Haloduric Anaerobes in the Sulfide Muds of a Saline Lagoon

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The lagoonal estuarine system on the Central East Coast of Florida consists of the Indian River, the Indian River Lagoon, the Banana River, and encompasses the Kennedy Space Center. The system extends from Ponce de Leon Inlet near New Smyrna Beach to the St. Lucie Inlet. The mean water depth is approximately six feet except for the width of the Intracoastal Waterway. The salinity, depending on rainfall and fresh water runoff, ranges between 12 and 36 parts per thousand. Such water movement as occurs is generally wind driven (SCHNEIDER ET AL, 1973).

Studies of sulfide ion concentrations in the sediments of the Indian River near Delespine and near Vero Beach by AKIMOTO (1971), demonstrated small amounts, frequently less than 4 mg. per cent, in all of the samples.

In a study of man-made channels and canals of the west central coast of Florida, BETZ (1971) cultured the sediments for Clostridium perfringens using a selective medium developed by MARSHALL, ET AL, (1965) for the study of food contamination and reported their recovery from most samples. C. perfringens is a gram-positive, rod shaped, spore forming, obligate anaerobe that produces hydrogen sulfide in suitable culture media, and is known to cause gas gangrene. SHERMAN (1972) also isolated gram-positive, spore forming, rod shaped, anaerobic organisms from sulfide sediments collected on or near the Kennedy Space Center. These believed to be Clostridium species, produced hydrogen sulfide in culture, using ethion, a commercial pesticide, as the only source of sulfur. It seemed reasonable, therefore, to anticipate the isolation of Clostridium species among the haloduric bacteria, and to ascribe to them a role in the microbiology of the lagoons.

METHODS AND MATERIALS

Surveys of the lagoonal waters in and around the Kennedy Space Center were conducted during 1972 to locate areas of relatively high bacterial populations. When a representative area was encountered, studies of water columns and underlying sediments were conducted in order to determine the predominant types of bacteria and to gain some insight into their activities.

Water samples were collected in sterile plastic, 100 ml. bottles six inches below the surface to minimize the expectancy of airborne contaminants. The capped bottles were immersed, uncapped, allowed to fill, and were recapped underwater.

Water column studies required that samples be collected from the bottom sediments and from the water at two-foot intervals between the

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bottom and the surface. The water samples were collected from the pre-selected depths in pre-sterilized self-sealing glass bottles. A twenty-foot length of monofilament fishing line was attached to a rubber stopper. The stopper was placed in the mouth of a three-ounce glass medicine bottle and secured with two rubber bands as shown in Figure 1. In subsequent experiments, a small rubber or plastic ball will replace the stopper. The bottle was then lowered to the preselected depth in the holding device also shown in Figure 1.

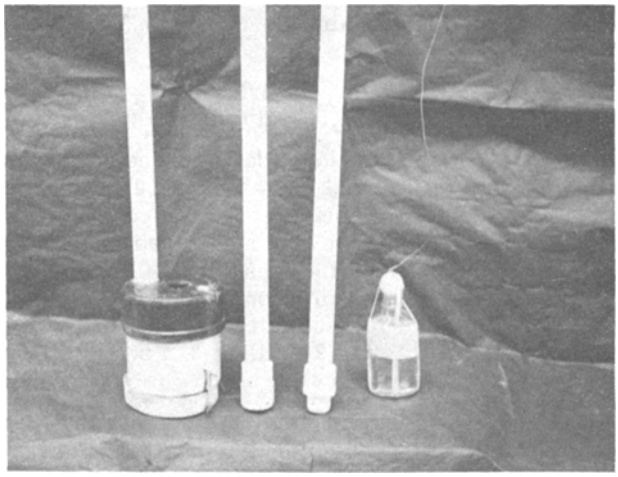

Figure 1. A collection bottle and sampling device for collecting sterile water samples at preselected depths.

The holding device was constructed of PVC plastic and fiberglass screening. A suitable (6 inches) length of four-inch diameter PVC pipe was capped at one end and a hole was drilled through the cap. A precut ring of the same pipe was covered with fiberglass screening and was attached to the bottom of the device by using additional fiberglass screening as a flexible hinge. The device was then attached to the end of one of a series of three-foot lengths of three-quarter inch diameter PVC pipe. The opposite end of that length was equipped with a screw-type connector. Additional lengths were fitted with alternating male and female screw-type connectors. When used, each length was marked at one-foot intervals.

