Occurrence of Purple Sulfur Bacteria in a Sewage Treatment Lagoon

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The ecology of purple sulfur bacteria in a sewage oxidation lagoon was investigated. Chemical changes in the lagoon were investigated by monitoring biochemical oxygen demand (BOD₅), sulfide, sulfate, phosphate, total carbohydrates, volatile acids, alkalinity, and pH. Lagoon water temperatures were observed daily. Microbial ecological relationships were deduced by enumerating coliforms, total bacteria other than anaerobes [Tryptone Glucose Extract (TGE) agar], methane formers such as Methanobacterium formicicum, sulfate reducers, purple sulfur bacteria, and algae. Finally, two strains of purple sulfur bacteria were characterized. Two populations, purple sulfur bacteria and total bacteria (TGE agar), reached maximal concentrations in the warmest part of the 1967 summer. Purple sulfur bacteria reached maximal numbers as concentrations of sulfate and volatile acids were depleted, whereas carbohydrates and alkalinity remained unchanged. Low sulfate levels, which were not limiting for sulfate reducers, may be attributable to storage of sulfur within purple sulfur bacteria. No biological, chemical, or physical agent was linked to the removal of coliforms. The increase of algae in the late summer of 1967 may have been related to the low organic content of the lagoon during this period. Although lagoon pH (7.7 to 8.2) was favorable for purple sulfur bacterial growth, temperatures and sulfides were not optimal in the lagoon for these organisms. Chromatium vinosum and Thiocapsa floridana (the predominant lagoon purple sulfur organism in 1967 and 1968) utilized certain carbohydrates, amino acids, volatile acids, and Krebs cycle intermediates. Also purple sulfur bacteria lowered BOD levels as demonstrated by the growth of T. floridana in sterilized sewage.

The advent of the sewage oxidation lagoon as a means of waste treatment has prompted research in the area of lagoon efficiency. Reduction of two parameters of pollution, enteric bacteria and organic substrates, has been most intensively studied in the last 20 years. Other major investigations, as evidenced by the review of Fitzgerald and Rohlich (2), have included studies of inorganic substrate concentrations and the role of algae in lagoons. Until recently (3), not even the major populations of oxidation lagoon had been identified. Therefore, the microbial interactions in oxidation lagoons remain largely unexplored.

The appearance of high populations of purple sulfur bacteria in a local oxidation sewage lagoon receiving municipal and industrial wastes initiated this ecological investigation. These organisms are capable of an autotrophic mode of nutrition while utilizing sulfide and certain other substrates as electron sources in the photosynthetic process (19).

Three areas of study warranted investigation. First, several parameters of the lagoon, including the biochemical oxidation demand (BOD₅), sulfide, sulfate, phosphate, total carbohydrates, volatile acids alkalinity, pH, and water temperatures, were monitored throughout the summers of 1967 and 1968. Second, possible ecological relationships between various organisms were investigated by enumerating total heterotrophic bacteria [Tryptone Glucose Extract (TGE) agar], coliforms, sulfate reducers, methane bacteria as Methanobacterium formicicum, purple sulfur bacteria, and algae. Finally, isolates from two genera of purple sulfur bacteria, Chromatium vinosum and Thiocapsa floridana, were characterized by determining optimum pH, sulfide, and temperature levels required for growth. 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.

**MATERIALS AND METHODS**

**Aquatic system.** The primary cell of the two-cell sewage oxidation lagoon at Grafton, N.D., was the environment which was studied. The primary and secondary lagoons, each 70 acres in area and containing about 70 million gal, were designed for treatment of the domestic wastes of Grafton. However, the introduction of potato wastes from local processing industries grossly affected the environment of the primary cell and allowed a new major population, purple sulfur bacteria, to thrive. (Approximately 18,000 lb/day of BOD$_5$ were introduced into the primary cell during the September to June processing season.) Duplicate sampling of the primary cell was from the effluent man hole. Continuous discharge to the secondary cell allowed easy access for sampling.

**Pure culture studies.** Two species of purple sulfur bacteria, *C. vinosum* and *T. floridana*, were isolated from lagoon waters by Hans Trüper, Woods Hole Oceanographic Institute, Woods Hole, Mass., by enrichment techniques (27). These cultures were used for pure culture studies. Inocula of purple sulfur bacteria were prepared by growing the organisms in 160-ml bottles of Pfennig's medium (18) with a trace element supplement. After incubation of 1 week at 25°C with illumination [60-w incandescent bulb at a distance of 18 inches (46 cm)], profuse growth developed. Routinely, 5 × 10$^3$ organisms were added to 60-ml tubes for the following substrate studies.

