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An Ecologic Study of an Overloaded Oxidation Lagoon Containing High Populations of Purple Sulfur Bacteria

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AN ECOLOGIC STUDY OF AN OVERLOADED OXIDATION LAGOON CONTAINING HIGH POPULATIONS OF PURPLE SULFUR BACTERIA

by

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This dissertation submitted by Harvey W. Holm in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota is hereby approved by the Faculty Advisory Committee under whom the work has been done.

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ABSTRACT

Concomitant with the addition of large amounts of potato starch wastes to the Grafton, North Dakota, municipal sewage lagoon was the development of a new major microbial population, the purple sulfur bacteria. This study attempts to define ecologic relationships of the lagoon, with special emphasis placed on the action of purple sulfur bacteria in the lagoon.

Chemical changes in the lagoon were investigated by monitoring Biochemical Oxygen Demand (BOD), sulfide, sulfate, phosphate, pyruvate, total carbohydrates, volatile acids, alkalinity, and pH. Lagoon water temperatures were observed daily. Microbial ecologic relationships were deduced by enumerating coliforms, fecal coliforms, enterococci, total bacteria (TGE agar), methane formers, sulfate reducers, purple sulfur bacteria, and algae. Finally, two strains of purple sulfur bacteria were characterized metabolically. Optimum pH, temperature, and sulfide levels were determined and the utilization of certain organic substrates was investigated and correlated with organic substrate changes in the lagoon.

The following observations summarize this ecologic study:

1. Two populations, purple sulfur bacteria and total bacteria, reached maximal concentrations in the warmest part of the 1967 summer. During 1968 no correlation of temperature values and microbial growth was observed.

2. Purple sulfur bacteria reached maximal numbers as concentrations of sulfide and volatile acids were depleted. Formic acid, which was not utilized by the isolated strains of Thiocapsa floridana and Chromatium vinosum, remained as the major volatile acid constituent in August of 1968, while levels of acetic, butyric, and propionic acids were depleted. Decreases in carbohydrate and alkalinity values in 1968 may also be related to purple sulfur populations. Low sulfate levels observed during the purple phase may be attributable to storage of sulfur within purple sulfur bacteria.

3. Populations of methane bacteria were low during the early portion of the summer when optimal volatile acid levels were present. Removal of sulfide, which inhibits methane bacteria, by purple sulfur bacteria, probably aided the development of these organisms.

4. No biological, chemical, or physical agent was linked to the removal of coliforms, fecal coliforms, and enterococci.

5. Increases of algal populations in the latter parts of summers 1966 and 1967 may have been related to the low organic content of the lagoon during these periods.

6. Populations of sulfate reducing bacteria, the contributors of sulfide to the lagoon, were not limited by depletion of sulfate (Shreve, 1967).

7. Sulfide concentrations of 45 - 60 mg/l, pH values of 7.5 - 8.0, and incubation temperatures of 25 - 30°C were optimal for maximal growth

of Thiocapsa floridana and Chromatium vinosum. Although lagoon pH (7.7 - 8.2) was favorable for purple sulfur growth, temperatures and sulfides were always minimal in the lagoon for these organisms. However, these organisms can grow at temperatures as low as 16°C and in sulfide concentrations of 1 - 5 mg/l.

8. Chromatium vinosum utilized succinate, pyruvate, fumarate, malate, glycolic acid, hexanoic acid, histidine, glucose, fructose, lactose, and sucrose, while Thiocapsa floridana, in addition to the substrates metabolized by Chromatium vinosum, used methionine, benzoic acid, maltose, valerate, propionate, and acetate.

9. Purple sulfur bacteria materially lowered BOD levels, as demonstrated by the growth of Thiocapsa floridana in sterilized sewage.

10. An absence of a direct correlation between BOD removal and a specific physical or chemical parameter in the lagoon was evident in this study. In 1967, maximal BOD reduction occured when populations of purple sulfur bacteria and total bacteria (TGE agar) were high.

INTRODUCTION

Comprehensive ecologic studies of microbial populations in aquatic environments are few in number. The majority of the early studies were primarily qualitative observations of the major genera of plankters (algae, fungi, protozoans, rotifers, and others) found in waters. Later, more extensive aquatic studies related population densities to the chemical and physical environments.

The omission of bacterial observations by early workers is understandable. The lack of a variety of reliable, differential media rendered enumeration of most species a difficult, if not impossible task. However, differential media for the quantitation of enteric pathogens or pathogen-related organisms allowed the enumeration of this group of organisms.

The advent of the sewage oxidation lagoon as a means of waste treatment prompted research in the areas of lagoon efficiency. Reduction of two parameters of pollution, numbers of enteric organisms and organic substrate levels, have been most intensively studied in the last twenty years.

The appearance of high populations of purple sulfur bacteria in a local oxidation sewage lagoon receiving municipal and industrial wastes initiated this ecologic investigation. Three areas of study warranted investigation. First, several chemical parameters of the lagoon,

including the Biochemical Oxygen Demand (BOD), sulfide, sulfate, phosphate, pyruvate, total carbohydrates, volatile acids, alkalinity, and pH were monitored weekly. Secondly, possible ecologic relationships between various organisms were investigated by enumerating total bacterial populations, coliforms, fecal coliforms, enterococci, sulfate reducers, methane formers, purple sulfur bacteria, and algae. Finally, purple sulfur bacteria were characterized metabolically by determining optimum pH, sulfide, and temperature levels. The ability of these organisms to utilize organic substrates was also determined and correlations between these organic substrates and those available in the lagoon were attempted.

HISTORICAL

Sewage Treatment Oxidation Lagoons

Although the first sewage treatment pond in North Dakota has been in existance for forty years, the vast majority of such facilities have been constructed during the last two decades. Fessenden, North Dakota, in 1928, constructed what has been considered the first oxidation lagoon by diking off a portion of a small lake to receive its sewage. Although the lagoon worked satisfactorily, no more lagoons were built until 1948, when Maddock, North Dakota, constructed a ten acre pond to treat raw sewage from a population of 1,000.

The success of the Maddock facility drew statewide attention, and after other North Dakota communities constructed efficient lagoons, neighboring states also began to utilize oxidation lagoons to treat sewage (Hopkins and Neel, 1956).

Today, approximately 175 lagoons are in operation in North Dakota (Olson, et al., 1968), while hundreds more are in use throughout the central and western states and Canada (Hopkins and Neel, 1956).

Reasons for Use of Oxygen Lagoons

Three factors dictate the use of oxidation lagoons in the sparsely populated Midwest: low cost of construction; ease of operation and maintenance; and efficiency.

The low cost of oxidation lagoons renders them acceptable to the small community in which per capita cost of conventional treatment is much greater than in large communities (Bartsch and Allum, 1957). New sewage ponds may be built for 1/8 - 1/2 the cost of a conventional plant. These cost variances depend on the price of land, topography, price of excavation, length of outfall sewers (Hopkins and Neel, 1956), flow measurement devices, and seeding and fencing of the property (Howells and Dubois, 1959). In the Howells and Dubois study, lagoons of the Midwest cost about $1,000 per acre, while Nemero and Bryson (1963) estimated the cost at $15 per capita ($1,500 per acre), which was about 1/5 of a primary sewage treatment plant.

Generally little maintenance work is necessary with lagoons. Cutting the grass, protecting washed banked areas with stone, and eliminating weed growth are the common tasks of upkeep. Pests, such as muskrats, may have to be removed periodically. On rare occasions sludge may have to be removed (Parker, et al., 1959). Howells and Dubois estimated the cost of maintenance and operation at $250 - 500 per year for a 10 acre lagoon (1959).

The efficiency of oxidation lagoons is well documented. Fitzgerald and Rohlich (1958), in analyzing data from various workers, observed that a BOD of about 150 mg/l could be effectively lowered to

20 mg/l with a detention time of 20 days under proper environmental factors. Nemerow and Bryson (1963) observed a BOD reduction of 69.3 per cent in one pond and 88.3 per cent for two ponds.

Coliform reduction, another parameter of lagoon efficiency, is also documented. Fitzgerald and Rohlich (1958) in a summary of 13 studies, observed that in all cases bacterial counts have been lowered to less than 1 per cent of the original concentration. Hok referenced by Higgins (1965) reported that 99.9999 per cent of Salmonella abortis Equi is removed by 41 days at 10°C. At 20 - 30°C the equivalent is removed in 6 days.

Theory of Oxidation Lagoons

The stabilization mechanism in sewage lagoons is primarily the interaction of bacteria and algae which remove objectionable characteristics of sewage. Physical and chemical conditions of the environment affect directly the effectiveness of the biological system of the lagoon.

In an oxidation lagoon, primarily two biological systems are functional, with both groups mutually beneficial. Primary heterotrophs feed directly on organic material introduced with the waste (Pipes, 1961). These organisms are probably the most beneficial since they serve to metabolize the vast majority of the organic wastes. This group of organisms is composed chiefly of bacteria,

but a few protozoa also function as primary heterotrophs. There has been no detailed study of the predominant bacterial species occurring in oxidation lagoons responsible for BOD reduction but it is presumed that faculatative aerobic species would be mainly responsible (Parker, 1962). Halverson et al. (1968) studied the oxidation of substrates by mixed populations as a function of seasonal change. They mentioned that although biotic types may vary, "it is not so evident that the physiological activity of the indigenous population associated with a domestic waste disposal unit remains reasonably constant". However, Halverson gave no evidence as to the types of organisms present. In a much needed microbial study of oxidation lagoons, Gann et al. (1968) found that 85 per cent of the bacterial populations of stabilization ponds is composed of Pseudomonas-Achromobacter-Flavobacterium group. Achromobacter was most numerous, followed by Pseudomonas and Flavobacterium.

The presence of secondary heterotrophs may also be expected in an oxidation lagoon. These organisms, such as protozoans, feed on primary heterotrophic bacteria (Pipes, 1961), or like the methane formers, use end porducts of primary heterotrophic metabolism (McCabe and Eckenfelder, 1958).

Probably of all the organisms in oxidation lagoons, the role of algae has been most extensively studied. It is generally conceded that these autotrophs are the primary contributors of the oxygen used by heterotrophic bacteria in the oxidation of organic substrates to CO_2, H_2O, and NH_3. Algae also utilize CO_2 as a carbon source which may cause extreme pH changes (Pipes, 1961). The summary of many studies of algal populations show there is no correlation between which species become dominant in a pond and the geographical location of the pond, the degree of BOD loading, and the degree and type of sewage pretreatment (Fitzgerald and Rohlich, 1958). However, Neel and Hopkins (1956) observed a seasonal variation in algal populations. Species of Chlorella, Scenedesmus, and Euglena are the most frequently reported algae in lagoons (Fitzgerald and Rohlich, 1961).

Two physical factors, temperature and light, play major roles in lagoon efficiency. According to the van't Hoff-Arhenius relationship, there is an approximate doubling of biological activity for every 10°C rise in temperature (Lamanna and Mallette, 1959). One would then conclude that lagoons should be designed in which biologic activities are maximal for the desired populations (Pipes, 1961). Halverson's work supports this assumption (1968). He found the highest biologic

activity occurs with populations of organisms from the lagoon at 23°C, the highest temperature of the lagoon.

Light is of ultimate importance for oxygenation lagoons. Reportedly, photosynthesis is dependant of light intensity in the range of 500 - 5,000 foot candles, but the rate of photosynthesis decreases at either extreme of light intensities. The design of oxidation lagoons allows for maximal light intensity since the water is no more than 3.5 - 4.0 feet deep. However, Bartsch and Allum (1957) reported that no light penetrated beyond 0.4 meters in one observed pond.

Three chemical factors influence the microbial action of the lagoon: nutrition factors, toxic products, and pH effects. An imbalance of any of these factors may grossly affect the lagoon's population and possibly its efficiency (Pipes, 1961).

Organic and inorganic nutrients in a sewage lagoon are seldom scarce. According to Pipes (1961) the nutritional requirements may be classified thusly: 1) an energy source; 2) the macronutrients carbon, hydrogen, oxygen, nitrogen, phosphorous, potassium and sulfur; 3) micronutrients such as iron, magnesium, calcium, boron, zinc, copper, manganese, cobalt and molybdinum; and 4) certain growth factors such as vitamins. If the quantities of nutrients are not adverse to a given population, the physical factors, light and temperature become the limiting growth factors. Usually sewage

treatment facilities are concerned only with levels of carbon, nitrogen and phosphorous. Obviously, since the objective of lagoons is to remove organic carbon, nitrogen and phosphorous must not be limiting to allow effective treatment (Isaac, 1960). Ratios of BOD:N of 17:1 and BOD:P of 100:1 are considered optimum for stabilization (Sawyer, 1956). Quantities of micronutrients and growth factors are not usually limiting since in mixed populations organisms that can grow on the factors available will be selected (Pipes, 1961).

The pH range has great effects on microbial activities. For most bacteria the optimum pH is in the range of 6.5 - 7.5, with the minimum being around 5 and the maximum near 8.5. Biologic activity is usually greatly impaired at extreme pH's (Isaac, 1960).

Physiology of Purple Sulfur Bacteria

Members of Thiorhodaceae, the purple sulfur bacteria, were among some of the first organisms described morphologically, since they are relatively large organisms that are readily observed with a light microscope (Breed et al., 1957). However, the unique metabolic characteristics of these organisms attracted much research in the first half of the twentieth century. Truper (1964) reported in his extensive literature review that Engelmann, in 1883, noted the photosynthetic abilities of these organisms and that Winogradsky observed the anaerobic nature of these organisms.

Van Niel (1931) showed that if purple sulfur bacteria are grown in an illuminated minimal medium containing sulfide and CO_2, the CO_2 is reduced and the sulfide is oxidized. This led to van Niel's concept of bacterial photosynthesis involving the photolysis of water. He proposed that a reducing fragment designated as (H) and an oxidizing fragment (OH) were produced and that the latter was involved in the oxidation of sulfide to sulfur. The proposed sequence was

1) $4\ H_2O + \text{light} \longrightarrow 4H + 4(OH)$

2) $CO_2 + 4(H) \longrightarrow (CH_2O) + H_2O$

3) $(OH) + 2H_2S \longrightarrow 4H_2O + 2S$

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4) $CO_2 + 2H_2S + \text{light} \longrightarrow (CH_2O) + H_2O + 2S$

This work by van Niel also showed that purple sulfur bacteria are autotrophs since the only carbon source was CO_2.

Fuller et al. (1961) showed that CO_2 is fixed by two pathways in Chromatium; one involving ribulose diphosphate carboxylase and a second involving carboxylation of P-enolpyruvate.

Van Niel observed (1931) that sulfur, sulfite, and thiosulfate may also serve as electron donors in the above process. In another observation, van Niel showed that purple sulfur bacteria could grow in media devoid of oxidizable sulfur substrates, but containing methylene blue or reduced indigo carmine. He also obtained good growth in organic media containing peptone, yeast extract, sodium lactate or sodium pyruvate.

Muller (1933) expanded substrate studies to show that succinate, acetate, malate and butyrate can also be used as organic substrates by Chromatium sp. He proposed that nearly all of the carbon goes to cellular material rather than to metabolic products. The photosynthetic process was also believed to be involved in the metabolism of purple sulfur bacteria in organic media.

Fuller et al. (1961) studied the metabolism of Chromatium strain D. They observed that after short time fixation, aspartate and phosphoglycerate are the primary products. All enzymes of the glyoxylate cycle except malic dehydrogenase are present in acetate-grown Chromatium. Isocitritase is induced when acetate is the carbon source. The glyoxylate cycle is completed by the decarboxylation of malate to pyruvate and the carboxylation of pyruvate to oxaloacetate. Also activities of enzymes which participate in carbohydrate synthesis in the "Calvin photosynthetic cycle" have been determined.

Few studies have been done on the types of organic substrates that can be utilized by purple sulfur bacteria. Muller (1933) observed that Chromatium (van Niel's type) and Thiocystis sp. utilize lactate, succinate, malate, and butyrate as organic substrates. He observed that the substrate is probably all converted to cellular material. He also concluded that these compounds could act as electron donors in the photosynthetic process.

Osnitskaya and Chudina (1963) showed that Chromatium vinosum may use malic, fumaric, succinic, oxalic, glyoxalic, pyruvic and lactic acids as carbon sources. Acetic acid combined with one of the above is also consumed.

Shaposhnikov et al. (1960a) (1961b) observed that acetic acid and propionic acid may be used as carbon sources by Chromatium vinosum.

May and Stahl (1967) studied organic substrate utilization in two cultures identified as Chromatium and Thiopedia. They observed that their strain of Thiopedia grew on fructose, glucose, acetate, pyruvate and propionate when bicarbonate was present, but grew only on pyruvate when bicarbonate was absent. No growth was observed in lactate or formate. Their Chromatium species grew in all of the before-mentioned substrates with bicarbonate, but only grew in pyruvate when bicarbonate was absent.

Truper observed that two strains of Chromatium vinosum and two strains of Ectothiorhodospira mobilis utilized a variety of organic substrates.

Ecology of Purple Sulfur Bacteria

The primary ecological observation during the first half of the 20th century was that sulfide is usually present where purple sulfur bacteria develop in marine and fresh water environments. In 1917 as noted by Marki (1951), a red color existed in a transition zone between the aerated and anaerobic zones of the Swiss mountain lake, Lago Ritom.

This color was attributed to populations of Chromatium okenii. Isachenko et al. (1939) reported a layer of Chromatium covering the H_2S-rich hypolimnion of Lake Mogilnoe. Tokuda reported (1940) the presence of Chromatium minus, Chromatium globosum, and Chromatium spadix and Rhodospirillum brevis in Japanese lakes at depths where H_2S is found.

Of marine waters, the Black Sea probably received the most intensive early studies. Issatschenko (1926) stated that at a depth of 200 meters the sulfide level begins and increases to a maximum of 6.54 cc/l of water at a depth of 2,300 meters. A vibro was isolated that converted sulfate to sulfide, and other bacteria were found that produced sulfide in the presence of proteins. Sulfur springs also harbor purple sulfur bacteria. Tarowaska (1933) in a comprehensive study of 29 sulfur pools observed that strains of Thiopolycoccus and Chromatium could thrive in springs that varied in sulfide levels from less than 2 mg to 100 mg/l; a pH range of 6.6 - 10 and in a temperature range of 0° to 75°C.

The observation of purple sulfur bacteria in polluted or stagnant waters began in the 1930's. Hama (1933) reported the observation of two Thiospirillum species and three Chromatium species from sewage polluted waters. Hansen et al. (1952) also reported the presence of high populations of Chromatium okenii in waters containing organic matter and H_2S.

Mitis (1940) characterized a body of stagnant water in a dead river branch of the Danube. He found that the water was eutrophic with a small amount of nitrates and high amounts of phosphates. There was a sulfide layer near the bottom covered by a "plate" of Chromatium okenii. The water was three meters deep and was divided into three biotops: 1) littoral - This bottom zone was composed of sialis larvae, Oligochaeta, Chironomidae larvae, and submerged plants; 2) pelagic - This middle zone contained Flagellatae, Diatomeae, Rotatoria, Corethra plumicornis, the sulfide zone and the Chromatium okenii layer; and 3) facial - This zone, the water surface, contained Gerridae and Poduridae.

Barkley et al. (1943) reported the appearance of high populations of blue green algae (Phormidium tenue and Oscillatoria chalybea) on the surface of oil waste water in the Luling field of Texas, while a layer of Chromatium developed under the algae layer. Similar populations developed after fourteen weeks in collected samples of travertine when incubated in the laboratory.

May and Stahl (1967) reported the presence of Chromatium and Thiopedia in five sewage treatment ponds and two rat waste treatment ponds near Pullman, Washington.

Cooper (1963) observed purple sulfur bacteria in a rendering waste pond and a petrolium refinery waste pond in California. Chromatium and Thiopedia species were observed.

Another observation on the ecology of purple sulfur bacteria is that these organisms develop in waters containing little or no oxygen (Ruttner, 1955). However, Ruttner also observed concentrations of Chromatium, Lamprocystis, and Thiospira reaching 10^5 per ml even though the mean O_2 level was 3 - 4 mg/l and immediately above the bottom it was 0.5 mg/l. Hurlbert (1967) reported that oxygen had no great detrimental effect on motility and substrate utilization of Chromatium strain D. He suggested this may play a role in the development of these organisms in sewage ponds, where the necessary light only penetrates the upper surfaces of the pond and where oxygen levels could possibly be harmful to purple sulfur bacteria.

Few studies have been completed on optimum temperatures for purple sulfur bacteria, but Turowska (1933) observed certain strains in waters ranging in temperature from 0 - 75°C. Bethge (1952) observed that maximal populations of Chromatium minus and Thiopolycoccus developed after ice cover formed on the ponds near Berlin. (Although the primary effect may not be due to temperatures in this case, apparently low temperatures may not be detrimental.) However, May and Stahl (1967) observed that under laboratory conditions, the optimum temperature range for Chromatium and Thiopedia is 28 - 32°C.

Classification of Purple Sulfur Bacteria

Until only recently, classification of purple sulfur bacteria was based only on morphological descriptions of these organisms (Breed et al.,

1957) (Skerman, 1959). Most of the descriptions are the classical ones given by Winogradsky, Molish, Perty, and Cohn near the end of the nineteenth century.

However, recent investigators have recognized the fact that microscopic observations alone may be inadequate for positive identification. Manten (1942) was the first person to isolate Chromatium okenii, a motile organism about 8 - 12μ in length. He observed that upon continued transfer the organisms gradually decreased in size to 3 - 4μ in length. The decrease in size was attributed to a loss of some factor that was originally present in the mud sample. By variation of culture conditions for the pure strain, it appeared that in a mineral medium containing about 0.4 per cent sodium thiosulfate and 0.05 per cent sodium malate, bacteria developed of nearly the same size as those of the enrichment culture. Media with different concentrations of these two substrates yielded organisms ranging in length from 1 - 15μ. Manten therefore cautioned against describing an organism morphologically without simultaneous indication of the cultural conditions.

Petrova (1959) isolated a species of Chromatium and studied the morphology as a function of the composition of the medium. He observed that in the presence of lactate, acetate, pyruvate, and mucic acid the cells were enlarged, but in the presence of glucose, butyrate and isovaleric acids the cells were smaller than when grown on a mineral medium.

Morphology also was affected by the organic substrate. Lactic acid produced clostridial-shaped cells while mucic acid formed round cells. Chains of cells were sometimes formed (lactic and acetic acids), and tetrads of cells were formed in glucose. Many amino acids were toxic at 0.1 per cent levels but at 0.001 per cent levels affected only morphology. Morphology did not change in the non-toxic amino acids glutamate, aspartate, and arginine.

Petrova concluded that his Chromatium species depending on the cultural conditions, could be classified as C. minus, C. vinosum, C. minutissimum, and even to another genus Rhabdochromatium.

Obviously, more definite criteria are needed for positive identification of species of purple sulfur bacteria. Among the recent techniques used for classification are: 1) substrate utilization (Truper, 1968); 2) pigment content (Schmidt et al., 1965); 3) nucleic acid base ratios (Truper, 1967).

Isolation of Purple Sulfur Bacteria

Van Niel, in his extensive review of the literature on purple sulfur bacteria (1931), suggested that attempts to isolate purple sulfur bacteria by several dilutions in liquid media were inadequate. He criticized Bavendamm's work in which purportedly pure cultures of these organisms were obtained by the serial dilution method. Instead, van Niel offered a

refined technique of his own in which 2.5 per cent agar was combined with inorganic constituents (NH_4Cl - 0.1 per cent; K_2HPO_4 - 0.05 per cent; $MgCl_2$ - 0.02 per cent; and NaCl if necessary). Sodium sulfide and $NaHCO_3$ were added aseptically after the agar-salt solution cooled to 45°C.

