THE BACTERIOLOGY OF A BENCH-SCALE LABORATORY ACTIVATED SLUDGE PLANT OPERATED WITH TOTAL CELL RECYCLE

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Dedicated to my wife and daughters

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INTRODUCTION

Biological stabilization of waste from industrial and domestic sources is used extensively to degrade complex organic material into simpler compounds. Ideally, the organic material should be converted to volatile compounds which are returned to the atmosphere--CO_2 in aerobic treatment and methane and CO_2 in anaerobic treatment. The treated effluent can then be introduced into bodies of water with minimal effect on the ecology of the receiving body of water. Producing a minimal effect on the receiving body of water is the primary goal of wastewater treatment. There are three commonly used methods of aerobic biological treatment: the trickling filter, the oxidation pond, and the sludge process.

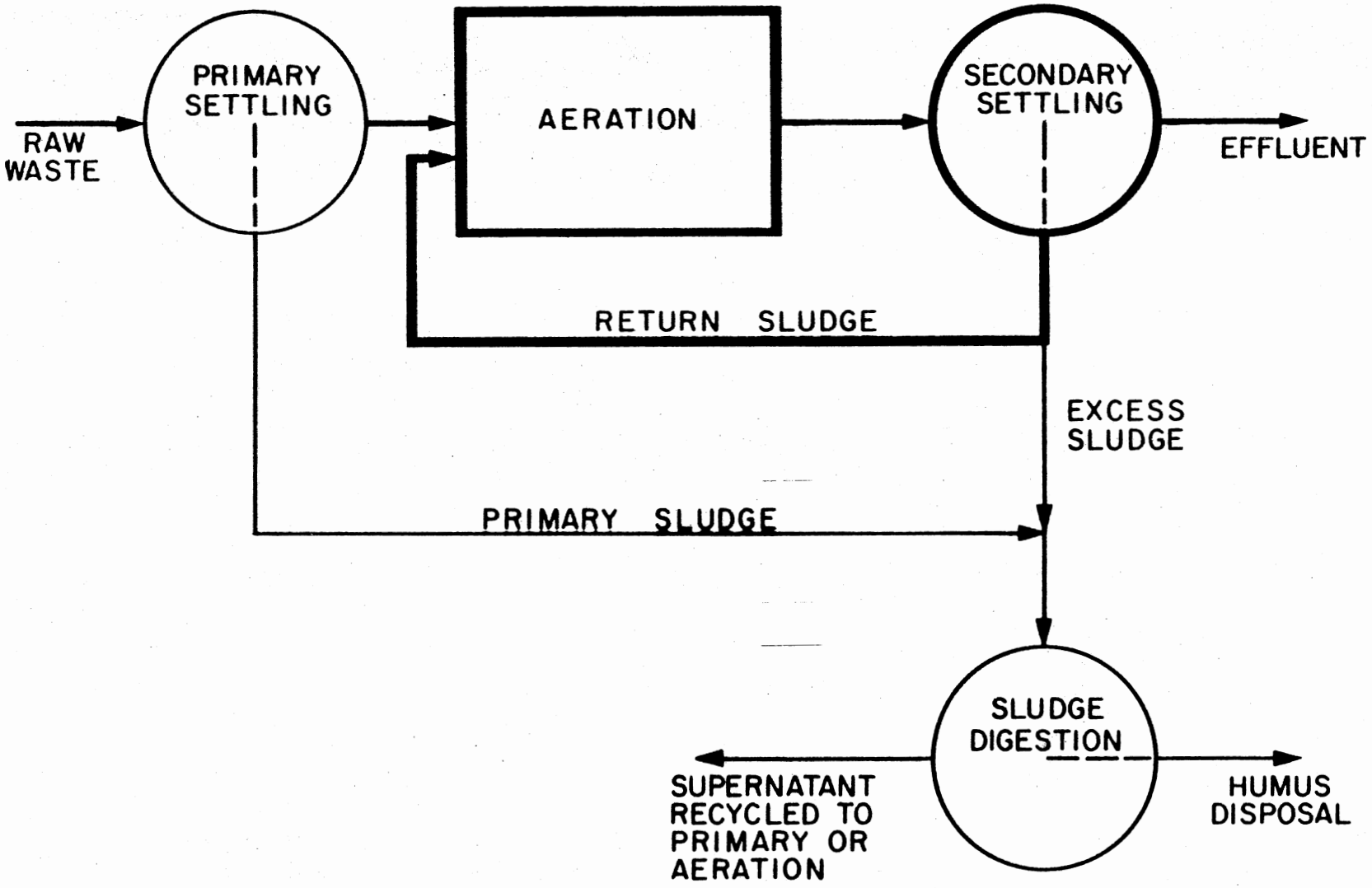
The activated sludge process was developed in England by Adern and Lockett (1914). It is the most versatile of the biological treatment processes. The main advantages of the activated sludge process, in addition to its versatility, include the rapid removal of organics and the ease of separating purified water from the biological solids. The activated sludge process is rather simple. A sludge can be developed by merely aerating a biodegradable waste for a period of time until a large mass of settleable solids forms. The masses of settleable solids or biological flocs are formed as a result of the growth of microorganisms, using the organic waste products as substrates for energy and growth,

in the presence of dissolved oxygen. The settleable solids or biological floc is actually the activated sludge.

A schematic diagram of the activated sludge process is presented in Figure 1. The wastewater, separately or after mixing with return sludge, enters the aeration tank. As the wastewater flows along the aeration tank, it is aerated and mixed. In newer, "completely mixed" aerators, incoming waste and sludge are immediately dispersed throughout the aerator by vigorous aeration. The microorganisms aerobically stabilize the organics in the aeration tank. The overflow from the aerator is channeled into the secondary sedimentation tank. In the sedimentation tank, the biological flocs are allowed to settle. This results in the separation of the biological flocs from the purified effluent. The efficiency of removal of solids is generally above 90 percent. A portion of the settled sludge is returned to the incoming wastewater. The excess sludge is further treated, usually by anaerobic digestion, before final disposal.

One major modification that has been developed for the activated sludge process is the extended aeration or total oxidation process. The extended aeration process differs from the basic activated sludge process in that it is operated without wasting sludge. This is accomplished by recycling all of the sludge to the aeration tank. The extended aeration process is represented by the heavy lines in Figure 1. A longer detention time provides for autooxidation of biological solids. Porges et al. (1953) theorized that total oxidation was possible under appropriate conditions. The idea of total oxidation has been a subject of controversy. Several researchers, Kountz and Fourney (1959), Symons and McKinney (1958), Busch and Myrick (1960), and Washington and

Figure 1. Representation of the Activated Sludge Process. The heavy lines represent the extended aeration process with total sludge recycle.


PRIMARY
SETTLING
AERATION
SECONDARY
SETTLING
RAW
WASTE
EFFLUENT
RETURN SLUDGE
EXCESS
SLUDGE
PRIMARY SLUDGE
SLUDGE
DIGESTION
SUPERNATANT
RECYCLED TO
PRIMARY OR
AERATION
HUMUS
DISPOSAL

Symons (1962) concluded that total oxidation was theoretically impossible. The concern of the above researchers was the accumulation of "inert" materials. They pointed out that extracellular polysaccharides often accumulate as waste products of metabolism. These polysaccharides, since they are waste products, would be expected to be nonbiodegradable. Gaudy et al. (1970) conducted long-term studies in which return of all biological solids was ensured by centrifugation of the effluent. Based on this 3-year study, it was proved that total oxidation was theoretically sound and that solids would not build up continually in an extended aeration-total oxidation unit. One of the most expensive parts of the activated sludge process, the disposal of excess sludge, therefore theoretically can be eliminated. Gaudy and Gaudy (1971) presented a detailed discussion of the biological concepts involved in the design and operation of the activated sludge process.

