleaned thoroughly before use and each medium was autoclaved separately. This avoided the possible contamination of any of the media by substances derived from different media.

Pseudomonads have been reported (Favero, 1971) to multiply waters with extremely low nutrient concentrations and to be capable of using many different carbon sources, including alcohols and other organic solvents commonly used in the chemical laboratory. Nevertheless it was necessary to monitor pseudomonads as they had been found in this study to degrade or tolerate many constituents of coke oven effluent. Pseudomonads usually constituted a major proportion of the heterotrophic bacteria in the activated sludge. The measures described above to minimise background growth were therefore of considerable importance.

5.12.1 Results.

Usually it was not possible to eliminate background growth on the agar media and colony counts of 10^2 - 10^5 were normally recorded. The use of sterile deionised water in the media minimised the amount of background growth. Incubation of all the plates and repli-dishes in a room freed from volatile organic compounds also assisted in minimising background counts.

Key to Tables 29-31 and Figs 22-25.

Key to Tables 29-31 and Figs 22-25.

Media:

No.	Medium
1	1% thiosulphate minerals agar.
3	0.02% ammonium thiocyanate minerals agar.
7	thiocyanate + succinate agar.
4	King's medium B
5	0.1% cetrimide agar.
13	plate count agar
14	nutrient agar.
6	casitone + glycerol + yeast extract agar.
10	'Pseudocel' agar.
9	King's medium A.
8	thiocyanate + phenol agar.
12	aeration tank supernatant agar.
15	background count agar.

Types of bacteria:

Type	Description
THIO	Thiobacilli
Gp'O'	'Trantwein-types' non-thiobacilli
T	total bacteria
FL	fluorescent pseudomonads
N.FL	non-fluorescent bacteria
'B'	
PH	pseudomonads forming phenazine pigments
N.P	[illegible] pigmented bacteria grown on medium 9
P.S.	bacteria whose colony-morphology resembled Pseudomonas stutzeri on medium 8 or 9.

Figures = Numbers of bacteria per ml of activated sludge.

ND = type not detected.

Table 29. Viable bacterial counts in relation to performance of the laboratory scale treatment plant.

1:1v/v dilution of coke oven effluent was being treated in (i), (ii) and (iii).

* Figs = Nos. of viable bacteria per ml.

Tests		Plant Performance: (i) Inefficient Purification	(ii) Efficient Purification	(iii) Efficient Purification
Viable bacteria:				
MEDIA	BACTERIA			
1	THIO	1.1×10^4 *	ND	5×10^7
	Gp 'O'	2.6×10^7	6×10^6	1.7×10^8
	T	2.6×10^7	6×10^6	2.2×10^8
3	THIO	5×10^4	ND	1.6×10^8
	Gp 'O'	3.7×10^7	1.5×10^8	3.9×10^8
	T	3.7×10^7	1.5×10^8	5.5×10^8
7	FL	8.8×10^6	ND	ND
	N.FL	1.2×10^6	8×10^7	2×10^6
	T	10^7	8×10^7	2×10^6
4	FL	10^7	4×10^4	1.3×10^2
	N.FL	3.5×10^7	1.5×10^8	9×10^8
	T	3.5×10^7	1.5×10^8	9×10^8
8	P.S.	ND	ND	ND
	T	9×10^7	6×10^6	6×10^7
1,3,7, 4,8	Combined Total	1.98×10^8	3.92×10^8	1.7×10^9
Condition of sludge:				
Bulked or well settled		bulked	well settled	well settled
free-living ciliates		absent	numerous	small numbers
% sludge volume		1	23	25
suspended solids concentration mg/l		320	4,040	4,980
Sludge age, days		9.3	3.3	2.1
Aeration time, h		60	20	7.5
% CNS destruction		89	>99	>99
Supernatant liquid in aeration tank: colour, Hazen units		65	10	3

Table 30. Bacterial counts on dispersed activated sludge from aeration tank of laboratory scale plant treating coke oven effluent.

Media	Days / Types	1	4	12	20	22	28
1	THIO Gp'O' T	6×10^{6}	6.7×10^{7}	1.2×10^{7}	1.1×10^{7}	9.2×10^{6}	4×10^{6}
3	THIO Gp'O' T	2×10^{8}	2×10^{8}	1.1×10^{8}	3×10^{8}	6.7×10^{7}	10^{9}
7	FL N.FL 'B' T	3×10^{3} 9×10^{7}	4×10^{2} 3.5×10^{7}	ND 4.8×10^{8}	ND 6.4×10^{8}	ND 9.4×10^{8}	ND 2.9×10^{8}
4	FL N.FL T	10^{5} 1.9×10^{8} 1.9×10^{8}	1.2×10^{3} 8×10^{7} 8×10^{7}	3.2×10^{4} 1.2×10^{8} 1.2×10^{8}	4×10^{3} 10^{8} 10^{8}	10^{3} 3.8×10^{7} 3.8×10^{7}	1.4×10^{2} 1.1×10^{8} 1.1×10^{8}
5	FL N.FL T						
13	T	7×10^{7}	3.3×10^{7}	6×10^{7}	3.7×10^{8}	4.6×10^{8}	7.4×10^{8}
14	T						
6	FL N.FL T						
10	FL PH N.P. T						
9	PH P.S. T	ND ND 2×10^{8}	10^{2} ND 7×10^{7}	ND ND 9.9×10^{7}			
8	T P.S.						
12	T						
15	T	2.1×10^{5}	1.7×10^{5}	4×10^{4}	10^{6}	2×10^{5}	3.5×10^{5}

(Cont'd)

Table 30 (cont'd)

Media	Days / Types	36	41	48	56	60	67	82	88
1	THIO Gp'O' T	$2x10^7$	$1.6x10^7$	$2x10^7$	$3.6x10^7$	$4x10^5$	$7.3x10^5$	$2.1x10^8$	$9x10^7$
3	THIO Gp'O' T	$4x10^8$	$3x10^7$	10^7	$6x10^6$	10^5	$3.1x10^5$	$1.3x10^9$	$6.7x10^8$
7	FL N.FL 'B' T	ND $2x10^9$	ND $9.4x10^7$	ND $3x10^8$	$3x10^3$ $7.5x10^8$	$2.6x10^3$ $8.5x10^6$	$3x10^2$ $4.1x10^8$	$4x10^3$ $3x10^9$	$3.8x10^3$
4	FL N.FL T	$4.6x10^2$ $9.3x10^6$ $9.3x10^6$	ND $8.3x10^5$ $8.3x10^5$	$2x10^2$ $7x10^5$ $7x10^5$	$3x10^2$ $1.3x10^6$ $1.3x10^6$	$2x10^2$ $4.4x10^5$ $4.4x10^5$	$4x10^4$ $9x10^6$ $9x10^6$	$2.2x10^4$ $2.6x10^7$ $2.6x10^7$	
5	FL N.FL T								
13	T	$2x10^9$	$3x10^8$	$2.1x10^8$	$2.3x10^8$	$5.4x10^7$	$4x10^7$	$5.9x10^7$	
14	T								
6	FL N.FL T								
10	FL PH N.P. T		ND ND $4.3x10^4$ $4.3x10^4$	ND ND $1.6x10^4$ $1.6x10^4$	$1.4x10^2$ 10^2 $6x10^4$ $6x10^4$				
9	PH P.S. T								
8	T P.S.								
12	T	$3x10^7$	$6.7x10^6$	$4.4x10^7$	$4x10^7$	$9x10^3$	$6x10^5$	$4x10^6$	
15	T	$6x10^5$	$3x10^5$	$1.2x10^4$	10^4	$9x10^2$		$7x10^5$	

(Cont'd)

Table 30 (Cont'd).

Media	Days / Types	92	98	128	133	136	165
1	THIO Gp'O' T	$1.7x10^8$	$1.8x10^8$	$1.2x10^8$	$6x10^7$	$6x10^7$	$1.3x10^7$
3	THIO Gp'O' T	$5.4x10^8$	$6.4x10^8$	$5x10^8$	$5x10^8$	$5.3x10^8$	$2.6x10^8$
7	FL N.FL 'B' T	10^2 $6x10^8$	ND $9x10^8$	ND $9x10^7$	ND $4x10^7$	$3x10^2$ $4x10^8$	ND $3x10^8$
4	FL N.FL T	48 $2.2x10^5$ $2.2x10^5$	ND $7.3x10^4$ $7.3x10^4$	$5x10^2$ $9x10^5$ $9x10^5$	10^3 $9x10^5$ $9x10^5$	$4x10^3$ $2x10^6$ $2x10^6$	$3x10^3$ $1.1x10^6$ $1.1x10^6$
5	FL N.FL T						
13	T	$3.1x10^8$	$4x10^8$	$9x10^7$	$2x10^8$	$5x10^8$	$2x10^8$
14	T						
6	FL N.FL T						
10	FL PH N.P. T						
9	PH P.S. T						
8	T P.S.						
12	T	$9x10^7$	$2.2x10^8$	$3x10^8$	$5x10^8$	$6x10^7$	$9x10^7$
15	T	$6x10^4$	$3x10^5$	10^6	$2x10^5$	$4x10^4$	$3x10^4$

(Cont'd)

Table 30 (Cont'd)

Media	Types \ Days	198	206	218	231	244	252
1	THIO	$5x10^6$	$1.5x10^5$	$2.5x10^6$	$3x10^5$	$1.5x10^4$	ND
	Gp'O'	$8x10^6$	$4.5x10^6$	$5x10^7$	$2x10^8$	$2.2x10^7$	10^8
	TOTAL	$1.3x10^7$	$4.7x10^6$	$5.3x10^7$	$2x10^8$	$2.2x10^7$	10^8
3	THIO	ND	ND	$2x10^6$	$3x10^5$	$2.8x10^5$	$3x10^4$
	Gp'O'	10^7	$1.2x10^7$	$4.3x10^7$	$1.3x10^8$	$2.2x10^7$	$9x10^7$
	TOTAL	10^7	$1.2x10^7$	$4.4x10^7$	$1.3x10^8$	$2.2x10^7$	$9x10^7$
7	FL	$2x10^4$	$6.7x10^4$	$3x10^5$	$6x10^5$	$1.1x10^6$	$7x10^6$
	N.FL						
	'B'						
	T	$9.5x10^6$	$8x10^7$	$3x10^7$	$5.3x10^6$	$1.6x10^7$	$4x10^7$
4	FL	$4.5x10^5$	$4x10^6$	$2x10^7$	$5x10^5$	$1.3x10^7$	$2x10^7$
	N.FL	$2.5x10^6$	$3x10^6$	$3x10^6$	$1.5x10^8$	$1.2x10^7$	$2x10^7$
	T	$3x10^6$	$7x10^6$	$2.3x10^7$	$1.5x10^8$	$2.5x10^7$	$4x10^7$
5	FL	$4x10^5$	$1.5x10^6$	10^6	$5x10^4$	$1.5x10^5$	
	N.FL	$9x10^5$	$1.5x10^6$	$9x10^6$	$4.5x10^7$	$1.4x10^6$	
	T	$1.3x10^6$	$2.5x10^5$	10^7	$4.5x10^7$	$1.6x10^6$	
13	T	$1.5x10^6$	$1.7x10^5$	$5x10^5$	$1.2x10^8$	$2.7x10^7$	
14	T						
6	FL						
	N.FL						
	T						
10	FL	10^2	10^5				
	PH	$3.5x10^5$	$8x10^5$				
	N.P.	10^2	10^2				
	T	$3.5x10^5$	$9x10^5$				
9	P.S.						ND
	PH						
	T						
8	P.S.						ND
	T						
12	T	10^4	10^4	$1.2x10^8$	$3x10^6$	$3x10^6$	10^7
15	T	$4x10^2$	$9x10^3$	10^2	ND	10^4	$2.5x10^4$