The mechanics of the isolation were as follows. A small inoculum was added to one melted tube of agar. Serial dilutions were made to 10^{-6} - 10^{-8} levels. After solidification, the agar tubes were covered with a mixture of sterile paraffin and paraffin oil. The tubes were incubated in the light for a period of about seven days. From tubes that contained 8 - 10 colonies subcultures were made. The agar was removed from the tube by cutting the end of the tube and forcing the agar column into a sterile petri dish. Here, agar slices were formed by cutting the agar with a sterile spatula, the spatula being flamed prior to each cut. Small quantities of each colony were microscopically observed for signs of contamination. Supposedly pure colonies were then transferred to melted agar tubes and the complete process was repeated. Oftentimes four such transfers were necessary to obtain a pure culture.

Other techniques for isolation of pure cultures have been reported. Skerman, (1959) using essentially the same medium

as van Niel, reportedly was successful in isolating Chromatium species from streak plates incubated anaerobically. May and Stahl (1967) isolated purple sulfur bacteria by using van Niel's medium in pour plate preparations, also incubated in an anaerobic atmosphere. Isolated colonies were then transferred to shake tubes and van Niel's technique was used for isolation of anexic cultures.

Pfennig (1965) developed a new medium for the isolation of purple sulfur bacteria. His medium was supplemented with a trace element solution and vitamin B_{12}. This medium has been used by Truper (1967) to isolate many strains of purple sulfur bacteria.

MATERIALS AND METHODS

Grafton Sewage Lagoon

Grafton, North Dakota, treats its sewage in a waste stabilization lagoon. The physical design of the lagoon is that of a primary cell, which receives raw sewage, and a secondary cell which receives partially treated wastes from the primary cell and discharges its effluent, after summer treatment, into the Park River. These two cells, each about 70 acres in area and 3 - 4 feet deep, are adequately large to treat the city's wastes and were designed for a 5-day Biochemical Oxidation Demand (BOD) loading of 30 pounds per acre per day. The average municipal waste load is about 800,000 gallons per day with a 5-day BOD loading of 3,000 pounds per day. However, during three-fourths of the year, from September to May, two potato processing companies discharge their wastes into the primary lagoon. The starch plant has a flow of about 200,000 gallons per day with a load of 12,000 pounds of 5-day BOD per day and the flake plant contributes a daily flow of 300,000 gallons per day with a 5-day BOD of 3,000 pounds per day (Vennes et al., 1966). Thus these processing plants contribute four-fifths of the organic wastes for three-fourths of the year. This added waste overloads the Grafton lagoon (about 250 pounds of five-day BOD per acre per day on the

primary cell) resulting in a period of highly disagreeable odors most probably due to the anaerobic characteristics of the overloaded lagoon.

During the past few years the highly loaded primary cell has developed three visually distinct phases in its annual summer ecologic transitions. The initial stage is the anaerobic phase, which continues from early spring to July; the second stage is the purple sulfur bacterial phase, so named because a new population of autotrophic organisms appears here for periods of one month or longer, and finally, the algal phase, in which the BOD values are low and the green color of the algae predominates until fall freeze-up.

Sampling Procedure

Sampling was usually done on a weekly basis in 1966 and 1968, while in 1967 samples were collected semi-weekly.

Special samples - Several determinations necessitated special handling of raw sewage samples at the lagoon site. Fifty-ml samples of the primary cell were preserved with 1.0 ml zinc acetate solution (2N) for sulfide determinations (Orland, 1965). (The preparation of this solution, as well as following ones, may be found in Appendix II.) Samples on which pyruvate determinations were attempted were collected in 20 ml volumes, acidified with 5.0 ml of a 6 per cent perchloric acid solution (Bergmeyer, 1965) and placed on ice until returned to

the laboratory for analysis. Mud samples were collected with the aid of a glass tube having an interior diameter of 15 mm and were stored on ice until sulfate reducers were enumerated.

Routine samples - Three-liter effluent samples of the primary cell were collected. These were placed on ice and returned to the laboratory where microbial determinations, pH values and alkalinity levels were punctually determined. Two-liter samples were immediately frozen on which the remaining tests were performed later. Composite raw samples of the total waste entering the primary cell were made on a 24-hour basis.

Microbial Determinations

Sewage samples for all microbial determinations were serially diluted in 99-ml bottles of buffered water (Orland, 1965).

Enteric organisms - The membrane filter technique was routinely used for enumeration of coliforms, fecal coliforms, and fecal streptococci. Apparatus utilized in this technique included a vacuum pump, a Millipore glass filter, a filter holder, and sterile 0.45 μ Millipore filters, type HA (Millipore Filter Corp., Bedford, Massachusetts). Coliforms were determined on Endo broth; fecal streptococci on enterococcus agar; and fecal coliforms on mFC broth and Endo broth. These media were purchased from Difco Laboratories, Detroit, Michigan, and were prepared to company specifications. The

mFC plates were incubated at 45°C for 24 hours, the Endo plates were incubated at 35°C for 24 hours, and the Enterococcus agar plates were incubated at 35°C for 48 hours.

Total bacteria - Estimates of mixed populations of bacteria were obtained by three techniques. Populations were estimated with the millipore technique, using Tryptone Glucose Extract broth (TGE) as the growth medium (Difco Laboratories, Detroit, Michigan). The plates were incubated at 35°C for 24 hours. Secondly, TGE pour plates were used to estimate mixed microbial populations. Three dilutions in the expected range were plated in duplicate and incubated at 20°C for 48 hours. Finally, estimates of non-purple sulfur bacterial populations, were determined by finding the ratio of non-purple sulfur bacteria to purple sulfur bacteria of known densities by doing differential counts on slides stained with crystal violet (Conn, 1957).

Purple sulfur bacteria - Populations of purple sulfur bacteria were estimated by direct and agar shake counts. A Neubauer Hemocytometer was used to count populations of purple sulfur bacteria. Those samples containing high numbers of purple sulfur bacteria were diluted 1:10 with buffered water before counting. The phase microscope was used at a magnification of 430 diameters for this counting procedure. Pfennig's medium with modifications by Truper (1967) was also used to enumerate purple sulfur bacteria. Appropriate dilutions of sewage were

inoculated into 9.0 ml samples of Pfennig's medium. After the agar solidified, each tube was covered with a layer of vaspar (50 per cent vaseline and 50 per cent paraffin) and incubated at 25°C in the light for two weeks after which pink colonies were counted.

Sulfate reducers - The method and medium of Postgate (1963) was used to determine sulfate reducers. Dilutions of sewage and mud were prepared in buffered water. Five tubes of medium for each of three dilutions were inoculated with 1.0 ml quantities and the tubes were incubated at room temperature for two weeks. Populations were estimated with the aid of a Most Probable Number (MPN) table (Orland, 1965). In 1967, bacterial numbers were estimated by counting black colonies in agar shakes (Postgate, 1966).

Methane bacteria - The procedure and medium of Hungate et al. (1954) was used to estimate populations of methane bacteria. Four and one-half-ml quantities of Hungate's medium were dispensed to sterile test tubes in which oxygen had been replaced by carbon dioxide. After the medium hardened, 0.1 ml of a 0.5 per cen solution of hydrogen sulfide was added and a mixture of 80 per cent hydrogen - 20 per cent carbon dioxide replaced the carbon dioxide. The tubes were remelted in a boiling water bath and cooled to 45°C at which time 0.5 ml of sewage was diluted by transferring 0.5 ml each time in a carbon dioxide atmos-

phere. The carbon dioxide was replaced by the 80 per cent hydrogen - 20 per cent carbon dioxide mixture and the tubes were incubated at 37°C for seven days.

Algae - Total algal populations were determined by counting them in a Neubauer Hemocytometer. Dominant populations of algae were identified (Orland, 1965).

Chemical Determinations

Alaklinity - Alkalinity was determined by titrating 50-ml lagoon samples to an endpoint of pH 4.0 with standard 0.02N hydrochloric acid (Orland, 1965).

pH - The pH of the lagoon samples was determined with a Leeds and Northrup pH meter (Orland, 1965).

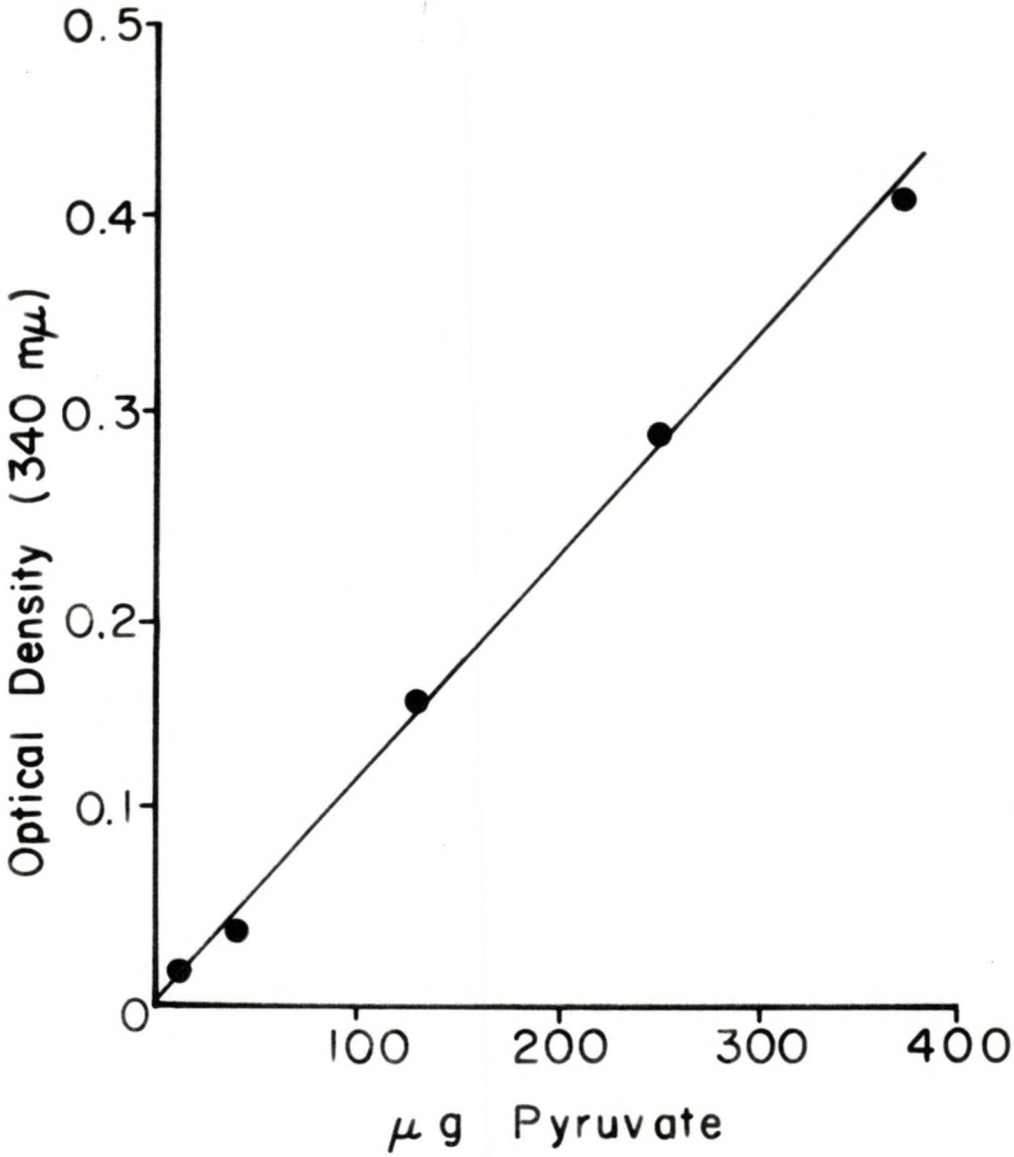
Sulfides - Sulfides were determined with the methylene blue colorimetric technique (Orland, 1965). This method is based on the reaction which takes place between paraaminodimethylanaline, ferric chloride, and sulfide ion which results in the formation of methylene blue. Seven and one-half-ml quantities of the zince acetate-preserved sewage were placed into each of two test tubes. The first test tube received 0.5 ml of the amine-sulfuric acid solution and the second tube received 0.5 ml of one + one sulfuric acid solution. After five minutes, 1.6 ml ammonium phosphate solution was added. The second tube was titrated to the blue color of the first tube with a standard methylene

blue solution, where the sulfide level as mg/l equalled the ml of standard methylene blue used in titration times 21.2.

Sulfates - Sulfate levels were determined by the gravimetric method with ignition of residue (Orland, 1965). Cation interference was removed from 10 ml centrifuged sewage samples by adding them to a 250 ml Erlenmeyer flask containing 90 ml of water and 2 g of Dowex 50W-X8 resin which had been acidified with 3N nitric acid (J. T. Baker Chemical Co., Philipsberg, New Jersey) and stirring for 10 minutes on a Magnestir (Aloe Scientific, St. Louis, Missouri). The sample was filtered through glass wool and rinsed with 50 ml of distilled water. The pH was adjusted to 4.5 with 6N HCl and two additional ml of the acid were added. Warm $BaCl_2 \cdot 2H_2O$ solution was added to the boiling sample solution until all precipitation ended and then two ml were added in excess. The precipitate was digested at 85°C for two hours after which the sample was filtered into a pre-weighed Gooch crucible, ignited at 800°C for one hour, cooled, and weighed.

Pyruvate - The pyruvate determination was completed enzymatically (Bergmeyer, 1965). Quartz cuvettes received 0.75 ml 0.02M tris buffer, 2 ml acidified sample, 0.06 ml 0.1 M DPNH and .19 ml water. The control cuvette received all except the DPNH. After reading at 340 mμ against the control (Beckman DB Spectrophotometer), two γ of lactic dehydrogenase (Cal Biochem, Los Angeles, California) were added to the experimental cuvette and the reading at 340 mμ was

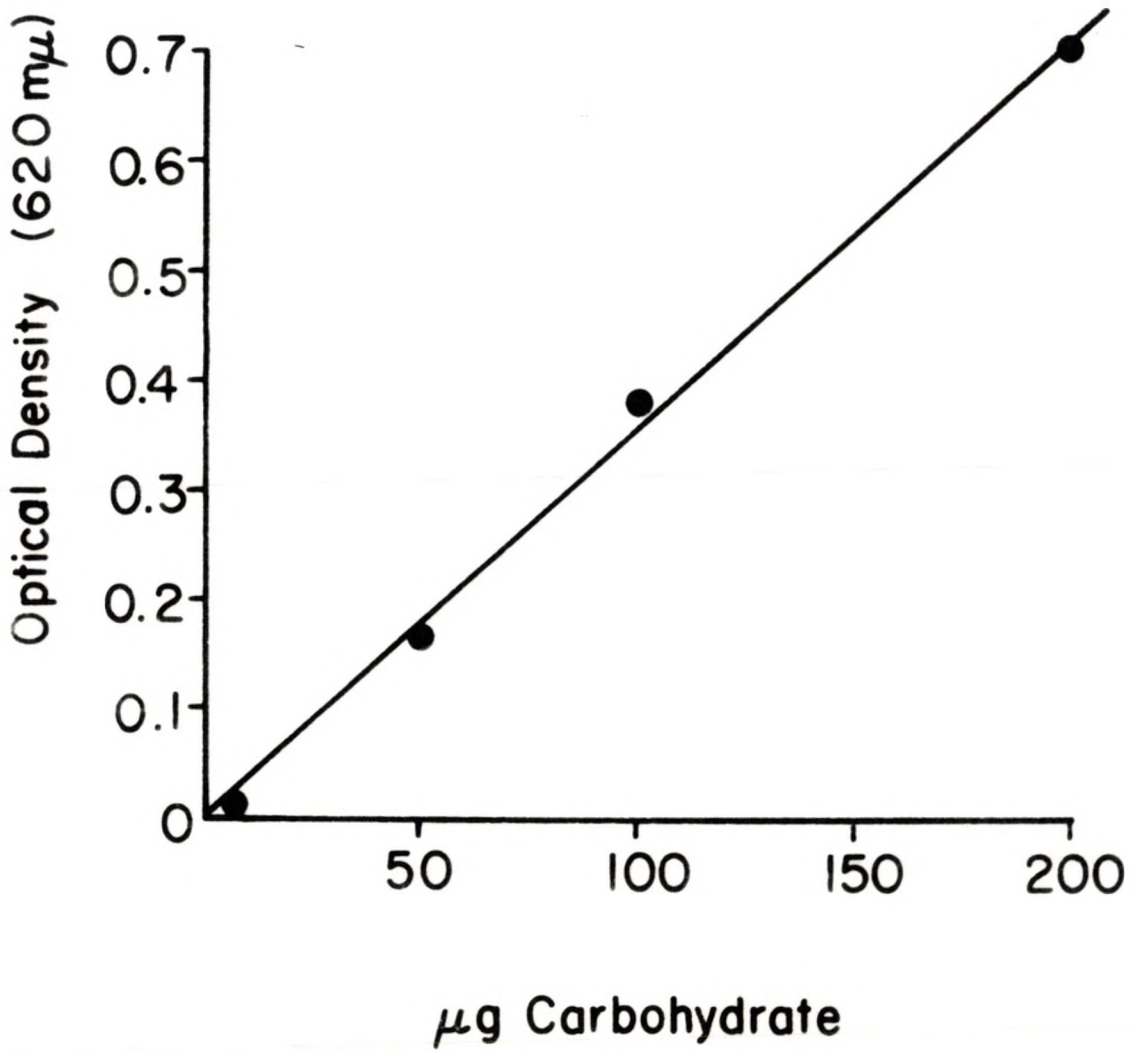
Figure 1. Reference curve for pyruvate (Bergmeyer, 1965).


0.5
0.4
0.3
0.2
0.1
0
Optical Density (340 mμ)
100
200
300
400
μg Pyruvate

again observed after three minutes. The decrease of optical density due to the oxidation of DPNH was measured and compared to a standard curve (Figure 1).

Total carbohydrates - Total carbohydrates were quantitated with Dreywood's anthrone reagent (Morris, 1948). Two ml of centrifuged sewage, 3.0 ml of distilled water, and 10 ml of the anthrone reagent were added to a test tube. After 10 minutes the color was measured photometrically (Coleman Junior Spectrophotometer) against a reagent-water blank at 620 mu and compared to a glucose standard curve (Figure 2).

Total volatile acids - Total volatile acids were determined by the column chromatography method (Orland, 1965). Ten ml of centrifuged sewage samples were acidified with 10N H_2SO_4 to the red color of thymol blue. Five ml samples of this acidified sewage were spread uniformly over the surface of 10 grams of silicic acid, which was previously packed in a fritted glass crucible. After the sample was drawn into the silicic acid by briefly applying suction from a vacuum pump, 50 ml of the chloroform-butanol reagent was added and drawn into the silicic acid, again with the vacuum pump. The flask collecting the chloroform-butanol reagent was removed and the organic acid level was determined by titrating with 0.0197N sodium hydroxide in methanol to the phenophthalein endpoint.


Optical Density (620 mμ)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
50
100
150
200
μg Carbohydrate

Biochemical Oxygen Demand - The BOD was determined according to the procedure in Standard Methods (Orland, 1965). Aliquots of sewage were placed in 300-ml glass-stoppered bottles and filled to capacilty with dilution water. The dilution water had been previously prepared by adding 1.0 ml each of phosphate buffer, magnesium sulfate, calcium chloride, and ferric chloride for each liter of water. River water seed was also added to compose one-fifteenth of the total volume and the dilution water was aerated for two hours. The samples and blanks were incubated at 20°C for five days and the amount of oxygen remaining in each bottle was determined with a Dissolved Oxygen Meter, A. 1672 (Southern Analytical, Firmly Road, Camberley, Surry) after adding 0.5 ml dosing solution per 100 ml sample to remove ionic interference.

Gas chromatography - Quantitation of individual short chain fatty acids was attempted with a Barber-Colman gas chromatograph. Sewage samples of 1,500 ml were clarified by centrifugation. After adjusting the pH to 8.6 to form non-volatile salts of the acids, the volume was reduced to 150 ml in a laboratory evaporator (Murtaugh and Burch, 1965). The concentrated samples were placed in an ether extraction apparatus and 5.0 ml of 5N HCl acid were added to the samples. This aqueous sample was covered with Spectro grade ether (Eastman Organic Chemicals, Rochester, New York) and side arm flasks containing 80 ml of Spectro grade ether were heated to 70°C. The extraction lasted 24 hours after

which the ether was evaporated, concentrating the short chain fatty acids in 0.5 ml distilled water. Five-ml samples were placed in the Chromosorb 101 column which was used at 150°C, with the argon gas at 14 psi, the hydrogen gas at 19 psi, and air at 60 psi. Curves from the samples were compared to fatty acid controls to determine qualitatively and quantitatively the presence of individual short chain fatty acids.

Phosphate - Phosphates were measured by the aminonaphthol-sulfonic acid method (Orland, 1965). Seven and one-half ml aliquots of filtered sewage were placed in test tubes. One tenth ml of 0.8 per cent bromine water was added to remove interferring sulfide. One ml of the molybdate solution was added, the tubes were mixed, and 1.0 ml of the sulfonic acid solution was added, and the tubes were mixed again. After 5 minutes, the optical density was recorded at 690 mμ against a blank prepared as above except that the molybdate was replaced by a strong acid solution. In all instances blanks and standards were run with the samples (Figure 3).

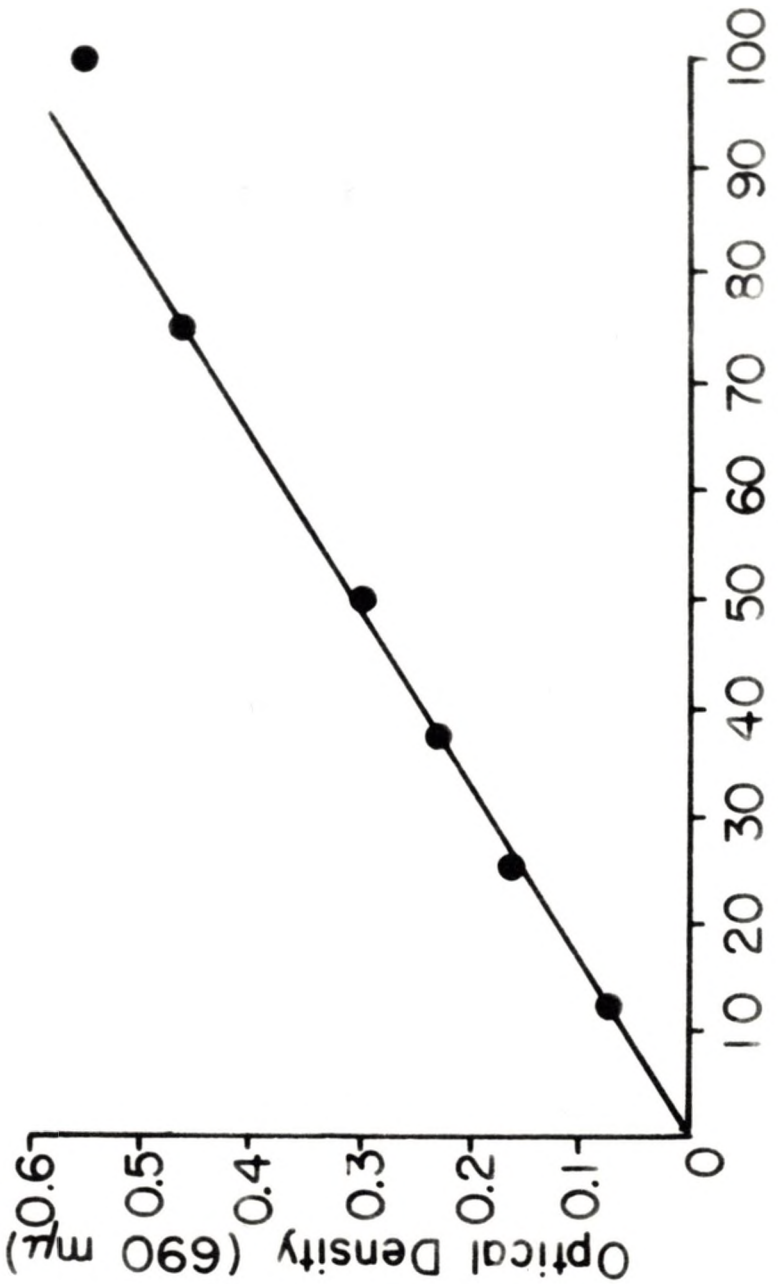
Physical Determinations

Laggon temperatures were taken daily at 9:00 a.m. The thermometer was placed six inches underwater for one minute prior to reading.