The ecology of the activated sludge process has not been studied extensively. The activated sludge ecosystem is dynamic, with ever-changing populations of microorganisms. These populations can be best described as heterogeneous. The interaction between the various populations is very important, as are the growth characteristics of the various species. The rate of waste conversion is related directly both to the growth characteristics of the biomass and to the design and operation of the system. Growth characteristics of the biomass have been an area of great interest since the pioneer work of Monod (1950). The work of Monod and the theory of continuous culture have been applied to a wide array of fields of research, including activated sludge.

The activated sludge process is actually a continuous mixed culture composed of heterogeneous populations that are continually stabilizing

organic waste. The heterogeneous nature of the populations allows for adaptation of the process for the treatment of a wide array of organic wastes. Also, this characteristic allows for adaptation to disturbances commonly called "shock loads." The ecosystem, due to its heterogeneous nature, also has the capability of producing undesirable effects, for example, the production of a poorly settling floc by filamentous organisms such as Sphaerotilus natans (Pipes, 1967). Additional studies in the areas of identification of the types of microorganisms present, their interrelationships, growth kinetics, and responses to various environmental changes are needed on a wide variety of plants.

The bacteria are responsible for the degradation of the majority of the organic material in the activated sludge process. Most of the research has been directed toward the aerobic heterotrophs, since this group appears to be of the greatest importance in the stabilization of waste. It is impossible to cultivate many of the bacteria normally found in activated sludge on ordinary culture media. Many different types of media have been used; therefore, the reported organisms and the numbers present will vary. Also, the composition of the wastewater being purified will be an important factor in determining what species are present.

Allen (1944) recommended using nutrient agar for isolating bacteria from activated sludge. He found that most of the bacteria in sludge were Gram-negative rods belonging to the genera Pseudomonas, Flavobacterium, and Achromobacter. Fecal bacteria and sporeformers were found in small numbers. Allen (1944) also reported viable counts of 10^8 - 10^{10} per ml of homogenized sludge from a plant treating domestic waste. Jasewicz and Porges (1956) investigated activated sludge

treating dairy waste and isolated strains on nutrient agar plus skim milk in the following proportions: 26 percent Alcaligenes, 34 percent Flavobacterium, 14 percent Micrococcus and 16 percent Pseudomonas. van Gils (1964), using tryptone glucose agar for isolation, reported that activated sludge grown on sewage of domestic origin belonged primarily to the family Achromobacteraceae. In addition, members of the Pseudomonadaceae and Corynebacteriaceae were found to occur. These results generally agree with those of Allen (1944) and Jasewicz and Porges (1956). van Gils (1964) also found, in an activated sludge produced in a laboratory plant fed with mineral medium with glucose and ammonium sulfate, many strains belonging to the Achromobacteraceae. About half of these strains belonged to the genus Flavobacterium; the other half were of the genus Achromobacter. Representatives of the genera Alcaligenes and Lophomonas were only a small portion of the population; the majority of the strains isolated from the laboratory sludge consisted of coccoid strains. The coccoid strains included Micrococcaceae but the majority were unidentifiable Gram-negative, capsulated egg-shaped bacteria. A few filamentous organisms were also isolated. One of the filamentous bacteria was identified as Nocardia.

van Gils (1964) reported viable counts on ammonium sulfate glucose agar and on tryptone glucose agar to be 10^8 bacteria per ml from homogenized laboratory sludge fed with glucose-ammonium sulfate mineral medium and 10^8 using tryptone glucose agar for homogenized activated sludge produced on waste from domestic origin. These counts for domestic waste sludge are comparable with those of Allen (1944).

Pioneering work in the isolation of bacteria from activated sludge was conducted by Butterfield (1935); he isolated Zoogloea ramigera. It

was accepted for many years that Zoogloea ramigera was the bacterium responsible for both the stabilization of the organic matter and the formation of flocs. McKinney and Weichlein (1953), using nutrient agar, isolated 72 bacterial strains, in addition to Zoogloea ramigera, which were capable of floc production. Dias and Bhat (1964) isolated over 300 bacterial strains from seven different sources by plating on sewage extract agar. Gram-negative bacteria of the genera Zoogloea and Comamonas predominated. A large number required either vitamins or amino acids, or both, for growth. Coliforms were rarely isolated. These results are quite different from those of Allen (1944) and van Gils (1964). These differences are probably due to the types of media used, source of sludge, and variations in technique. Prakasam and Dondero (1967a) compared several media and stated that activated sludge extract agar gave higher viable counts than did nutrient agar, iron-peptone agar, sewage agar, basal medium, Taylor's medium, or casitone-yeast extract autolysate medium. Only half of the total number of the developed colonies could be subcultured on richer standard media.

Pipes (1966), in a review of the taxonomic work on activated sludge, stated that the following aerobic heterotrophs are found in activated sludge: Achromobacter, Alcaligenes, Bacillus, Bacterium, Comomonas, Flavobacterium, Microbacterium, Pseudomonas, and Zoogloea. Benedict and Carlson (1971) isolated Acinetobacter, Alcaligenes, Brevibacterium, Caulobacter, Comomonas, Cytophaga, Flavobacterium, Hyphomicrobium, Microbacterium, Pseudomonas and Sphaerotilus in studies utilizing raw sewage agar and glutamate-urea agar and activated sludge from domestic waste treatment.

It is evident that no single medium will reliably support growth

of all types of bacteria present in activated sludge (Lighthart and Oglesby, 1969). Prakasam and Dondero (1967) reported higher counts with activated sludge agar. Pike et al. (1972) compared casitone-glycerol-yeast extract agar with activated sludge agar and found it to be superior. A large proportion of the microscopically visible bacteria in liquors from sewage treatment plants are not recoverable by viable counting methods (Unz and Dondero, 1970). Many of these discrepancies can possibly be attributed to difficulties in distinguishing inert particles from bacteria under the microscope, cultural techniques, and low viability of bacteria. Pike et al. (1972), in an attempt to standardize procedures, recommended, after extensive work, a series of procedures including the use of casitone-glycerol-yeast extract agar as a standard medium for isolation and enumeration of bacteria in activated sludge. The use of an easily prepared medium, such as casitone-glycerol-yeast extract agar, for routine plating of microorganisms present in activated sludge seems to be essential since all media tested thus far are selective, at least to some degree.

Bacteria other than those mentioned above have also received much attention by researchers. A number of conditions have been associated with bulking of sludge (poorly settling sludge) but the most common of these is related to the growth of filamentous microorganisms in the sludge. This condition has been described by Pipes (1967).

The study of filamentous microorganisms has been generally restricted to a few genera and in particular Sphaerotilus. The term "Sphaerotilus" has been used by many to describe all filamentous microorganisms. Many other filamentous microorganisms have been isolated and identified from activated sludge. Eikelboom (1975), in an extensive work on 1,100

activated sludge samples, distinguished and characterized 26 different types. In a study of 34 cases of filamentous bulking, Pipes (1978) found the dominant filamentous bacteria to belong to the genera Bacillus, Sphaerotilus, Beggiatoa, Arthrobacter, and Brevibacterium. It is difficult to differentiate the various filamentous microorganisms, since most of them are very difficult to isolate and grow in pure culture. Consequently, much of the taxonomic work is based on morphological characteristics. Continued and extensive taxonomic work is needed in this area since the filamentous microorganisms are thought to cause bulking of activated sludge.

Activated sludge plants are occasionally plagued with the production of a viscous scum or foam. Microscopic examinations of such scums and foams reveal that they are composed of a mass of hyphae. Lechevalier and Lechevalier (1974) studied the microbiology of scums and foams produced by activated sludge, and found that the hyphae present are predominately that of the actinomycte Nocardia amarae. Also, N. amarae was isolated from the mixed liquor of the activated sludge plants studied.