(Cont'd)

Table 30 (continued)

Media	Types \ Days	254	258	275	283	315	320
1	THIO Gp'O' T	1.1×10^{4} 2.6×10^{7} 2.6×10^{7}	3.5×10^{5} 1.6×10^{8} 1.6×10^{8}	10^{4} 1.6×10^{7} 1.6×10^{7}	3.5×10^{5} 3.1×10^{7} 3.1×10^{7}	4×10^{5} 8.5×10^{7} 8.5×10^{7}	ND 4.1×10^{7} 4.1×10^{7}
3	THIO Gp'O' T	5×10^{4} 3.7×10^{7} 3.7×10^{7}	2×10^{4} 1.3×10^{8} 1.3×10^{8}	5×10^{2} 10^{7} 10^{7}	ND 5×10^{7} 5×10^{7}	8×10^{4} 3.4×10^{8} 3.4×10^{8}	ND 1.2×10^{8} 1.2×10^{8}
7	FL N.FL 'B' T	8.8×10^{6} 1.2×10^{6} ND 10^{7}	7×10^{6} 1.3×10^{7} ND 2×10^{7}	10^{4} 4.5×10^{6} ND 4.5×10^{6}	ND 1.3×10^{7} ND 1.3×10^{7}	ND 3.6×10^{7} ND 3.6×10^{7}	ND 4×10^{7} 7×10^{7} 1.1×10^{8}
4	FL N.FL T	10^{7} 3.5×10^{7} 4.5×10^{7}	2×10^{7} 3.4×10^{7} 5.4×10^{7}	9×10^{6} 4.1×10^{7} 5×10^{7}	1.1×10^{7} 3×10^{7} 4×10^{7}	4.5×10^{6} 7.3×10^{6} 1.2×10^{7}	4.5×10^{6} 3.6×10^{7} 4.1×10^{7}
5	FL N.FL T	3.5×10^{6} 2.5×10^{6} 6×10^{6}	4.3×10^{6} 2×10^{5} 4.5×10^{6}	5×10^{6} 10^{5} 5.1×10^{6}	2.8×10^{5} 3×10^{4} 3.1×10^{5}	5×10^{3} 5×10^{4} 5.5×10^{4}	6×10^{5} 2.9×10^{5} 8.9×10^{5}
13	T	3.7×10^{7}	4.5×10^{7}	6×10^{6}	3×10^{7}	7.3×10^{6}	4×10^{7}
14	T			5.8×10^{6}	7.5×10^{7}	8×10^{6}	6×10^{7}
6	FL N.FL T				10^{7} 2.6×10^{7} 3.6×10^{7}	9.3×10^{6} 1.3×10^{7} 2.2×10^{7}	7.5×10^{6} 6.5×10^{6} 1.4×10^{7}
10	FL P.H. N.P. T	10^{5} 10^{5} ND 10^{5}	3×10^{2}	2×10^{6} ND			
9	P.S. PH T	ND 3.3×10^{5}	ND 7×10^{2}	ND 10^{2}	ND ND	ND ND	ND ND
8	P.S. T	ND 9×10^{4}	ND 5.9×10^{4}	ND 6.4×10^{2}	ND ND	ND ND	ND ND
12	T	2.5×10^{7}	4×10^{7}	9×10^{6}	5.5×10^{6}	7×10^{6}	4×10^{6}
15	T	10^{4}	2.5×10^{4}	ND	ND	10^{3}	2×10^{3}

(Continued)

Table 30. Bacterial counts on dispersed activated sludge from aeration tank of laboratory scale plant.

Media	Types \ Days	340	349	355	359	367	370
1	THIO Gp'O' T	ND 10^7 10^7	ND 5×10^7 5×10^7	6×10^4 2.6×10^7 3.2×10^7	2×10^4 1.2×10^8 1.2×10^8	5×10^7 1.7×10^6 2.2×10^8	6×10^5 3×10^7 3×10^7
3	THIO Gp'O' T	ND 10^7 10^7	ND 1.5×10^8 1.5×10^8	ND 6×10^7 6×10^7	ND 10^8 10^8	1.6×10^8 3.8×10^8 5.5×10^8	3×10^3 1.7×10^8 1.7×10^8
7	FL N.FL 'B' T	ND 2×10^6 1.1×10^8 1.1×10^8	ND 3×10^7 5×10^7 8×10^7	ND 1.4×10^6 10^6 2.4×10^6	ND 7×10^6 2×10^8 2×10^8	ND 2×10^6 1.7×10^7 2×10^6	ND 4×10^6 2×10^6 6×10^6
4	FL N.FL T	3×10^4 9×10^7 9×10^7	4×10^4 1.5×10^8 1.5×10^8	10^4 3×10^8 3×10^8	7×10^2 2.3×10^8 2.3×10^8	1.3×10^2 9×10^8 9×10^8	2×10^4 6×10^7 6×10^7
5	FL N.FL T						
13	T						
14	T						
6	FL N.FL T	ND 3.9×10^9 3.9×10^9	4×10^4 3.1×10^8 3.1×10^8	10^4 3×10^8 3×10^8	ND 1.1×10^8 1.7×10^8	ND 7.3×10^9 7.3×10^9	2×10^5 3×10^8 3×10^8
10	FL PH N.P. T						
9	PH P.S. T	ND ND 10^6	ND ND 2×10^6	ND ND 9×10^5	ND ND 2×10^6	ND 4×10^5 9×10^5	ND 7×10^6 10^7
8	T P.S.	4×10^6 ND	6×10^6 ND	1.4×10^6 ND	10^4 ND	6×10^7 10^2	2.4×10^8 10^8
12	T						
15	T	6×10^3	10^4	9×10^4	7.3×10^5	4×10^4	6×10^4

(Continued)

Table 30. Bacterial counts on dispersed activated sludge from aeration tank of laboratory scale plant.

Media	Days / Types	372	378	381	383	387	391	393
1	THIO Gp'O' T	3.5×10^6 3×10^7 3.4×10^7	3×10^6 9×10^7 9.3×10^7	10^7 5×10^7 6×10^7	1.5×10^7 3.3×10^8 3.5×10^8	8×10^8 1.3×10^8 1.3×10^8	4×10^6 3.1×10^8 3.1×10^8	2×10^6 4.3×10^8 4.3×10^8
3	THIO Gp'O' T	ND 2×10^8 2×10^8	7×10^6 1.1×10^8 1.1×10^8	ND 9×10^7 9×10^7	ND 3×10^8 3×10^8	2×10^2 8×10^7 8×10^7	4×10^4 9×10^7 9×10^7	10^4 7×10^7 7×10^7
7	FL N.FL 'B' T	ND 7×10^3 1.3×10^8 1.3×10^8	ND 2×10^7 1.1×10^9 1.1×10^9	ND 2×10^7 8×10^8 8.2×10^8	ND 4×10^7 10^6 4.1×10^7	ND 8×10^7 10^6 8×10^7	ND 2×10^7 1.1×10^7 3.1×10^7	ND 7×10^7 10^7 8×10^7
4	FL N.FL T	2×10^5 5×10^7 5×10^7	6×10^4 2×10^7 2×10^7	ND 4×10^7 4×10^7	ND 9×10^7 9×10^7	10^2 6×10^7 6×10^7	ND 2.5×10^8 2.5×10^8	ND 5.8×10^8 5.8×10^8
5	FL N.FL T							
13	T							
14	T							
6	FL B.FL T	7×10^4 2.9×10^8 2.9×10^8	ND 7×10^8 7×10^8	ND 4×10^8 4×10^8	ND 4.1×10^8 4.1×10^8	ND 2.1×10^8 2.1×10^8	ND 5.3×10^8 5.3×10^8	ND 9×10^8 9×10^8
10	FL PH N.P. T							
9	PH P.S. T	ND ND 9×10^7	ND ND 1.5×10^9	ND ND 6×10^8	ND ND 4.2×10^8	ND ND 5×10^7	ND ND 3.2×10^8	ND ND 10^8
8	T P.S.	3×10^8 8×10^7	1.1×10^9 ND	10^8 ND	3×10^8 ND			
12	T							
15	T	10^4	9×10^3	3×10^4	10^5	2×10^4	7×10^3	6×10^4

Table 31. Bacterial counts on dispersed activated sludge returned to the aeration tank of the full scale treatment plant.