Pure Culture Studies

Pure cultures of two species of purple sulfur bacteria, Chromatium vinosum and Thiocapsa floridana, were isolated by Dr. Hans Truper of Woods Hole Oceanographic Institute, Woods Hole, Massachusetts. Truper

Figure 3. Reference curve for total phosphates (Orland, 1965).


Optical Density (690 mμ)
0
0.1
0.2
0.3
0.4
0.5
0.6
10 20 30 40 50 60 70 80 90 100
μg Phosphate

identified the organisms and characterized their pigments and nucleic acid base ratios. Truper reported that these organisms appeared identical to strains isolated elsewhere (1967). Optimum pH, sulfide, and temperature values were characterized for these two cultures. Also, organic substrate utilization by these organisms was investigated.

Inocula of purple sulfur bacteria were prepared by growing the organisms in 160 ml bottles of Pfennig's medium (Truper, 1967) with a trace element supplement. After incubation of one week at 25°C with illumination (60 watt incandescent bulb at a distance of 18 inches) profuse growth occurred and sulfide levels were depleted. Routinely, 5.0 ml aliquots of these cells, adjusted with Pfennig's salt solution so that 1.0 ml contained 10^7 organisms, were added to 60 ml tubes used in the before-mentioned studies.

Sulfide levels - For each organism, two identically prepared sets of tubes received Pfennig's medium with concentrations of sulfide ranging from zero to 300 mg/l. One set of tubes per species was inoculated and incubated at room temperature in the light for one week, after which microbial counts were completed. Sulfide levels were determined on the duplicate sets of prepared tubes.

Optimum pH levels - Varying pH levels were prepared in Pfennig's complete medium by adding aliquots of 1N HCl or 1N NaOH. For each

determination a duplicate tube was made from which the pH was read. Growth was estimated after one week by microscopic enumeration.

Optimum temperature - Pfennig's complete medium containing 20 mg/l of sulfide was placed in 60 ml tubes and inoculated with 5.0 ml of the desired organism. The tubes were incubated at 5°C, 15°C, 25°C, 30°C, and 37°C in the light. After one week, growth was measured by direct counts in a Neubauer Hemocytometer.

Substrate utilization - The ability of purple sulfur bacteria to use organic substrates was determined by incorporation of the substrate into a modification of Pfennig's medium. Four sets of determinations were done per substrate: growth in an organic substrate + a minimal salt medium, growth in an organic substrate + a minimal salt solution + bicarbonate; growth in an organic substrate + the minimal salt medium + sodium sulfide; and growth in a medium containing the organic substrate + Pfennig's complete medium (salts + sulfide + bicarbonate).

Generally the method of May and Stahl (1967) was used for substrate utilization. In all cases except those pointed out specifically, the final concentration of substrate was 0.1 per cent. The tubes were prepared as follows: thirty ml of solution I ($CaCl_2$) was sterilized in 60 ml glass tubes. After cooling, 18 ml of solutions II and III were added to those tubes receiving the bicarbonate carbon source; those tubes not receiving the bicarbonate carbon source received salt solution II diluted up to volume with distilled water. At this time 2.0 ml of the sulfide solu-

tion (Solution IV) was added to the tubes that received this electron donor. Finally, the filter-sterilized substrate was added and the tubes were inoculated with the desired organism. The tubes were then filled to near capacity to minimize oxidation effects on the substrates and organisms. The tubes were incubated at 25°C in the light (60 watt bulb at 18 inches) for a period of two weeks with growth changes observed daily.

Estimates of growth were determined by reading at 650 mu (Truper, 1968) and counting in a Neubauer Hemocytometer. Substrate utilization was also determined in those tubes showing growth.

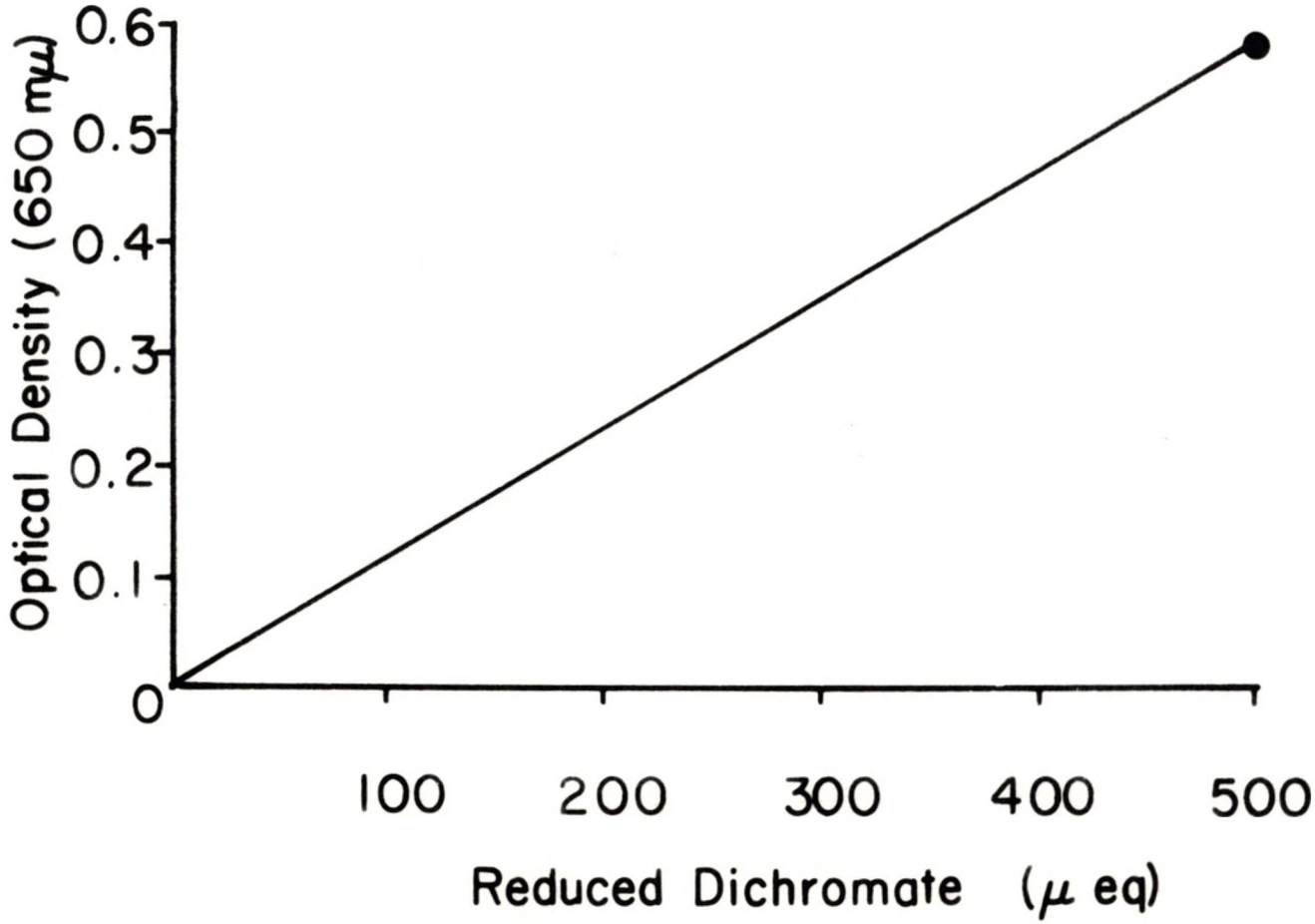
The initial level of each substrate was determined from three tubes prepared identically to the substrate sample receiving the inoculum. Final substrate levels in those tubes in which growth was observed were obtained at the termination of the experiments from aliquots removed from the growth tubes. Carbohydrate levels were determined with the anthrone method (Morris, 1948); fatty acids by the silicic acid method, (Orland, 1965); and non-volatile compounds by the sodium dichromate method for oxidation of organic substrates (Johnson, 1949).

Most organic substrates when heated with acidified dichromate are oxidized (Neish, 1952). The reduced dichromate may be measured directly at 650 mu because of the green color of the trivalent chromium ion. A solution of dichromate of known normality (0.097N) was used and two controls were run with the samples. One control, containing 2.0 ml of water + 5.0 ml of the sodium dichromate solution, was used to set the

spectrophotometer (Coleman Junior) at 0 optical density. The other blank received 2.0 ml of water + 5.0 ml of the sodium dichromate solution and excess sodium bisulfate to reduce all the dichromate. This served as a standard, giving the optical density for a known concentration of trivalent chromium. Along with the controls, 2.0 ml substrate samples were combined with 5.0 ml dichromate, and all tubes were boiled for 20 minutes. After cooling to room temperature, the optical density for each substrate sample was determined. A graph of optical density versus milliequivalents of dichromate reduced was plotted and the amount of dichromate reduced by the unknown sample was determined by reference to this graph (Figure 4). The amount of compound that reduces one milliequivalent of dichromate varies with the compound, but generally a value of 7 mg/meq dichromate reduced is used. However, several compounds have the true factor published and these values were used wherever possible (Appendix II, Neish, 1952).

Substrates that were added in a final concentration of 0.1 per cent included succinate, pyruvate, fumarate, malate, glycollate, lactate, citrate, acetate, glutamic acid, histidine, methionine, threonine, aspartic acid, fructose maltose, lactose, and sucrose. Benzoic acid, valeric acid, butyric acid, propionic acid, isobutyric acid, isovaleric acid, hexanoic acid, and formic acid were added in a final concentration of 0.5 per cent.

Figure 4. Reference curve for reduced dichromate. Added information may be found in the text.


Optical Density (650 mμ)
0.6
0.5
0.4
0.3
0.2
0.1
0
100
200
300
400
500
Reduced Dichromate (μ eq)

Growth in Sewage

The abilities of pure cultures of purple sulfur bacteria to materially lower BOD values was investigated. A culture of Thiocapsa floridana was inoculated into 500 ml centrifuged and filtered sewage to which sulfide of a final concentration of 20 mg/l had been added. The flask was incubated at 25°C for three weeks, after which BOD and population levels were obtained to compare with initial levels.

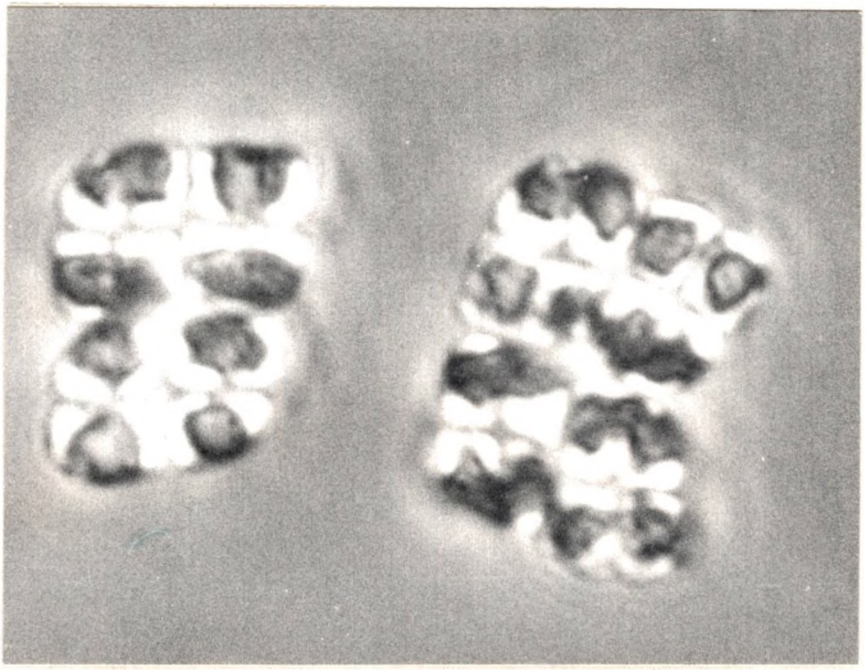
Confirmation of pure cultures was made by microscopic observation in all the previously mentioned experiments. Growth in organic substrates was confirmed by transferring 5 ml of the sample tube to new tubes containing the same substrate to ensure that growth was not due to a carry-over of substrate from the original inoculum solution.

RESULTS

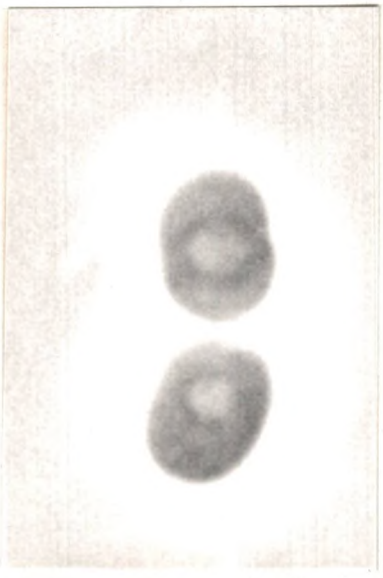
Lagoon Microbial Populations

Attempts were made to enumerate populations of organisms that may play key roles in the ecology of the lagoon. Special emphasis was placed on the ecology of the purple sulfur bacteria.

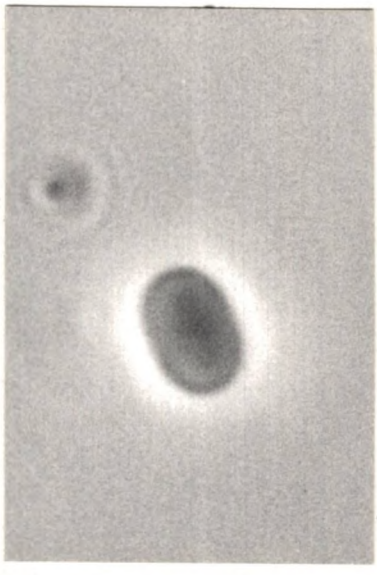
Purple Sulfur Bacteria - Purple sulfur bacteria were enumerated by direct counts in 1966, and by direct counts and agar shake tubes in 1967 and 1968 (Tables 1 and 2). (Data presented in this section are obtained from the weekly determinations tabulated in Appendix I.) In 1966, when the purple phase was of relatively short duration, a maximal population of 2×10^7/ml was present. The majority of these organisms were Thiopedia, a genus as yet not isolated (Figure 5). In 1967 the populations increased to 2×10^8 with the majority of the bacteria being of the genus Thiocapsa (Figure 5). Truper isolated T. floridana and C. vinosum (Figure 5) in 1967 and provided pure cultures for additional studies. As noted on Tables 1 and 2, the agar shake method provides population estimates about one log lower than estimates obtained with direct counts. This discrepancy may be explained in part by the fact that many Thiopedia were present and these organisms may not grow in this artificial medium. The purple phase was greatly prolonged in 1968 due to the cool summer and the higher BOD loading.



A. Thiopedia



B. Thiocapsa floridana



C. Chromatium vinosum

TABLE 1*

MICROBIAL POPULATIONS IN THE GRAFTON PRIMARY LAGOON - 1966, 1967, 1968

Organism	Pre-purple Phase[a]		
	1966	1967	1968
Purple Sulfur Bacteria/ml			
Direct Counts	-	8×10^5	1×10^7
Agar Shakes	-	8×10^4	7×10^5
Sulfate Reducers/ml			
Water	-	10^3	1×10^6
Mud	-	10^4	2×10^6
Methane Bacteria/ml	-	-	9×10^3
Bacteria/ml			
TGE Agar	5×10^6	1×10^6	2×10^6
Millipore Technique	-	8×10^4	2×10^4
Direct Counts	-	-	-
Coliforms/ml	2×10^2	4×10^4	8×10^3
Fecal Coliforms/ml	-	2×10^2	1×10^3
Enterococci/ml	1×10^2	1×10^2	3×10^2
Algae/ml	-	9×10^4	6×10^4

[a]The values listed for the pre-purple phase were recorded three weeks prior to the appearance of the purple phase.

[b]The purple phase was still present when the survey was terminated (September 11, 1968), but the populations of purple sulfur bacteria had commenced to decline.

*This table is compiled from data in Tables 19 - 27 (pp. 110 - 125).

TABLE 1 - continued

Initial Purple Phase			Terminal Purple Phase		
1966	1967	1968	1966	1967	1968
1×10^4	4×10^7	3×10^8	3×10^4	8×10^7	1×10^8
-	3×10^6	5×10^7	-	9×10^5	-
-	10^5	2×10^4	-	10^2	2×10^5
-	10^5	8×10^3	-	10^5	3×10^5
-	5×10^5	2×10^4	-	7×10^6	6×10^5
4×10^6	6×10^6	6×10^6	9×10^6	2×10^7	1×10^6
-	4×10^5	4×10^4	-	8×10^3	5×10^4
-	6×10^7	-	-	3×10^7	-
3×10^2	2×10^3	8×10^3	4×10^1	3×10^3	6×10^4
-	2×10^3	3×10^3	-	1×10^4	2×10^4
4×10^1	2×10^2	3×10^2	1×10^1	9×10^1	3×10^4
2×10^4	8×10^4	9×10^4	3×10^4	2×10^5	2×10^4

TABLE 2*

MAXIMAL POPULATION LEVELS OF PURPLE SULFUR BACTERIA IN 1966, 1967, 1968

Purple Sulfur Bacteria/ml	Year		
	1966	1967	1968
Direct Counts	2×10^7	2×10^8	5×10^9
Agar Shakes	-	1×10^7	6×10^8

*This table is compiled from data in Tables 19 and 20 (pp. 110 - 113).

Enteric Bacteria - Examination of Table 1 reveals a small decrease in enterococcus populations from May to August. The 1968 populations remained higher than those of the previous two years.

The coliform populations were quite stabile throughout the summer, with nearly a log increase during the purple phase. The high populations in 1968 are probably due to the increased amounts of organic substrate.

Mixed Bacterial Populations - Mixed populations were estimated primarily with two media. Standard plate counts were completed with TGE agar. Counts were also obtained with mTGE broth using the Millipore technique. Usually, the standard plate counts provided estimates about two logs higher than the Millipore technique. Probably the fact that the agar plates were incubated at temperatures near that of the lagoon caused these variances. Another possibility is that the Millipore technique enumerates only bacteria that can survive in an aerobic atmosphere (Table 1).

In 1966 and 1967 populations in TGE agar reached maximal values during July. This coincided with the maximal lagoon temperature period. However, the 1968 populations were quite constant throughout the summer.

Direct counts on crystal violet-stained preparations provided the highest estimate of non-purple sulfur bacteria. During July of 1967 the lagoon was about equally populated with purple sulfur and other bacteria (Table 1).

Sulfate Reducers - Populations of sulfate reducing bacteria were estimated from mud and water samples during 1967 and 1968 (Table 1). The mud samples usually contained slightly higher populations than the lagoon water. In 1967, sulfate reducers reached maximal numbers in the purple and algal phases. The 1968 populations of sulfate reducers were about two logs higher than in 1967. Also, there was a decrease in numbers from May to the middle of July, and then a gradual increase occurred.

Algae - Algae were enumerated in 1967 and 1968 by direct counts (Table 1). The population in 1967 was about 10^4/ml until August, when a near log increase occurred. The population in 1968 (through September 11) remained in the range of 10^4/ml. During each year the populations were primarily Scenedesmus and Chlorella. These genera are often the algae present in lagoons (Hopkins and Neel, 1956).

Methane Bacteria - Methane bacteria were enumerated during 1967 and 1968 (Table 1). In 1967, population determinations were incomplete, but values of 10^6 appeared average. The 1968 levels did not approach those of 1967 until September. This may be related to the presence of sulfide throughout the summer of 1968. McCabe and Eckenfelder (1957) reported that sulfide is especially detrimental to the methane bacteria that play an important role in anaerobic substrate utilization.

Lagoon Chemical Factors

BOD Reduction - The reduction of organic loads is the primary function of sewage oxidation lagoons. Table 3 presents BOD data indicating lagoon efficiency during 1966, 1967, and 1968. One may note a steady decrease of BOD values throughout the summer, beginning with the anaerobic phase and termination with the algal phase (Figure 6). During 1968 the BOD was initially higher in the spring compared to years 1966 and 1967, and no algal phase developed during the summer. However, there was a steady decrease of the BOD in both the anaerobic and the purple phases.

Organic Substrates - The quantitation of two specific organic substrates, carbohydrates and volatile acids, provided interesting results. Table 3 shows the carbohydrate levels in the primary lagoon during 1967 and 1968. Although the BOD loading during 1968 was higher than in 1967, there was little difference in the compared carbohydrate levels. No gross change in substrated levels was apparent in 1967, but a slight reduction of carbohydrate levels appeared during the purple phase of 1968.

Total volatile acid levels of 1967 and 1968 provide an obvious contrast (Table 3). Maximal values were a log higher in 1968, which may correlate with the presence of a higher organic load. A parallelism

Figure 6. BOD reduction during 1966, 1967, and 1968.