Protozoa clearly play an important role in the efficient operation of activated sludge plants. Protozoa are plentiful in activated sludge. It is not uncommon to find numbers in the order of 50,000 cells per ml in the mixed liquor of activated sludge plants. Calculations based on such numbers indicate that protozoa constitute approximately five percent of the dry weight of the suspended solids in the mixed liquor (Curds, 1973). Curds (1973) reported that four classes of protozoa are present in activated sludge. These in order of decreasing frequency, were the Ciliates, the Rhizopodea, the Phytomastigophorea, and the

Actinopodea. Based upon the relative abundance and frequency of the Ciliates, they are considered to be the most important group. The two Ciliates, Vorticella and Opercula, are responsible to a great degree for the success of the treatment process (Curds and Fey, 1969). Vorticella and Opercula have been shown to be responsible for the removal of Escherichia coli from wastewater (Curds and Fey, 1969). Curds (1973) showed that activated sludge produced a turbid effluent in the absence of protozoa. Ciliated protozoa are therefore responsible for decreasing the bacterial population in the clarifier supernatant, ensuring a clear effluent (Pillai and Subrahmanyan, 1944). Thus, oxygen-consuming bacteria are not released into the receiving body of water. Additional studies on the abundance, distribution, and ecological relationships between protozoa and bacteria are warranted.

Fungi, due to their versatility in decomposing an extremely wide variety of organics, play an important role in the decomposition and stabilization of waste. The detailed systematic studies of Cooke and Pipes (1970) were the first to describe quantitatively the fungal populations in activated sludge. The fungi most commonly found in their studies belonged to the genera Geotrichum, Trichosporon, and Penicillium. Geotrichum and Penicillium were isolated from all 19 activated sludge units examined.

Due to the absence of light, algae do not become established readily in activated sludge. Rotifers have been suggested as indicators of a very high level of purification, since they predominate after all of the protozoa have died of starvation (McKinney, 1957). Nematode worms, Oligochaete worms and Chironomid larvae may also be found rarely (Curds, 1973). The role of these higher forms is not thought to be important.

Further research in systems where they are found should be conducted to determine their role in the ecosystem.

More work is needed in the field of microbial ecology to determine the organisms present and their roles and interrelationships in a variety of activated sludge plants. This information, hopefully, can be useful to help solve some of the operational problems of wastewater treatment plants, such as bulking sludge and excess foam and scum. The present investigation was undertaken to characterize the bacterial populations that develop in a bench-scale pilot plant operated with total cell recycle.

CHAPTER II

MATERIALS AND METHODS

Description of the Bench-scale Laboratory Activated Sludge Plant Operated With Total Cell Recycle

The bench-scale laboratory plant investigated in this study is part of a research project conducted in the Bioenvironmental Engineering laboratories at Oklahoma State University, Stillwater, Oklahoma. The plant was operated with glucose-ammonium sulfate minimal medium with a chemical oxygen demand (COD) of approximately 500 mg/l as the feed. The composition of the glucose-ammonium sulfate minimal medium used as feed for the plant is shown in Table I.

The rate of feed was eight liters per day with an adjusted pH of 7. The detention time in the aerator was 24 hours. The liquor in the 12-liter aerator was adjusted periodically to pH 7. The settling tank had an 18-liter capacity. The settled sludge was returned to the aerator at a rate of two liters per day.

Isolation and Enumeration of the Predominant Bacteria

Samples were collected on the following dates: 8-29-78, 11-8-78, 1-18-79, 2-6-79, and 2-20-79. Each sample was collected from the

TABLE I

COMPOSITION OF GLUCOSE AMMONIUM SULFATE MINIMAL MEDIUM

Glucose	500 mg/l
$(NH_4)_2SO_4$	250 mg/l
$MgSO_4 \cdot 7H_2O$	50 mg/l
$FeCl_3$	0.25 mg/l
$CaCl_2$	3.75 mg/l
$MnSO_4 \cdot H_2O$	5 mg/l
Phosphate buffer, pH 7.6	
(KH_2PO_4, 38.5 gm/l and K_2HPO_4, 124.5 gm/l)	10 ml/l
Tap water	100 ml/l
Distilled water	to volume

aerator in a sterile test tube.

Each sample was diluted 1:10 with sterile buffered dilution water and blended for four minutes at the high setting to release the bacteria from the flocs. The blender used was an Osterizer Galaxie dual range 14. The blender container was disinfected by rinsing with 70 percent ethanol, air drying and rinsing with sterile buffered dilution water immediately before blending each sample.

Quantitative dilutions of each sample were prepared in sterile buffered dilution water in preparation for plating. The buffered dilution water, used for dilution of samples, was prepared by dissolving 16 gm KH_2PO_4 in 250 ml distilled water. The pH was adjusted to 7.2 with 1.0 N NaOH. Distilled water was added to make the total volume 500 ml. The above stock was used to prepare buffered dilution water by adding 1.25 ml of the stock buffer to one liter of distilled water, and dispensing in dilution blanks. The buffered dilution water was sterilized by autoclaving for fifteen minutes at 15 pounds pressure (121C).

Each dilution (10^{-6}, 10^{-7}, and 10^{-8}) was plated in quadruplicate by plating 0.1 ml of each dilution on a pre-dried casitone-glycerol-yeast extract agar (CGYA) surface and spreading with a sterile bent glass rod. CGYA agar medium contained: casitone, 5 gm; glycerol, 5 gm; yeast extract, 1 gm; agar (Difco), 20 gm, and one liter distilled water. The final pH was 7.2. Plates were prepared with 20 ml of medium per plate and were incubated at 37C over-night to dry the surface and to check for contamination.

All spread plates were incubated at 30C for three days. Colonies were enumerated daily by using a darkfield Quebec colony counter. Colonies of each colonial type present on countable plates were selected

and transferred with a sterile needle to casitone-glycerol-yeast extract broth (CGYB) which has the same formula as CGYA without the agar. Each isolate was incubated in a slanted position on a reciprocal shaker at 30C. Incubation was continued for each isolate until growth in CGYB produced visible turbidity. Then each culture was streaked on CGYA and incubated at 30C until colonies formed. Isolated colonies were selected and restreaked on CGYA to ensure purity. Wet mounts and Gram stains were utilized to check the purity of each isolate and to determine the cellular morphology of each isolate.

Cultivation of Isolates

Stock cultures of each of the isolates used in these studies were prepared by inoculating tryptic soy agar (TSA) (Difco) slants from isolated colonies. The inoculated slants were incubated at 30C for 24 to 48 hours and stored at 4C. Stock cultures were transferred to new TSA slants monthly. For most experiments, cells used as inoculum were grown on TSA slants over-night and washed off with sterile 0.85 percent saline solution.

Characterization of the Isolates

The colonial morphology of each isolate was described according to its appearance on TSA plates. The cellular morphology and motility of each isolate were determined by observing wet mounts using phase-contrast microscopy. The Gram reaction of each isolate was determined by Gram staining cultures 24 hours old.

The biochemical properties of the isolated organisms were determined by subjecting each isolate to a series of tests. The series of

tests included glycerol and glucose fermentation broths containing bromthymol blue as pH indicator. Phenol red was also used as pH indicator for some tests. Other broths used were nitrate, urea, and methyl red-Voges Proskauer (MRVP). These broths were used respectively to test each isolate's ability to reduce nitrate and produce urease, and to determine its pattern of fermentation. Readings were taken at 24 and 48 hours and one week for the fermentation broths and nitrate broth. Urease production and the VP portion of the MRVP test were checked after 48 hours and the MR portion of the MRVP test after one week.

Gelatin, starch, and tributyrin plates were inoculated and examined for hydrolysis after 48 hours. Tributyrin plates were checked again after four days.

To test the ability of each isolate to utilize glucose, acetate and citrate as sole carbon and energy sources, the following media were inoculated and read after 48 and 72 hours: glucose and acetate minimal media and Simmon's citrate agar (Difco).

The composition of the glucose and acetate minimal media used was: $(NH_4)_2SO_4$, 0.5 gm; $MgSO_4 \cdot 7H_2O$, 0.1 gm; $FeCl_3 \cdot 6H_2O$, 0.5 gm; $CaCl_3 \cdot 2H_2O$, 7.5 mg; KH_2PO_4, 3 gm; Na_2HPO_4, 6 gm; carbon and energy source (glucose or sodium acetate), 1 gm; tap water, 100 ml; distilled water, 700 ml, and agar (Difco), 20 gm.

Kligler's iron agar (Difco) was inoculated to determine the production of hydrogen sulfide. Readings were taken after 24 and 48 hours.