Sediment samples were obtained by dragging a flat blade anchor and collecting the sediment sample adhering to it in a plastic bag. At difficult sampling sites, a length of three-quarter inch diameter PVC pipe served as a simple coring device. The sample was then expressed from the pipe into a plastic bag. Sediment samples collected simultaneously for sulfide ion analyses were alkalinized immediately with sodium hydroxide pellets in order to trap volatile hydrogen sulfide as non-volatile sodium sulfide, thus preventing any loss during transport and preliminary treatment.

The samples were ordinarily processed in a mobile laboratory in the sampling area. Inoculations were initiated within four hours of collection. When the mobile laboratory was not available, the samples were refrigerated in an ice chest and returned to the laboratory facilities at Florida Institute of Technology for processing. When transport was required, the time elapsed between collection and processing rarely exceeded six hours.

The bacteriological methods used routinely were:

1. 25 Tube MPN (most probable number) of total bacteria (PORTER, 1950)
2. Microscopic examination of Gram-stained smears made from cultures (MANUAL OF MICROBIOLOGICAL METHODS, 1957)
3. Multiple (5) Tube Presumptive Coliform MPN (STANDARD METHODS, 1971)
    a. EMB - E. coli, confirmation
    b. E.C. - E. coli, fecal types

Using sterile techniques and standard procedures, 10 ml. of each water sample were diluted in ten-fold series from 10^{-2} through 10^{-6}, inclusively, in suitable sterile water blanks. When sediments were studied, one gram, net weight, of each sample was suspended in 100 ml. of sterile distilled water and diluted in ten-fold series from 10^{-3} through 10^{-7}, inclusively. One ml. aliquots of each dilution were then used to inoculate tubes containing 5 ml. of NIH Thioglycollate medium (Difco) and 10 ml. aliquots to inoculate tubes containing 20 ml. of 3/2 strength Lactose Broth (Difco).

In addition to the above, SS agar (Difco), Sabourad's agar (Difco), and Nutrient agar (Difco) plates were inoculated with one ml. aliquots of each diluted sample collected from the Banana Creek area during the water column study. The inocula were allowed to dry before the plates were inverted and incubated.

Bacterial hydrogen sulfide production was determined by placing a strip of sterile lead acetate paper in the neck of each thioglycollate tube which had been inoculated and in which growth had already occurred. The paper was held in place by the plastic cap. Upon reincubation at room temperature for 24 hours, the strips were inspected for the appearance of black lead sulfide.

Cultures in which H_2S was produced were streaked on petri plates containing NIH Formula Thioglycollate (Difco) medium which had been hardened by adding enough agar to yield a 1.5 per cent preparation. The plates were then incubated anaerobically for 48 hours at 35°C. in a disposable system (Gas Pak, BBL). Fresh tubes of fluid thioglycollate medium were inoculated with representative isolated colonies. The subcultures were checked for purity by microscopic examination of gram-stained preparations, and then forwarded to an independent laboratory for confirmation of generic identification.

The alkalinized sediment samples were placed in suitable containers and dried in an oven at 100°C. for 48 hours. Twenty-five grams of each dried sediment were weighed out and processed as described by AKIMOTO (1971).

The chemical procedures for sulfide ion analyses of the sediments were conducted according to STANDARD METHODS (1971) as modified by AKIMOTO (1971), wherein the alkalinized sample was acidified with concentrated hydrochloric acid to liberate gaseous hydrogen sulfide. The hydrogen sulfide gas was bubbled through a cadmium chloride solution, trapped as cadmium sulfide, and then titrated iodometrically.