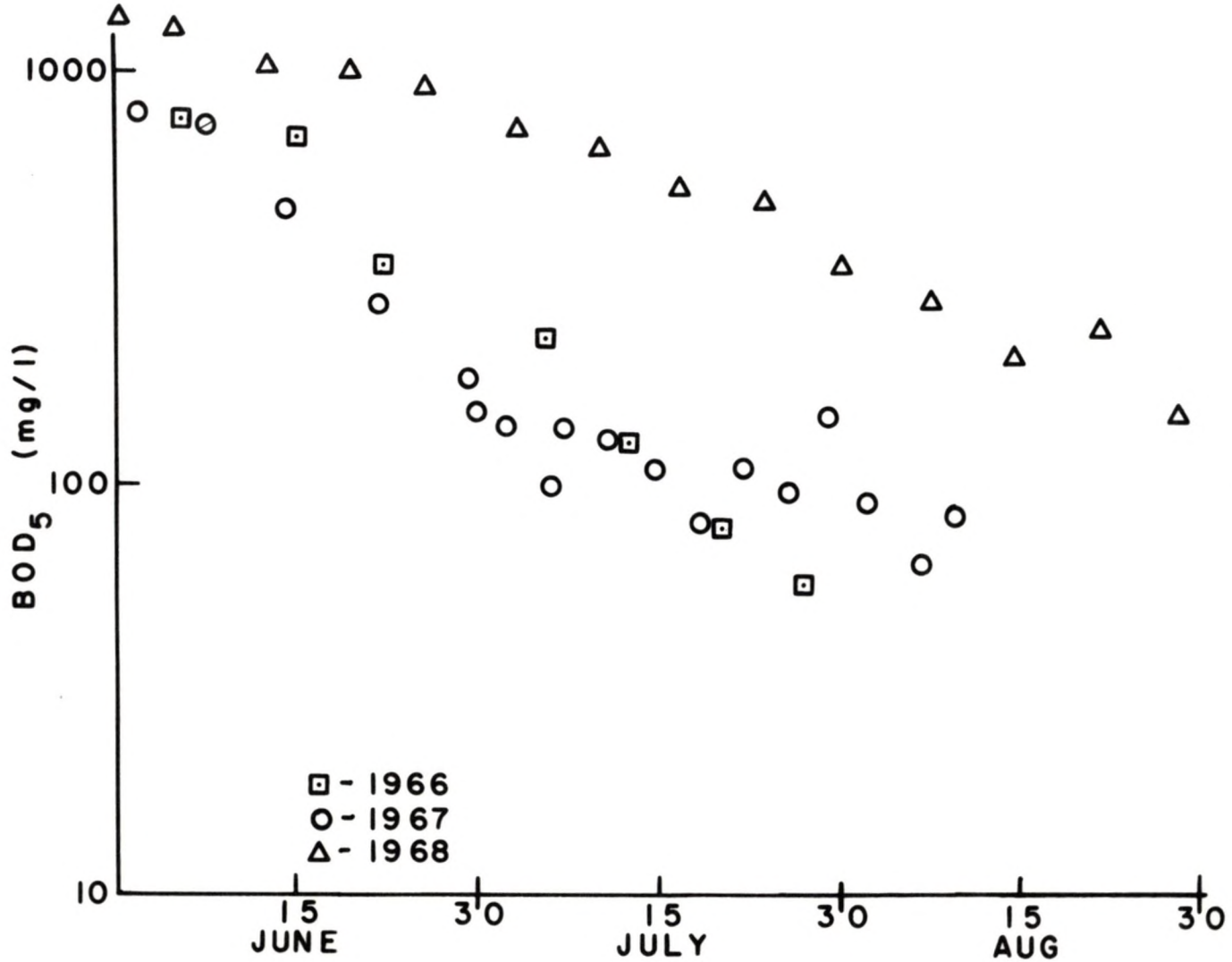

1000
100
10
BOD5 (mg/l)
□ - 1966
○ - 1967
△ - 1968
15
JUNE
30
15
JULY
30
15
AUG
30

TABLE 3*

ORGANIC SUBSTRATE LEVELS IN THE GRAFTON PRIMARY LAGOON - 1966, 1967, 1968

Substrate (mg/l)	Pre-purple Phase[a]			Initial Purple Phase			Terminal Purple Phase		
	1966	1967	1968	1966	1967	1968	1966	1967	1968[b]
BOD_5	671	688	747	217	195	545	77	97	119
Carbohydrates	-	20	16	-	14	15	-	36	5
Volatile Acids	-	47	768	-	51	484	-	7	15

[a]The values listed for the pre-purple phase were recorded three weeks prior to the appearance of the purple phase.

[b]The purple phase was still present when the survey was terminated (September 11, 1968). However, the population of purple sulfur bacteria had commenced to decline.

*This table is compiled from data in Tables 28 - 32 (pp. 126 - 133).

between the two years does exist, however. Minimal values of volatile acids were detected during the presence of high populations of purple sulfur bacteria (July of 1967; August of 1968).

Individual short chain volatile acids were quantitated by gas chromatography. Table 4 summarizes these data. Early in the summer the greatest variety of detectable volatile acids were present. Acetic acid and formic acid were present in highest concentration in the pre-purple phase, while propionic acid, butyric acid, and others were present in lesser amounts. By the end of the purple phase, however, only formic acid remained in an appreciable concentration, while a small amount of acetic acid was detectable.

Attempts to quantitate pyruvate from the lagoon failed. Apparently any pyruvate that is released by the cells is immediately utilized by organisms in the lagoon.

Phosphate - Inorganic phosphate determinations were made throughout the summers of 1967 and 1968. The BOD reduction (a reflection of microbial growth) indicated that phosphate was present in adequate amounts for organic substrate utilization (Table 5). The phosphate levels tended to vary throughout both summers, but they were in the range reported in other lagoons (Hopkins and Neel, 1956). Although phosphates may accumulate in anaerobic waters, no such phenomenon occurred in this lagoon.

TABLE 4*

VOLATILE ACID LEVELS IN THE GRAFTON PRIMARY LAGOON - 1968

Volatile Acids	Pre-purple Phase[a]		Initial Purple Phase		Terminal Purple Phase[b]	
	%	mg/l	%	mg/l	%	mg/l
Formate	14.6	112	55.7	270.6	72.2	10.9
Acetate	77.1	592	44.3	214.4	27.8	4.2
Propionic	-	-	-	-	-	-
Butyrate	8.3	63.7	-	-	-	-
Others	trace	trace				

[a]The values listed for the pre-purple phase were recorded three weeks prior to the appearance of the purple phase.

[b]The purple phase was still present when the survey was terminated (September 11, 1968). However, the populations of purple sulfur bacteria had commenced to decline.

*This table is compiled from data in Tables 31 - 33 (pp. 130 - 134).

TABLE 5*

INORGANIC SUBSTRATE LEVELS IN THE GRAFTON PRIMARY LAGOON - 1966, 1967, 1968

Substrate	Pre-purple Phase			Initial Purple Phase			Terminal Purple Phase		
	1966	1967	1968	1966	1967	1968	1966	1967	1968[a]
Phosphate (mg/l)	-	32	24	-	18	23	-	38	17
Sulfate (mg/l)	-	461	465	-	304	80	-	670	676
Sulfide (mg/l)	0.7	10.6	1.2	0.2	3.8	1.4	0.2	0.1	0
pH	8.1	7.9	7.7	8.2	8.3	8.4	8.2	8.3	7.7
Alkalinity (mg/l)	-	719	1,040	-	925	970	-	985	650

[a]The purple phase was still present when the survey was terminated (September 11, 1968), but the populations of purple sulfur bacteria had commenced to decline.

*This table is compiled from data in Tables 28 - 32 (pp. 126 - 133).

Alaklinity - Total alkalinity values of the lagoon were obtained by titration of the aquatic samples to an end point of pH 4.0 with 0.02 N H_2SO_4. It was thought that photosynthetic affects might be reflected in the quantities of alkalinity, since CO_2 utilization would decrease bicarbonate levels. Year 1967 provided a period of relatively constant alkalinity values which were somewhat unexpected since algae and purple sulfur bacteria utilize bicarbonate. Hopkins and Neel (1956) reported a sharp reduction of alkalinity values during the summer months in a Nebraska lagoon. That lagoon had an average alkalinity value of 447 mg/l, which is somewhat below the levels obtained at Grafton. The alkalinity values of 1968 contrast with those of 1967. The period of high populations of photosynthetic organisms (purple sulfur bacteria) coincides with the decrease of total alkalinity values. Perhaps the higher population of these organisms in 1968 contributed to this phenomenon.

Sulfate - Sulfate levels were monitored in 1967 and 1968. Since purple sulfur bacteria release their sulfate as the final oxidation product in sulfur metabolism (van Niel, 1931), lagoon levels of this ion were determined to relate to this biological process. Table 5 shows representative sulfate levels of 1967. One may observe that minimal sulfate levels were obtained during the purple phase. This was probably due to the uptake of sulfur compounds by the high population of purple sulfur bacteria. The increased levels of sulfate in August may be attributed to cellular release

of the ion. In 1968, sulfate levels were markedly lower than in 1967. However, 1968 level followed the pattern of 1967, since minimal values were obtained during the peak purple sulfur population.

Sulfide - Sulfide levels were measured in 1966, 1967, and 1968 since sulfide may be utilized by purple sulfur bacteria as an electron donor. Cursory examination of Table 5 reveals that sulfides are at maximal levels prior to the development of purple sulfur bacteria, and disappear during the purple phase. Sulfide levels may also decrease by physical oxidation processes. However, the lagoons are always anaerobic when the purple sulfur bacteria appear, so probably these organisms play an active role in depleting sulfides.

pH - The pH values of the lagoon were measured throughout the three-year study. Low pH values in the anaerobic periods of the year (Table 5) are attributed to production of organic acids during anaerobic metabolism and probably to a lack of CO_2 utilization by algae and purple sulfur bacteria. Values above pH 7.8 result from photosynthesis, which removes CO_2 from bicarbonate and produces quantities of monocarbonate (Hopkins and Neel, 1956). The pH levels during the purple phase, which is in the optimal pH range for many purple sulfur bacteria, may actually be stabilized by the metabolism processes of purple sulfur bacteria. The high pH values in the algae phase is due to algal metabolism.

Physical Factors

Temperatures - The water temperatures are summarized in Table 6. During 1966 and 1967, water temperatures reached 20 - 22°C prior to the purple phase, and reached 22 - 25°C during the purple phase. However, in 1968, the water temperatures seldom reached 20°C. The cool summer possibly prolonged the purple sulfur bacteria phase by decreasing the rate of organic substrate utilization.

Pure Culture Studies

Cultures of T. floridana and C. vinosum were isolated from the primary cell by Dr. Hans Truper and made available from metabolic studies.

Temperature Effects - As seen on Table 7, both C. vinosum and T. floridana were able to grow over a relatively wide temperature range. Optimal growth was achieved in the 25 - 30°C range. Other researchers have also found that optimum growth of strains of purple sulfur bacteria occurs at these temperatures (May and Stahl, 1967). The fact that good growth occurs at 16°C is undoubtedly an important factor in developing high populations in lagoons, especially during 1968 when the water temperatures remained between 15 and 20°C.for the majority of the summer.

pH Effects - T. floridana grows in a wider pH range than does C. vinosum, but both have optimum values of peak pH 7.5 (Table 8). Reports

TABLE 6*

LAGOON WATER TEMPERATURES - 1966, 1967, 1968

Year	Water Temperatures[a] (°C)		
	Pre-purple Phase	Initial Purple Phase	Terminal Purple Phase
1966	17	21	22
1967	16	22	21
1968	16	21	16

[a]Water temperatures were recorded at 9:00 a.m. daily.

*This table is compiled from data in Tables 28 - 30 (pp. 126 - 129).

TABLE 7*

GROWTH OF CHROMATIUM VINOSUM AND THIOCAPSA FLORIDANA AT VARIOUS TEMPERATURES[a]

Temperature	Growth[b]	
	C. vinosum	T. floridana
5°C	-	-
16°C	++	++
25°C	+++	+++
30°C	+++	+++
37°C	-	-

[a]Final concentrations of 0.1% bicarbonate and 0.05% sodium sulfide were present in Pfennig's medium.

[b]Growth was estimated by direct counts. Densities similar to Pfennig's basic medium control at 25°C (10^6 bacteria/ml) are indicated by a minus sign; higher populations are indicated by plus signs (+, one log increase; ++, two log increase; +++, three log increase; and ++++, four log increase).

*This table is compiled from data in Table 45 (p. 151).

TABLE 8*

GROWTH OF CHROMATIUM VINOSUM AND THIOCAPSA FLORIDANA AT VARIOUS pH LEVELS[a]

pH	Growth[b]	
	C. vinosum	T. floridana
6.1	-	+
6.3	-	+
6.6	-	+
6.8	+	+
7.1	+	++
7.5	++	+++
7.8	+++	+++
8.2	++	+++
8.5	+	+

[a]Final concentrations of 0.1% bicarbonate and 0.05% sodium sulfide were present in Pfennig's medium. The various pH levels were obtained by adding aliquots of 1N HCl and 1N NaOH.

[b]Growth was estimated by direct counts. Densities similar to Pfennig's basic medium control (pH 7.5, 10^6 bacteria/ml) are indicated by a minus sign; higher populations are indicated by plus signs (+, one log increase; ++, two log increase; +++, three log increase; and ++++, four log increase).

*This table is compiled from data in Table 46 (p. 152).

TABLE 9*

GROWTH OF CHROMATIUM VINOSUM AND THIOCAPSA FLORIDANA AT VARIOUS SULFIDE LEVELS[a]

Sulfide (mg/l)	Growth[b]	
	C. vinosum	T. floridana
1.6	+	-
5.0	+	+
8.0	+	+
16.0	+	+
24.0	+	+
32	++	+
40	++	+
48	+++	++
56	+++	+++
64	++	++
80	+	+
128	-	-

[a]Pfennig's medium was used with a final concentration of 0.1% bicarbonate. Varying aliquots of sterile 1.5% $Na_2S \cdot 9H_2O$ solution were added to the tubes.

[b]Growth was estimated by direct counts. Densities similar to Pfennig's basic medium control (10^6 bacteria/ml) are indicated by a minus sign; higher populations are indicated by plus signs (+, one log increase; ++, two log increase; +++, three log increase; and ++++, four log increase).

*This table is compiled from data in Table 47 (p. 153).

of lower pH optima for C. okenii (pH 6.7) have been reported (Postgate, 1966); thus all purple sulfur bacteria do not have this high pH optimum for growth.

Substrate Utilization - Several groups of compounds were investigated to determine their utilization by purple sulfur bacteria. The substrate studies included sulfide, carbohydrates, fatty acids, amino acids, and non-volatile organic acids. The ability of purple sulfur bacteria to utilize the organic compounds as electron donors was also investigated.

Optimum Sulfide Concentrations - Table 9 indicates the optimal sulfide levels for the two organisms. Ranges of 32 - 64 mg/l for C. vinosum and of 48 - 64 mg/l for T. floridana provide dense growths (About 10^8/ml) in one week at 25°C. Lagoon sulfide levels did not reach this density, but the organisms thrived at lower concentrations as also observed in Table 9.

Organic Substrate Utilization - Table 11 summarizes the carbohydrate study. Glucose, fructose, maltose, lactose, and sucrose are utilized by T. floridana while C. vinosum utilized all but maltose. No sugar could simultaneously act as both a carbon source and electron donor as noted by observing growth with the organic substrate alone. However, C. vinosum used all but maltose as electron sources and T. floridana

TABLE 10*

CONTROLS FOR THE SUBSTRATE UTILIZATION STUDY - GROWTH OF CHROMATIUM VINOSUM AND THIOCAPSA FLORIDANA IN PFENNIG'S BASIC MEDIUM WITH ADDITIONS OF BICARBONATE AND SULFIDE

Substrate[a] Additions	Growth[b]	
	C. vinosum	T. floridana
-	-	-
$NaHCO_3$	-	-
$Na_2S \cdot 9H_2O$	-	-
$NaHCO_3 + Na_2S \cdot 9H_2O$	++	++

[a]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: bicarbonate, 0.1%; and sodium sulfide, 0.05%.

[b]After incubation in the light at 25°C for two weeks, growth was estimated by direct count and optical densities at 650 mu. Pfennig's basic medium control (10^6 bacteria/ml) was used as the reference with higher populations indicated by plus signs (+, one log increase; ++, two log increase, +++, three log increase; and ++++, four log increase).

*Tables 10 - 14 are compiled from data in Tables 36 - 44 (pp. 138 - 150).

TABLE 11

UTILIZATION OF CARBOHYDRATES BY CHROMATIUM VINOSUM AND THIOCAPSA FLORIDANA

Substrate[a] Additions	Growth[b] C. vinosum	T. floridana
Glucose	–	–
+$NaHCO_3$	+	++
+$Na_2S \cdot 9H_2O$	–	++
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	+++	+++
Fructose	–	–
+$NaHCO_3$	+	++
+$Na_2S \cdot 9H_2O$	–	++
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	+++
Maltose	–	–
+$NaHCO_3$	–	–
+$Na_2S \cdot 9H_2O$	–	+
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	+++
Lactose	–	–
+$NaHCO_3$	++	++
+$Na_2S \cdot 9H_2O$	++	+
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	+++	+++
Sucrose	–	–
+$NaHCO_3$	++	–
+$Na_2S \cdot 9H_2O$	–	++
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	+++	+++

[a]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: carbohydrate, 0.1%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

[b]After incubation in the light at 25 °C for two weeks, growth was estimated by direct counts and optical densities at 650 mu. Densities similar to Pfennig's basic medium control (10^6 bacteria/ml) are indicated by a minus sign with higher populations indicated by plus signs (+, one log increase; ++, two log increase, +++, three log increase; and ++++, four log increase).

utilized glucose, fructose, and lactose as electron sources. C. vinosum utilized lactose as a carbon source when sulfide was present, and T. floridana utilized all of the sugars in this manner. The substrate that consistantly provided the most growth was lactose.

The amino acid study provides some interesting results (Table 12). Histidine was utilized by both organisms as an electron donor and a carbon source, while methionine was utilized as a carbon source by T. floridana. Methionine inhibited the growth of C. vinosum and aspartic acid inhibited T. floridana.

The role of the short chain fatty acids was only that of electron donor (Table 13). C. vinosum could utilize only hexanoic acid, while T. floridana utilized valeric acid, propionic acid, hexanoic acid, and acetic acid. In all cases growth was sparse.

Of all the substrates tested, the most profuse growth occurred with several of the non-volatile organic acids (Table 14). Pyruvate and fumarate acted as electron donors and carbon sources for both organisms. Succinate, malate, and glycolic acid were utilized as electron donors by both organisms, and benzoic acid acted as an electron donor for T. floridana.

Growth in Sewage - A culture of T. floridana was inoculated into a 500 ml flask of centrifuged, filtered sewage and incubated for two weeks. As seen in Table 15, growth of the organisms occurred with concomitant

TABLE 12

UTILIZATION OF AMINO ACIDS BY CHROMATIUM VINOSUM AND THIOCAPSA FLORIDANA

Substrate[a] Additions	Growth[b] C. vinosum	T. floridana
Glutamic Acid	–	–
+$NaHCO_3$	–	–
+$Na_2S \cdot 9H_2O$	–	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++
Histidine	+	++
+$NaHCO_3$	+	++
+$Na_2S \cdot 9H_2O$	+	++
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	+++
Methionine	–	–
+$NaHCO_3$	–	–
+$Na_2S \cdot 9H_2O$	–	+
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	–	++
Threonine	–	–
+$NaHCO_3$	–	–
+$Na_2S \cdot 9H_2O$	–	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++
Aspartic Acid	–	–
+$NaHCO_3$	–	–
+$Na_2S \cdot 9H_2O$	–	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	–

[a]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: amino acids, 0.1%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

[b]After incubation in the light at 25°C for two weeks, growth was estimated by direct counts and optical densities at 650 mu. Densities similar to Pfennig's basic medium control (10^6 bacteria/ml) are indicated by a minus sign with higher populations indicated by plus signs (+, one log increase; ++, two log increase, +++, three log increase: and ++++, four log increase).

TABLE 13

UTILIZATION OF VOLATILE ORGANIC ACIDS BY CHROMATIUM VINOSUM AND THIOCAPSA FLORIDANA

Substrate[a] Additions	Growth[b]	
	C. vinosum	T. floridana
Valeric Acid	–	–
+$NaHCO_3$	–	+
+$Na_2S \cdot 9H_2O$	–	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++
Butyric Acid	–	–
+$NaHCO_3$	–	–
+$Na_2S \cdot 9H_2O$	–	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	+++	++
Propionic Acid	–	–
+$NaHCO_3$	–	+
+$Na_2S \cdot 9H_2O$	–	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++
Isobutyric Acid	–	–
+$NaHCO_3$	–	–
+$Na_2S \cdot 9H_2O$	–	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++
Hexanoic Acid	–	–
+$NaHCO_3$	+	+
+$Na_2S \cdot 9H_2O$	–	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++

-- continued --

TABLE 13 - continued

Substrate[a] Additions	Growth[b]	
	C. vinosum	T. floridana
Isovaleric Acid	-	-
+$NaHCO_3$	-	-
+$Na_2S \cdot 9H_2O$	-	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++
Formic Acid	-	-
+$NaHCO_3$	-	-
+$Na_2S \cdot 9H_2O$	-	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	-	-
Acetic Acid	-	-
+$NaHCO_3$	-	+
+$Na_2S \cdot 9H_2O$	-	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++

[a]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: volatile acids, 0.05%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

[b]After incubation in the light at 25°C for two weeks, growth was estimated by direct counts and optical densities at 650 mu. Densities similar to Pfennig's basic medium control (10^6 bacteria/ml) are indicated by a minus sign with higher populations indicated by plus signs (+, one log increase; ++, two log increase, +++, three log increase; and ++++, four log increase).

TABLE 14

UTILIZATION OF NON-VOLATILE ORGANIC ACIDS BY CHROMATIUM VINOSUM AND THIOCAPSA FLORIDANA

Substrate[a] Additions	Growth[b]	
	C. vinosum	T. floridana
Succinate	-	-
+$NaHCO_3$	+	+
+$Na_2S \cdot 9H_2O$	-	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++
Pyruvate	++	++
+$NaHCO_3$	++	++
+$Na_2S \cdot 9H_2O$	++	++
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	+++	++
Fumerate	+	++
+$NaHCO_3$	++	++
+$Na_2S \cdot 9H_2O$	+++	++
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	+++	++
Malate	-	-
+$NaHCO_3$	+	+
+$Na_2S \cdot 9H_2O$	-	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++

-- continued --

TABLE 14 - continued

Substrate[a] Additions	Growth[b]	
	C. vinosum	T. floridana
Glycolic Acid	-	-
+$NaHCO_3$	+	+
+$Na_2S \cdot 9H_2O$	-	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++
Benzoic Acid	-	-
+$NaHCO_3$	-	+
+$Na_2S \cdot 9H_2O$	-	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	++	++

[a]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: non-volatile acids, 0.1%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

[b]After incubation in the light at 25°C for two weeks, growth was estimated by direct counts and optical densities at 650 mu. Densities similar to Pfennig's basic medium control (10^6 bacteria/ml) are indicated by a minus sign with higher populations indicated by plus signs (+, one log increase; ++, two log increase, +++, three log increase: and ++++, four log increase).

TABLE 15

UTILIZATION OF SEWAGE AS A GROWTH MEDIUM BY THIOCAPSA FLORIDANA

	Concentrations	
	Initial	Final
Carbohydrate (mg/l)	43	38
BOD (mg/l)	2,535	1,975
Volatile Acids (mg/l)	1,710	1,316
Sulfide (mg/l)	20	0
Bacteria/ml	5×10^5	3×10^9

decreases in the BOD, volatile acids, and sulfide levels. No perceptible change occurred in the carbohydrate level. It is obvious from this experiment that purple sulfur bacteria take an active role in organic substrate utilization.

DISCUSSION

The contribution of large quantities of potato starch wastes to the primary cell of the Grafton, North Dakota, sewage lagoon initiated the appearance of high populations of purple sulfur bacteria. Aside from the general interest in the development of a "pink lagoon" (Figure 7), this investigation was directed towards defining the role of these purple sulfur bacteria in lagoon ecology.

Lagoon Microbial Ecology

Other workers (Cooper, 1963; May and Stahl, 1967) have defined the major role of purple sulfur bacteria in lagoons as one of biological deodorizers. Microbial ecologic studies, ignored by these workers and presented in this study, relate populations of methane formers, sulfate reducers, coliforms, enterococci, total bacteria (TGE agar and broth), algae, and purple sulfur bacteria in order to define possible ecologic relationships among these organisms.

The possible antagonistic role of purple sulfur bacteria was investigated by observing total bacterial populations throughout the summer. The possibility that purple sulfur bacteria could utilize some substrate required by other major lagoon populations existed since during the pink phase of the lagoon, purple sulfur bacteria made up the major populations of the lagoon. The production of toxic metabolites by purple sulfur bacteria is, however, unlikely since others (Shaposhnikov and Kondrat'eva, 1960) have

Figure 7. Photograph of the Grafton Primary lagoon during the purple sulfur phase.