Media	Types \ Days	1	18	29	40	47	69
1	THIO Gp'O' T	5.7×10^{7} 10^{4} 5.7×10^{7}	ND 5.9×10^{7} 5.9×10^{7}	ND 9.5×10^{7} 9.5×10^{7}	ND 5.7×10^{7} 5.7×10^{7}	ND 7.5×10^{7} 7.5×10^{7}	ND 4×10^{6} 4×10^{6}
2	THIO Gp'O' T	9×10^{4} 5×10^{7} 5×10^{7}	ND 1.3×10^{8} 1.3×10^{8}	ND 8.5×10^{7} 8.5×10^{7}	ND 5×10^{7} 5×10^{7}	ND 1.1×10^{8} 1.1×10^{8}	ND 10^{7} 10^{7}
7	FL N.F. 'B' T	ND 10^{4} ND 10^{4}	10^{4} 10^{4} ND 2×10^{4}	5.5×10^{5} 5.9×10^{7} 6×10^{5} 6×10^{7}	2.5×10^{5} 1.6×10^{7} 8.5×10^{6} 2.5×10^{7}	1.2×10^{6} 10^{8}	9×10^{4} 5×10^{6}
4	F N.F. T	1.5×10^{4} 10^{5} 1.2×10^{5}	1.6×10^{7} 10^{8} 1.2×10^{8}	1.1×10^{7} 7×10^{7} 1.5×10^{8}	4×10^{6} 8.5×10^{6} 1.3×10^{7}	7×10^{6} 4×10^{7} 4.7×10^{7}	
5	FL N.FL T	10^{4} 2×10^{4} 3×10^{4}	1.2×10^{8} 4.8×10^{5} 2×10^{7}	3.5×10^{5} 2×10^{6} 2.3×10^{6}	2×10^{6} 2×10^{6} 4×10^{6}	5×10^{5} 4×10^{5} 9×10^{5}	
13	T						
14	T		1.2×10^{8}	1.4×10^{8}			
6	FL N.F. T			1.2×10^{7} 8.6×10^{7} 9.8×10^{7}	3.8×10^{6} 9.8×10^{6} 1.4×10^{7}	7.5×10^{6} 8×10^{6} 1.6×10^{7}	
10	FL PH N.P. T						
9	P.S. PH T	ND ND 1.1×10^{5}	ND ND 7×10^{6}	ND ND 2×10^{7}	ND ND 1.5×10^{7}	ND ND 9×10^{6}	ND ND
8	P.S. T	ND 10^{6}	ND 3.4×10^{7}	ND 4.5×10^{7}	ND 2×10^{7}	ND 3×10^{7}	ND 9×10^{5}
12	T						
15	T	2×10^{4}	2.3×10^{5}	3×10^{2}	1.8×10^{2}	ND	7×10^{3}

FIG 23

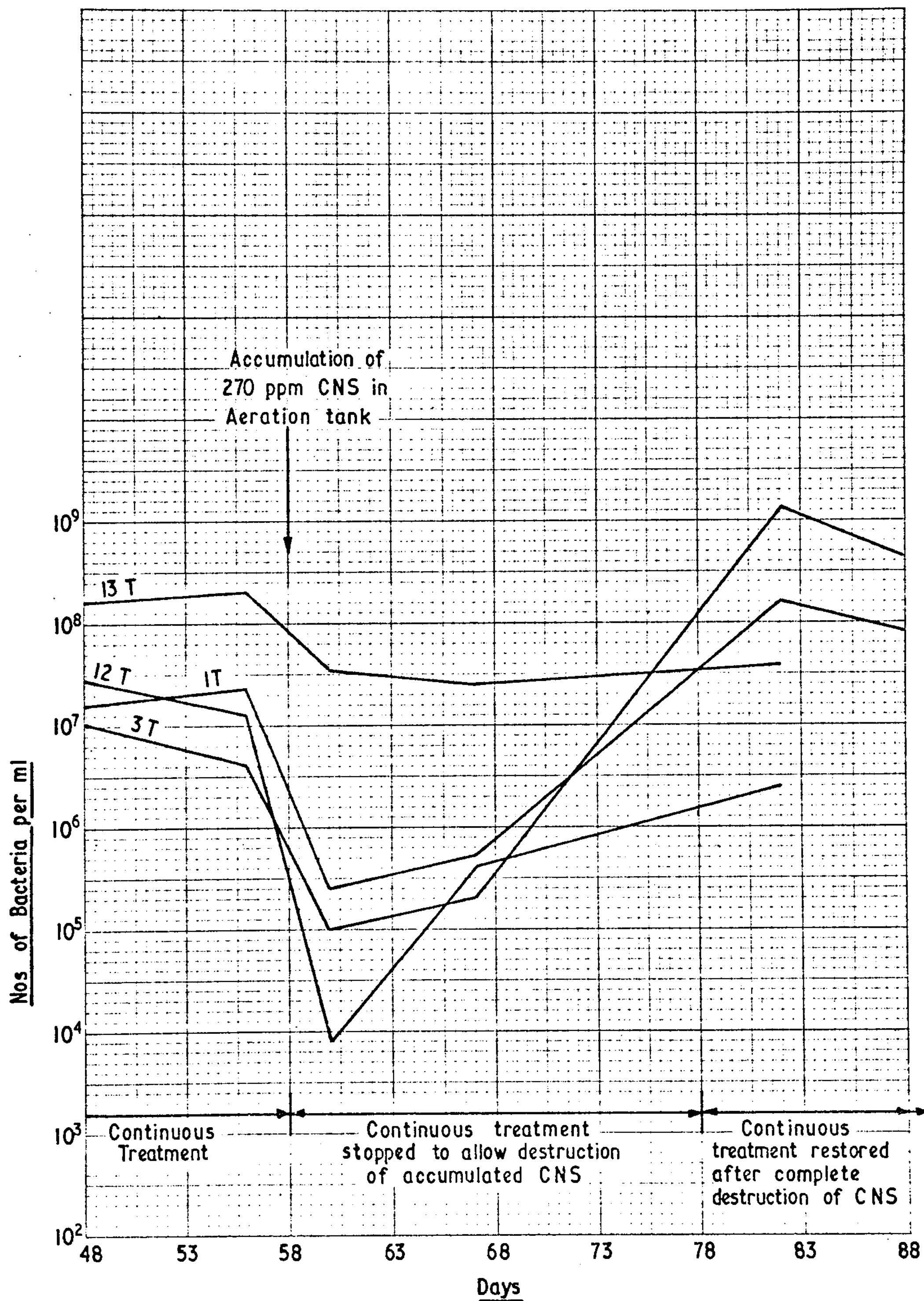


FIG 24 LABORATORY SCALE PLANT : RELATION BETWEEN COMPOSITION OF FEED LIQUOR, NUMBERS OF THIOBACILLI AND THE RATE OF TREATMENT