RESULTS

During the preliminary survey, the highest bacterial counts were obtained from samples collected in Banana Creek. The site profiles in Figure 2, were developed from subsequent water column studies. It is evident in Figure 2 that the sediment samples taken at each site in Banana Creek and at selected sites immediately west of the mouth of Banana Creek had higher bacterial counts than did the water samples taken above them. The highest MPN, 1.6×10^8 per gram, was obtained from the sediment sample collected at the mouth of a waste water outfall, east of State Road 3. Coliforms were cultured from the sediments collected at the 4 sites located east of State Road 3; 2 sites located west of State Road 3 in Banana Creek; and 1 site in the open lagoon. The respective MPN's of coliforms were 10^6, 10^4, 10^3, 10^4, 10^4, 10^5, and 10^3 per gram, wet weight, of sediment sample. The water sample taken from the mouth of the waste water outfall also had a presumptive coliform count of 10^3 per 100 ml. of sample. E. coli, per se was not cultivated from any sample collected in the Banana Creek area. Neither were Salmonella nor Shigella cultivated from any sample on a medium selective (SS Medium, Difco) for these genera.

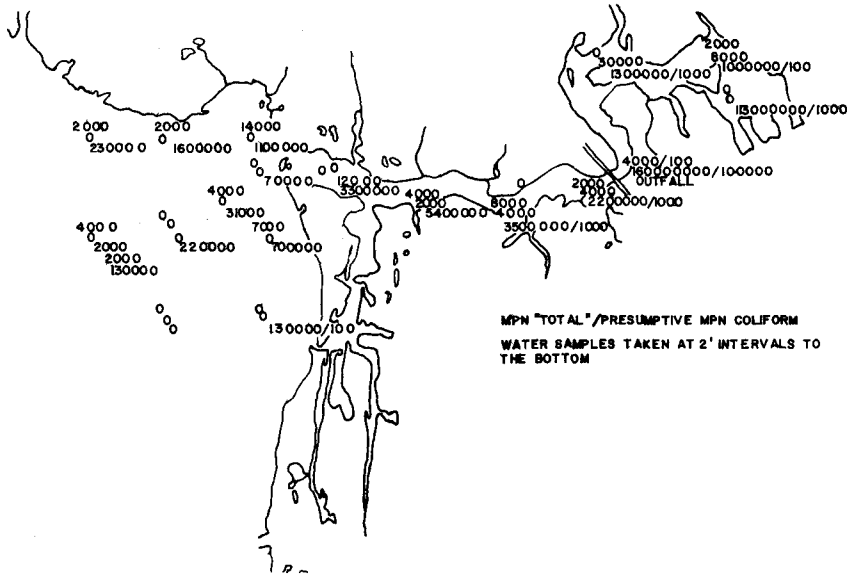

Figure 2. Distribution of haloduric bacteria (MPN) and coliforms (Presumptive MPN) in Banana Creek and adjacent waters.

The sediments were grouped according to geological type, regardless of the height of water above the site, as follows:

1. "Sulfide muds" - any sediment that offered olfactory evidence of hydrogen sulfide. Usually grey clays or black to brown silty sands.
2. "Fine sands" - usually fine uniform sands having little evident plant materials regardless of color.
3. "Sands" - fine to medium grained sands with some shell fragments.

Their distribution throughout the Banana Creek area is shown in Figure 5. Those designated as sulfide muds generally had bacterial populations an order of magnitude greater than those designated as fine sands, and two orders of magnitude greater than those designated as sand. These data are summarized in Figure 3. Smears were made from the mixed cultures cultivated from the sulfide sediments and stained by Gram's method (MANUAL OF MICROBIOLOGICAL METHODS, 1957). The predominent organism was a gram-positive, spore-forming rod that was anaerobic in culture. When streaked on thioglycollate agar (1.5 per cent) plates, they produced colonies that were irregular, raised, and erose.

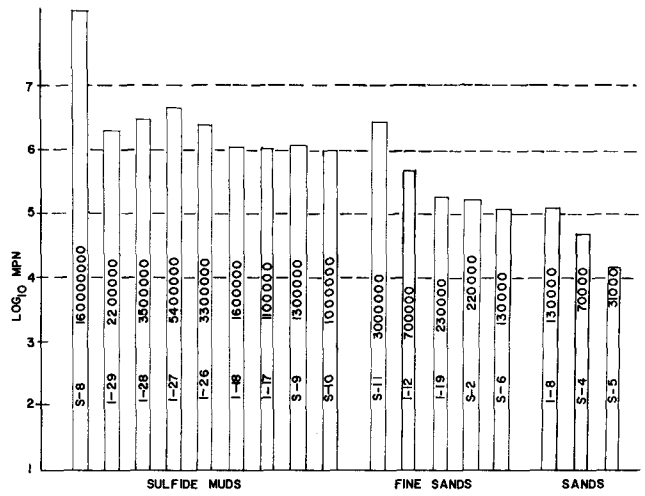

Figure 3. Bacterial populations (MPN) of sediment types regardless of water depth.