A variety of organic substrates were incorporated into Pfennig's basal medium to examine the potential of a given substrate as a carbon source or an electron donor, or both, for the tested organisms. The substrate studies followed the protocol of May and Stahl (7), in which four sets of determinations were completed for each test compound. These sets included organic substrate plus Pfennig's salt solution, organic substrate plus the salt solution plus 0.1% bicarbonate, organic substrate plus salt solution plus 0.05% sulfide, and organic substrate plus Pfennig's complete medium (salts, sulfide, and bicarbonate). Additionally, a control with basal medium plus bicarbonate and sulfide was included. Organic substrates which were tested in a final concentration of 0.1% included succinate, pyruvate, fumarate, malate, glycolate, lactate, citrate, acetate, glutamate, histidine, methionine, threonine, aspartate, fructose, maltose, lactose, sucrose, and glucose. Valerate, butyrate, propionate, isobutyrate, hexanoate, isovalerate, and formate were added in final concentrations of 0.05%. Growth was compared to controls which received none of the previously mentioned substrates (organic substrates, bicarbonate, or sulfide). Additional physiological studies included determination of the optimal pH range, temperature range, and sulfide concentrations for the test cultures. In addition, *T. floridana* was grown in filter-sterilized sewage supplemented with hydrogen sulfide (20 $\mu$g/ml). The flask was incubated at 25°C for 3 weeks after which the BOD$_5$, sulfide, volatile acids, total carbohydrates, and *T. floridana* populations were determined and compared to the initial levels.

**Lagoon populations.** The Millipore filter technique employing Endo broth was used to enumerate coliforms. (Equipment was supplied by the Millipore Filter Corp., Bedford, Mass., and media were obtained from Difco Laboratories, Detroit, Mich.) Total viable counts of heterotrophic bacteria other than anaerobes were estimated with TGE agar provided by Difco. Comparative direct and viable counts (18) of purple sulfur bacteria were also obtained.

Populations of sulfate reducers were estimated by the method of Postgate (20). Population estimates were completed by using a most-probable-number table (14).

Methane bacteria with colonies similar to *M. formicicum* were determined with a technique and medium devised by Myroie and Hungate (12).

Total algal numbers were determined by microscopic direct counts utilizing a Neubauer hemocytometer. Dominant populations of green algae were identified (14).

Purple sulfur bacteria were enumerated by direct counts with a Neubauer hemocytometer or a Levy counting chamber and phase microscopy. Stained preparations using the Maneval capsule stain were also utilized for size determinations.

**Lagoon chemistry.** The titrimetric method for total alkalinity (which is equivalent to buffering capacity of the lagoon and is a measure of the total carbonate-bicarbonate present in the system), the glass electrode method for lagoon pH, the methylene blue colorimetric technique for sulfides, the gravimetric method for sulfates, the sillicic acid column chromatography method for total volatile acids, the technique for the 5-day BOD$_5$, and the aminonaphtholsulfonic acid method for total soluble phosphates were used as described in *Standard Methods for the Examination of Water and Wastewater* (14).

Total carbohydrates were determined with anthrone reagent (9).

Quantitation of individual short-chain fatty acids was completed with a Barber Coleman gas chromatograph (model 10) equipped with a Chromosorb 101 column (carrier gas, helium; column temperature, 150°C; thermal conductivity detector). Alkaline centrifuged sewage was concentrated in a laboratory evaporator (11), after which fatty acids were extracted with ether (13).

Water temperatures were recorded daily at 9:00 AM at a water depth of 6 inches (15 cm).

**RESULTS**

**Pure culture studies.** Two pure cultures of purple sulfur bacteria, *T. floridana* (2 to 3 μm, coccoid, encapsulated organisms) and *C. vinosum* (2 to 4 μm, motile organisms), were used in laboratory studies as test organisms representative of the metabolic characteristics of purple sulfur bacteria of the lagoon. *T. floridana*, represented by the direct counts of purple sulfur bacteria in Fig. 1B and 2B, was the predominant organism in the lagoon in 1967 and 1968.

*T. floridana* exhibited a wider pH range than
did *C. vinosum* (Table 1), but both organisms had a pH optimum of 7.5 to 8.2. Both organisms grew in high sulfide concentrations (Table 2). It is important to note, however, that growth was also supported with low sulfide concentrations.

Table 3 demonstrates that *T. floridana* and *C. vinosum* grew over a wide temperature range (16 to 30°C), temperatures which one would expect in the lagoon during the summer.