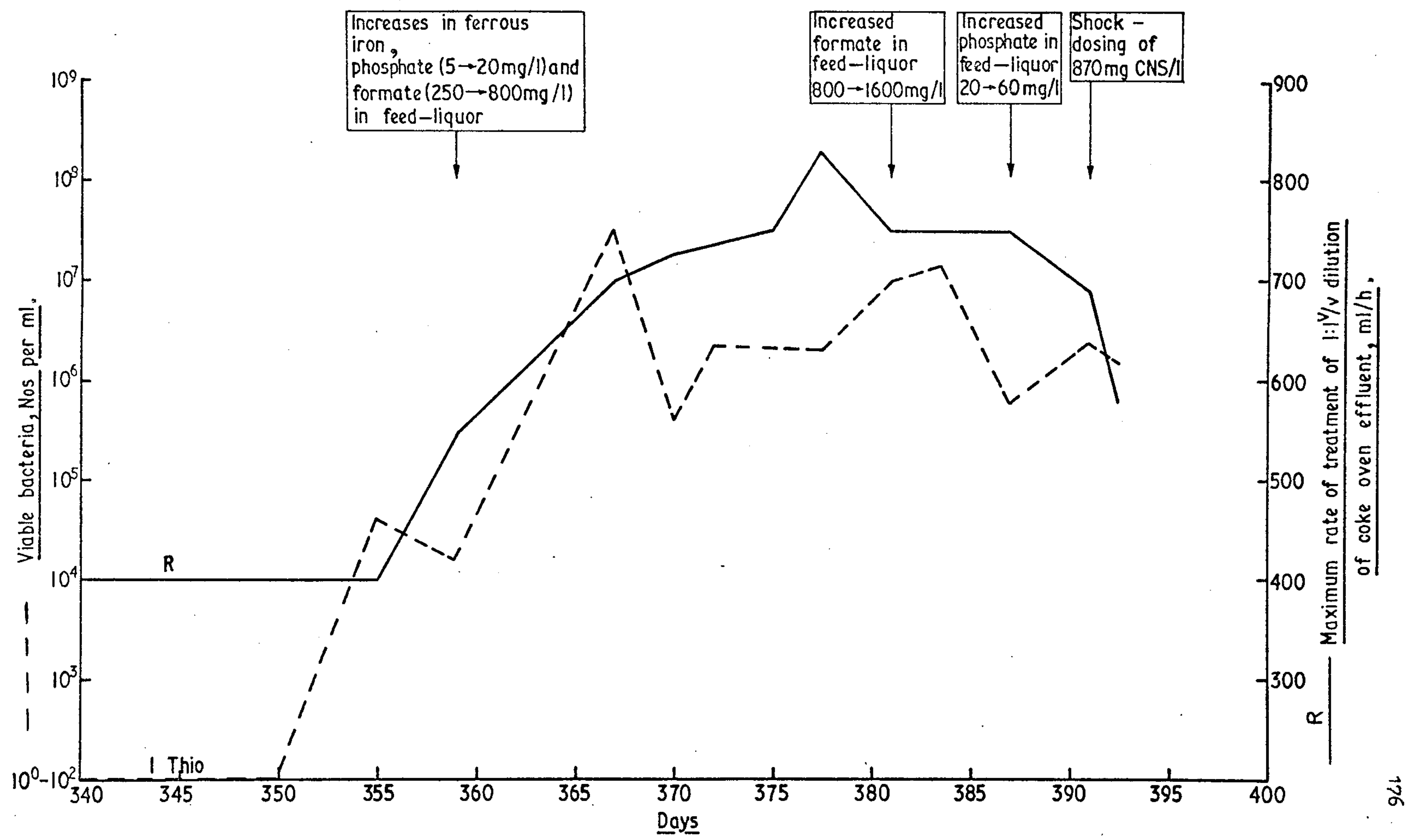


FIG 25

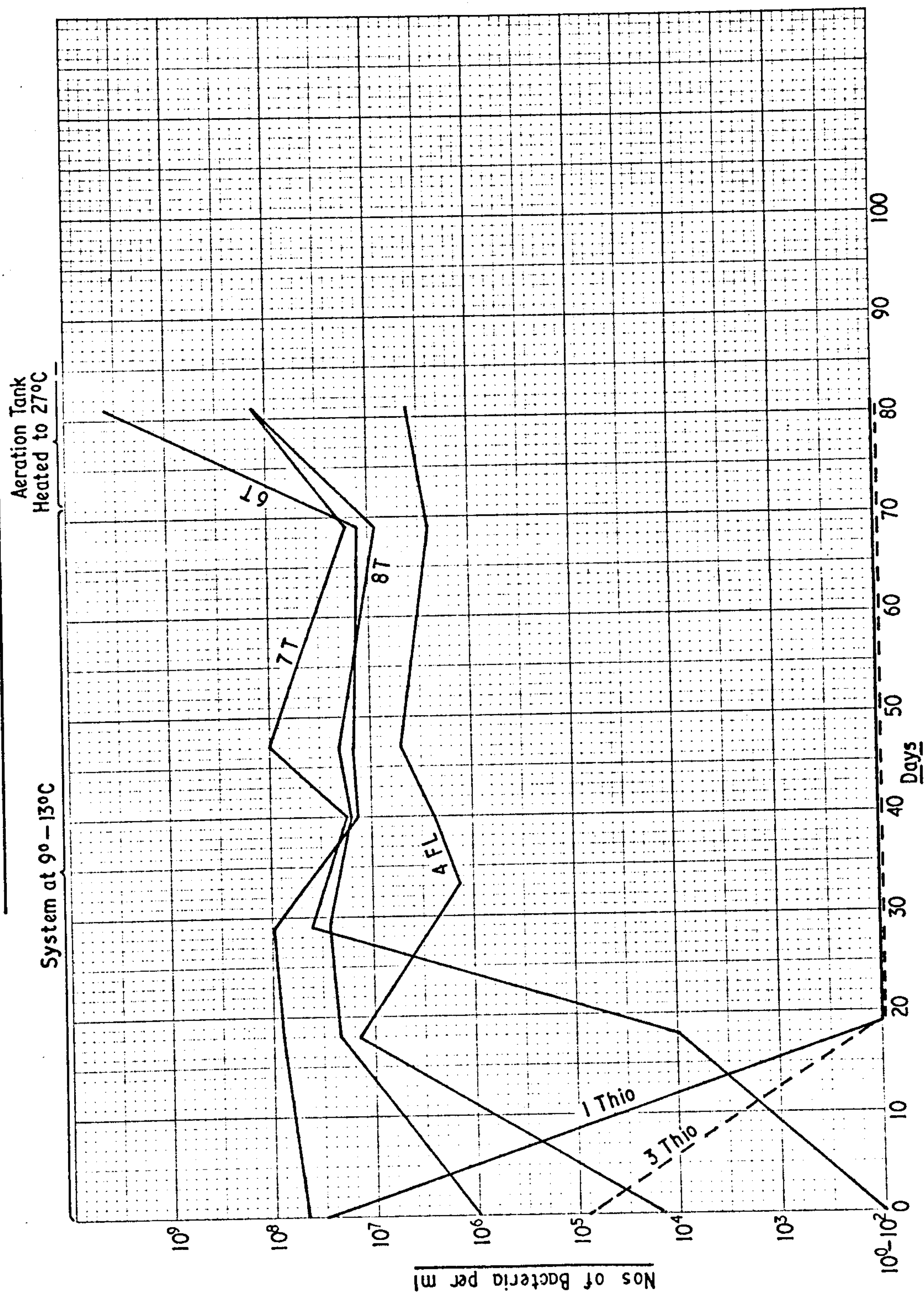
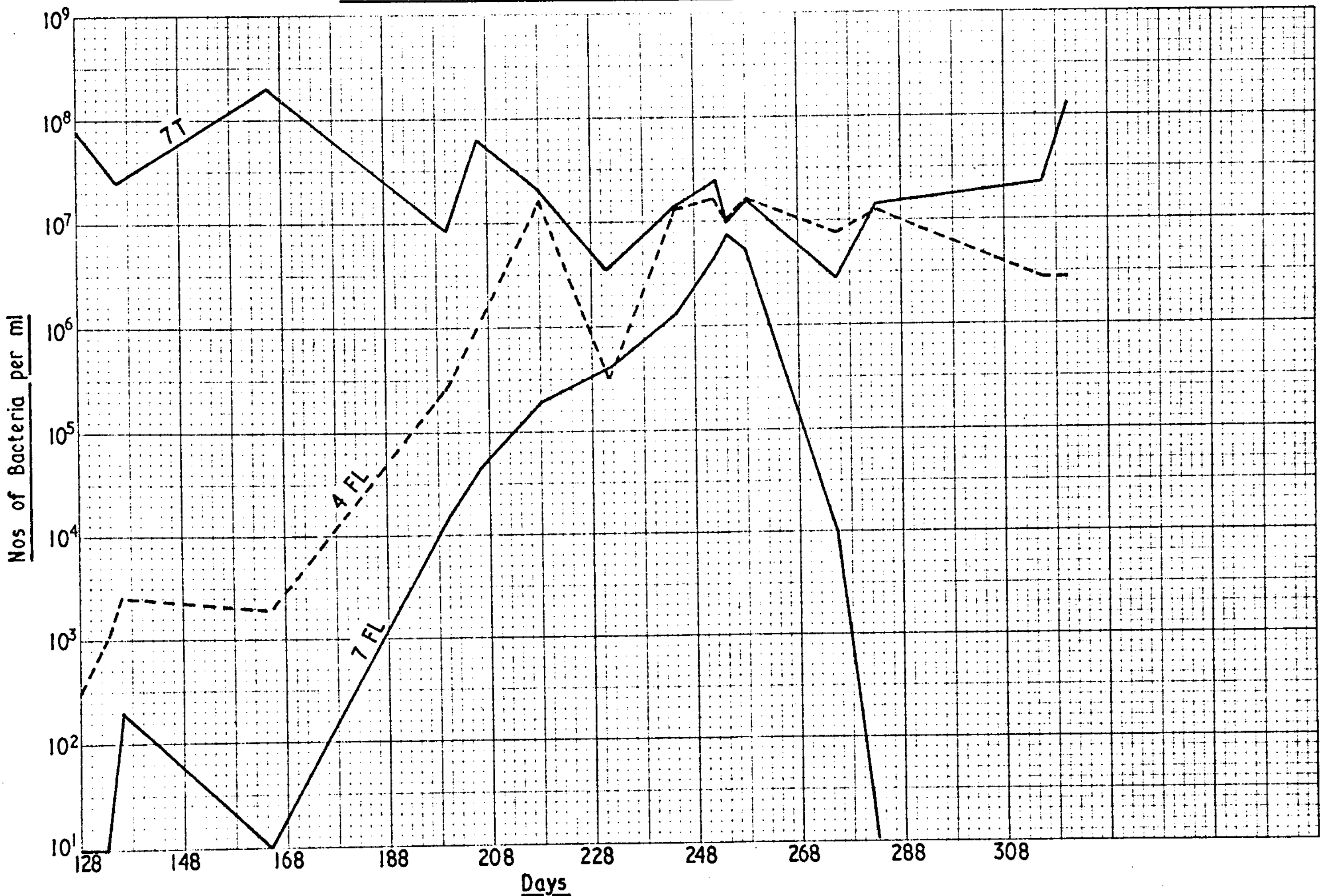


FIG 26

CHANGES IN NUMBERS OF FLUORESCENT PSEUDOMONADS AND TOTAL BACTERIA DETECTED ON THIOCYANATE + SUCCINATE AGAR



6. Discussion.

The destruction of thiocyanate in activated sludge systems purifying coke oven effluent has been attributed to the activity of thiobacilli (Abson and Todhunter, 1958), Pseudomonas stutzeri (Stafford et al, 1969), Achromobacter sp., and Hyphomicrobium sp, (Mather, 1971). The present investigation confirmed that four species of bacteria derived from the laboratory scale activated sludge systems were capable of degrading thiocyanate in pure cultures. The two fluorescent isolates, 4 and 5, described in section 4 resembled Pseudomonas putida; this species had not previously been reported to destroy thiocyanate. The isolation in section 4 of Thiobacillus denitrificans and T. thioparus on an autotrophic thiocyanate medium had been anticipated in view of the earlier confirmation that these species could utilise thiocyanate (De Kruyff, et al 1957). The isolation of a thiocyanate-destroying species of Achromobacter, isolate 3, appeared to support Mather's claim that species of this genus might contribute to the removal of thiocyanate in activated sludges treating coke oven effluent. The species of Micrococcus, isolate 6, was found to be tolerant of 200 mg of thiocyanate per litre; however pure cultures of this species failed to destroy thiocyanate.

When the six isolates of bacteria had been identified and some of their growth characteristics had been determined in section 4, it was necessary to attempt to assess the contribution of each species to the removal of thiocyanate during the activated sludge treatment of coke oven effluent. This was carried out using the programme of bacterial monitoring detailed in section 5. The thiobacilli and fluorescent pseudomonads were readily detected and enumerated in view of their colonial features on thiosulphate minerals agar and on King's medium B respectively. Non-fluorescent colonies which gave a rapid oxidase positive reaction and were also resistant to 0.1% cetrimide were

tentatively identified as pseudomonads which could therefore be monitored readily. However for confirmation of their identity a number of additional tests were necessary as shown in Table 20 on page 113. Nevertheless, monitoring of the non-fluorescent pseudomonad, Pseudomonas stutzeri, was facilitated by its characteristic wrinkled colony form on King's medium A and on the phenol + thiocyanate medium 7. The grouping of isolates within the genus Achromobacter also required several tests given in Table 16 on page 108, all of which could not be included in the programme of frequent bacterial monitoring. Consequently although a species of Achromobacter had been found in this study to destroy thiocyanate, bacteria belonging to this genus were not monitored. The detection of Micrococcus spp in activated sludge was facilitated by their characteristic yellow colonial form on the heterotrophic media, together with their microscopic appearance as Gram positive cocci arranged in cubical packets.

The monitoring of populations of bacteria in the laboratory scale and full scale treatment plants led to an indication of the possible roles of some of the different species of thiocyanate destroying bacteria during biological treatment. Surface drop viable counts using King's medium B and cetrimide agar showed that pseudomonads developed to 10^5-10^7 viable organisms per ml in the coke oven liquor fed to the laboratory scale plant, provided that the pH was lowered from 11 to 7.5-8.5 and inorganic phosphate was added. Raw coke oven liquors remained sterile. The feed-liquor therefore contained a continuous inoculum of pseudomonads which were adapted to coke oven effluent. Several isolates of pseudomonads obtained from the feed liquor were found to oxidise phenol as the sole source of carbon. The stability of the purification process may have been improved by the continuous inoculation of an acclimated population of pseudomonads. Biological treatment plants purifying wastes containing inhibitory

substrates such as thiocyanate, phenol and ammonia may show greater stability if fluctuations in the numbers of viable pseudomonads removing these substances can be minimised by the continuous or intermittent addition of acclimated bacteria.

Bacterial monitoring of the laboratory scale plant showed that large numbers of fluorescent pseudomonads were present in the aeration tank when the supernatant liquid gave a large ultra violet absorbance between 225 mμ and 325 mμ. A large proportion of the total numbers of fluorescent pseudomonads were capable of destroying thiocyanate at this time; (Fig 26, page 178). The presence of catechol in the feed-liquor may have stimulated the growth of fluorescent pseudomonads capable of using thiocyanate. This possibility was supported by the observation that thiocyanate destruction by washed cell suspensions of isolate 5 appeared to be stimulated by catechol which showed a peak absorbance of ultra violet light at 274 mμ. However, in addition to catechol, other constituents of coke oven effluent such as phenol, cresols and quinol might have stimulated fluorescent pseudomonads. The ability of species of pseudomonads to utilise aromatic compounds which absorb ultra violet light within the range 225-325 mμ has been reported by Tabak et al (1964), Ornston et al (1966), Stanier et al (1966), Beveridge et al (1969), Walker et al (1969) and Bayly et al (1973).