A drop of three percent H_2O_2 was placed on isolated colonies grown on TSA plates to determine catalase activity. Cytochrome oxidase activity was determined by placing one drop of a freshly prepared one

percent solution of dimethyl-p-phenylenediamine-HCl on isolated colonies grown on TSA plates.

Urea broth was filter-sterilized. All other media used for the cultivation, characterization, and enumeration of the isolates were sterilized by autoclaving for fifteen minutes at 15 pounds pressure (121C). All incubations were at 30C.

Microscopic Observations of the Mixed Liquor

Microscopic observations of samples taken from the aerator were conducted with phase-contrast microscopy on the following dates: (1) 8-29-78, (2) 10-31-78, (3) 11-9-78, (4) 1-18-79, (5) 2-6-79, (6) 2-20-79, (7) 3-19-79, and (8) 4-5-79.

CHAPTER III

EXPERIMENTAL RESULTS

Isolation and Enumeration of the Predominant Bacteria

The bench-scale laboratory activated sludge plant was sampled on five different occasions to isolate the predominant bacteria present. Viable cell counts were obtained by the spread plate technique on CGYA incubated at 30C for 72 hours. The viable cell count for each isolation, expressed as an average of four replica plates is shown in Table II.

The bacteria used in these studies were isolated from dilution plates used to determine viable counts. Selected colonies of each colonial type present on countable plates were subcultured in CGYB. Pure cultures of twenty-five bacteria were obtained from isolation #1, 8-29-78; twenty-two from isolation #2, 11-8-78; twenty-three from isolation #3, 1-18-79; twenty-three from isolation #4, 2-6-79; and twenty-four from isolation #5, 2-20-79. A total of 118 colonies were selected for isolation; only one failed to grow when subcultured in CGYB.

Characterization of Isolates

The overall characteristics of the population at each sampling were determined by compiling the biochemical and morphological characteristics of all isolates. A summary of the characteristics of the isolates

TABLE II

VIABLE CELL COUNTS

Isolation #	Date	Bacteria/ml
1	8-29-78	1.7×10^9
2	11- 8-78	4.7×10^9
3	1-18-79	3.8×10^8
4	2- 6-79	2.1×10^9
5	2-20-79	6.6×10^8

Viable count is the average of four replicate plates.

at each isolation, expressed as percent of positives, is shown in Tables III and IV. A composite total of the five isolations, expressed as percent of positives, is also included in Tables III and IV.

In order to determine the number of groups present in each isolation, characteristics that were of diagnostic value for grouping isolates in each sample were determined from the morphological and biochemical properties of each isolate. The characters of diagnostic value for grouping isolates in each isolation are presented in Tables V - IX. A total of 46 groups was isolated in the five isolations.

To determine whether the same group was present in more than one isolation, the morphological and biochemical characteristics of each group isolated were tabulated. The results are shown in Tables X - XIV. By comparing the characteristics of each of the 46 groups with each other, it was determined that group #1 in isolation #3 had the same characteristics as group #11 of isolation #4 and group #1 of isolation #5. Since these groups have the same characteristics, they are probably in the same group taxonomically. Several different groups have the same biochemical characteristics, but different morphological characteristics. The differences between these groups are shown in Table XV. All other groups isolated have at least one biochemical and one morphological difference. Those groups with morphological and/or biochemical differences are considered to be different groups; therefore 44 different groups were isolated.

Additional information observed for the isolates, but not included in Tables III - XV, is as follows: (1) giant cocci three to four times as large as the other cocci were present in some older cultures of isolates with a cell cycle; (2) all the cocci isolated were

TABLE III

MORPHOLOGICAL CHARACTERISTICS OF ISOLATES AS PERCENT OF POSITIVES

	Isolation Number					Total
	1	2	3	4	5	
Number of isolates tested	25	22	23	23	24	117
Colonial Morphology						
Colony elevation flat	0	0	13	0	8	4
Colony elevation raised	100	100	87	100	92	96
Colony margin entire	60	50	78	70	75	67
Colony margin irregular	40	50	22	30	25	33
Colony pink/red	4	0	0	0	0	1
Colony yellow/orange/brown	8	9	83	87	63	50
Colony cream/grey/white	88	91	17	9	38	49
Colony gelatinous-watery	0	50	43	30	17	28
Exopigment-water soluble	0	0	0	4	0	1
Cell Morphology						
Branching present	0	5	43	39	54	28
Rods only	4	0	0	0	0	1
Cocci only	48	9	0	0	0	11
Rods and cocci	48	91	100	100	100	88
Motility	8	68	57	65	8	41

TABLE IV

BIOCHEMICAL CHARACTERISTICS OF ISOLATES AS PERCENT OF POSITIVE REACTIONS

	Isolation Number					
	1	2	3	4	5	Total
Number of isolates tested	25	22	23	23	24	117
Catalase	100	100	100	100	100	100
Oxidase	0	55	52	43	42	38
Acid from Glucose						
Aerobic	48	0	5	0	0	11
Anaerobic	12	9	5	13	21	12
Acid from Glycerol, Aerobic	0	0	0	0	13	3
Hydrolysis Tests						
Urea	0	0	0	0	0	0
Tributyrin	96	59	96	100	92	89
Starch	4	0	0	13	25	8
Gelatin	4	77	22	30	17	30
Growth on Minimal Media						
Citrate	48	18	74	74	63	55
Glucose	40	100	91	100	100	86
Acetate	40	86	91	100	100	83
Nitrate Reduction						
$NO_3^- \rightarrow NO_2^-$	84	45	57	61	79	65
$NO_3^- \rightarrow NH_4^+$	4	0	0	0	0	1
$NO_3^- \rightarrow N_2$	0	0	26	5	4	7
Methyl Red	0	9	9	13	8	6
Voges-Proskauer	0	0	13	13	17	9
H_2S Production	0	0	0	0	0	0

TABLE V

CHARACTERS OF DIAGNOSTIC VALUE FOR GROUPING ISOLATES IN ISOLATION #1

	Group #						
	1	2	3	4	5	6	7
Number of isolates in each group	10	4	7	1	1	1	1
Colonial Morphology							
Colony margin entire	-	+	+	+	+	+	+
Colony margin irregular	+	-	-	-	-	-	-
Colony pink/red	-	-	-	-	-	+	-
Colony yellow/orange/brown	-	-	-	+	+	-	-
Colony cream/grey/white	+	+	+	-	-	-	+
Cell Morphology							
Cocci only	-	+	+	-	-	-	+
Rods only	-	-	-	-	-	+	-
Rods and cocci	+	-	-	+	+	-	-
Motility	-	-	-	-	+	+	-
Acid from Glucose							
Aerobic	+	-	-	-	+	-	-
Anaerobic	-	+	-	-	-	-	-
Hydrolysis Tests							
Tributyrin	+	+	+	+	+	-	+
Starch	-	-	-	-	-	+	-
Gelatin	-	-	-	-	-	+	-
Growth on Minimal Media							
Citrate	+	-	-	-	-	+	-
Glucose	+	-	-	-	-	-	-
Acetate	+	-	-	-	-	-	-
Nitrate Reduction							
$NO_3^- \rightarrow NO_2^-$	+	+	+	+	+	+	-
$NO_3^- \rightarrow NH_4^+$	-	-	-	-	-	-	+

TABLE VI

CHARACTERS OF DIAGNOSTIC VALUE FOR GROUPING
ISOLATES IN ISOLATION #2

	Group #				
	1	2	3	4	5
Number of isolates in each group	11	2	7	1	1
Colonial Morphology					
Colony margin entire	-	+	+	+	+
Colony margin irregular	+	-	-	-	-
Colony orange/brown	-	-	-	+	+
Colony cream/grey/white	+	+	+	-	-
Colony gelatinous-watery	+	-	-	-	-
Cell Morphology					
Cocci only	-	+	-	-	-
Rods and cocci	+	-	+	+	+
Branching present	-	-	-	+	-
Motility	+	-	+	+	+
Oxidase	+	-	-	-	+
Acid from Glucose, Anaerobic	-	+	-	-	-
Hydrolysis Tests					
Tributyrin	+	+	-	-	-
Gelatin	+	-	+	+	-
Growth on Minimal Media					
Citrate	+	+	-	+	-
Acetate	+	-	+	+	+
Methyl Red	-	+	-	-	-