Grafton Primary Lagoon

shown that only trace amounts of end products (carbon dioxide, lactate, and pyruvate) are released by organisms grown in a medium containing volatile acids. No apparent inhibition of lagoon populations occurred upon development of purple sulfur bacteria (Table 1). In fact, a near log increase of total bacteria (TGE agar) was noted during July of 1967 (the period of high purple sulfur bacterial concentrations). These population levels of total bacteria were comparable to populations of bacteria in lagoons not containing purple sulfur bacteria (Gann et al., 1968; Halverson et al., 1968).

Populations of coliforms and enterococci appeared to vary insignificantly throughout the summer (Table 1), suggesting that the advent of purple sulfur bacteria did not hinder these organisms. Of interest, however, was the apparent reduction in numbers of these organisms during the primary cell treatment. During the summer, the primary cell received about 800,000 gallons of sewage per day (Vennes et al., 1966). This quantity was diluted in the 70 acre lagoon containing about 70 million gallons of primary liquids. Therefore, roughly a hundred-fold dilution of the raw sewage occurred upon entering the primary cell. It follows that the microbial populations were diluted at the same rate as the raw sewage: coliform densities of 10^5/ml in raw sewage were diluted to 10^3/ml upon entering the primary lagoon, and these levels were detectable throughout the summer. There appeared to be a constant removal of coliforms, then, that equaled the daily contribution of coliforms by the raw

sewage. A similar phenomenon was apparent with the populations of enterococci. No toxic metabolic products from algae have been linked to this inhibition (Fitzgerald and Rohlich, 1958). It is significant, considering the high organic loading, that increases of coliforms and enterococci did not occur.

Methane bacterial populations were surprisingly low in early summer (Table 1). These organisms are obligate anaerobes which utilize alcohols and volatile acids in the production of methane (McCabe and Eckenfelder, 1958). They thrive in a pH range of 6.4 - 7.2; below pH 6.0 and above pH 8.0 the growth rate falls off rapidly. The lagoon water temperature never approached the optimum temperature (37 - 45°C) for methane organisms (Mylroie and Hungate, 1955). One might expect maximal populations of methane bacteria when the pH, volatile acids, and temperatures are optimum. Contrarily, maximal populations developed in August when the pH was 8.0 - 8.2 and the water temperature was 15 - 17°C. Factors other than the above probably limited the development of the organisms early in the summer when pH and volatile acids were optimum and the temperature of the water was 16°C. The inhibitory factor may be sulfide, which is known to be detrimental to methane bacteria (McCabe and Eckenfelder, 1958). Our findings were consistent with this observation in that when sulfides were depleted, probably by purple sulfur bacteria, methane bacteria reached maximal levels.

Populations of sulfate reducers were variable in 1967 and 1968, which may indicate that no single factor controlled their numbers. Sulfate reducers undoubtedly contributed sulfide which was utilized by purple sulfur bacteria. Sulfate reducers also produce fatty acids, especially acetic acid, from alcohols, which may also be utilized by purple sulfur bacteria (Postgate, 1965).

Conversely, purple sulfur bacteria may assist the growth of sulfate reducers by removing sulfides and organic acids, since these products of metabolism may in high concentrations be inhibitory to certain sulfate reducers (e.g. Desulfovibrio desulfuricans) (Ghose and Wiken, 1955).

A near log increase of algae occurred near the end of the purple phase (years 1966 and 1967). This increase may be related to the low organic substrate concentrations found in the lagoon at this time of year, since generally algae grow most abundantly in media containing a total of only 100 mg/l of required ions (Hutchinson, 1967). This increase of algal population did not occur in the observed period of 1968, since the substrate level was still relatively high when the experiments were terminated. Probably the primary bacterial contribution, then, is the removal of organic substrate by heterogenous lagoon populations allowing the development of algae. An interesting observation was that a major algal population of the Grafton lagoon, Chlorella, is able to grow in concentrated

media (Hutchinson, 1967). However, Parker and Skerry (1968) attributed low algal populations in heavily loaded lagoons to decreased light penetration due to rise of bottom sludge solids.

Relation of Lagoon Microbial Populations to Physical Factors

Since substrate levels in lagoons are not stabile, it is difficult to specifically relate population densities to temperature differences. Coliforms, fecal coliforms, enterococci, methane bacteria and sulfate reducers failed to reflect temperature changes.

Algae populations in the Grafton lagoon reached maximal levels in the late summer (years 1966 and 1967), but in 1968 the algal population was constant throughout the summer. There appeared to be no direct correlation of algal population with lagoon temperature.

Two populations, purple sulfur bacteria and total bacteria (TGE agar), reflect seasonal temperature changes. For example, purple sulfur bacteria appeared in the lagoons when temperatures reached 20 - 22°C. Total bacteria increased to maximal levels in 1967 when temperatures were highest in the summer, but they remained at constant levels during 1968 when water temperatures were slightly lower than 1967.

Relationships of Microbial Populations to Inorganic Agents

Phosphates appeared to play minimal roles in the lagoon ecology. Phosphate remained at a level (20 - 40 mg/l) that was not limiting for microbial growth. The continuous supply of raw sewage probably provided enough phosphate to replace that leaving the lagoon in microbial cells.

Alkalinity values did not reflect the utilization of bicarbonate by the algae or purple sulfur bacteria in 1967. Probably bicarbonate was continually regenerated by anaerobic respiration or was leeched from the anaerobic mud (Hutchinson, 1957). The slight decrease in alkalinity in 1968 could be due to utilization of bicarbonate by the high populations of purple sulfur bacteria (Hopkins and Neel, 1956).

The decreased levels of sulfate in the lagoon during the purple sulfur phase were undoubtedly a result of sulfur storage within the purple sulfur bacteria. After the purple phase terminated, high levels of sulfate were once again detected in the lagoon. At no time, however, did the sulfate level become limiting for sulfate reducers, since concentrations above 50 mg/l have little effect on the rate of sulfide production (Shreve, 1967).

Sulfide was probably involved in several biological processes of the lagoon. First, a correlation between the sulfide level and the onset of the purple phase was demonstrated each year. Sulfide levels disappeared during the purple phase, although small quantities of sulfide persisted in the lagoon in 1968 until September. The sulfide was probably contributed by the metabolism of proteinaceous wastes and the biological reduction of sulfate, which may have been prolonged by the lower lagoon temperatures observed in 1968.

As mentioned previously, sulfide may inhibit methane bacteria and sulfate reducers (Ghose and Wiken, 1955; McCabe and Eckenfelder, 1958).

Generally the pH increased from about 6.7 in the spring to 8.2 by fall. The pH rise may be attributed to utilization of volatile acids by lagoon bacteria, and utilization of carbon dioxide by algal and purple sulfur bacteria, resulting in a saturation deficiency of carbon dioxide (Hutchinson, 1967). The pH rise was favorable for purple sulfur bacteria and sulfate reducers, but was not optimal for methane bacterial growth.

Relationship of Organic Substrates to Environmental Conditions

BOD removal rates (calculations are appended) were determined for the periods of linear BOD removal (June 1 through July 12, 1966; May 31 through July 8, 1967; and May 29 through August 28, 1968). No significant differences were noted among these slopes (-19.1, 1966; -18.6, 1967; and -14.2, 1968).

Although temperature directly affects microbial activity (Lamanna and Mallette, 1963), these data do not establish that temperature variations significantly alter rates of BOD removal. For example, the average water temperature during the period of linear BOD removal in 1966 was 22°C, while in 1967 the average was 18°C.

Year 1968 also supports the observation that water temperatures do not materially change the rate of BOD reduction. Two 22-day periods, (June 12 through July 3, and July 24 through August 14) having mean temperatures of 16°C and 21°C respectively had similar removal rates (slope values for the periods were -16.4 and -15.6). During much of this time

the lagoon level was increasing because no effluent was being discharged from the secondary cell. From June 18 to August 5 the lagoon level rose 0.85 feet (Fossum, 1968). One might assume that some BOD reduction during this period was due to dilution, since the influent (800 - 900 thousand gallons per day) contained only about 150 mg/l BOD, while the BOD of the lagoon ranged from 1,020 mg/l (June 19) to 295 mg/l (August 7).

To test the assumption that dilution reduced the BOD, the BOD removal was calculated by two methods: Firstly, BOD removal was calculated from the above data in terms of mg/l; and secondly, BOD removal was calculated as total pounds of BOD removed from the lagoon during the same time interval (June 19 through August 7). The data indicate that 71 per cent of the BOD was removed when calculated as mg/l. The BOD removal calculated by pounds of BOD removed yielded somewhat different results and are as follows: On June 19, the lagoon contained 1,020 pounds of BOD per million pounds of lagoon water and on August 7 the lagoon contained 295 pounds of BOD per million pounds of lagoon water. Since the lagoon contained 70 million gallons (525 million pounds of sewage waters on June 18 and 89 million gallons (667.5 million pounds) on August 7, the total BOD present June 18 was equal to 535,500 pounds and the total BOD in the primary cell August 7 was 196,900 pounds. The amount of BOD removed was about 63 per cent of that present June 18. Therefore, about 8 per cent of the BOD reduction recorded as mg/l was probably due to a dilution phenomenon.

Since BOD reduction in the lagoon does not reflect temperature differences as do determinations in BOD removal in the laboratory (Eckenfelder, 1966), factors other than temperature must affect the rate of BOD removal in the Grafton primary lagoon.

One explanation of the prolonged BOD removal period in 1968 is that the BOD load was constantly being regenerated by lagoon sediments (benthic layer). Early in 1968 the BOD load was 1300 - 1500 mg/l -- a value substantially higher than the 600 - 800 mg/l BOD observed in the spring of 1967. Therefore, a much larger amount of sediment was probably formed in 1968. Meron et al. (1965) reported that a two hour settling period results in about a 35 per cent reduction of BOD and an 8 hour period allows up to 50 per cent BOD reduction in domestic sewage plants. It is obvious that an influent containing 1,500 mg/l BOD (spring of 1968) will result in a deposit of significant amounts of organic substrate.

Theoretically four possibilities exist involving the fate of the settled volatile solids (Oswald, 1960). First, if the temperature is near 4°C or if the pH is below 5.5, little decomposition of organic sediment occurs. Secondly, if the bottom temperature is high, acid decomposition occurs, with a production of sulfide from sulfates. Thirdly, methane fermentation may become established. Methane fermentation requires a pH of 6.8 - 7.2, anaerobic conditions, relatively high temperatures, available volatile acids, and the essential organisms. Acid conditions and low temperatures may

delay or entirely inhibit the onset of methane fermentation, but if fermentation is in progress, acid conditions seldom occur since the methane organisms utilize the volatile acids. The final possibility is that the organic solids may be resuspended in the lagoon by physical means.

In a lagoon such as the primary cell at Grafton, one may visualize all four of the previously mentioned fates of sediments occurring during the year. Throughout the winter, sediments were deposited on the lagoon bottom while small amounts of substrate were removed by organic decomposition. Secondly, although lagoon temperatures were not high, organic acids and sulfide appeared during the summer. Also evident during the summer was the production of gases, presumably methane and others, from the bottom of the lagoon, indicating action by methane bacteria. Mixing and circulation probably continued throughout the summer months since the large surface area to volume ratio of the lagoon exposed much water to the forces of the wind. Therefore the bottom sediments may have acted as an organic substrate reserve which continuously contributed substrate to the lagoon BOD. This could have minimized observable temperature affects in the lagoon, especially during years of high BOD loading.

Decreasing rates of BOD removal due to the presence of non-easily oxidizable substrates may have also contributed to the lack of apparent temperature affects in the lagoon. As Eckenfelder noted (1966), "as the composition of the waste changes, the overall removal rate may also change because of the variation in the removal rate of particular constituents and

in their initial concentration." One may visualize the utilization of easily oxidizable substrates early in the year when water temperatures were relatively low (15 - 16°C) and utilization of non-readily oxidizable substrates later in the year when water temperatures reached 20 - 22°C, the end result being a constant BOD reduction rate throughout the summer.

Another factor which could have affected the BOD removal was the emergence of a possible antagonistic population in the lagoon. The only population monitored in the lagoon which increased greatly was the purple sulfur organisms. However, these organisms removed toxic substrates (sulfide), volatile acids, short chain non-volatile acids, and carbohydrates and metabolically utilized them.

In fact, as seen in Figure 6, there appeared to be a steady decrease in BOD throughout the summer until a level of 100 - 150 mg/l was reached. At this point much fluctuation of BOD values was apparent. The constant rate of BOD removal, even in periods of high populations of purple sulfur bacteria (July of 1967 and August of 1968), suggested that these bacteria do not hinder lagoon efficiency. It is also significant that different populations of purple sulfur bacteria were dominant in 1966 compared to 1967 and 1968 while the rates of BOD removal during these years were comparable. The major population in 1966 was Thiopedia, while 1967 and 1968 had high populations of Thiocapsa.

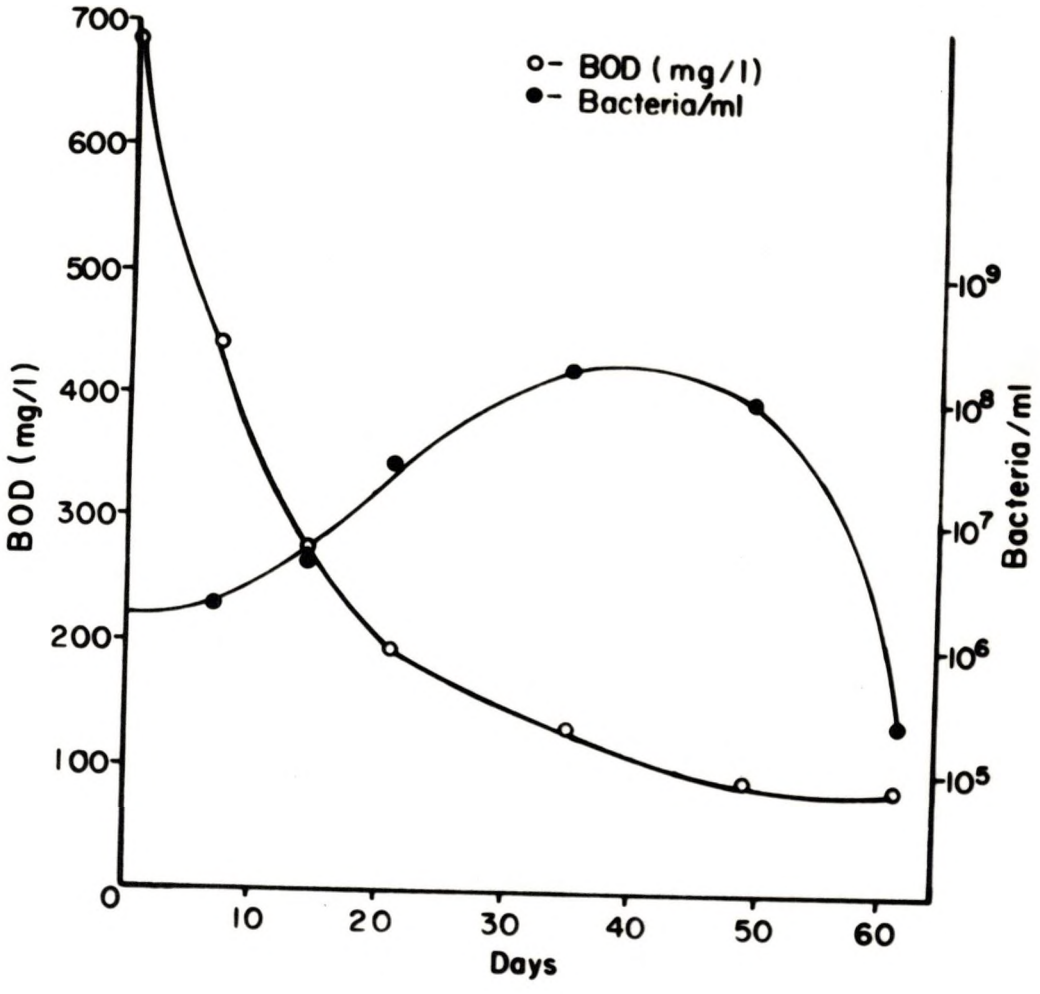
Eckenfelder (1966), in designing a model for lagoon changes observed, in laboratory experiments, that maximal cellular mass was reached con-

comitant with the onset of decreased rate of BOD removal. He attributed the rapid decrease of cellular mass, after exhaustion of substrate, to the conversion of stored carbohydrates to cellular protoplasm.

The kinetics of the Grafton lagoon may be likened to this model of Eckenfelder. A plot of BOD reduction and major microbial populations (purple sulfur plus total bacteria) yielded results comparable to that reported by Eckenfelder (Figure 8). This probably indicates that organic substrates were removed by purple sulfur bacteria and stored in the bacterial cellular protoplasm.

The presence of toxic substances in the lagoon could also be a factor in the slow rate of BOD removal of 1968. Volatile acids monitored in 1967 and 1968 varied materially between the two years. These acids, produced by the anaerobic breakdown of cellulose and carbohydrates by lagoon heterotrophs (McCabe and Eckenfelder, 1958), were a log higher in 1968 compared to 1967 values, probably because of the higher BOD and organic substrate load of 1968. These levels, in the range of 500 mg/l were not toxic to purple sulfur bacteria (Truper, 1968). Since methane bacteria, in the absence of sulfide, can utilize volatile acids in high concentrations (4,000 mg/l, Rudolfs and Amberg, 1952; 2,000 mg/l, Keefer and Urtes, 1962), the volatile acid levels did not inhibit these organisms. It is also unlikely that the populations of bacteria which produced the volatile acids were inhibited by these metabolic products since Imhoff tanks routinely contain high levels of volatile acids (2,000 mg/l) without harmful

Figure 8. Plot of BOD removal versus combined major bacterial populations, 1967. Bacterial numbers were obtained by summing the purple sulfur bacteria and the total bacteria (TGE).


o- BOD (mg/l)
●- Bacteria/ml
BOD (mg/l)
Bacteria/ml
Days
700
600
500
400
300
200
100
0
10
20
30
40
50
60
10^9
10^8
10^7
10^6
10^5

effects to the microbial population (McCabe and Eckenfelder, 1958). One must suspect that the observed decrease of volatile acids was primarily a result of purple sulfur metabolism, since the purple sulfur bacteria vastly outnumbered the methane organisms, and the pH was above the recorded optimum for methane bacteria. It was unlikely that other populations materially reduced volatile acids, since in Imhoff tanks volatile acids accumulate when methane bacteria are absent even though other populations remain (McCabe and Eckenfelder, 1958). Volatile acid levels may have also decreased by some other mechanism, such as evaoporation, but the fact that the greatest decrease occurred at pH 8.1 – 8.4 when stabile salts of the acids were present (Neisch, 1957), suggests that biological removal of these compounds was the prime reaction.

The disappearance of specific volatile acids is noteworthy (Table 4). Levels of propionic, butyric and acetic acid decreased dramatically during the purple phase. Only formic acid and small amounts of acetic acid remained by the end of the purple phase. The lack of utilization of formic acid by T. floridana and C. vinosum may be reflected in these lagoon results.

Since methane bacteria readily utilize formic acid as a carbon source, one would expect low levels of this acid if methane bacteria were active in volatile acid removal (McCabe and Eckenfelder, 1958). Hence, methane bacteria probably played only a minor role in volatile acid and BOD removal.

The presence of sulfide could decrease the lagoon efficiency. However, the detectable level of sulfide in 1968 was lower than most estimates of toxic levels. Reported values for inhibiting levels of sulfide vary, but complete inhibition of biological growth has been recorded for sulfide concentrations of 25 mg/l (Eckenfelder and O'Connor, 1961), and 50 mg/l (Parker and Skerry, 1968). Partial inhibition of metabolism probably occurs with lower levels of sulfides. Also, sulfide concentrations probably were much higher in the bottom sediments where methane bacteria normally thrive (Postgate, 1965).

Correlation Between Pure Culture Studies and the Lagoon Environment

The metabolic characteristics of certain strains of purple sulfur bacteria have been observed. Optimum pH, sulfide, and temperature values have been determined for Chromatium and Thiopedia species (May and Stahl, 1967), while Truper listed organic substrates utilized by several purple sulfur bacteria (1968). The relationships of these parameters to environmental situations, however, are undefined.

The temperature range in which T. floridana and C. vinosum exhibit growth (16 - 30°C) correlated well with summer lagoon temperatures (15 - 25°C).

The relatively high pH optimum (7.5 - 8.0) for the pure cultures of T. floridana and C. vinosum was consistent with the pH values in the lagoon when purple sulfur bacteria develop.

Lagoon sulfide levels never reached the optimal concentrations (45 - 60 mg/l) for the pure cultures of purple sulfur bacteria. However, both organisms grew in sulfide levels of 1 - 5 mg/l.

Utilization of organic substrates was confirmed with pure cultures of T. floridana and C. vinosum. Certain carbohydrates (glucose, fructose, lactose, maltose, and sucrose), volatile acids (valeric, propionic, hexanoic, and acetic acids), amino acids (histidine and methionine), and non-volatile acids (succinate, pyruvate, fumarate, malate, glycolate, and benzoate) were utilized by these organisms. However, only carbohydrate and volatile acid levels were monitored in the lagoon.

No correlation between total carbohydrate level and purple sulfur bacterial populations was found in 1967, while a decrease in carbohydrates was observed in the purple phase of 1968. The high population of purple sulfur bacteria in 1968 may be related to the reduced carbohydrate levels.

Volatile acids decreased rapidly as purple sulfur bacteria increased. Two volatile acids available in the lagoon, propionic and acetic acids, were utilized by T. floridana, the dominant organism in the lagoon, and were reduced to trace amounts by the end of the purple phase. Formic acid, which was not utilized by these organisms in the laboratory, remained in highest concentration in the lagoon at the end of the purple phase. The probability that lagoon purple sulfur bacteria utilized other volatile acids exists since Truper (1968) reported utilization of butyrate by purple sulfur bacteria.

Although organic substrates have long been known to be metabolized by purple sulfur bacteria, the relationship of the organic electron donor to the bacterial photosynthetic process is not completely understood.

Several investigators (Stanier et al., 1959; Gest, 1966; and Mahler and Cordes, 1966) believe that ATP is produced in purple sulfur bacteria by the process of cyclic phosphorylation. It is entirely possible that the conversion of organic substrates to carbon dioxide and hydrogen in the Krebs cycle is facilitated by this energy produced by cyclic phosphorylation. The ATP in some way effects the reoxidation of reduced pyridine nucleotide by liberation of hydrogen, thereby allowing the operation of the cycle in the absence of an external terminal electron acceptor (Gest, 1966).

Autotrophic growth by photosynthetic bacteria, however, requires a source of reducing power for the ultimate reduction of carbon dioxide to carbohydrate (Mahler and Cordes, 1966). In the classical studies of purple sulfur metabolism, reduced sulfur compounds, especially sulfide, were observed to fulfill this function. The mechanism of reduction of carbon dioxide was likened to that mechanism used by green plants, where a reductant and an oxidant are formed from a molecule of water, with oxygen liberated and hydrogen used in the reduction of carbon dioxide.

The mechanism of reduction of carbon dioxide by organic molecules is even less understood. Early work by van Niel (1931) suggested that the organic substrate probably was intimately involved in the electron transfer to carbon dioxide, and this work was substantiated by Foster (1940) who

isolated an organism which performed an incomplete photoxidation of isopropanol, yielding carbohydrate and acetone. However, new evidence suggests that organic substrates may also contribute reducing power by being oxidized in the common metabolic pathways (Truper, 1964; Gest, 1966). Hence it is entirely possible that the source of reducing power is a metabolic product of the provided organic substrates (Gest, 1966). It is important to note that not all strains of purple sulfur bacteria can utilize organic substrates in the absence of reduced sulfur compounds (Pfennig, 1967).