Before interpreting the results of bacterial monitoring, it is necessary to take into account the changes which occur in viable counts and the dominant species, apparently independent of the composition of the coke oven effluent being treated. The difficulty in assigning a particular biochemical role to a species of bacteria during biological treatment may be increased by the possibility that the ability to utilise a given compound may be transferred between different species. For example, Dunn et al (1973) found that the ability of a strain of Pseudomonas putida to metabolise naphthalene

could be acquired by conjugation or transduction. The metabolism of strains of pseudomonads in biological treatment plants might be influenced by similar interactions; the chemical composition of the waste being treated would exert a selective pressure on these changes. Consequently, in order to determine if catechol stimulates the development, in activated sludge, of fluorescent pseudomonads capable of using thiocyanate it would be necessary to apply a more extensive programme of bacterial and chemical monitoring to several biological treatment plants. Surveys of this type may show that knowledge of the dominant species of *Pseudomonas* in activated sludge could indicate the composition of the waste undergoing purification, signifying increasing concentrations of compounds which might suppress thiocyanate destruction.

Evidence that fluorescent pseudomonads were capable of utilising very low concentrations of nitrogen sources arose in this study during the testing of isolates for the ability to destroy thiocyanate, (section 4.4.14). Growth developed even when precautions were taken to exclude volatile organic compounds from the laboratory atmosphere. In these tests the only source of nitrogen added to the medium was thiocyanate. It was possible that the low growth yields of 10^5-10^6 cells per ml developed at the expense of not more than 1 mg per litre of thiocyanate. If so, this would indicate a high efficiency of conversion of thiocyanate-nitrogen into cellular structures. A drop of 1 mg thiocyanate per litre may have occurred without this being detected by chemical analysis. Hutchinson et al (1969) found difficulty in obtaining an absence of growth in nitrogen free media despite precautions to exclude possible sources of nitrogen contamination. The difficulty in eliminating background growth emphasised the importance of chemical tests to confirm if thiocyanate had been destroyed by the isolates (section 4).

The role of fluorescent pseudomonads in thiocyanate destruction in the biological treatment plant was uncertain in view of the inability of isolates 4 and 5 (section 4) to use thiocyanate in the absence of organic sources of carbon. However, isolate 5 destroyed thiocyanate when it was inoculated into sterile activated sludge with phenol as the only added source of organic carbon, (page 115). Although the use of sterile activated sludge provided some of the conditions which resembled those present in the aeration tank, pure cultures of isolate 5 were used in the tests; isolate 5 might have shown different biochemical activity when influenced by interactions with populations of other species of micro-organisms in continuous culture.

Despite the rapid rate of thiocyanate destruction achieved in pure cultures by isolate 5, the bacterial monitoring described in section 5 provided evidence that the successful purification of coke oven effluent could be achieved in the absence of viable fluorescent pseudomonads. Furthermore the period of rapid thiocyanate destruction which permitted a reduction in the minimum aeration time from twenty hours to seven hours was accompanied by rapid increases in total heterotrophs, non-fluorescent pseudomonads and thiobacilli, but the numbers of fluorescent pseudomonads did not increase. Nevertheless the ability of the fluorescent pseudomonad, isolate 5, to destroy thiocyanate in the presence of ammonia, chloride (Table 22 , page 114), cresol, aniline, pyridine and catechol (page 117) and to use thiocyanate and phenol simultaneously indicated that this isolate might be capable of contributing significantly to the removal of thiocyanate in activated sludge plants purifying coke oven effluents. The stability of the purification process could usefully be improved by the intermittent or continuous inoculation of an acclimated culture of isolate 5. Alternatively a culture of the bacterium may be included in the activated sludge used to "seed" biological treatment plants during their commissioning periods. A specimen of isolate 5 was therefore submitted

as NCIB 11198, to the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen.

A significant characteristic of isolate 5 was its ability to use thiocyanate and phenol simultaneously. In this respect the isolate resembled Pseudomonas stutzeri (N.C.I.B. 10331) isolated by Stafford et al (1969). The ability of species of bacteria such as isolate 5 and P. stutzeri to obtain energy from the degradation of more than one major constituemt of coke oven effluent may provide these species with a selective advantage over other species such as thiobacilli which have a more restricted range of substrates, particularly when fluctuations in purification-efficiency give rise to the accumulation of thiocyanate and phenol in the aeration tank.

Medium 7, which contained high concentrations of thiocyanate and phenol was expected to serve as a selective medium for the detection of Pseudomonas stutzeri. However, none of the colonies recovered on medium 7 showed the characteristic wrinkled colonial form of P. stutzeri. Subculturing of several of the isolates onto King's medium A again failed to detect this species. Furthermore the failure to detect either P. stutzeri or thiobacilli on several occasions when thiocyanate destruction was complete indicated that other species of micro-organisms were then responsible for degrading thiocyanate. Achromobacter spp or Hyphomicrobium spp might then have contributed to thiocyanate destruction. Further work is needed to confirm the contribution of these and possibly other species of micro-organisms to the overall removal of thiocyanate from coke oven effluents. However the present programme of bacterial monitoring showed that Micrococcus spp and phenazine-forming pseudomonads such as Pseudomonas aeruginosa were usually not detected in the treatment plants.

At the time when two aeration vessels in series were recommended for the biological treatment of coke oven effluents (Abson and Todhunter, 1958) it was suggested that species of bacteria which

destroyed phenol were active mainly in the first aeration vessel and that thiocyanate destruction by a different group of species, namely species belonging to the genus Thiobacillus, occurred only in the second aeration tank. The present investigation confirmed that the oxidation of phenol was established before thiocyanate destruction became rapid, but the testing of pure cultures of isolate 5 (section 4) showed that phenol and thiocyanate could be destroyed simultaneously by this single species of bacteria. Furthermore, in the treatment plants used in the present investigation the thiobacilli often remained in small numbers or were not detected although efficient destruction of thiocyanate was maintained.

The testing of washed resting cell suspensions of isolate 5 (page 116), indicated that when a source of organic carbon, such as phenol, was available at a relatively high concentration as an alternative source of carbon to thiocyanate, a delay occurred before thiocyanate destruction occurred. A similar delay in the destruction of thiocyanate was observed using mixed cultures of activated sludge, described on page 76. Further evidence of a delaying effect on thiocyanate destruction caused by active phenol oxidising bacteria arose from chemical and bacteriological tests carried out using the full scale treatment plant during starting operations (Table 31, page 174). Owing to difficulties in controlling the rate of flow of feed liquor at this time, excessively large volumes of coke oven effluent were pumped intermittently into the aeration tank. The biological oxidation of phenol was rapidly stimulated by these additions, although thiocyanate destruction was delayed. The high activity of phenol oxidising bacteria was shown by chemical tests which confirmed the substantial destruction of phenol, although thiocyanate accumulated to 300 mg per litre and was removed only slowly during a period of three days. Inefficient thiocyanate destruction appeared to be caused by the introduction into the aeration vessel of abnormally large amounts of phenol, either

in high concentrations in the feed-liquor, or as abnormally large doses of coke oven effluent. However, since phenol did not accumulate in the treatment plant, this compound was not immediately considered to have caused thiocyanate to accumulate.

It was possible that the increased rate of phenol oxidation delayed thiocyanate destruction. The rapid destruction of phenol was accompanied by an increase in the viable count of heterotrophic bacteria, including pseudomonads, which were capable of using phenol, (Fig 25, page 177). At the same time an increase occurred in the turbidity of the final effluent; this was caused by the presence of greater numbers of dispersed bacteria which might have become dislodged from the flocs owing to their increased activity in oxidising phenol. This indicated that increased viable counts of heterotrophic bacteria might sometimes have indicated a decline in the efficiency of thiocyanate destruction, when phenol was rapidly used possibly as an alternative source of carbon. However, during carefully controlled operation of the plant, when the flow rate and composition of the feed liquor were constant, viable counts of pseudomonads were normally between 10^7 and 10^9 per ml during efficiency purification. Cell densities of a similar order, i.e. 10^7 viable bacteria per ml, were also required before thiocyanate destruction was detected using pure cultures of isolate 5; it was therefore possible that an optimum cell density may have been required.

The prolonged lag periods which often occurred before thiocyanate was destroyed by isolates 4 (page 110) and 5 might have been caused by an inhibitory effect of thiocyanate on growth until cell yields of 10^7 bacteria per ml had developed. An inhibitory effect of this nature might have resembled prolonged lag periods described by Andrews (1968) who proposed a mathematical model, incorporating an inhibition function, for use in the design and operation of continuous flow biological systems utilising inhibitory substrates. The

application of mathematical modelling to the biological treatment of coke oven effluent may be valuable in view of the inhibitory effect of thiocyanate on the bacteria that utilise it.

Tests using the laboratory scale plant, page 62, showed that in addition to delaying thiocyanate destruction, high influent concentrations of phenol sometimes appeared to caused sludge-bulking which in turn resulted in a decline in the extent to which thiocyanate was removed during biological treatment.

The above observations using pure cultures of isolate 5 and the laboratory scale and full scale treatment plants indicated that although phenol was normally oxidised rapidly, the efficiency of thiocyanate destruction might have been diminished owing to the preferential oxidation of phenol, the washout from the system of dispersed bacteria and sludge-bulking.

During the investigation of the full scale plant, it was not determined whether one species or a number of different species were responsible for the oxidation of both phenol and thiocyanate; thiobacilli were normally absent while pseudomonads were in large numbers. The delay in thiocyanate destruction could therefore have been caused by the use of thiocyanate in preference to phenol by one or more species of bacteria other than thiobacilli, or alternatively thiocyanate destruction by one species may have been suppressed by the activity of different species which were actively oxidising phenol. The latter type of interaction between different species was reported by Jones et al (1972). However Jones observed the suppression of thiocyanate destruction by *Thiobacillus thioparus* in mixed cultures, whereas this species was rarely detected in the present study.

Deterioration in the performance of the laboratory scale plant which were caused by sludge bulking, page 58, were not shown by drops in viable bacterial counts until a substantial amount of activated sludge had been lost from the system. By this time the deterioration

was shown by the conventional test for residual thiocyanate concentration in the aeration tank, together with the microscopic appearance, page 69 , and drops in the percentage sludge volume and suspended solids concentration.

The lack of sensitivity of counts of viable bacteria in providing an early indication of deteriorating sludge settlement was attributed to three factors: Firstly, a moderate drop in the numbers of viable bacteria, e.g. from 10^8 to 10^7 per ml, often occurred without a corresponding decline in plant performance. Consequently a ten percent loss of the viable bacterial content of the activated sludge flocs could occur without the recording of abnormally low viable bacterial counts. Secondly, since all the viable counts included dispersed and flocculated bacteria, the degree of flocculation in the treatment plant was not determined. A condition of poor sludge-settlement involving dispersion of the bacteria from the flocs could therefore remain undetected by bacterial monitoring until substantial washout of the deflocculated cells occurred. Thirdly, decreased rates of thiocyanate destruction in the laboratory system were often caused by increases in the proportion of poorly settled fungal flocs. During the early stages of the development of fungal flocs the numbers of viable bacteria were not reduced. Microscopic examination of the flocs, Fig 5 page 65 , was then of greater value than bacterial monitoring in detecting the decline in sludge quality due to bulking.