TABLE VII

CHARACTERS OF DIAGNOSTIC VALUE FOR GROUPING ISOLATES IN ISOLATION #3

	Group #							
	1	2	3	4	5	6	7	8
Number of isolates in each group	6	3	2	4	1	1	1	5
Colony Morphology								
Colony margin entire	-	+	+	+	+	+	+	+
Colony margin irregular	+	-	-	-	-	-	-	+
Colony yellow/orange/brown	+	+	+	+	+	-	-	+
Colony cream/grey/white	-	-	-	-	-	+	+	-
Colony gelatinous-watery	+	-	-	-	-	-	-	+
Cell Morphology								
Branching present	+	+	-	-	-	-	-	-
Motility	-	-	+	+	+	+	+	+
Oxidase	-	-	-	+	+	+	+	+
Acid from Glucose, Anaerobic	-	-	+	-	-	-	-	-
Gelatin Hydrolysis	-	-	+	+	-	-	-	-
Growth on Minimal Media, Citrate	+	+	-	+	+	+	-	+
Nitrate Reduction								
$NO_3^- \rightarrow NO_2^-$	+	+	+	+	-	-	+	-
$NO_3^- \rightarrow N_2$	-	-	-	-	-	+	-	+
Methyl Red	-	-	+	-	-	-	-	-
Voges-Proskauer	-	-	+	+	-	-	-	-

TABLE VIII

CHARACTERS OF DIAGNOSTIC VALUE FOR GROUPING ISOLATES IN ISOLATION #4

	Group #											
	1	2	3	4	5	6	7	8	9	10	11	12
Number of isolates in each group	2	1	1	1	1	1	1	1	2	4	7	1
Colony Morphology												
Colony margin entire	+	+	+	+	+	+	+	+	+	+	-	-
Colony margin irregular	-	-	-	-	-	-	-	-	-	-	+	+
Colony yellow/orange/brown	+	+	-	+	+	+	-	-	-	+	+	+
Colony cream/grey/white	-	-	+	-	-	-	+	+	+	-	-	-
Colony gelatinous-watery	-	-	-	-	-	-	-	-	-	-	+	+
Exopigment-water soluble	-	-	-	-	-	-	-	+	-	-	-	-
Cellular Morphology												
Branching present	-	-	+	+	-	-	-	-	-	+	+	+
Motility	+	+	+	+	-	-	+	+	+	+	-	-
Oxidase	-	+	-	+	-	-	+	+	-	+	-	-
Acid from Glucose, Anaerobic	+	+	-	-	-	-	-	-	-	-	-	-
Hydrolysis Test												
Starch	-	-	+	+	-	+	-	-	-	-	-	-
Gelatin	+	-	-	-	-	+	-	+	+	-	-	+
Growth on Minimal Media, Citrate	-	+	+	+	+	-	+	+	-	+	+	+
Nitrate Reduction												
$NO_3^- \rightarrow NO_2^-$	+	+	-	-	+	-	+	-	+	+	+	+
$NO_3^- \rightarrow N_2$	-	-	-	-	-	-	-	+	-	-	-	-
Methyl Red	+	+	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	+	+	-	-	-	-	-	-	-	-	-	-

TABLE IX

CHARACTERS OF DIAGNOSTIC VALUE FOR GROUPING ISOLATES IN ISOLATION #5

	Group #													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Number of isolates in each group	4	6	1	2	1	1	1	1	1	1	1	1	1	2
Colony Morphology														
Colony elevation flat	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Colony elevation raised	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Colony margin entire	-	+	+	+	+	+	+	+	+	+	+	+	+	-
Colony margin irregular	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Colony yellow/orange/brown	+	+	-	+	+	-	-	-	-	+	+	+	-	-
Colony cream/grey/white	-	-	+	-	-	+	+	+	+	-	-	-	+	+
Colony gelatinous-watery	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Cell Morphology														
Branching present	+	+	+	-	-	-	-	+	+	+	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Oxidase	-	-	+	+	-	-	+	-	+	+	+	-	+	+
Acid from Glucose, Anaerobic	-	-	-	-	+	+	-	-	-	-	-	+	-	+
Acid from Glycerol, Aerobic	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Hydrolysis Tests														
Tributyrin	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Starch	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	+	+	-	+	-	-	-	-	+
Growth on Minimal Media, Citrate	+	+	+	-	-	-	+	-	-	+	-	-	+	+
Nitrate Reduction														
$NO_3^- \rightarrow NO_2^-$	+	+	+	+	+	+	-	+	+	-	+	-	+	-
$NO_3^- \rightarrow N_2$	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	+	+	-	-	-	-	-	-	-	+

TABLE X

CHARACTERISTICS OF EACH GROUP ISOLATED IN ISOLATION #1

	Group #						
	1	2	3	4	5	6	7
Number of isolates in each group	10	4	7	1	1	1	1
Morphological Characteristics							
Colonial Morphology							
Colony elevation flat	-	-	-	-	-	-	-
Colony elevation raised	+	+	+	+	+	+	+
Colony margin entire	-	+	+	+	+	+	+
Colony margin irregular	+	-	-	-	-	-	-
Colony pink/red	-	-	-	-	-	+	-
Colony yellow/orange/brown	-	-	-	+	+	-	-
Colony cream/grey/white	+	+	+	-	-	-	+
Colony gelatinous-watery	-	-	-	-	-	-	-
Exopigment-water soluble	-	-	-	-	-	-	-
Cell Morphology							
Branching present	-	-	-	-	-	-	-
Rods only	-	-	-	-	-	+	-
Cocci only	-	+	+	-	-	-	+
Rods and cocci	+	-	-	+	+	-	-
Motility	-	-	-	-	+	+	-
Biochemical Characteristics							
Catalase	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-
Acid from Glucose							
Aerobic	+	-	-	-	+	-	-
Anaerobic	-	+	-	-	-	-	-
Acid from Glycerol, Aerobic	-	-	-	-	-	-	-
Hydrolysis Tests							
Urea	-	-	-	-	-	-	-
Tributyrin	+	+	+	+	+	-	+
Starch	-	-	-	-	+	-	-
Gelatin	-	-	-	-	+	-	-
Growth on Minimal Media							
Citrate	+	-	-	-	+	-	-
Glucose	+	-	-	-	-	-	-
Acetate	+	-	-	-	-	-	-
Nitrate Reduction							
$NO_3^- \rightarrow NO_2^-$	+	+	+	+	-	+	-
$NO_3^- \rightarrow NH_4^+$	-	-	-	-	-	-	+
$NO_3^- \rightarrow N_2$	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-	-	-
H_2S Production	-	-	-	-	-	-	-

TABLE XI

CHARACTERISTICS OF EACH GROUP ISOLATED IN ISOLATION #2

	Group #				
	1	2	3	4	5
Number of isolates in each group	11	2	7	1	1
Morphological Characteristics					
Colonial Morphology					
Colony elevation flat	-	-	-	-	-
Colony elevation raised	+	+	+	+	+
Colony margin entire	-	+	+	+	+
Colony margin irregular	+	-	-	-	-
Colony pink/red	-	-	-	-	-
Colony yellow/orange/brown	-	-	-	+	+
Colony cream/grey/white	+	+	+	-	-
Colony gelatinous watery	+	-	-	-	-
Exopigment-water soluble	-	-	-	-	-
Cell Morphology					
Branching present	-	-	-	+	-
Rods only	-	-	-	-	-
Cocci only	-	+	-	-	-
Rods and cocci	+	-	+	+	+
Motility	+	-	+	+	+
Biochemical Characteristics					
Catalase	+	+	+	+	+
Oxidase	+	-	-	-	+
Acid from Glucose					
Aerobic	-	-	-	-	-
Anaerobic	-	+	-	-	-
Acid from Glycerol, Aerobic	-	-	-	-	-
Hydrolysis Tests					
Urea	-	-	-	-	-
Tributyrin	+	+	-	-	-
Starch	-	-	-	-	-
Gelatin	+	-	+	+	-
Growth on Minimal Media					
Citrate	+	+	-	+	-
Glucose	+	+	+	+	+
Acetate	+	-	+	+	+
Nitrate Reduction					
$NO_3^- \rightarrow NO_2^-$	+	+	+	+	-
$NO_3^- \rightarrow NH_4^+$	-	-	-	-	-
$NO_3^- \rightarrow N_2$	-	-	-	-	-
Methyl Red	-	+	-	-	-
Voges-Proskauer	-	-	-	-	-
H_2S Production	-	-	-	-	-