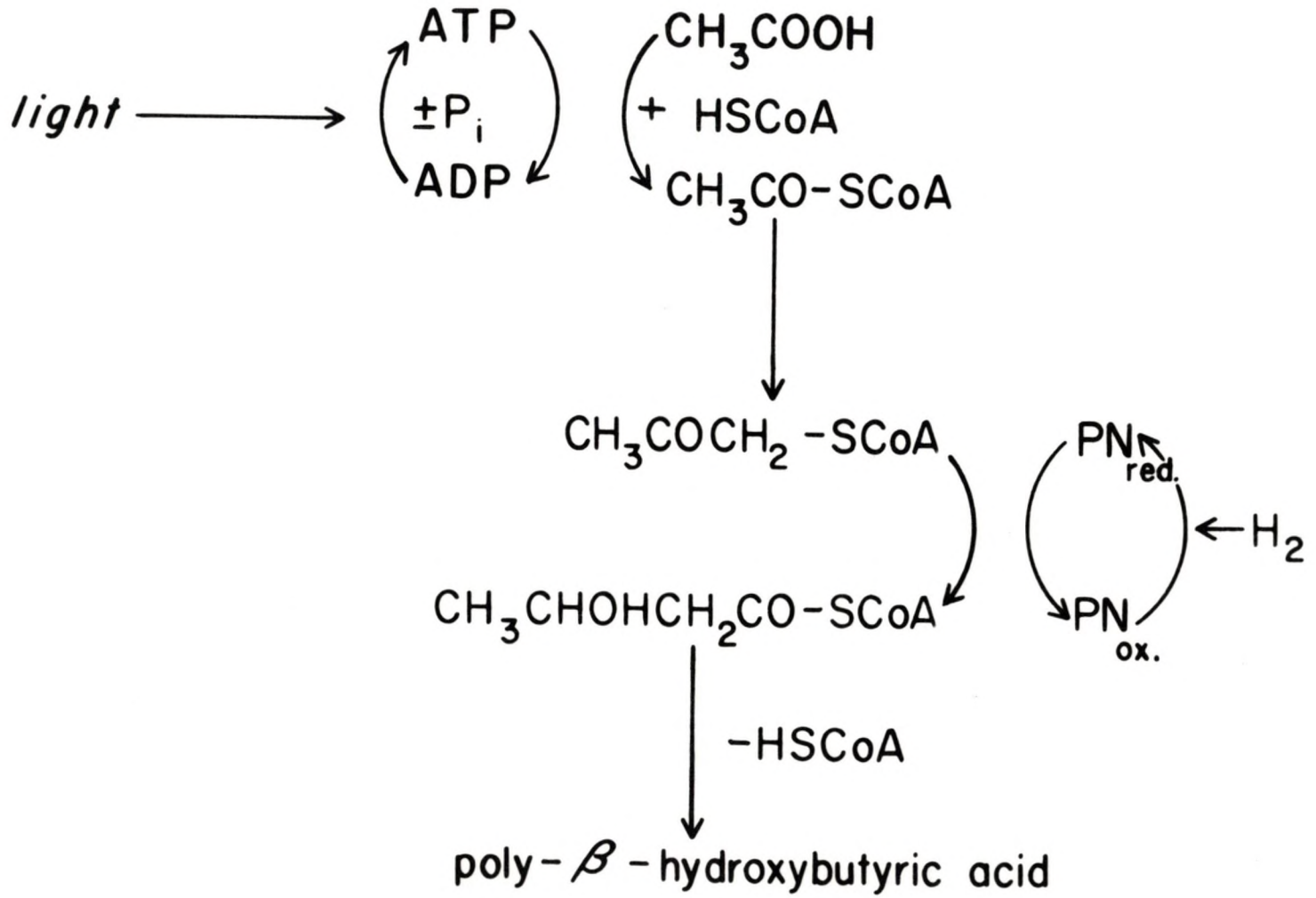
Purple sulfur bacteria apparently contain the Krebs cycle and the Calvin photosynthetic cycle (Truper, 1964; Fuller et al., 1961). However, certain organisms may contain incomplete cycles, since Fuller (1961) reported that a Chromatium species had no detectable malate dehydrogenase.

Stanier et al. (1959) investigated the nature of organic substrate utilization by the non-sulfur purple organism, Rhodospirillum rubrum. They noted that the main function of organic substrates in bacterial photosynthesis was to serve as readily assimilable sources of carbon. They also provide a source of reducing power for carbon dioxide, but the fixation of carbon dioxide, although qualitatively important for the growth of purple bacteria with organic substrates, is usually of minor quantitative importance as a source of cellular carbon. These conclusions are based on observations of labeled substrate studies (acetate, butyrate,

and succinate) with R. rubrum. Stanier observed two products of organic substrate utilization: poly-β-hydroxybutyric acid and a polysaccharide. Acetate and butyrate form large amounts of poly-β-hydroxybutyric acid and succinate yields the polysaccharide. It was observed that carbon dioxide is necessary for the utilization of stored poly-β-hydroxybutyric acid. It is believed that by the conversion of acetate to polyesters, the harmful fatty acids cannot accumulate and cause damage. This stored polymer may act as the stored reserve of reducing substances used for later carbon dioxide fixation. The process of storing organic polymers appears to be analagous to the storage of sulfur granules in purple sulfur bacteria. Stanier noted that in the photometabolism of acetate, organic substrates do not necessarily act as a photoreductant of carbon dioxide. The reductive synthesis may be coupled either with oxidation of part of the acetic acid, or as seen on Figure 9, with uptake of molecular hydrogen or some other reducing agent.

Carbohydrates have been reported utilizable by purple sulfur bacteria as electron donors (May and Stahl, 1967; Truper, 1968), and also as carbon sources (Fuller et al., 1968). Carbohydrates taken from the medium are probably utilized in the same manner as those synthesized for reserve energy (Stanier et al., 1959). It is presumed that electrons from the oxidation of sugars are used to reduce the diphosphopyridine nucleotide (DPN) in those sugars providing growth with bicarbonate (glucose,

Figure 9. Proposed mechanism of acetate utilization by Rhodospirillum rubrum (Stanier et al., 1961).


light
ATP
±P$_i$
ADP
CH$_3$COOH
+ HSCoA
CH$_3$CO-SCoA
CH$_3$COCH$_2$-SCoA
CH$_3$CHOHCH$_2$CO-SCoA
PN red.
PN ox.
H$_2$
-HSCoA
poly-β-hydroxybutyric acid

fructose, and lactose by Thiocapsa; glucose, fructose, and sucrose, by Chromatium). Apparently all sugars were utilized by Thiocapsa when sulfide was provided as an electron donor; while only lactose was utilized in this manner by Chromatium.

Since enzymes of the Krebs cycle are present in Chromatium (Truper, 1964), succinate, fumarate, pyruvate, and malate were tested for microbial utilization (Table 14). All of these compounds were readily utilized by both Chromatium and Thiocapsa, as evidenced by the profuse growth when provided with bicarbonate. Pyruvate and fumarate may act as both a carbon source and an electron donor with these organisms. The inability of these organisms to use succinate as the only carbon source is somewhat perplexing since several researchers report this function for the compound (Pfennig, 1967; Hurlbert and Lascalles, 1964). However, Truper (1968) reported that Ectothiorhodospira mobilis produces only slight growth when incubated with carbon dioxide and succinate, so our results are probably not without precedent.

Ormerod et al. (1961) reported that with certain amino acids, hydrogen is evolved during photosynthetic growth. The mechanism of utilization is not known, but Omerod concluded that hydrogen evolution is a product of photometabolism of accesory electron donors. In this study, only histidine, used as an electron donor and a carbon source by both organisms, and methionine, used as a carbon source by T. floridana,

proved to be of value as organic substrates. The observation that certain amino acids were toxic to purple sulfur bacteria agrees with the results of Petrova (1959).

Volatile acids were investigated as possible substrates for purple sulfur bacteria. The usable acids, used by T. floridana, were functional only as electron donors. Propionate probably was combined with carbon dioxide by the organism to form succinate. Acetic acid may have entered the glyoxylate cycle (Fuller et al., 1961), or formed poly-β-hydroxy-butyric acid (Stanier et al., 1959). Although many purple sulfur bacteria utilize acetate as the sole carbon source (Pfennig, 1967; Fuller et al., 1961) these organisms, like those studied by May and Stahl (1967) and Hurlbert and Lascelles (1963) could not grow on acetate alone. Hexanoic acid may undergo β-oxidation before entering the Krebs cycle.

Although the previously discussed experiments indicated specific substrate utilization by the purple sulfur bacteria, the efficiency of these organisms as BOD removers was still not certain. However, Table 15 distinctly shows that purple sulfur bacteria materially lowered BOD values. Volatile acids and carbohydrates were among organic substrates utilized. The sulfide dropped to undetectable levels which is analagous to lagoon situations when purple sulfur bacteria are present.

SUMMARY

The observation of "blooms" of purple sulfur bacteria in the primary cell of a local sewage lagoon prompted an ecologic study of the lagoon during 1966, 1967, and 1968. Four facets of the problem were investigated: Firstly, several chemical parameters (BOD, total carbohydrates, total volatile acids, individual volatile acids, pyruvate, sulfide, sulfate, phosphate, pH, and alkalinity) were monitored weekly. Secondly, a physical factor, water temperatures, was recorded and related to biological activity of the lagoon. Thirdly, microbial populations (total bacteria, algae, methane formers, sulfate reducers, coliforms, fecal coliforms, enterococci, and purple sulfur bacteria) were noted and probably ecologic relationships among the various organisms and the observed chemical and physical parameters of the lagoon were deduced. Finally, two genera of purple sulfur bacteria isolated from the lagoon were characterized metabolically. Optimum pH, temperature, and substrate levels (sulfide, carbohydrates, amino acids, volatile acids, and non-volatile acids) were observed and correlated with certain lagoon parameters (pH, water temperatures, sulfides, carbohydrates, and volatile acids).

The following observations summarize this ecologic study:

1. Rates of BOD removal could not be linked directly to temperature differences. Several factors including BOD loading, toxic

substances, and BOD regeneration from bottom sediments probably dimished temperature affects.

2. Two populations, purple sulfur bacteria and total bacteria, reached maximal concentrations in the warmest part of the 1967 summer. During 1968 no correlation of temperature values and microbial growth was observed.

3. Purple sulfur bacteria reached maximal numbers while concentrations of sulfide and volatile acids were low. Formic acid, a volatile acid not utilizable by these strains of T. floridana and C. vinosum, remained as the major volatile acid constituent in August of 1968, while levels of acetic, butyric, and propionic acids were depleted. Slight decreases in carbohydrate and alkalinity values in 1968 may also be related to purple sulfur populations. Low sulfate levels during the purple phase are attributable to storage of sulfur within the purple sulfur bacteria.

4. Populations of methane bacteria were low during the portion of the summer when optimal volatile acid levels were present. It is likely that the removal of sulfide by purple sulfur bacteria aided the development of these organisms in August of 1968.

5. Coliforms, fecal coliforms, and enterococci do not find the lagoon an ideal habitat even when high organic loads are present. No biological, chemical, or physical agent was linked to the removal of these organisms from the lagoon.

6. Algal populations were stabile during the anaerobic and purple phases of the lagoon. Algal increases during the latter parts of summers 1966 and 1967 may be related to the low organic content of the lagoon during that period.

7. Sulfate reducing bacteria contribute sulfide, which inhibits the growth of methane bacteria while enhancing the growth of purple sulfur bacteria. The numbers of sulfate reducing bacteria were not limited by depletion of sulfate.

8. Sulfide concentrations of 45 - 60 mg/l, pH values of 7.5 - 8.0, and incubation temperatures of 25 - 30°C are optimal for maximal growth of T. floridana and C. vinosum. Although lagoon pH (7.7 - 8.2) was favorable for purple sulfur growth, temperature and sulfides were always limiting in the lagoon for these organisms. However, these organisms can grow at temperatures as low as 16°C and in sulfide concentrations of 1 - 5 mg/l.

9. C. vinosum utilized succinate, pyruvate, fumarate, malate, glycolic acid, hexanoic acid, histidine, glucose, fructose, lactose, and sucrose, while T. floridana, in addition to the substrates metabolized by C. vinosum, used methionine, benzoic acid, maltose, valerate, propionate, and acetate. Carbohydrates and volatile acids, both monitored in the lagoon, decreased markedly during the emergence of purple sulfur bacteria.

10. Purple sulfur bacteria materially lowered BOD levels, as demonstrated by the growth of T. floridana in sterilized sewage.

11. An absence of a direct correlation between BOD removal and a specific physical, biological, or chemical parameter in the lagoon was evident in this study. However, such results would not be unexpected in an ecologic situation. Since the lagoon contained a multitude of organisms which had varied metabolic capabilities, organic substrates probably were removed by different populations during the summer. Also, since BOD represents a wide variety of oxidizable organic substrates, the removal of a single group of compounds, such as volatile acids, probably would not be evident in the overall rate of BOD reduction.

APPENDIX I.

The following tables contain data used to plot the graphs in Figures 1 - 5. Additional experimental details are found in the legends accompanying the figures and in the text.

Also found in this section are data incorporated into Tables 1 - 15 and the statistical analysis of the BOD removal curves.

TABLE 16

REFERENCE CURVE FOR PYRUVATE

µg Pyruvate	Optical Density (340 mµ)
0	0
12.6	0.02
50.4	0.04
126	0.16
252	0.29
378	0.41

TABLE 17

REFERENCE CURVE FOR CARBOHYDRATE

µg Glucose	Optical Density (620 mµ)
0	0
5	0.02
50	0.17
100	0.38
200	0.70

TABLE 18

REFERENCE CURVE FOR INORGANIC ORTHOPHOSPHATE ASSAY

µg Phosphate	Optical Density (690 mµ)
0	0
12.5	0.08
25.0	0.16
37.5	0.23
50.0	0.30
75.0	0.46
100.0	0.55

TABLE 19

MICROBIAL POPULATIONS[a] IN THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1966

Date	Phase	Bacteria/ml				Algae/ml
		Purple Sulfur	Total	Coliform	Enterococci	
13May66	Anaerobic	-	2.0×10^6	5.7×10^2	4.6×10^2	-
17May66		-	9.5×10^5	4.4×10^2	6.7×10^3	-
1Jun66		-	6.5×10^5	5.0×10^1	3.0×10^2	-
15Jun66		-	4.9×10^6	2.3×10^2	1.1×10^2	-
22Jun66		-	4.7×10^7	1.6×10^3	2.7×10^2	-
6Jul66	Purple	1.0×10^4	4.0×10^6	2.9×10^2	4.3×10^1	2×10^4
12Jul66		2.0×10^7	5.3×10^6	2.7×10^3	2.1×10^3	5×10^4
20Jul66		3.3×10^4	9.1×10^6	4.3×10^1	1.4×10^1	3×10^4

-- continued --

TABLE 19 - continued

Date	Phase	Bacteria/ml				Algae/ml
		Purple Sulfur	Total	Coliform	Enterococci	
27Jul66	Algae	-	1.0×10^6	4.0×10^2	8.7×10^1	3.2×10^5
3Aug66		-	7.0×10^5	1.6×10^2	3.9×10^2	-

[a]Purple sulfur bacteria and algae were estimated by direct counts; total bacteria were estimated by TGE agar pour plates; coliforms were determined in Endo broth; and enterococci were estimated on Enterococcus agar.

TABLE 20

ESTIMATES OF PURPLE SULFUR BACTERIA IN THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1967, 1968

1967			1968		
Date	Direct Counts	Agar Shakes	Date	Direct Counts	Agar Shakes
18May	2×10^6	-	9May	-	-
24May	5×10^5	2×10^2	15May	3×10^5	-
31May	3×10^5	-	21May	4×10^5	-
7Jun	8×10^5	8×10^4	29May	8×10^6	-
14Jun	1×10^6	6×10^4	5Jun	7×10^5	-
21Jun	6×10^6	2×10^6	12Jun	3×10^6	4×10^4
28Jun	4×10^7	3×10^6	19Jun	2×10^6	9×10^4
6Jul	2×10^8	1×10^7	26Jun	3×10^7	4×10^5
12Jul	2×10^8	1×10^7	3Jul	1×10^7	7×10^5
19Jul	2×10^8	7×10^6	10Jul	3×10^7	1×10^5
26Jul	8×10^7	9×10^5	17Jul	3×10^7	9×10^5
2Aug	5×10^6	-	24Jul	3×10^8	5×10^7
9Aug	9×10^5	-	31Jul	3×10^9	6×10^9
16Aug	2×10^5	-	7Aug	3×10^9	2×10^8

-- continued --

TABLE 20 - continued

1967			1968		
Date	Direct Counts	Agar Shakes	Date	Direct Counts	Agar Shakes
			14Aug	4×10^9	6×10^8
			21Aug	3×10^9	2×10^8
			28Aug	5×10^9	6×10^8
			4Sep	4×10^9	-
			11Sep	1×10^8	-

TABLE 21

POPULATIONS OF ENTERIC[a] ORGANISMS IN THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1967

Date	Phase	Bacteria/ml		
		Coliform	Fecal Coliform	Enterococci
18May67	Anaerobic	2.1×10^4	1.5×10^3	1.2×10^3
24May67		2×10^3	2.9×10^2	1.1×10^3
31May67		6.0×10^2	3.2×10^2	1.1×10^2
7Jun67		4.4×10^4	1.7×10^2	9.5×10^1
14Jun67		1.5×10^3	6.7×10^2	2.8×10^2
21Jun67		1.8×10^3	1.2×10^3	2.6×10^2
28Jun67	Purple	2.2×10^3	1.5×10^3	2.0×10^2
6Jul67		2.3×10^3	2.4×10^3	3.0×10^2
12Jul67		6.2×10^3	3.6×10^3	4.0×10^2
19Jul67		3.5×10^3	9.1×10^2	9.0×10^1
26Jul67		3.4×10^3	1.2×10^4	9.2×10^1
2Aug67	Algae	7.0×10^3	4.9×10^3	1.0×10^2
9Aug67		2.2×10^3	8.4×10^2	6.7×10^1
16Aug67		4.4×10^3	1.7×10^3	1.0×10^1

[a]The Millipore technique was used for enumeration of organisms (coliforms, Endo broth; fecal coliforms, mFC broth; and enterococci, Enterococcus agar).

TABLE 22

POPULATIONS OF ENTERIC[a] ORGANISMS IN THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1968

Date	Phase	Bacteria/ml		
		Coliform	Fecal coliform	Enterococci
9May68	Anaerobic	1.3×10^4	3.4×10^3	7×10^3
15May68		7.8×10^3	1.9×10^3	1.6×10^3
21May68		8×10^3	5.1×10^3	1.5×10^3
29May68		1.3×10^4	3.8×10^3	3.0×10^2
5Jun68		1.2×10^4	3.0×10^3	2.1×10^2
12Jun68		8.3×10^3	3.8×10^3	3.0×10^2
19Jun68		3.3×10^3	6.7×10^2	1.9×10^2
26Jun68		2.4×10^4	1.1×10^4	4.8×10^2
3Jul68		8.4×10^3	1.4×10^3	2.7×10^2
10Jul68		1.2×10^4	1.8×10^3	3.1×10^2
17Jul68		8.4×10^3	1.7×10^3	3.2×10^2

-- continued --

TABLE 22 - continued

Date	Phase	Bacteria/ml		
		Coliform	Fecal coliform	Enterococci
24Jul68	Purple	8.3×10^3	3.1×10^3	2.8×10^2
31Jul68		8.9×10^3	2.5×10^3	2.0×10^3
7Aug68		1.1×10^4	4.3×10^3	3.4×10^2
14Aug68		9.8×10^3	3.1×10^3	3.0×10^2
21Aug68		4.9×10^3	3.1×10^3	3.0×10^2
28Aug68		7.1×10^3	1.4×10^3	1.8×10^2
4Sep68		1.1×10^5	9.3×10^3	-
11Sep68		5.8×10^4	2.0×10^4	3.4×10^4

[a]The Millipore technique was used for enumeration of organisms (coliforms, Endo broth; fecal coliforms, mFC broth; and enterococci, Enterococcus agar).

TABLE 23

ESTIMATES OF MIXED BACTERIAL POPULATION OF THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1967

Date	Phase	Bacteria/ml		
		mTGE[a]	TGE[b]	Direct Counts[c]
18May67	Anaerobic	5.5×10^4	4.3×10^6	-
24May67		5.5×10^5	1.9×10^6	-
31May67		3.8×10^3	1.1×10^6	-
7Jun67		8.6×10^3	1.3×10^6	-
14Jun67		1.0×10^4	1.8×10^6	-
21Jun67		8.9×10^3	8.2×10^5	4.3×10^7
28Jun67	Purple	3.5×10^5	6.0×10^6	6.2×10^7
6Jul67		5.0×10^4	1.6×10^7	1.3×10^8
12Jul67		6.6×10^3	1.8×10^7	1.2×10^8
19Jul67		1.5×10^4	5.0×10^7	1.4×10^8
26Jul67		8.7×10^3	2.1×10^7	2.8×10^7

-- continued --

TABLE 23 - continued

Date	Phase	Bacteria/ml		
		mTGE[a]	TGE[b]	Direct Counts[c]
2Aug67	Algae	5.0×10^3	1.5×10^7	1.4×10^7
9Aug67		3.1×10^4	5.8×10^5	3.1×10^7
16Aug67		4.3×10^3	2.0×10^5	4.3×10^6

[a]Bacterial populations were estimated with the Millipore technique using Tryptone Glucose Extract broth incubated for 24 hours at 35°C.

[b]Bacterial populations were determined with Tryptone Glucose Extract agar pour plates incubated for 48 hours at 30°C.

[c]Direct counts were completed on crystal violet-stained preparations.

TABLE 24

ESTIMATES OF MIXED BACTERIAL POPULATIONS IN THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1968

Date	Phase	Bacteria/ml	
		mTGE[a]	TGE[b]
9May68	Anaerobic	7.7×10^8	5.5×10^6
15May68		2.2×10^5	1.8×10^6
21May68		1.3×10^5	2.7×10^6
29May68		7.4×10^4	2.1×10^6
5Jun68		1.5×10^4	1.0×10^6
12Jun68		2.1×10^4	9.1×10^5
19Jun68		1.2×10^4	3.7×10^6
26Jun68		9.5×10^4	9.9×10^5
3Jul68		1.9×10^4	1.6×10^6
10Jul68		2.0×10^4	3.2×10^6
17Jul68		1.8×10^4	2.1×10^5

-- continued --

TABLE 24 - continued

Date	Phase	Bacteria/ml	
		mTGE[a]	TGE[b]
24Jul68	Purple	4.2×10^4	6.1×10^6
31Jul68		1.9×10^4	3.2×10^6
7Aug68		2.1×10^4	4.3×10^5
14Aug68		1.1×10^4	1.7×10^6
21Aug68		3.1×10^4	1.0×10^5
28Aug68		2.0×10^4	2.2×10^5
4Sep68		3.2×10^5	1.1×10^6
11Sep68		4.8×10^4	1.1×10^6

[a]Bacterial populations were estimated with the Millipore technique using Tryptone Glucose Extract broth.

[b]Bacterial populations were determined with Tryptone Glucose Extract agar pour plates.