In the present study microscopic examination of the activated sludge proved to be a valuable operational aid. The gradual development of fungal flocs shown in Figs 5 and 12 gave an advance warning of deteriorating sludge quality. This deterioration eventually resulted in the accumulation of thiocyanate in the aeration tank if remedial action was not taken. On three successive occasions it was possible to eliminate the sludge bulking problem by increasing the dilution of the feed liquor using clean dilution

water. This remedy can be recommended for the operation of full scale plants, but the extra dilution water may not always be available. Furthermore the increased volume of final effluent from the biological treatment plant may exceed the volume required for coke quenching. Surplus final effluent may then be discharged to waterways. Strict control of this practice would be required particularly if biological purification was ineffective at the time of discharging.

The isolation, from the laboratory scale plant, of Thiobacillus thioparus and T. denitrificans using an autotrophic thiocyanate medium had been anticipated in view of the earlier confirmation that these species destroyed thiocyanate (De Kruyff, et al 1957). However, the sensitivity of thiobacilli to inhibition by constituents of coke oven effluent, such as oxidised catechol (Hutchinson et al 1964) appeared to be illustrated in the present study by the elimination of thiobacilli in the full scale plant and by their apparently rare occurrence in the laboratory scale plant.

During the programme of bacterial monitoring, the main feature used to identify and enumerate colonies of thiobacilli was the deposition of elemental sulphur. However, thiobacilli might sometimes have been active in using thiocyanate in the liquid environment of the biological treatment plant, but their ability to form sulphur-depositing colonies on solid media may have been impaired, due possibly to slow adaption to the change in physiological conditions during primary isolation on the solidified selective media.

Large numbers of thiobacilli were detected during a brief period of three weeks, using the laboratory scale system purifying coke oven effluent, (Fig 24 , page 176). The rapid increase in viable counts of thiobacilli occurred at the same time as an increase in sludge volume and a significant improvement in the settling property of the activated sludge (see page 140). Although the numbers of viable thiobacilli declined shortly after the initial increase in numbers,

the beneficial effect on purification persisted. This might have been due to the improvement in sludge quality brought about by pronounced flocculation of thiobacilli which entered the declining phase of growth. The decline in the viable counts of thiobacilli might have been caused by an increase in the proportion of cells utilising thiocyanate primarily for maintenance energy. As suggested by Jones (1975), although such cells might have remained non-viable, they might nevertheless have retained the ability to contribute to the biochemical activity of the activated sludge. Furthermore the increased biomass of flocculated micro-organisms offered a larger surface area for the adsorption of thiocyanate from the supernatant liquid.

The conditions of biological treatment which had been established preceding the increase in thiobacilli (page 176) could have favoured their development. For instance, a high rate of treatment had been established which prevented the accumulation in the system of inhibitory concentrations of compounds such as dihydric phenols and pyridine bases; the supernatant liquid from the aeration tank showed a relatively low ultra violet absorbance between 225 mμ and 325 mμ. Furthermore, the water used to dilute the coke oven effluent did not contain high concentrations of inhibitory materials. The development of thiobacilli may also have been favoured by the low pH of the feed liquor which enabled inorganic phosphate and ferrous iron to be retained in solution. The large increase in thiobacilli might have been caused by a combination of these factors.

Further work should be carried out to determine if, under similar conditions to those described above, the improvement in purification and the high counts of thiobacilli may be repeated. The required combination of operational conditions may then be established. However despite the possibility that sludge settlement may be improved by the presence of a large population of thiobacilli, careful control of the

process would be required to maintain the optimum conditions for thiobacilli in view of their sensitivity to inhibition. The present investigation indicated that although the ratio of pseudomonads to thiobacilli was usually very high in activated sludges which were efficiently purifying coke oven effluent, this ratio might, under the conditions described above, be reversed without detrimental effects on the efficiency of purification.

Organic compounds present in coke oven effluent were, at the outset of this study, considered generally to have an inhibitory effect on thiobacilli, particularly T. thioparus and T. denitrificans which were regarded by Rittenberg (1969) to be obligately chemolithotrophic. However, certain organic compounds such as formate (Youatt, 1954) and succinate (Vishniac, 1952), had previously been reported to be utilised by T. thioparus. Hall (1968) had recorded high rates of treatment and increased yields of rapidly flocculating activated sludge during the biological treatment of Corby coke oven liquors containing up to 1,000 mg ammonium formate per litre, but the species of bacteria which were dominant in the sludge were not determined at that time. In the present study, a rapidly settling activated sludge was again formed when an exceptionally high concentration of formate was present in the feed liquor, (page 64). Since a rapid increase in the number of thiobacilli was also recorded at this time, the possibility was considered that formate might have been utilised by the thiobacilli. The presence of 400 mg formic acid per litre in batch cultures of N.C.I.B. 8370 caused a suppression of both thiocyanate destruction and viable counts, (page 106). Nevertheless during continuous culture, the rapid oxidation of formate as it entered the system could have maintained a low residual concentration in the aeration tank. Additional tests should therefore be carried out to investigate the effects of different concentrations of formate on growth and thiocyanate destruction by thiobacilli in continuous culture.

The influence of organic compounds on the thiobacilli has been studied in greater detail since the isolation of facultatively autotrophic thiobacilli; T. intermedius (London, 1963) and T. perometabolis (London and Rittenberg, 1967). Smith et al (1967) considered that reduced NAD dehydrogenase plays a key role in heterotrophic metabolism and proposed that the absence of this enzyme in T. thioparus was the basis of its obligate chemoautotrophy. However Johnson and Abraham (1969) foundevidence for an active NAD dehydrogenase enzyme in one strain of T. thioparus. It was therefore suggested that the inability to couple the breakdown of organic substrates with the generation of ATP might be attributed to the absence of coupling between NAD dehydrogenase activity and phosphorylation. The same workers also reported the apparent absence from T. thioparus of phosphofructokinase and αketoglutarate dehydrogenase. The absence of phosphofructokinase would prevent subtrate-level phosphorylation using certain substrates which, in heterotrophs, normally enter the glycolytic pathway. The lack of αketoglutarate dehydrogenase in the thiobacilli causes a break in the tricarboxylic acid cycle at the point where αketoglutarate would be converted into succinate.

Evidence has however been presented for both substrate level phosphorylation (Trudinger, 1967) and electron transport phosphorylation (Kelly and Syrett, 1964) in thiobacilli. The importance of substrate level phosphorylation was emphasised by Johnson et al (1969) who reported that in the strictly autotrophic thiobacilli, the main sources of energy as ATP and reducing power as NADH, are usually reduced inorganic sulphur compounds; during thiosulphate oxidation approximately one mole of phosphate may be esterified to ADP for each mole of sulphate produced (Trudinger, 1967). The reduction of carbon dioxide to carbohydrates and other cell components by thiobacilli requires both ATP and NADH (Johnson et al, 1968) which are generated during the oxidation of reduced inorganic sulphur compounds. Pan et al (1972)

grew T. thioparus in dialysis culture (Borichewski et al, 1966) on a glucose mineral salts medium lacking any inorganic energy source such as thiosulphate. Pan suggested that under normal conditions the accumulation of growth-inhibiting substances may prevent growth on heterotrophic substrates such as glucose.

Despite the dependence of T. thioparus on sources of reduced inorganic compounds to provide a substantial proportion of the energy required for optimal growth, certain organic compounds may be incorporated into the cells. Since Cooper et al (1966) reported that shorter retention times and the complete oxidation of thiocyanate could be achieved by adding certain organic compounds, the possibility that thiobacilli may utilise these compounds is considered in this discussion. Heterotrophs are likely to be stimulated to a greater extent than thiobacilli, but thiobacilli may also incorporate certain organic compounds.

Cooper et al (1974) found that the addition to activated sludge plants of 5-10 mg glycine per litre could bring about improvements in biological treatment. Kelly (1967a) reported that glycine was incorporated into Thiobacillus neapolitanus; 1.7% of the total cellular carbon was calculated to have been derived from glycine. However, Kelly observed that although certain amino-acids may contribute to biosynthesis in T. neapolitanus, they could not be utilised unless energy was obtained from the chemolithotrophic oxidation of thiosulphate. Further work by Kelly (1967b) showed that other organic compounds, namely acetate, pyruvate and formate, could be incorporated into T. neapolitanus. However, their use was probably restricted to biosynthesis; formate was incorporated into purines. The degradation of pyruvate, acetate and formate appeared to occur only to a limited degree. However, acetate could replace carbon dioxide as the major source of cellular carbon.

Using activated sludge purifying a synthetic solution of ammonium

thiocyanate, Woodward et al (1974) found that pyruvate was not oxidised but was incorporated into cell structures. Since the bacteria in the activated sludge were found to resemble thiobacilli, the results of Woodward's investigation appeared to agree with the results of Kelly's (1967) work on the use of pyruvate by thiobacilli.

The work of Johnson et al (1969) provided evidence against the possibility that *T. thioparus* might be stimulated by pyruvate or acetate when thiosulphate was available as an exogenous energy source. Johnson found that the presence of thiosulphate suppressed the uptake of pyruvate and acetate. Since thiosulphate is always present in the feed liquor to biological systems purifying coke oven effluent, a stimulating effect of pyruvate or acetate on *T. thioparus* appears unlikely.

As described above, the absence of phosphofructokinase in *T. thioparus* would appear to preclude the possibility of stimulating this species using substrates such as glucose which are normally metabolised through the glycolytic pathway in heterotrophs. The beneficial effects of additions of glucose which were reported by the British Carbonisation Research Association (1973) were therefore unlikely to have involved a stimulation of *T. thioparus*. Nevertheless, recent experiments carried out by Taylor et al (1969) showed that a different growth factor proposed by Cooper et al (1966), namely para-hydroxybenzoic acid, could be assimilated by a species of *Thiobacillus*. A detailed identification of this species was not given and the ability to degrade thiocyanate was not investigated. However, the isolate was shown to be capable of oxidising thiosulphate to sulphate autotrophically.

The effects of organic compounds in stimulating heterotrophs during the biological treatment of coke oven effluent appeared to be substantiated in this study by the apparent stimulation of fluorescent pseudomonads by catechol (page 178), the development of fungal

filaments following the addition of alanine (page 78) and the growth of fungal flocs in the presence of high influent concentrations of phenol (page 65). Consideration should therefore be given to the ways in which micro-organisms generally utilise the organic growth factors recommended by the British Carbonisation Research Association (1966 and 1972). The growth factors described below include glucose, pyruvic acid, para-aminobenzoic acid, para-hydroxybenzoic acid, anthranilic acid, glycine and alanine:

The catabolism of glucose has a central role in the metabolism of many micro-organisms. Differences have been observed between different species in the relative importance of the two main pathways for the breakdown of glucose; namely the Embden Meyerhof Pathway (E.M.P.) and the pentose phosphate pathway. All the enzymes of the pentose phosphate pathway, which provides pentoses and $NADPH_2$, were demonstrated in Thiobacillus thioparus (Johnson et al, 1969). A third pathway of glucose metabolism was found to operate in species of Pseudomonas (Entner and Doudoroff, 1952 and De Ley, 1962).