The predominant organism was a purple sulfur bacterium, *T. floridana* (indicated by the purple sulfur counts in Fig. 1B and 2B), which also grew in filter-sterilized sewage supplemented with sulfide (Table 4). Along with the development of a high purple sulfur bacterial population in the sewage, the BOD₅ and volatile acids were lowered, and the sulfide was depleted.

Utilization of organic substrates by the pure cultures of *C. vinosum* and *T. floridana* is summarized in Table 5. Several observations may be made from these data. First, both organisms have the ability to utilize an array of organic com-
pounds, including sugars, short-chain organic acids, and amino acids. Second, as observed in columns 4 and 8, certain substrates apparently inhibit growth of purple sulfur bacteria. (Formate inhibits both organisms, methionine inhibits C. vinosum, and aspartate inhibits T. floridana.) Third, not all of the organic substrates are utilized in the same manner. For example, as observed in columns 1 and 5, pyruvate, fumarate, and histidine support growth without additional carbon or electron donor sources present; whereas, as shown in columns 2 and 6, a multitude of compounds ultimately may act as electron donors with bicarbonate as a carbon source, and, as seen in columns 3 and 7, certain compounds apparently are utilized as carbon sources if sulfide is present. It is thus apparent that these organisms have the metabolic capabilities of not only autotrophic growth, but also heterotrophic growth, and probably may be best described as exhibiting both a heterotrophic and autotrophic mode of nutrition.

**Lagoon ecology.** Figures 1 to 3 summarize the

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**Fig. 2. Microbial, chemical and physical changes occurring in the Grafton primary lagoon during the summer of 1968.**
lagoon relationships observed during the summers of 1967 and 1968 in the Grafton primary lagoon. The numbers of purple sulfur bacteria given are from data on direct counts. The viable counts of these bacteria were usually about one log lower than direct counts.

Figures 1A and 2A show an increase in lagoon pH from about 7 in May of each year to 8 to 8.2 later in the summer. In 1968, the higher pH was not reached until about 1 month after that of 1967. During both summers, water temperatures reached their maximum (20 to 25°C) near the end of July.

No perceptible differences in phosphate concentrations were noted when the numbers of purple sulfur bacteria were increasing (Fig. 1B, 2B).

Although algae increased about one log unit at the end of the summer of 1967 (Fig. 1B), algal numbers remained constant throughout the summer of 1968 (Fig. 2B). The predominant algal populations during both years were Scenedesmus and Chlorella.

In Figures 1C and 2C, we note a decrease of volatile acids as purple sulfur bacterial numbers increased. Since these organisms have the capability of utilizing these substrates, volatile acid decreases could reflect microbial utilization by purple sulfur organisms. In 1967, the purple sulfurs reached densities of $10^9$ organisms per ml when the volatile acids were in the range of 80 μg/ml, whereas in 1968, when volatile acids approached 1,000 μg/ml, the purple sulfur population reached $5 \times 10^9$ organisms per ml.

Figure 1D and 2D show that, as purple sulfur bacteria increased, sulfide was depleted and sulfate was lowered. Maximal numbers of purple sulfur organisms were noted at a time when sulfide utilization or disappearance was occurring.
Table 4. Utilization of sewage as a growth medium by Thiocapsa floridana

| Determination                  | Concnsa  |
|-------------------------------|----------|
|                               | Initial  | Final   |
| Carbohydrates (µg/ml)         | 43       | 38      |
| BOD (µg/ml)                   | 2,535    | 1,915   |
| Volatile acids (µg/ml)        | 1,710    | 1,316   |
| Sulfide (µg/ml)               | 20       | 0       |
| T. floridana (bacteria/ml)    | \(5 \times 10^5\) | \(3 \times 10^3\) |

a Sewage was filter-sterilized after which sulfide (20 µg/ml) and the inoculum (\(5 \times 10^5\) bacteria/ml) were added. The inoculated sewage was incubated in the light at 25 C for 3 weeks.

Figure 3 illustrates a possible relation of acetate to the purple sulfur bacteria and of formate to the methane bacteria. As purple sulfur bacteria increased in numbers, a dramatic decrease of acetate was noted. Formate, which can be utilized by methane bacteria but which does not support growth of the tested purple sulfur bacteria (Table 5), was depleted as methane bacteria increased. However, since the technique used for enumerating the methane bacteria (specifically *M. formicicum*) probably does not reflect total methane bacteria present and since other organisms may utilize formate, the above suggestion requires more definitive experimentation to establish the suggested relation.