TABLE 25

POPULATIONS OF SULFATE REDUCERS AND ALGAE - 1967

	Phase	Sulfate Reducers/ml[a]		Algae/ml
		Water	Mud	
18May67	Anaerobic	10^1	10^3	1.0×10^5
24May67		10^2	10^3	6.3×10^4
31May67		10^3	10^4	5.1×10^4
7Jun67		10^3	10^4	9.0×10^4
14Jun67		10^2	10^4	8.3×10^4
21Jun67		10^4	10^5	5.4×10^5
28Jun67	Purple	10^5	10^5	7.5×10^4
6Jul67		10^4	10^5	1.9×10^5
12Jul67		10^4	10^6	6.2×10^4
19Jul67		10^2	10^5	5.0×10^5
26Jul67		10^2	10^5	2.3×10^5
2Aug67	Algae	10^3	10^4	4.0×10^5
9Aug67		10^4	10^5	1.0×10^6
16Aug67		10^2	10^4	3.4×10^5

[a]Sulfate reducers were counted in agar deeps (Postgate's medum).

[b]Algae were estimated by direct counts.

TABLE 26

POPULATIONS OF SULFATE REDUCERS AND ALGAE IN THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1968

Date	Phase	Sulfate Reducers/ml[a]		Algae/ml[b]
		Water	Mud	
9May68	Anaerobic	1.2×10^6	4.3×10^7	4.3×10^4
15May68		2.3×10^6	1.6×10^7	5.5×10^4
21May68		2.3×10^6	3.1×10^6	7.0×10^4
29May68		2.4×10^6	5.4×10^6	4.5×10^4
5Jun68		5.4×10^6	1.6×10^7	4.3×10^4
12Jun68		3.5×10^6	9.2×10^6	4.0×10^4
19Jun68		2.4×10^6	1.6×10^7	2.5×10^4
26Jun68		3.5×10^6	3.5×10^6	2.6×10^4
3Jul68		1.3×10^6	2.4×10^6	6.0×10^4
10Jul68		3.3×10^4	5.0×10^4	6.3×10^4
17Jul68		4.9×10^3	2.0×10^2	6.5×10^4

-- continued --

TABLE 26 - continued

Date	Phase	Sulfate Reducers/ml[a]		Algae/ml[b]
		Water	Mud	
24Jul68	Purple	2.4×10^4	7.9×10^3	8.5×10^4
31Jul68		3.5×10^4	5.4×10^4	6.5×10^4
7Aug68		4.9×10^4	5.4×10^5	6.0×10^4
14Aug68		1.4×10^4	4.9×10^4	2.0×10^4
21Aug68		3.5×10^4	3.5×10^5	3.5×10^4
28Aug68		2.4×10^4	3.5×10^4	4.3×10^4
4Sep68		1.6×10^5	5.4×10^5	5.3×10^4
11Sep68		2.1×10^5	3.2×10^5	2.3×10^4

[a]MPN values (95% confidence levels) were obtained by using Postgate's liquid medium.

[b]Algae were estimated by direct counts.

TABLE 27

POPULATION ESTIMATES OF METHANE BACTERIA - 1967, 1968

1967		1968	
Date	Bacteria/ml	Date	Bacteria/ml
18May67	-	9May68	-
24May67	-	15May68	-
31May67	-	21May68	-
7Jun67	-	29May68	4.9×10^4
14Jun67	-	5Jun68	6.3×10^4
21Jun67	4.3×10^5	19Jun68	1.2×10^4
28Jun67	5.0×10^5	26Jun68	7.4×10^4
6Jul67	6.2×10^5	3Jul68	9.3×10^3
12Jul67	3.4×10^6	10Jul68	1.6×10^4
19Jul67	7.8×10^6	17Jul68	2.6×10^3
26Jul67	6.7×10^6	24Jul68	1.6×10^4

-- continued --

TABLE 27 - continued

1967		1968	
Date	Bacteria/ml	Date	Bacteria/ml
2Aug67	9.1×10^5	31Jul68	1.2×10^4
9Aug67	4.3×10^5	7Aug68	8.9×10^4
16Aug67	6.3×10^5	14Aug68	2.0×10^5
		21Aug68	3.4×10^5
		28Aug68	4.3×10^5
		4Sep68	5.6×10^6
		11Sep68	6.3×10^5

[a]Methane bacteria were estimated with Hungate's medium.

TABLE 28

BOD_5, SULFIDE, pH AND WATER TEMPERATURE VALUES OF THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1966

Date	Phase	pH	BOD_5 mg/l	Sulfide mg/l	Temp. °C.
13May66	Anaerobic	7.2	714	3.18	8
17May66		7.3	765	0.81	8
1Jun66		7.7	865	0.70	18
15Jun66		8.1	671	0.72	17
22Jun66		8.1	325	8.50	24
6Jul66	Purple	8.2	217	0.23	21
12Jul66		8.1	125	0.08	27
20Jul66		8.2	77	0.17	22
27Jul66	Algae	8.8	57	0.08	21
3Aug66		8.6	66	-	-

TABLE 29

BOD_5, SULFIDE, pH, AND WATER TEMPERATURE VALUES OF THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1967

Date	Phase	Water Temperatures °C	pH	BOD_5 mg/l	Sulfide mg/l
18May67	Anaerobic	-	7.0	1,640	1.04
24May67		-		748	7.42
31May67		16	7.4	783	10.20
7Jun67		16	7.9	688	10.60
14Jun67		19	8.3	445	14.80
21Jun67		16	8.3	275	5.30
28Jun67	Purple	22	8.3	195	3.82
6Jul67		19	8.1	98	trace
12Jul67		17	8.0	133	trace
19Jul67		24	8.1	83	0.64
26Jul67		22	8.3	97	0.10
2Aug67	Algae	21	8.3	93	0.21
9Aug67		18	8.9	88	trace
16Aug67		23	8.4	32	trace

TABLE 30

BOD_5, SULFIDE, pH, AND WATER TEMPERATURE VALUES OF THE PRIMARY CELL OF THE GRAFTON MUNICIPAL LAGOON - 1968

Date	Phase	pH	Water Temperatures °C	BOD_5 mg/l	Sulfide mg/l
May68	Anaerobic	7.1	-	1,470	2.1
15May68		7.1	15	1,490	10.5
21May68		7.2	15	1,355	10.0
29May68		7.1	14	1,380	7.2
5Jun68		7.4	19	1,330	5.1
12Jun68		7.4	15	1,097	8.4
19Jun68		7.6	18	1,020	4.3
26Jun68		7.7	16	920	1.6
3Jul68		7.7	16	747	1.23
10Jul68		7.4	16	680	0.3
17Jul68		7.9	22	520	1.8

-- continued --

TABLE 30 - continued

Date	Phase	pH	Water Temperatures °C	BOD_5 mg/l	Sulfide mg/l
24Jul68		8.1	21	545	1.4
31Jul68		8.1	20	355	1.0
7Aug68		8.2	22	295	1.0
14Aug68	Purple	7.8	17	200	1.3
21Aug68		7.8	16	245	0.9
28Aug68		8.0	16	150	0.7
4Sep68		7.9	15	110	0.15
11Sep68		7.7	16	119	0

TABLE 31

QUANTITATIVE DETERMINATIONS OF SEVERAL CHEMICAL PARAMETERS OF THE GRAFTON PRIMARY CELL - 1967

Date	(mg/l) Sulfate	Carbohydrate	Phosphate	Alkalinity	Volatile Acids
8May67	539	13.5	22	450	54.4
18May67	572	22	28	430	48.2
24May67	565	19	15	520	55.0
31May67	520	23	19	515	47.3
7Jun67	460.8	12	32	719	49.3
14Jun67	461	20	28	828	47.1
17Jun67	405	21	24	893	48.0
21Jun67	341.5	20	18	1,330	54.9
28Jun67	304.5	14	18	925	51.3
1Jul67	370.4	17	16	1,065	83.1
6Jul67	448.5	19	24	910	47.3
8Jul67	316.9	15	18	735	29.3
12Jul67	432.1	18	12	805	23.4
15Jul67	308.6	14	16	875	18.9
19Jul67	625.5	22	18	920	15.4
22Jul67	522.6	21	22	860	9.5

-- continued --

TABLE 31 - continued

Date	(mg/l) Sulfate	Carbohydrate	Phosphate	Alkalinity	Volatile Acids
26Jul67	670.7	36	38	985	7.1
29Jul67	1,222.2	43	40	1,040	9.5
2Aug67	1,130	21	23	1.120	12.3
6Aug67	1,000	22	13	1,095	23.6
9Aug67	621	35	14	980	33.1
12Aug67	1,234.5	36	21	1,450	26
16Aug67	810.7	33	20	940	28

TABLE 32

QUANTITATIVE DETERMINATIONS OF SEVERAL CHEMICAL PARAMETERS OF THE GRAFTON PRIMARY CELL - 1968

Date	(mg/l)				
	Sulfate	Carbohydrate	Phosphate	Alkalinity	Volatile Acids
9May68	249	21	32	940	1,043
15May68	315	20	35	890	1,029
21May68	121	16	28	960	957
29May68	340	29	22	980	295
5Jun68	521	32	26	950	150
12Jun68	560	20	25	950	295
19Jun68	738	24	33	1,000	934
26Jun68	580	20	21	1,020	730
3Jul68	465	16	24	1,040	768
10Jul68	246	21	25	960	697
17Jul68	185	24	17	970	440
24Jul68	80	15	23	910	484
31Jul68	93	34	16	850	130

-- continued --

TABLE 32 - continued

Date	(mg/l)				
	Sulfate	Carbohydrate	Phosphate	Alkalinity	Volatile Acids
7Aug68	81	10	20	810	60
14Aug68	98	9	18	810	59
21Aug68	142	4	16	720	130
28Aug68	287	6	12	690	123
4Sep68	320	6	15	640	26
11Sep68	676	5	17	650	15

TABLE 33

VOLATILE ACID LEVELS[a] IN THE GRAFTON PRIMARY LAGOON - 1968

Date	Formate		Acetate		Propionate		Butyrate		Others
	%	mg/l	%	mg/l	%	mg/l	%	mg/l	
29May	4.6	13.6	76.3	225	1.5	4.4	17.6	51.9	trace
5Jun	31.7	47.5	45	67.5	–	–	23.3	34.9	–
12Jun	11.9	35.1	85	250.8	2.0	5.9	1.6	4.7	trace
19Jun	14.1	131.7	82.7	772	1.4	13.1	1.7	15.9	trace
26Jun	15.9	116.1	81.6	595	0.8	5.8	1.6	11.7	trace
3Jul	14.6	112	77.1	592	–	–	8.3	63.7	trace
10Jul	25.6	178.4	74.4	571.4	–	–	–		
17Jul	31.3	137.7	68.7	302.3	–	–	–		
24Jul	55.7	270.6	44.3	214.4	–	–	–		
31Jul	84.6	109.9	15.4	20	–	–	–		
7Aug	93.5	56	65	3.9	–	–	–		
14Aug	100	59	–	–	–	–	–		
21Aug	100	130	–	–	–	–	–		
28Aug	87.3	107	6.3	7.7	–	–	–		
4Sep	93.9	24	6.1	1.4	–	–	–		
11Sep	72.2	10.9	27.8	4.2	–	–	7.2	8.8	

[a]Individual volatile acids were quantitated by gas chromatography. Percentages of the total volatile acid levels were obtained by calculating the areas under the curves.

TABLE 34

TOTAL BACTERIA, pH, AND BOD VALUES FROM GRAFTPM RAW SEWAGE - 1966, 1967, 1968

Date	pH	BOD (mg/l)	Bacteria/ml	
			TGE[a]	mTGE[b]
13May66	6.6	2,009	1.2×10^8	-
26Jul66	7.4	193	1.0×10^7	-
25Aug66	7.2	152	2.5×10^7	-
18May67	6.4	1,640	1.8×10^8	9.8×10^6
31May67	7.7	110	3.0×10^7	2.5×10^5
7Jun67	7.6	130	2.7×10^7	1.6×10^6
21Jun67	7.8	112	2.1×10^7	7.1×10^5
6Jul67	6.8	1,860	1.8×10^7	1.5×10^6
22Jul67	-	264	-	-
2Aug67	7.6	133	1.5×10^7	5.3×10^4
16Aug67	7.5	92	2.0×10^7	1.2×10^5

-- continued --

TABLE 34 - continued

Date	pH	BOD (mg/l)	Bacteria/ml TGE[a]	Bacteria/ml mTGE[b]
9May68	7.1	420	6.8×10^7	6.9×10^6
21May68	7.3	190	3.2×10^7	4.6×10^6
5Jun68	7.6	175	3.3×10^7	1.2×10^6
19Jun68	7.6	205	3.7×10^7	8.1×10^6
3Jul68	7.6	150	2.3×10^7	1.5×10^6
17Jul68	7.6	85	2.0×10^7	1.8×10^6

[a]Total bacteria were estimated in Tryptone Glucose Extract Agar pour plates.

[b]Bacterial populations were estimated with the Millipore technique using Tryptone Glucose Extract broth.

TABLE 35

ESTIMATES OF ENTERIC ORGANISMS FROM GRAFTON RAW SEWAGE USING THE MILLIPORE TECHNIQUE - 1967, 1968

Date	Bacteria/ml		
	Coliform[a]	Fecal Coliform[b]	Enterocicci[c]
15May67	4.7×10^6	1.2×10^5	7.0×10^4
31May67	6.7×10^4	2.0×10^4	5.0×10^4
7Jun67	2.4×10^5	1.2×10^5	7.0×10^3
21Jun67	9.0×10^5	8.9×10^4	6.4×10^3
6Jul67	4.3×10^5	3.7×10^5	5.3×10^4
2Aug67	5.6×10^5	1.2×10^4	1.4×10^3
16Aug67	1.2×10^5	2.3×10^4	2.1×10^3
9May68	3.9×10^6	3.0×10^5	1.5×10^5
21May68	2.6×10^6	2.9×10^5	8.0×10^3
5Jun68	1.7×10^6	3.1×10^5	9.0×10^3
19Jun68	1.1×10^6	2.8×10^5	7.1×10^3

TABLE 36

CONTROLS FOR THE SUBSTRATE UTILIZATION STUDY - GROWTH OF *CHROMATIUM VINOSUM* AND *THIOCAPSA FLORIDANA* IN PFENNIG'S BASIC MEDIUM WITH ADDITIONS OF BICARBONATE AND SULFIDE

Organism	Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml
C. vinosum	-	0.01	2×10^6
C. vinosum	$NaHCO_3$	0.02	3×10^6
C. vinosum	$Na_2S \cdot 9H_2O$	0.02	2×10^6
C. vinosum	$NaHCO_3 + Na_2S \cdot 9H_2O$	0.24	8×10^8
T. floridana	-	0.01	9×10^5
T. floridana	$NaHCO_3$	0.03	2×10^6
T. floridana	$Na_2S \cdot 9H_2O$	0.02	2×10^6
T. floridana	$NaHCO_3 + Na_2S \cdot 9H_2O$	0.28	3×10^9

[a]Growth was estimated by direct counts and optical densities at 650 mu.

[b]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: bicarbonate, 0.1%; and sodium sulfide, 0.05%.

TABLE 37

UTILIZATION OF CARBOHYDRATES BY CHROMATIUM VINOSUM[a]

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Glucose	0.02	3×10^6	950
+$NaHCO_3$	0.16	5×10^7	470
+$Na_2S \cdot 9H_2O$	0.04	2×10^6	930
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.32	2×10^9	140
Fructose	0.03	5×10^6	900
+$NaHCO_3$	0.14	6×10^7	540
+$Na_2S \cdot 9H_2O$	0.04	7×10^6	890
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.25	2×10^8	130
Maltose	0.05	9×10^5	940
+$NaHCO_3$	0.05	4×10^6	910
+$Na_2S \cdot 9H_2O$	0.05	3×10^6	890
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.28	3×10^8	120
Lactose	0.05	7×10^6	900
+$NaHCO_3$	0.25	8×10^8	240
+$Na_2S \cdot 9H_2O$	0.05	1×10^8	270
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.19	7×10^7	180
Sucrose	0.05	8×10^5	910
+$NaHCO_3$	0.21	6×10^8	430
+$Na_2S \cdot 9H_2O$	0.03	6×10^6	910
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.33	2×10^9	210

[a]Growth was estimated by direct counts and optical densities at 650 mu; substrate utilization was determined with Dreywood's anthrone reagent.

[b]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: carbohydrates, 0.1%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

TABLE 38

UTILIZATION OF CARBOHYDRATES BY THIOCAPSA FLORIDANA[a]

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Glucose	0.02	4×10^6	830
$+NaHCO_3$	0.17	5×10^8	450
$+Na_2S \cdot 9H_2O$	0.11	1×10^8	540
$+NaHCO_3 + Na_2S \cdot 9H_2O$	0.26	6×10^9	240
Fructose	0.01	7×10^6	870
$+NaHCO_3$	0.19	6×10^8	440
$+Na_2S \cdot 9H_2O$	0.12	2×10^8	330
$+NaHCO_3 + Na_2S \cdot 9H_2O$	0.29	1×10^9	140
Maltose	0.05	3×10^6	910
$+NaHCO_3$	0.05	7×10^6	850
$+Na_2S \cdot 9H_2O$	0.08	4×10^7	350
$+NaHCO_3 + Na_2S \cdot 9H_2O$	0.26	1×10^9	150
Lactose	0.04	8×10^6	860
$+NaHCO_3$	0.47	3×10^8	330
$+Na_2S \cdot 9H_2O$	0.07	9×10^7	440
$+NaHCO3 + Na_2S \cdot 9H_2O$	0.53	6×10^{11}	130
Sucrose	0.02	2×10^6	930
$+NaHCO_3$	0.03	9×10^5	920
$+Na_2S \cdot 9H_2O$	0.18	1×10^8	560
$+NaHCO_3 + Na_2S \cdot 9H_2O$	0.33	8×10^9	340

[a]Growth was estimated by direct counts and optical densities at 650 mu; substrate utilization was determined with Dreywood's anthrone reagent.

[b]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: carbohydrates, 0.1%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

TABLE 39

UTILIZATION OF AMINO ACIDS BY CHROMATIUM VINOSUM[a]

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Glutamic Acid	0.03	6×10^6	–
+$NaHCO_3$	0.07	4×10^6	–
+$Na_2S \cdot 9H_2O$	0.04	1×10^6	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.14	1×10^8	780
Histidine	0.08	2×10^7	540
+$NaHCO_3$	0.09	7×10^7	560
+$Na_2S \cdot 9H_2O$	0.17	1×10^7	430
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.14	8×10^8	350
Methionine	0.07	5×10^5	–
+$NaHCO_3$	0.03	3×10^6	–
+$Na_2S \cdot 9H_2O$	0.04	2×10^6	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.02	3×10^6	–
Threonine	0.04	7×10^5	–
+$NaHCO_3$	0.05	2×10^6	–
+$Na_2S \cdot 9H_2O$	0.04	5×10^6	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.10	3×10^8	740
Aspartic Acid	0.07	2×10^6	–
+$NaHCO_3$	0.05	7×10^5	–
+$Na_2S \cdot 9H_2O$	0.03	9×10^5	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.10	2×10^8	710

[a]Growth was estimated by direct counts and optical densities at 650 mu; organic substrate utilization was determined by the dichromate method.

[b]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: amino acids, 0.1%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

TABLE 40

UTILIZATION OF AMINO ACIDS BY THIOCAPSA FLORIDANA[a]

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Glutamic Acid	0.03	3×10^6	–
+$NaHCO_3$	0.05	2×10^6	–
+$Na_2S \cdot 9H_2O$	0.02	5×10^6	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.08	7×10^7	790
Histidine	0.24	7×10^7	620
+$NaHCO_3$	0.33	2×10^8	490
+$Na_2S \cdot 9H_2O$	0.33	8×10^7	510
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.19	1×10^8	330
Methionine	0.06	6×10^6	750
+$NaHCO_3$	0.05	5×10^6	730
+$Na_2S \cdot 9H_2O$	0.06	9×10^7	370
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.17	3×10^8	350
Threonine	0.04	4×10^6	–
+$NaHCO_3$	0.02	2×10^6	–
+$Na_2S \cdot 9H_2O$	0.06	9×10^5	780
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.07	6×10^7	730
Aspartic Acid	0.02	4×10^5	–
+$NaHCO_3$	0.62	7×10^5	–
+$Na_2S \cdot 9H_2O$	0.03	6×10^5	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.02	6×10^6	–

[a]Growth was estimated by direct counts and optical densities at 650 mu; organic substrate utilization was determined by the dichromate method.

[b]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: amino acids, 0.1%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

TABLE 41

UTILIZATION OF VOLATILE ORGANIC ACIDS BY CHROMATIUM VINOSUM[a]

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Valeric Acid	0.02	8×10^5	-
+$NaHCO_3$	0.03	2×10^6	-
+$Na_2S \cdot 9H_2O$	0.04	1×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.34	3×10^9	330
Butyric Acid	0.04	7×10^5	-
+$NaHCO_3$	0.02	9×10^5	-
+$Na_2S \cdot 9H_2O$	0.05	3×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.30	1×10^9	350
Propionic Acid	0.03	3×10^6	-
+$NaHCO_3$	0.05	8×10^5	-
+$Na_2S \cdot 9H_2O$	0.03	4×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.15	4×10^7	410
Isobutyric Acid	0.06	6×10^5	-
+$NaHCO_3$	0.05	9×10^5	-
+$Na_2S \cdot 9H_2O$	0.05	3×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.22	2×10^8	420
Hexanoic Acid	0.04	1×10^6	-
+$NaHCO_3$	0.12	1×10^7	230
+$Na_2S \cdot 9H_2O$	0.06	4×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.14	4×10^8	200

-- continued --

TABLE 41 - continued

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Isovaleric Acid	0.02	7×10^5	-
+$NaHCO_3$	0.03	3×10^6	-
+$Na_2S \cdot 9H_2O$	0.03	1×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.26	2×10^8	320
Formic Acid	0.02	3×10^5	-
+$NaHCO_3$	0.03	7×10^5	-
+$Na_2S \cdot 9H_2O$	0.01	2×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.02	3×10^6	-
Acetic Acid	0.02	3×10^6	-
+$NaHCO_3$	0.02	2×10^6	-
+$Na_2S \cdot 9H_2O$	0.03	8×10^5	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.14	2×10^8	350

[a]Growth was estimated by direct counts and optical densities at 650 mu; substrate utilization was determined by the silicic acid method for volatile acids.

[b]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: volatile acids, 0.05%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

TABLE 42

UTILIZATION OF VOLATILE ORGANIC ACIDS BY THIOCAPSA FLORIDANA[a]

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Valeric Acid	0.02	1×10^6	-
+$NaHCO_3$	0.09	3×10^7	200
+$Na_2S \cdot 9H_2O$	0.05	3×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.20	1×10^8	250
Butyric Acid	0.03	5×10^5	-
+$NaHCO_3$	0.15	6×10^7	220
+$Na_2S \cdot 9H_2O$	0.06	3×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.15	1×10^8	240
Propionic Acid	0.05	3×10^6	-
+$NaHCO_3$	0.09	6×10^7	290
+$Na_2S \cdot 9H_2O$	0.03	3×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.08	4×10^7	400
Isobutyric Acid	0.02	5×10^5	-
+$NaHCO_3$	0.04	8×10^5	-
+$Na_2S \cdot 9H_2O$	0.02	3×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.17	6×10^7	270
Hexanoic Acid	0.05	6×10^6	-
+$NaHCO_3$	0.09	2×10^7	300
+$Na_2S \cdot 9H_2O$	0.02	2×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.12	1×10^8	240

-- continued --

TABLE 42 - continued

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Isovaleric Acid	0.05	4×10^6	-
+$NaHCO_3$	0.04	3×10^6	-
+$Na_2S \cdot 9H_2O$	0.05	5×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.05	5×10^7	350
Formic Acid	0.01	4×10^5	-
+$NaHCO_3$	0.02	5×10^5	-
+$Na_2S \cdot 9H_2O$	0.01	3×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.01	6×10^6	-
Acetic Acid	0.02	3×10^6	-
+$NaHCO_3$	0.07	4×10^7	220
+$Na_2S \cdot 9H_2O$	0.03	4×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.15	4×10^8	240

[a]Growth was estimated by direct counts and optical densities at 650 mu; substrate utilization was determined by the silicic acid method for volatile acids.