TABLE XII

CHARACTERISTICS OF EACH GROUP ISOLATED IN ISOLATION #3

	Group #							
	1	2	3	4	5	6	7	8
Number of isolates in each group	6	3	2	4	1	1	1	5
Morphological Characteristics								
Colony Morphology								
Colony elevation flat	-	-	-	-	-	-	-	-
Colony elevation raised	+	+	+	+	+	+	+	+
Colony margin entire	-	+	+	+	+	+	+	-
Colony margin irregular	+	-	-	-	-	-	-	+
Colony pink/red	-	-	-	-	-	-	-	-
Colony yellow/orange/brown	+	+	+	+	+	-	-	+
Colony cream/grey/white	-	-	-	-	-	+	+	-
Colony gelatinous-watery	+	-	-	-	-	-	-	+
Exopigment-water soluble	-	-	-	-	-	-	-	-
Cell Morphology								
Branching present	+	+	-	-	-	-	-	-
Rods only	-	-	-	-	-	-	-	-
Cocci only	-	-	-	-	-	-	-	-
Rods and cocci	+	+	+	+	+	+	+	+
Motility	-	-	+	+	+	+	+	+
Biochemical Characteristics								
Catalase	+	+	+	+	+	+	+	+
Oxidase	-	-	-	+	+	+	+	+
Acid from Glucose								
Aerobic	-	-	-	-	-	-	-	-
Anaerobic	-	-	+	-	-	-	-	-
Acid from Glycerol, Aerobic	-	-	-	-	-	-	-	-
Hydrolysis Tests								
Urea	-	-	-	-	-	-	-	-
Tributyrin	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-	-
Gelatin	-	-	+	+	-	-	-	-
Growth on Minimal Media								
Citrate	+	+	-	+	+	+	-	+
Glucose	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+
Nitrate Reduction								
$NO_3^- \rightarrow NO_2^-$	+	+	+	+	-	-	+	-
$NO_3^- \rightarrow NH_4^+$	-	-	-	-	-	-	-	-
$NO_3^- \rightarrow N_2$	-	-	-	-	-	+	-	+
Methyl Red	-	-	+	-	-	-	-	-
Voges-Proskauer	-	-	+	-	+	-	-	-
H_2S Production	-	-	-	-	-	-	-	-

TABLE XIII

CHARACTERISTICS OF EACH GROUP ISOLATED IN ISOLATION #4

	Group #											
	1	2	3	4	5	6	7	8	9	10	11	12
Number of isolates in each group	2	1	1	1	1	1	1	1	2	4	7	1
Morphological Characteristics												
Colony Morphology												
Colony elevation flat	-	-	-	-	-	-	-	-	-	-	-	-
Colony elevation raised	+	+	+	+	+	+	+	+	+	+	+	+
Colony margin entire	+	+	+	+	+	+	+	+	+	+	+	+
Colony margin irregular	-	-	-	-	-	-	-	-	-	-	+	+
Colony pink/red	-	-	-	-	-	-	-	-	-	-	-	-
Colony yellow/orange/brown	+	+	-	+	+	+	-	-	-	+	+	+
Colony cream/grey/white	-	-	+	-	-	-	-	-	-	+	+	+
Colony gelatinous-watery	-	-	-	-	-	-	-	-	-	-	+	+
Exopigment-water soluble	-	-	-	-	-	-	+	-	-	-	-	-
Cell Morphology												
Branching present	-	-	+	+	-	-	-	-	-	+	+	+
Rods only	-	-	-	-	-	-	-	-	-	-	-	-
Cocci only	-	-	-	-	-	-	-	-	-	-	-	-
Rods and cocci	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	-	-	+	+	+	+	-	-
Biochemical Characteristics												
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	+	-	+	-	-	+	+	-	+	-	-
Acid from Glucose												
Aerobic	-	-	-	-	-	-	-	-	-	-	-	-
Anaerobic	+	+	-	-	-	-	-	-	-	-	-	-
Acid from Glycerol, Aerobic	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis Tests												
Urea	-	-	-	-	-	-	-	-	-	-	-	-
Tributyrin	+	+	+	+	+	+	+	+	+	+	+	+
Starch	-	-	+	+	-	+	-	-	-	-	-	-
Gelatin	+	-	-	-	-	+	-	+	+	-	-	+
Growth on Minimal Media												
Citrate	-	+	+	+	+	-	+	+	-	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate Reduction												
$NO_3^- \rightarrow NO_2^-$	+	+	-	-	+	-	+	-	+	+	+	+
$NO_3^- \rightarrow NH_4^+$	-	-	-	-	-	-	-	-	-	-	-	-
$NO_3^- \rightarrow N_2$	-	-	-	-	-	-	-	+	-	-	-	-
Methyl Red	+	+	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	+	+	-	-	-	-	-	-	-	-	-	-
H_2S Production	-	-	-	-	-	-	-	-	-	-	-	-

TABLE XIV

CHARACTERISTICS OF EACH GROUP ISOLATED IN ISOLATION #5

	Group #													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Number of isolates in each group	2	6	1	2	1	1	1	1	1	1	1	1	1	2
Morphological Characteristics														
Colony Morphology														
Colony elevation flat	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Colony elevation raised	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Colony margin entire	-	+	+	+	+	+	+	+	+	+	+	+	+	-
Colony margin irregular	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Colony pink/red	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colony yellow/orange/brown	+	+	-	+	+	-	-	-	-	+	+	+	-	-
Colony creamy/grey/white	-	-	+	-	-	+	+	+	+	-	-	-	+	+
Colony gelatinous-watery	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Exopigment-water soluble	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cell Morphology														
Branching present	+	+	+	-	-	-	-	+	+	+	-	-	-	-
Rods only	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cocci only	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rods and cocci	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Biochemical Characteristics														
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	+	+	-	-	+	-	+	+	+	-	+	+
Acid from Glucose														
Aerobic	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anaerobic	-	-	-	-	+	+	-	-	-	-	-	+	-	+
Acid from Glycerol, Aerobic	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Hydrolysis Tests														
Urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tributyrin	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Starch	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	+	+	-	+	-	-	-	-	+
Growth on Minimal Media														
Citrate	+	+	+	-	-	-	+	-	-	+	-	-	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate Reduction														
$NO_3^- \rightarrow NO_2^-$	+	+	+	+	+	+	-	+	+	-	+	-	+	-
$NO_3^- \rightarrow NH_4^+$	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$NO_3^- \rightarrow N_2$	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	+	+	-	-	-	-	-	-	-	-
H_2S Production	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE XV

GROUPS THAT DIFFER ONLY MORPHOLOGICALLY

	Group #				
	1	2	5	11	1
Isolation number	3	3	4	4	5
Colony gelatinous-watery	+	-	-	+	+
Colony margin entire	-	+	+	-	-
Colony margin irregular	+	-	-	+	+
Branching present	+	+	-	+	+

	Group #		
	7	10	5
Isolation number	4	4	5
Branching present	-	+	+
Motility	+	+	-
Colony cream/grey/white	+	-	+
Colony yellow/orange/brown	-	+	-

Gram-positive; (3) all of the isolates that have a cell cycle were Gram-positive or Gram-variable.