Pyruvic acid occupies a key position in linking several metabolic pathways. For example, pyruvate links the glycolytic pathway of carbohydrate breakdown with the tricarboxylic acid cycle. Pyruvate serves as a precursor of amino-acids such as leucine and valine. In particular, pyruvate is converted into acetyl co-enzyme A. Pyruvic acid may also participate in the incorporation of ammonia into organic compounds such as L-alanine (Mortensen, 1962).

Para-aminobenzoic acid is known to serve as a growth factor for many micro-organisms, although Mather (1971) did not observe a stimulating effect on thiocyanate degradation using pure cultures. Para-aminobenzoic acid may be incorporated into dihydropteroate. This compound is a precursor of tetrahydropteroylglutamate (Rose, 1968) which functions as a co-factor in the transfer of one-carbon units, e.g. in the interconversion of glycine and serine (Davis et al 1964).

Para-hydroxybenzoate is a simple organic compound which was found to be extensively used by aerobic pseudomonads (Stanier et al, 1966) and more recently by a facultatively autotrophic species of Thiobacillus (Taylor et al, 1969). The mechanism of cleavage of protocatechuate, a product of the degradation of para-hydroxybenzoate, has been used as a criterion for characterising different species of pseudomonads (Stanier et al, 1966). Stanier reported that para-hydroxybenzoate and benzoate could be utilised for carbon and energy by 57% of the strains of fluorescent pseudomonads tested. Compounds such as succinate and acetyl co-enzyme A, which are commonly formed from the oxidation of aromatic compounds, may be oxidised in the tricarboxylic acid cycle.

Anthranilic acid participates in biosynthetic reactions leading from phosphoenol pyruvate and erythrose-4-phosphate to the biosynthesis of tryptophan (Rose, 1968).

Micro-organisms oxidise glycine in a number of ways. Glycine may participate in reactions leading to the replenishment of intermediates of the tricarboxylic acid cycle, such as malate. Glycine, together with succinyl co-enzyme A, also serves as a precursor for the biosynthesis of haemoproteins such as the cytochromes.

Beta-alanine functions with pantoate as a precursor of acetyl co-enzyme A. Alanine and glycine may also be synthesised into several different proteins.

Certain organic compounds such as phloroglucinol, pyrogallol and quinol which are sometimes encountered in carbonisation effluents, were found to be resistant to biological oxidation (Pankhurst, 1959). Other organic compounds including pyridine (page 56 of this study) and oxidised catechol (Hutchinson et al, 1964) inhibited the biological oxidation of thiocyanate. The toxic effect of pyridine, investigated in section 3.1.1.6, did not immediately cause an accumulation of thiocyanate. A gradual accumulation of dissolved coloured materials,

possibly polymerised phenols, preceded the suppression of thiocyanate oxidation. As pointed out by Callely et al (1971), high concentrations of these coloured materials in the liquid surrounding the flocs are generally considered to be inhibitory. Coloured substances which resist biodegradation may be absorbed by the activated sludge. The gradual loss of sludge caused by bulking in the laboratory scale plant probably aggravated the deterioration in plant performance. A once weekly determination of the concentrations of dissolved coloured materials which resist bacterial attack could supplement the daily tests for thiocyanate and phenol.

Pyridine might have been destroyed if it had been introduced into the system at a lower concentration, or if micro-organisms capable of metabolising pyridine had been active. Stafford and Callely (1970) reported that pyridine can be utilised by activated sludge-bacteria. Stafford found that a portion of the pyridine was oxidised completely to carbon dioxide; energy was obtained by the bacteria involved in this process. The remainder of the pyridine was incorporated as carbon and nitrogen in proteins and nucleic acids. The addition of a small concentration of glucose, 5 mg per litre, was found to have a beneficial effect in the treatment of coke oven wastes by permitting the destruction of pyridine (B.C.R.A. report 76, 1973). The experiments carried out by the B.C.R.A. showed that additions of glucose also facilitated the complete oxidation of 200 mg ammonia per litre without the need for tertiary treatment.

High inhibitory concentrations of ammonia, 1,000 mg per litre, in the feed liquor to the laboratory scale plant (section 3) probably suppressed the rate of thiocyanate destruction. This possibility was supported by the suppression of thiocyanate destruction caused by the addition of 300 mg ammonia per litre to washed resting cell suspensions of isolate 5. Nitrification did not occur in the laboratory scale plant.

The possible inhibition of nitrification by thiocyanate was investigated by Tomlinson et al (1966), who found that potassium thiocyanate at 300 mg per litre had no inhibitory effect on the oxidation of ammonia by activated sludge. However, Tomlinson observed the inhibition of nitrification by phenol, cresols and aniline at concentrations below 20 mg per litre.

Ammonia and phenol were used as the sole sources of nitrogen and carbon for the growth of fungi isolated from bulked activated sludge described in section 3.1.1. Feed liquor with high concentrations of phenol and ammonia may therefore contribute to poor plant performance due to sludge bulking.

The need for strict control of the operating conditions for the laboratory scale plant demonstrated the instability of the treatment process. Inhibition of thiocyanate degradation was shown by a prolonged lag period, during the initial stage of the acclimation of sewage sludge to coke oven effluent (Table 4 , page 41). The inhibitory effect was overcome, and greater growth yields of activated sludge developed, when the inoculum was exposed to progressively increasing concentrations of thiocyanate, instead of immediately dosing with the final high thiocyanate concentration. Further evidence for the inhibitory effect of thiocyanate at high concentrations arose from the experiments carried out in section 4 using washed resting cell suspensions of the fluorescent pseudomonad, isolate 5. Maximum growth yields were obtained using 200 mg thiocyanate per litre, but at 300-500 mg per litre, lower growth yields were obtained. Prolonged lag periods were observed in the present study when pure cultures of isolate 4 were tested for the ability to degrade thiocyanate. This could have been caused by an inhibitory effect of thiocyanate, although this was not expected at the low initial concentration of 20 mg per litre.

Experience gained during the operation of the laboratory scale plants enabled assessments to be made of additional factors which

might influence the activity of micro-organisms in the activated sludge. These additional factors included the concentration of chloride in the feed-liquor, the optimum range of pH values and the concentration of dissolved oxygen. The main criterion used to determine the effects of these factors was the rate of removal of thiocyanate from the liquid in the aeration tank.

The experiment in section 3.1.2, using synthetic coke oven effluents, showed that chloride concentrations which were commonly encountered in Corby coke oven effluent did not impair the treatment process when the concentration of chloride was increased progressively from 1,000 to 8,000 mg per litre. This result was consistent with Pankhurst's (1959) observation that inhibition by chloride could be overcome by acclimatisation. However Cooper and Catchpole (1966) found that high chloride concentrations were inhibitory. The implication of the present investigation and work done elsewhere is that concentrations of chlorides consistently in the range of 1,000 - 8,000 mg per litre will not normally adversely affect the biological treatment process.

After the development of an enrichment of thiobacilli active in the oxidation of thiosulphate, the main difficulty in inducing thiocyanate oxidation was the control of pH. Acidity developed due to the oxidation of elemental sulphur to sulphuric acid, after the supply of thiosulphate had been exhausted. Below pH 5.5 and above pH 7.5 the rate of destruction of thiocyanate was severely suppressed. This illustrated the restricted range of pH values required for the degradation of thiocyanate.

Excessively low pH values of 5.2 - 5.9 occurred during the oxidation of thiosulphate when this substance was present at 410 mg per litre in the feed liquor. Pearce et al (1975) reported that high concentrations of thiosulphate in excess of 300 mg per litre could result in acid conditions due possibly to the production of sulphur dioxide.

The dissolved oxygen concentration in the aeration tank was maintained in excess of the 1 mg per litre needed for the maximum rate of thiocyanate-destruction. However, mechanical failures of the aerator resulted in a rapid depletion of dissolved oxygen to below the critical concentration of 0.8 mg per litre, see Fig 4 on page 43. Maintenance of a dissolved oxygen concentration of at least 2-3 mg per litre in the aeration tank may permit a safety margin of approximately five minutes before severe oxygen-depletion occurred following failures of the aerator. Since the most critical resistance to oxygen-transfer is probably at the floc-surface, dissolved oxygen measurements in the aeration-tank mixed liquor may be higher than at the interface between the bacteria and their immediate surroundings. Provision of an excess of dissolved oxygen may compensate for these areas of oxygen-depletion and would allow for sudden increases in the oxygen demand of the waste.

It was concluded from the present investigation that the monitoring of populations of pseudomonads and thiobacilli, together with microscopic examination of the activated sludge and additional chemical tests for inhibitors of thiocyanate destruction, should be included in the programme of regular tests used to assess plant performance. The bacteriological monitoring technique developed in this study enabled a thiocyanate degrading strain of *Pseudomonas putida* to be recognised and enabled the ratio of pseudomonads to thiobacilli to be determined. The cultural methods used may confirm the results of more rapid biochemical monitoring techniques (Woodward et al, 1974). The surface drop technique may conveniently be used to assess, with greater accuracy, the efforts to improve process stability by adding organic compounds to stimulate heterotrophs such as pseudomonads in preference to the chemolithotrophic thiobacilli.

Appendix 1: Media used for the isolation of pure cultures (Section 4) and in the monitoring of bacterial populations (Section 5).

1. 1% thiosulphate minerals medium.

Na_2HPO_4	1.2 g
KH_2PO_4	1.8 g
$MgSO_4.7H_2O$	0.1 g
$(NH_4)_2SO_4$	0.1 g
$CaCl_2$	0.03 g
$FeCl_3.6H_2O$	0.02 g
$MnSO_4.4H_2O$	0.02 g
$Na_2S_2O_3.5H_2O$	10.0 g *
Deionised water	1,000 ml
pH	6.7

The medium was solidified using 12.0 g purified agar (Difco, 0.140-01) or 12.0 g Ionagar No. 2 (Oxoid L12). Sterilisation was by autoclaving for 15 minutes at 121°C, 15psi.

* For the testing of certain pure cultures in Section 4, the concentration of $Na_2S_2O_3.5H_2O$ was increased to 60 g per litre.

Unless otherwise stated, all other media were solidified and sterilised in the same way.

2. Acidified 1% thiosulphate minerals medium.

The composition was the same as medium 1, but the pH was adjusted to 4.1. At this low pH it was necessary to use 24 g of purified agar to solidify the medium.

3. 0.02% ammonium thiocyanate minerals medium.