**DISCUSSION**

Purple sulfur bacteria have long been associated with anaerobic, sulfide-containing zones in lakes (5, 25), and more recently these organisms have been associated with treatment facilities (1, 7). These organisms were shown to have the capabilities of utilizing certain organic compounds as electron donors or as carbon sources (7, 10, 15, 22, 23, 26). However, the action of these organisms in the lagoon environment has not previously been fully explored (1, 7).

Our data (Table 5) show that *T. floridana* and *C. vinosum* have the capabilities of utilizing many organic substrates, especially as electron donors. These data indicate that purple sulfur bacteria have the enzymatic capability for utilization of organic substrates, many of which are found in a lagoon. Additionally, the evidence of growth of *T. floridana* in filter-sterilized sewage further supports this assumption.

The significant removal of volatile acids concomitant with the emergence of purple sulfur bacterial growth provides environmental evidence that this group of organisms does utilize certain organic substrates in nature (Fig. 1C, 2C). Acetate, an organic substrate commonly utilized by purple sulfur bacteria, is removed from the lagoon environment during exponential increases in purple sulfur bacteria. Acetate-utilizing methanogenic bacteria may also be of importance. Other phenomena, such as evaporation, may also help deplete the lagoon of volatile acids, but most of these acids disappear at a time when the lagoon pH is near 8; therefore, the organic acids will be in their nonvolatile salt form (13). It is highly probable, then, that the removal of volatile acids from the lagoon is primarily a biologic process.

Although the test organisms have an optimum sulfide concentration of 45 to 60 µg/ml for growth, the organisms will grow at lesser concentrations found in the lagoon (Table 2; Fig. 1D,
### TABLE 5. Utilization of organic substrates by Chromatium vinosum and Thiocapsa floridana

| Substrate additions | Chromatium vinosum | Thiocapsa floridana |
|---------------------|--------------------|--------------------|
|                     | Substrate only | Substrate + HCO<sub>3</sub><sup>-</sup> | Substrate + HCO<sub>3</sub><sup>-</sup> + S<sup>2-</sup> | Substrate only | Substrate + HCO<sub>3</sub><sup>-</sup> | Substrate + HCO<sub>3</sub><sup>-</sup> + S<sup>2-</sup> |
| None                | -                | -                  | ++                | -                | -                  | -                  |
| Glucose             | -                | +                  | ++                | --               | ++                 | ++                 |
| Fructose            | -                | +                  | ++                | ++               | ++                 | ++                 |
| Maltose             | -                | -                  | ++                | -                | +                  | ++                 |
| Lactose             | -                | ++                 | ++                | ++               | ++                 | ++                 |
| Sucrose             | -                | +                  | ++                | ++               | ++                 | ++                 |
| Glutamate           | -                | -                  | ++                | -                | +                  | ++                 |
| Histidine           | +                | +                  | ++                | ++               | ++                 | ++                 |
| Methionine          | -                | -                  | -                 | -                | +                  | ++                 |
| Threonine           | -                | +                  | ++                | +                | ++                 | ++                 |
| Aspartate           | -                | -                  | ++                | -                | +                  | ++                 |
| Valerate            | -                | -                  | ++                | -                | +                  | ++                 |
| Butyrate            | -                | -                  | ++<sup>+</sup>    | -                | +                  | ++                 |
| Propionate          | -                | -                  | ++<sup>+</sup>    | -                | +                  | ++                 |
| Isovalerate         | -                | -                  | ++<sup>+</sup>    | -                | +                  | ++                 |
| Formate             | -                | -                  | -                 | -                | -                  | -                  |
| Acetate             | -                | -                  | ++<sup>+</sup>    | -                | --                 | --                 |
| Succinate           | -                | +                  | --<sup>+</sup>    | -                | +                  | ++                 |
| Pyruvate            | ++               | ++                 | ++<sup>+</sup>    | ++               | ++                 | ++                 |
| Fumarate            | +                | ++                 | ++<sup>+</sup>    | ++<sup>+</sup>   | ++                 | ++                 |
| Malate              | +                | ++                 | ++<sup>+</sup>    | ++<sup>+</sup>   | ++                 | ++                 |
| Glycolate           | -                | -                  | ++<sup>+</sup>    | -                | +                  | ++                 |

* Final concentrations of the additives were as follows: volatile acids, 0.05%; all other organic substrates, 0.1%; NaHCO<sub>3</sub>, 0.1%; and Na<sub>2</sub>S·9H<sub>2</sub>O, 0.05%.

b Growth was measured by direct counts. Densities similar to a basal medium control (which received 10<sup>8</sup> organisms/ml but contained no bicarbonate, sulfide, or organic substrates) are indicated by a minus sign; higher populations are indicated by plus signs (+, one-log increase; ++, two-log increase, and ++++, three-log increase). Growth in basal medium plus bicarbonate and sulfide gave a plus two (++) or two-log increase over basal medium.