Microscopic Observations of the Aerator

General microscopic observations to determine the diversity of forms present in the aerator were conducted on eight different occasions. A summary of the forms observed in the aerator is presented in Table XVI.

TABLE XVI

SUMMARY OF MICROSCOPIC OBSERVATIONS

	Observation #							
	1	2	3	4	5	6	7	8
Protozoa								
Flagellates, free swimming	+	+	+	+	+	+	+	+
Ciliates	+	-	-	-	-	-	+	-
Amoeboid with test grey to golden brown	+	-	-	-	-	-	+	+
Nematoda	+	-	-	-	-	-	-	-
Bacteria								
Filaments, with ectobacilli	+	+	+	+	+	+	+	+
Flat coccoid chains	+	+	+	+	-	-	+	+
Microcolonies of cocci	+	+	+	-	+	+	+	+
Free floating cocci	-	-	-	-	-	-	+	-
Giant dense cocci	-	+	+	-	+	+	+	+
Branched filaments	-	+	+	-	+	+	+	+
Sheathed filaments	+	-	-	-	+	+	-	-
Free swimming bacilli	+	+	+	+	+	+	+	+
Free floating coryneform rods	-	-	-	-	-	-	+	+
Flocs large with projecting filaments	+	+	+	+	+	+	+	-
Flocs small with few filaments	-	-	-	-	-	-	-	+

CHAPTER IV

DISCUSSION

The primary purposes of this investigation were to enumerate, isolate in pure culture, and identify and/or characterize the populations of the predominant bacteria in the bench-scale laboratory activated sludge plant. The ecosystem investigated may be described as a continuous culture system in which essentially all of the cells produced are recycled into the aerator.

The bacterial populations in the mixed liquor of this ecosystem are aggregated into flocs. The formation of rapidly settling flocs is essential for removing organic matter and the production of a clear effluent. The formation of flocs makes quantitative studies of the numbers and types of bacteria more difficult. The bacteria present in flocs must be separated and dispersed in order to conduct quantitative studies. The accuracy of the enumeration of the bacteria by the plate count technique relies on the assumption that the bacteria to be counted are in suspension as single cell units; therefore, the dispersal of the clumps of bacteria into unattached individuals is imperative.

Lighthart and Oglesby (1969) in studies using blenders to achieve floc dispersal, found that maximum dispersal occurred after four minutes blending time. Microscopic examination of each of the five samples blended for four minutes in this study showed the cells to be evenly dispersed with no evidence of clumping.

Careful choice of culture media must be made for enumeration, since no single medium can be expected to support the growth of all of the nutritional types that one would expect to encounter in activated sludge. Pike et al. (1972) evaluated and compared several media for plating activated sludge organisms. They found CGYA to be superior to activated sludge agar which Prakasam and Dondero (1967) had recommended as being superior. CGYA used for the isolations in this investigation was also somewhat selective. Microscopic observations of samples revealed that several forms, particularly filamentous forms, were observed but were not isolated.

The spread plate technique used in these investigations has been shown by Clark (1967) to give higher counts than other methods in studies of aquatic bacteria. The viable cell counts found in these studies are in the range reported by van Gils (1964) for a laboratory plant fed daily by "fill and draw" using mineral medium with glucose and ammonium sulfate. Although all media are somewhat selective, CGYA, due to the high viable counts obtained, was adequate for the isolation and enumeration of bacteria for this study. The bacteria that grew on high dilution CGYA plates were assumed to be the predominant bacteria present in the ecosystem.

An activated sludge ecosystem may be described as a continuous culture with the feedback of part of the cell yield. The system is continually being inoculated with microorganisms from the incoming wastewater. Only those organisms that can compete for the growth-limiting nutrients supplied in the wastewater will form the dominant populations. The composition of the growth-limiting substrate affects the outcome of competition in activated sludge. An important applied

example is that the sludge bulking organisms Geotrichum and Sphaerotilus have a competitive advantage over flocculating species, when the concentrations of nitrogen and phosphorus limit growth (Dias et al., 1968).

Pipes (1966) has suggested the importance in activated sludge of two factors that are commonly assumed to influence survival in this competitive environment: (1) ability to form storage products, and (2) low requirements for growth-limiting substrates. Organisms that have these properties would be considered to have a better chance of survival than organisms lacking these properties.

This study was conducted on an activated sludge system that was fed glucose minimal medium with all of the cell yield recycled. The substrate concentration for the system was approximately 500 mg/l, as shown in Table XVII. This concentration is quite low in relation to the concentration of cells, and would be selective for those microorganisms with low requirements for nutrients. Nutrients used by the organisms present in the system also become available upon the death and lysis of cells, since all of the cell yield is returned to the aerator. These cellular nutrients are evidently being utilized, since the recycled biomass of the unit remained relatively stable during the time of this study, as shown in Table XVII.

The high percentage of the isolates that were able to grow on minimal media and utilize inorganic nitrogen indicates that the mode of operation of the system is selective for organisms able to grow with limited nutrients. A large percentage of the organisms isolated in this study, therefore, meet at least one of the selective survival criteria of Pipes for activated sludge. The ability to form storage

TABLE XVII

OPERATIONAL DATA FOR THE UNIT

Date	X_R	$\bar{X}$	X_e	S_{et}	S_i	S_e	NO_3-N
8-29-78	9,750	2,127	25	36	526	26	40.3
11- 9-78	9,981	2,091	17	31	501	21	22.5
1-18-79	10,412	1,772	20	35	527	16	-
2- 6-79	10,596	1,753	9	61	549	34	-
2-20-79	10,700	1,834	6	69	569	33	-
3-19-79	7,723	1,669	7	40	533	25	10
4- 5-79	6,011	1,001	5	26	520	16	4.2

Each expressed as average (in mg/l) of approximately four days before and after the given date (Gaudy, 1979, unpublished data).

X_R - recycled biomass

$\bar{X}$ - steady state biological solids concentration in the aeration tank

X_e - biomass in effluent

S_{et} - total substrate concentration in effluent

S_i - substrate concentration in feed

S_e - soluble substrate concentration in effluent

NO_3-N - nitrate nitrogen

products was not examined.

Pilot plants operated in a manner similar to that used in this study generally nitrify readily. Unfortunately, the nitrifying information for the unit under study is limited, as shown in Table XVII. With nitrate available, organisms able to resort to nitrate as an electron acceptor may have a competitive advantage and be selected by this system due to conditions approaching anaerobiosis in the center of flocs and in the settling tank.

A number of bacteria isolated from activated sludge have been reported by Krul and Veeningen (1977) to synthesize dissimilatory nitrate reductase under aerobic conditions, independent of the nitrogen source present. This implies that as soon as aeration of the sludge ceases, nitrate reduction may be expected to occur, since nitrate reduction is an anaerobic process. Therefore growth, or at least endogenous respiration, of organisms capable of nitrate reduction can continue under the anaerobic conditions which may be established in the settling tank until the cell yield is harvested and returned to the aerator. This will give a competitive advantage to those organisms capable of nitrate reduction. The above information may help to explain why a high percentage of the organisms isolated in this study are nitrate reducers.

The majority of the isolates have respiratory metabolism. A possible explanation is that fermentative organisms are not as nutritionally versatile as respiratory bacteria. The amount of substrate in the aeration tank, due to the prolonged detention time, is very low, which would select for organisms with respiratory metabolism instead of those with fermentative metabolism.

In addition to a high percentage of isolates being able to reduce nitrate, the majority of the isolates were able to hydrolyse lipid. A significant percentage were also able to hydrolyze gelatin. The hydrolysis of lipid and gelatin thought to be merely incidental properties of the isolates of little selection value.