[b]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: volatile acids, 0.05%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

TABLE 43

UTILIZATION OF NON-VOLATILE ORGANIC ACIDS BY CHROMATIUM VINOSUM[a]

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Succinate	0.02	9×10^5	–
+$NaHCO_3$	0.12	2×10^7	380
+$Na_2S \cdot 9H_2O$	0.02	3×10^6	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.21	1×10^8	240
Pyruvate	0.21	7×10^8	150
+$NaHCO_3$	0.31	3×10^9	90
+$Na_2S \cdot 9H_2O$	0.24	6×10^7	130
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.20	5×10^8	110
Fumarate	0.08	4×10^7	670
+$NaHCO_3$	0.17	6×10^7	210
+$Na_2S \cdot 9H_2O$	0.24	1×10^9	310
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.18	6×10^7	290
Malate	0.02	7×10^6	–
+$NaHCO_3$	0.12	9×10^7	570
+$Na_2S \cdot 9H_2O$	0.04	5×10^6	–
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.13	4×10^8	410

-- continued --

TABLE 43 - continued

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Glycolic A	0.01	2×10^6	-
+$NaHCO_3$	0.11	8×10^7	710
+$Na_2S \cdot 9H_2O$	0.01	8×10^5	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.15	2×10^7	620
Benzoic Acid	0.01	6×10^5	-
+$NaHCO_3$	0.02	9×10^5	-
+$Na_2S \cdot 9H_2O$	0.09	3×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.08	2×10^8	720

[a]Growth was estimated by direct counts and optical densities at 650 mu; organic substrate utilization was determined by the dichromate method.

[b]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: non-volatile acids, 0.1%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

TABLE 44

UTILIZATION OF NON-VOLATILE ORGANIC ACIDS BY THIOCAPSA FLORIDANA[a]

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Succinate	0.02	1×10^6	-
+$NaHCO_3$	0.21	4×10^7	730
+$Na_2S \cdot 9H_2O$	0.04		-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.12	6×10^7	
Pyruvate	0.15	7×10^7	180
+$NaHCO_3$	0.33	4×10^8	210
+$Na_2S \cdot 9H_2O$	0.32	4×10^8	250
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.22	7×10^7	160
Fumarate	0.16	3×10^8	430
+$NaHCO_3$	0.20	4×10^8	210
+$Na_2S \cdot 9H_2O$	0.21	5×10^7	320
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.10	1×10^8	310
Malate	0.04	7×10^6	-
+$NaHCO_3$	0.16	8×10^7	480
+$Na_2S \cdot 9H_2O$	0.03	3×10^6	-
+$NaHCO_3$ + $Na_2S \cdot 9H_2O$	0.10	2×10^8	410

-- continued --

TABLE 44- continued -

Substrate[b] Additions	Absorbance 650 mu	Bacteria/ml	Final Organic Substrate Level (mg/l)
Glycolic Acid	0.02	-2×10^6	-
$+NaHCO_3$	0.11	5×10^7	630
$+Na_2S \cdot 9H_2O$	0.03	8×10^5	-
$+NaHCO_3 + Na_2S \cdot 9H_2O$	0.16	4×10^7	710
Benzoic Acid	0.01	9×10^5	-
$+NaHCO_3$	0.09	3×10^7	590
$+Na_2S \cdot 9H_2O$	0.03	5×10^6	-
$+NaHCO_3 + Na_2S \cdot 9H_2O$	0.13	3×10^8	630

[a]Growth was estimated by direct counts and optical densities at 650 mu; organic substrate utilization was determined by the dichromate method.

[b]All substrates were added to Pfennig's basic medium. Final concentrations of the additives were as follows: non-volatile acids, 0.1%; bicarbonate, 0.1%; and sodium sulfide, 0.05%.

TABLE 45

GROWTH OF *CHROMATIUM VINOSUM* AND *THIOCAPSA FLORIDANA* AT VARIOUS TEMPERATURES[a]

Temperature	Growth[b]	
	C. vinosum	*T. floridana*
5°C	8×10^5	2×10^6
16°C	9×10^7	1×10^8
25°C	3×10^9	9×10^8
30°C	4×10^9	3×10^9
37°C	3×10^6	4×10^6

[a]Final concentrations of 0.1% bicarbonate and 0.05% sodium sulfide were present in Pfennig's medium.

[b]Growth was estimated by direct counts. Densities similar to Pfennig's basic medium control at 25°C (10^6 bacteria/ml) are indicated by a minus sign; higher populations are indicated by plus signs (+, one log increase; ++, two log increase; +++, three log increase; and ++++, four log increase).

TABLE 46

GROWTH OF *CHROMATIUM VINOSUM* AND *THIOCAPSA FLORIDANA* AT VARIOUS pH LEVELS[a]

pH	Growth[b]	
	C. vinosum	*T. floridana*
6.1	2×10^6	7×10^6
6.3	1×10^6	9×10^6
6.6	3×10^6	8×10^6
6.8	7×10^6	2×10^7
7.1	7×10^6	9×10^7
7.5	2×10^8	2×10^9
7.8	1×10^9	1×10^9
8.2	4×10^8	3×10^9
8.5	5×10^7	3×10^7

[a]Final concentrations of 0.1% bicarbonate and 0.05% sodium sulfide were present in Pfennig's medium. The various pH levels were obtained by adding aliquots of 1N HCl and 1N NaOH.

[b]Growth was estimated by direct counts. Densities similar to Pfennig's basic medium control (pH 7.5c 10^6 bacteria/ml) are indicated by a minus sign; higher populations are indicated by plus signs (+, one log increase; ++, two log increase; +++, three log increase; and ++++, four log increase).

TABLE 47

GROWTH OF *CHROMATIUM VINOSUM* AND *THIOCAPSA FLORIDANA* AT VARIOUS SULFIDE LEVELS[a]

Sulfide (mg/l)	Growth[b]	
	C. vinosum	*T. floridana*
1.6	6×10^6	9×10^5
5.0	6×10^6	8×10^6
8.0	8×10^5	1×10^7
16.0	5×10^6	2×10^7
24.0	3×10^7	1×10^7
32	8×10^7	4×10^7
40	2×10^8	5×10^7
48	2×10^9	3×10^8
56	1×10^9	9×10^8
64	4×10^8	4×10^8
80	7×10^6	3×10^7
128	9×10^5	7×10^5

[a]Pfennig's medium was used with a final concentration of 0.1% bicarbonate. Varying aliquots of sterile 1.5% $Na_2S \cdot 9H_2O$ solution were added to the tubes.

[b]Growth was estimated by direct counts. Densities similar to Pfennig's basic medium control (10^6 bacteria/ml) are indicated by a minus sign; higher populations are indicated by plus signs (+, one log increase; ++, two log increase; +++, three log increase; and ++++, four log increase).

Calculations of BOD Removal Slopes (Goldstein, 1964).

$$b = \text{slope} = \frac{\Sigma xy - n\bar{x}\bar{y}}{\Sigma x^2 - n\bar{x}^2}$$

Year 1966

Date	x (Days)	y (BOD, mg/l)
1Jun	1	895
15Jun	15	671
22Jun	22	325
6Jul	36	217
12Jul	42	125

$\Sigma x = 116$ $\Sigma y = 2,233$

$\bar{x} = 23.2$ $\bar{y} = 446.6$

$\Sigma x^2 = 3,770$ $\Sigma y^2 = 1,419,605$

$\Sigma xy = 31,172$ $n = 5$

therefore $b = \frac{31,172 - (5)(23.2)(446.6)}{3,770 - 5(538)} = -19.1$

Year 1967

Date	x (Days)	y (BOD, mg/l)
31May	1	783
7Jun	8	688
14Jun	15	445
21Jun	22	275
28Jun	29	195
1Jul	32	158
6Jul	37	98
8Jul	39	133

$\Sigma x =$ 183 $\quad \Sigma y =$ 2,775

$\bar{x} =$ 22.8 $\quad \bar{y} =$ 346.9

$\Sigma x^2 =$ 5,529 $\quad \Sigma y^2 =$ 1,450,365

$\Sigma xy =$ 38,536 $\quad n =$ 8

therefore $b = \dfrac{38,536 - 8(22.8)(346.9)}{5,529 - 8(523.3)} = -18.6$

Year 1968

Date	x (Days)	y (BOD, mg/l)
29May	1	1,380
5Jun	8	1,330
12Jun	15	1,097
19Jun	22	1,020
26Jun	29	920
3Jul	35	747
10Jul	42	680
17Jul	49	520
24Jul	56	545
31Jul	63	355
7Aug	70	295
14Aug	77	200
21Aug	84	245
28Aug	91	150

$\Sigma x =$ 642 $\quad\quad$ $\Sigma y =$ 9,484

$\bar{x} =$ 45.86 $\quad\quad$ $\bar{y} =$ 677.4

$\Sigma x^2 =$ 40,276 $\quad\quad$ $\Sigma y^2 =$ 8,686,918

$\Sigma xy =$ 280,945 $\quad\quad$ $n =$ 14

therefore $b = \dfrac{280,945 - 642(677.4)}{40,276 - 14(2,103.1)} = \dfrac{153,945.8}{10,832.6} = -14.2$

Using

$$S^2_{yx} = \frac{1}{n-2} \left(\Sigma y^2 - \frac{(\Sigma y)^2}{n} - b\left[\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{n}\right]\right)$$

$$SS_x = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$$

$$S^2 = \frac{(n_1-2)S^2_{yx1} + (n_2-2)S^2_{yx2}}{(n_1-2) + (n_2-2)}$$

$$t = \frac{b_1 - b_2}{S\left(\frac{1}{SS_{x1}} + \frac{1}{SS_{x2}}\right)^{1/2}}$$

then, for 1966

$S^2_{yx} = 9,383$

$SS_x = 1,079$

for 1967

$S^2_{yx} = 4,087$

$SS_x = 1,343$

for 1968

$S^2_{yx} = 6,966$

$SS_x = 10,836$

thus $S^2_{66,67} = 5,852$

therefore $S = 76.5$

and $S^2_{66,68} = 7,449$

therefore $S = 86.31$

and $S^2_{67,68} = 6,006$

therefore $S = 77.5$

So, comparing 1966 and 1967

$t = .17$

Comparing 1966 and 1968

$t = 1.77$

Comparing 1967 and 1968

$t = 1.97$

Comparison of BOD Slopes During Different Times of Summer 1968

Period 1

Date	x (Days)	y (BOD, mg/l)
12Jun	1	1,097
19Jun	8	1,020
26Jun	15	920
3	22	747

$\Sigma x =$ 46 $\quad \Sigma y =$ 3,787

$\bar{x} =$ 11.5 $\quad \bar{y} =$ 946

$\Sigma x^2 =$ 747 $\quad \Sigma y^2 =$ 3,648,218

$\Sigma xy =$ 39,491 $\quad n =$ 4

$$b = \frac{3,941 - 11.5(4)(946)}{774 - 4(132.25)} = \frac{-4,025}{245} = -16.4$$

Period 2

Date	x (Days)	y (BOD, mg/l)
24Jul	43	545
31Jul	50	355
7Aug	57	295
14Aug	64	200

$\Sigma x = 214$ $\Sigma y = 1,395$

$\bar{x} = 53.5$ $\bar{y} = 348.75$

$\Sigma x^2 = 11,694$ $\Sigma y^2 = 550,075$

$\Sigma xy = 70,800$ $n = 4$

$$b = \frac{70,800 - 53.5(4)(348.75)}{11,694 - 4(2,862.25)} = \frac{-38,325}{245} = -15.6$$

Solving for S^2_{yx1}, S^2_{yx2}, SS_{x1}, SS_{x2}, and S^2 in the usual manner, we have

$S^2_{yx_1} = 1,214$

$S^2_{yx_2} = 1,809$

$SS_{x_1} = 245$

$SS_{x_2} = 245$

$S^2 = 1,512$

therefore $S = 38.88$

then

$$t = 0.224$$

Comparing these t values to those given in Goldstein (1964) for $(n_1 - 2 + n_2 - 2)$ degrees of freedom, we find that in no instance does the calculated t value exceed the given t value, and therefore we accept that the slopes are parallel in all cases.

APPENDIX II

MEDIA AND SOLUTIONS

Pfennig's Medium (Truper, 1967):

Solution I

distilled water	2,500	ml
$CaCl_2 \cdot 2H_2O$	1.3	g

Solution II

distilled water	67	ml
*trace elements	30	ml
**Vitamin B_{12}	3	ml
KH_2PO_4	1	g
NH_4Cl	1	g
$MgCl_2 \cdot 6H_2O$	1	g
KCl	1	g

Solution III

distilled water	900	ml
$NaHCO_3$	4.5	g

*distilled water	1,000	ml
EDTA-Na_2	500	mg
$FeSO_4 \cdot 7H_2O$	200	mg
$ZnSO_4 \cdot 7H_2O$	10	mg
$MnCl_2 \cdot 4H_2O$	3	mg
H_3BO_3	30	mg
$CoCl_2 \cdot 6H_2O$	20	mg
$CuCl_2 \cdot 2H_2O$	1	mg
$NiCl_2 \cdot 6H_2O$	2	mg
$Na_2MoO_4 \cdot 2H_2O$	3	mg

**distilled water	100	ml
Vitamin B_{12} (Cyanocobalamine Merk)	2	mg

Solution IV

distilled water	200	ml
$Na_2S \cdot 9H_2O$	3	g

Five hundred ml of Solution I was autoclaved separately in an Erlenmeyer flask. One hundred ml quantities of the remainder were distributed in 165 ml bottles and also autoclaved. Solution III was bubbled for 30 minutes with oxygen-free carbon dioxide (Hungate, 1950) until the pH was 6.2 (about 30 minutes). Solution II was added and the combined solutions were sterilized by filtration with a Millipore apparatus. This sterilized solution then was added aseptically in 55 ml amounts to the bottles containing Solution I. Solution IV was autoclaved and after cooling, 1.5 ml of sterile 2M H_2SO_4 was added slowly. The pH of the medium, usually near 7.0, was controlled by varying the amounts of acid added to the sulfide solution. Usually each bottle received 8 ml of the sulfide solution. Finally, the bottles were filled with the sterile $CaCl_2$ solution from Solution I and were stored in a dark area.

For tube dilutions, a solution of 0.1 per cent $CaCl_2$ and 2.4 per cent agar was prepared in distilled water, dispensed in 3 ml quantities in test tubes, and autoclaved. After cooling to 45°C, each tube received 6 ml of Pfennig's medium. Serial dilutions of purple sulfur bacteria were then made in liquid Pfennig's medium from which the agar shakes were inoculated.

Postgate's Medium for Sulfate Reducers (Postgate, 1963) - The medium was prepared by adding 0.5 g KH_2PO_4, 1.0 g NH_4Cl, 1.0 g $CaCl_2 \cdot 6H_2O$, 2.0 g $MgSO_4 \cdot 7H_2O$, 3.5 g Na lactate, 1.0 g yeast extract, 0.1 g ascorbic acid, 0.1 g thioglycollic acid, and 0.5 g $FeSO_4 \cdot 7H_2O$ to 1,000 ml of distilled water. Fifteen g of agar was added to the medium used for agar shakes. The pH was adjusted to 7.6 with concentrated NaOH and the medium was dispensed in 9 ml quantities in test tubes and autoclaved at 121°C for 15 minutes.

Hungate's Medium for Methane Bacteria (Hungate et al., 1954) - One g NH_4Cl, 0.4 g KH_2PO_4, 0.4 g K_2HPO_4 and 0.1 g $MgCl \cdot 6H_2O$ and 15 g Difco agar were placed in one liter of distilled water and sterilized in a flask containing a CO_2 atmosphere. After cooling, oxygen-free CO_2 was bubbled in the flask through a sterile cotton-plugged Pasteur pipette. Two ml of sterile 10 per cent $NaCO_3$ was added per 100 ml of medium.

Buffered Water (Orland, 1965) - Buffered dilution water was prepared by adding 34.0 g KH_2PO_4 to 500 ml distilled water, adjusting the pH to 7.2 with 1N NaOH and bringing the volume up to one liter with distilled water. This stock buffer was added in 1.25 ml amounts per liter of distilled water, dispensed in 99 ml dilution bottles, and autoclaved at 121°C for 15 minutes.

Oxygen-Free Cylinder Gas (Hungate, 1950) - Seventy-five g of metallic zinc was added to 50 ml of 3N HCl and stirred for 30 seconds; then $HgCl_2$ solution (2.5 ml of a saturated aqueous solution diluted to 50 ml) was added and the mixture was stirred for three minutes after evolution of the gas ceased. The zinc was washed by decantation and placed in the gas washing flask. Fifty grams of $CrK(SO_4)_2 \cdot 12\ H_2O$ were dissolved in 200 ml of water and placed in the washing flask. Ten ml of 5N H_2SO_4 were added and the flask was filled with the cylinder gas. After 24 hours the hydrogen evolved reduced the chromium and the solution was ready for use.

Reagents for Sulfide Determination (Orland, 1965): 1) Zinc acetate (2N) - This solution was prepared by dissolving 220 g $Zn(C_2H_3O_2) \cdot 2H_2O$ in 870 ml distilled water; 2) One + one sulfuric acid - Five hundred ml of concentrated H_2SO_4 was added to 500 ml distilled water; 3) Ferric Chloride - one hundred g $FeCl_3 \cdot 6H_2O$ were dissolved in 39 ml of distilled water; 4) Ammonium phosphate - four hundred g $(NH_4)HPO_4$ were dissolved in 805 ml distilled water; 5) Amine-sulfuric Acid Test Solution - Twenty-five ml amine-sulfuric acid stock solution (27.2 g paraaminodimethylanaline sulfate plus 50 ml concentrated H_2SO_4 brought up to a final volume of 100 ml with distilled water) was diluted with 975 ml one + one H_2SO_4; 6) Methylene Blue Solution - One g of dye was dissolved in distilled water and diluted to one liter. The methylene blue was standardized by determining the amount of sulfide in a sample as ZnS by a

titrimetic method (Orland, 1965) and comparing it to that received by the colorimetric procedure.

Reagents for Volatile Acid Determination (Orland, 1965): 1) Silicic Acid - Fines were removed by slurrying the acid in distilled water and decanting the supernate after settling for 15 minutes. The process was repeated three times, the silicic acid was dried at 103°C overnight, and stored in a dessicator; 2) Chloroform-butanol reagent - Three hundred ml chloroform, 100 ml n-butanol, and 80 ml 0.5N H_2SO_4 were mixed in a separatory funnel. The lower organic layer was drained through a filter paper into a dry bottle; 3) Thymol blue indicator - Eighty mg thymol blue was dissolved in 100 ml absolute methanol; 4) Phenolphthalein indicator - Eighty mg phenolphthalein was dissolved in 100 mg absolute methanol; 5) Standard sodium hydroxide - This was prepared in absolute methanol from concentrated NaOH in water.

Reagents for Total Carbohydrates (Morris, 1948): 1) Anthrone - Two g anthrone was dissolved in one liter of 95 per cent H_2SO_4; 2) Glucose standard - Exactly 0.100 g anhydrous glucose was dissolved in water and diluted to 1,000 ml.

Reagents for Phosphates (Orland, 1965): 1) Strong acid - Three hundred ml concentrated H_2SO_4 was added to 600 ml distilled water. After cooling, 4 ml of concentrated HNO_3 was added and the solution was diluted to one liter; 2) Ammonium-molybdate reagent - This was prepared by dis-

solving 31.4 g $(NH_4)6Mo_7O_4 \cdot 4H_2O$ in 200 ml distilled water. After cooling, 3.4 ml concentrated HNO_3 was added and the solution was diluted to one liter; 3) Aminonaphtholsulfonic acid reagent (ANSA) - Weighed separately were 0.75 g ANSA, 42 g anhydrous Na_2SO_3, and 70 g $Na_2S_2O_5$. The ANSA was ground with a small portion of the $Na_2S_2O_5$ in a mortar. The remaining salts were dissolved in 900 ml of distilled water. The ANSA was dissolved in this and diluted to one liter. The solution was stored in a brown bottle; 4) Stock phosphate - Anhydrous KH_2PO_4 (0.07165 g) was dissolved in water and diluted to one liter. One ml equalled 0.0500 mg of phosphate. The stock phosphate was diluted (100 ml up to 1,000 ml) with distilled water so that one ml equalled 50.0 ug phosphate.

Reagents for Sulfates (Orland, 1965): 1) Barium chloride - One hundred g of $BaCl_2 \cdot 2H_2O$ was diluted to one liter with distilled water and filtered through a Millipore filter (HA, .45u pore size); 2) Asbestos cream - Fifteen g of asbestos was diluted to one liter and then used to prepare the Gooch crucibles; 3) Silver nitrate-nitric acid reagent - Five hundred ml distilled water received 8.5 g $AgNO_3$ and 0.5 ml concentrated HNO_3.

Reagents for Pyruvate (Bergmeyer, 1965): 1) Reduced diphosphopyridine nucleodide (DPNH) (0.01M) - Sixty-six mg of DONH-Na_2 were dissolved in 10 ml of doubly distilled water; 2) lactic dehydrogenase -

(Cal Biochem, Los Angeles, California) The commercial preparation was used; 3) Tris(Hydroxymethyl)Aminomethane (0.02M) - This was prepared by dissolving 0.603 g of Tris in water and bringing the volume up to 250 ml with distilled water.

Reagents for BOD (Orland, 1965): 1) Phosphate buffer - This was prepared by dissolving 8.5 KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g NH_4Cl in 500 ml of water and diluting to one liter; 2) Magnesium sulfate solution - This was prepared by dissolving 22.5 g $MgSO_4 \cdot 7H_2O$ in distilled water and diluting to one liter; 3) Calcium chloride solution - Twenty-seven and one half g $CaCl_2$ were dissolved in distilled water and diluted to one liter; 4) Ferric chloride solution - This was prepared by dissolving 0.24 g $FeCl_3 \cdot 6H_2O$ in distilled water and diluting to one liter.

Dosing Solution (DO Meter equipment handbook, A1672, Southern Analytical, Firmley Road, Camberley, Surry): This solution was composed of 54.6 g sodium starch glycollate, 20 g $(NaPO_3)_6$, 300 g NaCl, 85 g K_2CO_3, 35 g KCl, 100 g KNO_3, and 60 g glycine in one liter of distilled water. This was heated to 50°C while stirring and made up to a final volume of 1,425 ml with distilled water.

Crystal Violet (Conn, 1957): Two g of crystal violet were dissolved in 20 ml of 95 per cent ethyl alcohol and 80 ml distilled water.

Oxidation Factors (Neish, 1952): The following table gives experimentally determined oxidation factors for certain substrates used in the study.

Compounds	Mgms. of compound per Meq. dichromate reduced
Glucose	7.26
Lactic acid	19.1
Succinic acid	26.4
Citric acid	9.65
Formic acid	23
Butyric acid	4.86
Malic acid	9.94

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