The composition was the same as medium 1, except that 10 g of $Na_2S_2O_3.5H_2O$ was replaced by 0.2 g NH_4CNS.

4. King's medium B.

proteose peptone (Oxoid L46)	20.0 g
glycerol	10.0 g
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar No. 3 (Oxoid CM 39)	15.0 g
Deionised water	1,000 ml
pH	7.2

5. Cetrimide agar.

The composition was the same as medium 4, but cetrimide (cetyl trimethyl ammonium bromide) was added, giving a final concentration of 1 g per litre.

6. C.G.Y. agar (casitone + glycerol + yeast extract).

Casitone (Difco 0259-01)	5.0 g
glycerol	5.0 g
yeast extract (Oxoid L 20)	1.0 g
agar No. 3 (Oxoid CM 39)	13.0 g
pH	7.2

Autoclaved for 15 minutes at 121°C.

7. Thiocyanate + succinate agar.

Basal medium :	K_2HPO_4	1.0 g
	KH_2PO_4	1.0 g
	$MgSO_4.7H_2O$	0.2 g
	$CaCl_2$	0.2 g
	$FeCl_3.6H_2O$	0.1 ml of 60% w/v solution in deionised water.
	Deionised water	1,000 ml
	pH	6.7
	Ionagar No. 2 (Oxoid)	12.0 g

0.36 g KCNS and 6.95 g disodium succinate were sterilised separately, and added to the sterilised basal medium.

8. Thiocyanate + phenol agar.

The composition was the same as medium 7, but the 6.75 g disodium succinate was replaced by 0.47 g phenol. The phenol medium used in Section 4 contained 0.264 g $(NH_4)_2SO_4$ in place of KCNS.

9. King's medium A.

bacto-peptone (Difco)	20.0 g
DL-alanine	2.0 g
Sodium citrate	10.0 g
K_2SO_4	8.6 g
KCl	1.4 g
$MgSO_4.7H_2O$	1.4 g
bacto agar (Difco)	15.0 g
deionised water	1,000 ml
pH	7.2

10. Pseudocel agar. B.B.L. 11553

A medium designed to show resistance to 0.03% cetrimide and the formation of both fluorescein and phenazine pigments.

11. C.L.E.D. medium.

peptone	4.0 g
'lab lemco'	3.0 g
trytone	4.0 g
lactose	10.0 g
L-cystine	0.128 g
Brom-thymol blue	0.02 g
agar	15.0 g
deionised water	1,000 ml
pH	7.3

12. Aeration tank supernatant agar.

Aeration tank liquid	100 ml filtered through Whatman No. 1 filter paper.
Ionagar No. 2	1.2 g

Medium 12 was supplemented with 0.023 g KCNS per 100 ml, when required.

13. Plate count agar (Oxoid CM 325).

14. Nutrient agar (Oxoid CM 3).

15. Coke oven effluent medium :

This medium provided coke oven effluent constituents in the highest concentrations present in the feed liquor used in continuous biological treatment. Liquors from the coke ovens at Corby were mixed in the following proportions : spent still liquor 520 ml, devil liquor 80 ml, benzol plant condensate 40 ml. To this mixture were added 320 ml distilled water, giving a dilution of 2:1 v/v coke oven liquors : water. The mixture was then adjusted to pH 6.6 using 10% caustic soda. 1 ml of a minerals nutrient solution, Table 3 , page 39 , solution No. 1, was then added and the volume was brought to 1 litre. The medium was solidified when required, using 3% Ionagar No. 2 (Oxoid). The concentrations of thiocyanate and phenol in this medium were 530 mg/l and 980 mg/l respectively. Autoclaving at 121°C/15psi/30 minutes did not alter these concentrations.

16. Background count agar.

Na_2HPO_4	1.2 g	$FeCl_3.6H_2O$	0.02 g
KH_2PO_4	1.8 g	$MnSO_4.4H_2O$	0.02 g
$MgSO_4.7H_2O$	0.1 g	Deionised water	100 ml
$(NH_4)_2SO_4$	0.1 g	Purified agar	12 g
$CaCl_2$	0.03 g	pH	6.7

Appendix 2. Methods used for chemical estimation of coke oven effluent constituents.

P.V. 4h. Oxygen absorbed from $^N/80$ $KMnO_4$ acidified with dilute H_2SO_4, during 4h at 27^oC.

Cyanide. Adjusted pH of sample using lead acetate-zinc acetate buffer-solution. Distilled into NaOH solution and determined cyanide in distillate by modified Aldridge's method using p-phenylene diamine.

Thiocyanate. (1) Using iron alum for CNS > 5 mg/l. (2) using modified Aldridge's method in absence of cyanide for CNS < 5 mg/l.

Phenol. Reaction with antipyrin in alkaline solution, using potassium ferricyanide as oxidising agent. Range 0.1 - 5 mg/l for undiluted sample.

Thiosulphate. Sulphide removed with $PbCO_3$. Filtered off $PbCO_3$ + PbS. Added iodine and dilute H_2SO_4 acid to filtrate. Titrated excess iodine with sodium thiosulphate solution.

1 mg $^N/80$ thiosulphate solution = 1.4 mg $S_2O_3^{--}$

Chloride. Added H_2O_2 to boiling sample to oxidise CNS. Removed H_2O_2 by continued boiling Added potassium chromate and sodium carbonate. Filtered, titrated filtrate with $AgNO_3$ solution. Accuracy $\pm$ 1%.

Hydrogen Sulphide. Added cadmium acetate solution. Filtered precipitated cadmium sulphide and reacted with acidified iodine solution. Determined excess iodine with sodium thiosulphate solution. Accuracy $\pm$ 1%.

Formate. Added ammonia to sample. Evaporated to low volume, diluted and repeated evaporation to remove phenols. Added N.H_2SO_4 to adjust to pH 2. Steam distilled to collect 600 ml distillate. Added phenolphthalein and titrated with 0.1N NaOH. 1 ml NaOH = 4.6 mg HCOOH. Accuracy to 5% of true figure.

Ammonia. (1) Free. Distillation into H_2SO_4. Excess H_2SO_4 determined by titration with standard NaOH solution. (2) Fixed. NaOH added to liquid remaining after distillation of free-ammonia. Again distilled into H_2SO_4 and titrated excess acid with NaOH solution.

Nitrite. Diazotisation of sulphanilic acid in acid solution. Resulting diazo compound coupled with 1-naphthylamine-7-sulphonic acid, forming purple-pink azo dye. Range up to 30 µg nitrate as N.

Orthophosphate. Formation of molybdophosphoric acid in acid solution followed by reduction to molybdenum blue by stannous chloride. Range 5-100 µg PO_4.

KEY TO TABLES

No.		Page
1	Analysis of ammoniacal liquors.	2
2	Analysis of coke oven effluents at Corby steelworks.	38
3	Stock nutrients solutions for activated sludge.	39
4	Acclimation of sewage sludge to coke oven effluent.	41
5	Conditions specified for operation of the full scale plant.	45
6	Development of continuous treatment.	48
7	Changes in composition of spent still liquor.	50
8	First series of tests on laboratory scale plant treating 1:1 v/v dilution of coke oven effluent.	54
9	Inhibitory effect of aeration tank mixed liquor.	59
10	Second series of tests on laboratory scale plant treating 1:1 v/v dilution of coke oven effluent.	60
11	Effects of different liquor constituents on the minimum aeration-time.	72
12	Rates of destruction of thiocyanate and phenol in the presence of glycine and alanine.	76
13	Rates of destruction of thiocyanate and phenol in the presence of ammonium formate.	80
14	Testing of *Thiobacillus spp.*, isolates 1 and 2.	104
15	Testing of N.C.I.B. 8370: The effects of 400 mg formic acid per litre.	106
16	Testing of isolates 3a and 3b, *Achromobacter sp.*	108
17	Growth of isolate 4, *Pseudomonas putida*, on six different media.	109
18	Tests for thiocyanate destruction by isolate 4.	110
19	Test for thiocyanate destruction by isolate 4.	111
20	Testing of fluorescent pseudomonads, isolates 4 and 5.	113
21	Characteristic growth of isolate 4 on five different media.	114

KEY TO TABLES (Continued)

No.		Page
22	Growth of isolate 5, in seven different liquid media.	114
23	Growth of isolate 6 on six different media.	118
24	Effects of different methods of sludge-dispersion on bacterial counts.	123
25	Effect of different sonication times on surface drop bacterial counts.	125
26	Comparisons between six different media for the enumeration of fluorescent pseudomonads and total bacteria.	128
27	Test for accuracy of surface drop colony counts.	134
28	Growth of ten isolates from the laboratory scale plant on heterotrophic and autotrophic media.	161
29	Viable bacterial counts in relation to performance of the laboratory scale treatment plant.	166
30	Bacterial counts on dispersed activated sludge from aeration tank of laboratory scale plant.	167
31	Bacterial counts on dispersed activated sludge returned to the aeration tank of the full scale treatment plant.	174

KEY TO FIGURES.

No.		Page
1	Production and purification of coke oven effluent at Corby Steelworks.	3
2	Laboratory scale activated sludge plant treating coke oven effluent.	35
3	Cone-aerator for aeration tank.	40
4	Accumulation of thiocyanate in the aeration tank at low concentrations of dissolved oxygen.	43
5	Bulking of activated sludge.	65
6	Rapidly settling bacterial floc.	66
7	Rapidly settling bacterial flocs.	67
8	Dense bacterial flocs.	68
9	Tasselled bacterial flocs.	69
10	Destruction of thiocyanate at low pH values.	73
11	Dissolved oxygen-uptake and thiocyanate-destruction during recovery from a three-day period out of contact with thiocyanate.	75
12	Fungal filaments causing sludge-bulking during addition of alanine.	78
13	_Pseudomonas putida_ showing four polar flagella.	91
14	Batch cultures used to test for thiocyanate oxidation.	94
15	_Thiobacillus sp_, colony grown on 1% thiosulphate minerals agar.	99
16	_Thiobacillus sp_, colony grown on 0.02% ammonium thiocyanate minerals agar.	100
17	_Thiobacillus sp_, colony showing characteristic form on 1% thiosulphate minerals agar.	101

KEY TO FIGURES (continued).

No.		Page
18	Pseudomonas sp, colonies showing brown deposits on 1% thiosulphate minerals agar.	102
19	Effects of different sonication times on bacterial counts on activated sludge.	126
20	Use of a repli-dish for monitoring different populations of bacteria.	131
21	Monitoring of thiobacilli in activated sludge.	141
22	Surface drop viable count using thiocyanate + succinate agar.	153
23	Bacterial counts in relation to plant performance.	175
24	Relation between composition of feed-liquor, the numbers of thiobacilli and the rate of treatment.	176
25	Bacterial counts in the full scale plant.	177
26	Changes in numbers of fluorescent pseudomonads and total bacteria detected on thiocyanate + succinate agar.	178

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