### TABLE 6. Volatile acids of the Grafton primary lagoon during 1968

| Date       | Formate (µg/ml) | Acetate (µg/ml) | Propionate (µg/ml) | Butyrate (µg/ml) | Others |
|------------|----------------|----------------|-------------------|-----------------|--------|
| 29 May     | 13.6           | 225.0          | 4.4               | 51.9            | Trace  |
| 5 June     | 47.5           | 67.5           | 0                 | 34.9            | 0      |
| 12 June    | 35.1           | 250.8          | 5.9               | 4.7             | Trace  |
| 19 June    | 131.7          | 772.0          | 13.1              | 15.9            | Trace  |
| 26 June    | 116.1          | 595.0          | 5.8               | 11.7            | Trace  |
| 3 July     | 112.0          | 592.0          | 0                 | 63.7            | Trace  |
| 10 July    | 178.4          | 571.4          | 0                 | 0               | 0      |
| 17 July    | 137.7          | 302.3          | 0                 | 0               | 0      |
| 24 July    | 270.6          | 214.4          | 0                 | 0               | 0      |
| 31 July    | 109.9          | 20.0           | 0                 | 0               | 0      |
| 7 August   | 56.0           | 3.9            | 0                 | 0               | 0      |
| 14 August  | 59.0           | 0              | 0                 | 0               | 0      |
| 21 August  | 130.0          | 0              | 0                 | 0               | 0      |
| 28 August  | 107.0          | 6.3            | 7.7               | 0               | 0      |
| 4 September| 93.3           | 1.4            | 0                 | 0               | 0      |
| 11 September| 10.9          | 4.2            | 0                 | 8.8             | 0      |

* Volatile acids were quantitated with a Barber Coleman gas chromatograph (model 10) equipped with a Chromosorb 101 column.
A decrease in the alkalinity values may be expected with high populations of such carbon dioxide-utilizing organisms as purple sulfur bacteria and algae (Fig. 1B, 2B; 4). However, since the changes were not pronounced, especially in 1967, the bicarbonate may have been continuously regenerated by fermentation through formation of carbon dioxide. Phosphate levels never were limiting, nor did they reflect changes in microbial populations. The constant supply of phosphorus in the raw sewage may have masked phosphate uptake that could occur by microbial utilization.

The algal population increased about one log unit in 1967, but remained uniformly low in 1968. The higher BOD₅ load throughout much of 1968 may have prevented adequate light penetration into the lagoon, which can limit algal growth (17).

At least 10 species of bacteria have been characterized which produce methane as a terminal product of their metabolism, with two species, *M. formicicium* and *M. ruminantium* (8), occurring in the largest numbers in digesting sludge. Smith (24) found that at least three genera of methane bacteria, isolated in pure culture, occur in sludge in concentrations from 10⁴ to 10⁶/ml. All strains grew on hydrogen gas, whereas specific strains grew on acetate, methanol, and formate. Methane production in lagoons has been reported by Oswald (16) and others at temperature below the optimum mesophilic (32 to 37 °C) and thermophilic (50 to 55 °C) range suggested for sewage treatment plants. He suggested that the minimum temperature for methane production in lagoons is near 15 °C.

The results of our study indicate that methane organisms (specifically *M. formicicium*) are present and reproducing in the lagoon at times when temperature is at or above 15 °C. Although populations of this species of methane-producing organisms were highest when lagoon liquid pH was 8.0 or above, it is possible that the bottom or sludge pH was nearer the optimum pH of 7.0 for methane production. It is not known whether other methane producers were present in the lagoon. Formate utilization by *M. formicicium* could account for the disappearance of this substrate from the lagoon, although other methane bacteria can readily utilize this substrate. Other substrates for methane production are also present in the lagoon.

The decrease of BOD₅ values was notable during the development of purple sulfur bacteria (Fig. 1E, 2E). For example, in 1968 the BOD₅ was lowered from approximately 1,000 μg/ml in June to 100 μg/ml by September. During this
same period, volatile acids decreased from 900 to 75 μg/ml (Fig. 2C), and acetate decreased from 800 μg/ml to trace amounts in August. Since purple sulfur bacteria utilize these compounds, it is probable that these organisms play a positive role in the removal of organic substrates that contribute to the BOD₅. The significance of other methanogenic bacteria and strictly anaerobic heterotrophic bacteria in sewage treatment lagoons needs study.

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