The operational data, presented in Table XVII, reveal a significant reduction in the recycled biomass (autolysis) during the latter part of this study. Autolysis in pilot plants similar to the one investigated in this study occurs periodically. In the plant under study the relative numbers of a testate amoeboid protozoan increased dramatically during the period of autolysis. Only on one other occasion was this protozoan observed, and then only in small numbers. The testate amoeboid protozoan was identified, according to the keys provided in Edmondson (1959), as belonging to the genus Arcella in the family Arcellidae. Curds and Cockburn (1970) reported Arcella vulgaris in almost half of the activated sludge samples examined. No attempt was made to identify the Arcella observed in this study beyond the genus level. The size of the flocs was noticeably smaller during the period in which the Arcella population was high, although the sludge was settling rapidly and producing a clear effluent. A possible explanation is that amoeboid protozoa, due to their size, settle rapidly to the bottom. The Arcella observed were adhering to the smaller floc particles and therefore could be aiding in the settling of the flocs. The microscopic observations, as summarized in Table XVI, were not conducted to determine quantitatively the numbers of protozoa present. The very large populations of Arcella present during the last two observations were much greater than those found during the first

observation. Unfortunately, no isolation of bacteria was conducted during the period of autolysis.

The operational data, given in Table XVII, show that the unit was operating, during the period that isolations were obtained, in a stable manner, since the solids concentration in the aerator and the effluent remained relatively constant with only minor fluctuations.

In comparing the characteristics of each of the 46 groups with each other (see Tables X - XIV), it is apparent that only one group was reisolated; group #1 of isolation #3 was reisolated in isolations #4 and #5. Some groups differ only in one morphological characteristic, as shown in Table XV, which may not be a significant difference due to possible variabiiity in morphological characteristics of bacteria. Based on the characteristics of each isolate, most of the isolates were definitely not reisolated from succeeding isolations. A total of 44 different groups of bacteria were isolated in the five isolations. The populations of bacteria present in this ecosystem can thus be described as dynamic with shifting predominance patterns. Generally, the bacterial populations of the ecosystem were continually changing, although the system as a whole from the engineering standpoint remained stable. Additional studies such as those carried out by Jones (1977) in this laboratory to determine the factors influencing the predominance of microorganisms in this system and a variety of other systems would be of practical value in the understanding of problems such as bulking and the formation of scum in the activated sludge process.

The bacteria isolated in this study appear to belong to two major groups: (1) a group of Gram-positive cocci isolated in isolation #1 and #2 and comprising eleven percent of the isolates, and (2) a

coryneform group with a definite cycle of development isolated in each of the five isolations and comprising 88 percent of the isolates. One isolate was a Gram-negative rod that was not identified as to genus.

The Gram-positive cocci, groups #2, #3, and #7 of isolation #1 and group #2 of isolation #2 were identified as members of the genus Micrococcus in the family Micrococcaceae. Identification was accomplished by comparing their characteristics tablulated in Tables X and XI with the descriptions given in the eighth edition of Bergey's Manual.

Approximately 25 percent of the bacteria isolated by van Gils (1964) from a laboratory activated sludge system fed daily by "fill and draw" with mineral medium containing glucose and ammonium sulfate were identified as Gram-positive cocci of the genus Micrococcus.

The positive identification of the coryneform isolates is a difficult task, because the taxonomic demarcation lines often run close together in the coryneform group. Several numerical taxonomic studies such as that of Jones (1975) on named and unnamed coryneform organisms, reveals that no "clear cut" distinction can be made between the coryneform genera.

The coryneform group, which is characterized mainly on a morphological basis, is considered to include the genera Corynebacterium, Arthrobacter, Brevibacterium, Microbacterium, Cellulomonas, Listeria, Erysipelothrix, Mycobacterium, and certain species of Nocardia, but the equivocal definition of these genera causes difficulties in identification (Bansfield, 1972). Veldkamp (1970) in a review of coryneform bacteria, states that all coryneform bacteria during log-phase growth form irregularly-shaped cells, although the extent of pleomorphy may vary among the coryneforms. Even within one species, the degree

of pleomorphy may depend on cultural conditions. A cycle of development has also been shown to occur in representatives of all coryneform genera. But again, the extent of conversion of relatively large pleomorphic rods to very short rods or coccoid cells may vary widely within the group of coryneform bacteria and within a species may depend on cultural conditions. In a comparison of the results obtained in this study with numerical taxonomic studies on coryneform bacteria by Bansfield (1972), Jones (1975), and Davis and Newton (1969), the isolates appear to be more closely associated with the Arthrobacter group because of the presence in the isolates of the following characteristics in common with the Arthrobacter group: (1) a definite cell cycle; (2) limited branching in some groups but no extensive branching; (3) limited nutritional requirements; (4) Gram-positive or Gram-variable; (5) presence of giant cocci called "cystites" in some isolates; (6) formation of capsules by several of the isolates as shown by the gelatinous-watery colonies; (7) reports of Arthrobacter as common in activated sludge by van Gils (1964) and other workers; (8) the generally diverse physiological properties of the isolates: nitrate reduction, lipid hydrolysis, and growth on minimal media widespread by the isolates, and (9) the high percentage of strict aerobes among the isolates. Attempts to separate the coryneform isolates into separate genera failed, since they all appear to have characteristics that fit into the range of characteristics attributed to the genus Arthrobacter.

Extensive comparative numerical taxonomic studies, such as that of Jones (1975), would be required to confirm the position of each of the coryneform bacteria isolated in relation to other coryneform groups. None of the data accumulated in this study suggest that the coryneform

isolates do not belong to the Arthrobacter group. Additional tests, particularly cell wall analysis and determination of DNA base composition, are needed to confirm each isolate's taxonomic position.

The genus Arthrobacter has been described by Mulder (1964) as remarkably resistant to desiccation and starvation in both the cocci and rod states. Also, the genus Arthrobacter is a heterogeneous group that has considerable nutritional versatility. Clark (1972) and other workers have reported the frequent production of large amounts of polysaccharides both internally and externally by members of the genus Arthrobacter. The genus Arthrobacter, therefore, meets the selective survival criteria of Pipes (1966) for growth in activated sludge. The above listed properties of the genus Arthrobacter may help to explain the selection by this ecosystem for a wide variety of Arthrobacter forms.

The populations of bacteria present in the ecosystem are heterogenous as demonstrated by the large number of different groups isolated.

CHAPTER V

SUMMARY AND CONCLUSIONS

This study was primarily an attempt to isolate in pure culture, enumerate, and characterize the predominant bacterial populations present in a laboratory activated sludge plant operated with total cell recycle.

The utilization of CGYA, as a medium for isolation and enumeration, gave plate counts of 10^8 to 10^9 cell/ml which is in the range reported for activated sludge using various media by van Gils (1964) and Allen (1944).

The predominant bacteria of this ecosystem constitute a heterogeneous population, which is typical of activated sludge systems. The mode of operation of the system imposed a selective environment that selected for those organisms able to survive and grow under the conditions of low nutrients and total cell recycle. The predominant bacterial populations were found to be changing continuously, although the system as a whole from the engineering standpoint remained stable. The predominant bacterial populations showed a remarkable physiological diversity with the majority of the isolates being able to grow on minimal media, hydrolyze lipid and reduce nitrate. The selection for nitrate reducers by this system may be due to the establishment of anaerobic conditions in the settling tank and also in the center of flocs.

The predominant bacteria isolated belonged to two groups: a

coryneform group comprising the majority of the isolates, and a group of cocci. The cocci were identified as belonging to the genus Micrococcus. Much difficulty was encountered in the identification of the coryneform isolates due to the absence of clearly distinctive characteristics to separate the coryneform genera. Based on the available information, the coryneforms isolated were tentatively identified as members of the genus Arthrobacter. Additional research to separate clearly the coryneform genera is sorely needed. Both the genera Arthrobacter and Micrococcus are known to be resistant to adverse environmental conditions, such as starvation, desiccation, and reduced water potential, which may partially explain their predominance in this ecosystem.

Microscopic observations revealed the presence of large numbers of a testate amoeboid protozoan identified as a member of the genus Arcella during the period of autolysis that occurred after the last isolation. The large Arcella population is thought to aid in the settling of the smaller flocs that occurred during autolysis. This observation may have significance for future studies of the phenomenon of autolysis.

The methods utilized in these studies for the isolation and enumeration of the predominant bacteria in this ecosystem are recommended for other workers desiring to isolate aerobic heterotrophs from activated sludge systems.

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