Sediment samples were collected at nine sites approximating those sampled by AKIMOTO (1971) near Vero Beach. MPN's of total bacteria therein revealed that larger numbers of bacteria might be expected from sites in deeper waters. Samples collected at sites under 6 or more feet of water, for example, had total bacterial counts between 1.4×10^7 and 2.4×10^8 per gram, wet weight, whereas samples collected at sites under less than six feet had bacterial counts between 1.8×10^6 and 1.3×10^7 per gram, wet weight. As demonstrated in Figure 4, MPN's of sulfide producing bacteria, estimated as described from grown cultures, in sediments collected at sites six feet or more below the surface ranged between 4.5×10^3 and 1.3×10^5 per gram, wet weight, with a median of 1.3×10^4 per gram, wet weight. MPN's of sulfide producing bacteria obtained from samples taken from sites with water depths of less than six feet ranged from 2.0×10^3 and 7.0×10^4 per gram, wet weight, with a median of 8.0×10^3 per gram, wet weight, of sample. Similar results, shown in Table 1, were obtained during a study conducted near Delespine, Florida.

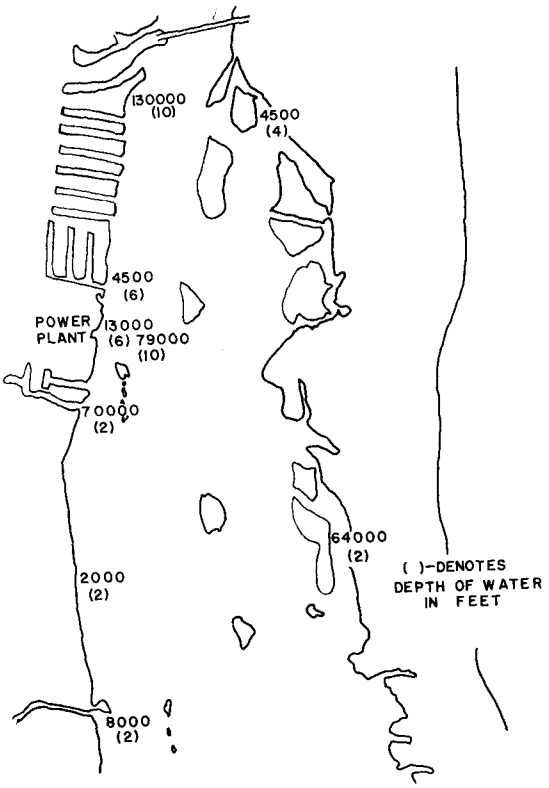

Figure 4. Distribution of sulfide producing bacteria (MPN) in Indian River sediments near Vero Beach, Florida

Table 1. Haloduric and haloduric sulfide producing bacteria (MPN) in 14 sediment samples collected near Delespine, Florida.

Water Depth	Median MPN and range of haloduric bacteria	Median MPN and range of haloduric sulfide producing bacteria
Shallow, less than 6 feet	1.3×10^5 (4.4×10^4 - 4.9×10^6)	1.1×10^4 (2.0×10^3 - 2.6×10^4)
Deeper, greater than 6 feet	1.1×10^6 (7.0×10^4 - 2.3×10^6)	2.0×10^4 (6.8×10^3 - 4.6×10^4)

The predominant organism cultured during these studies was a gram-positive, spore-forming rod similar to those encountered in the other areas studied. Therefore, cultures representative of the several sampling areas were sent to an independent laboratory for confirmation of identification. Nine of the 14 strains submitted were confirmed as members of the genus Clostridium. The remaining 5 were reported as facultative members of the genus Bacillus.

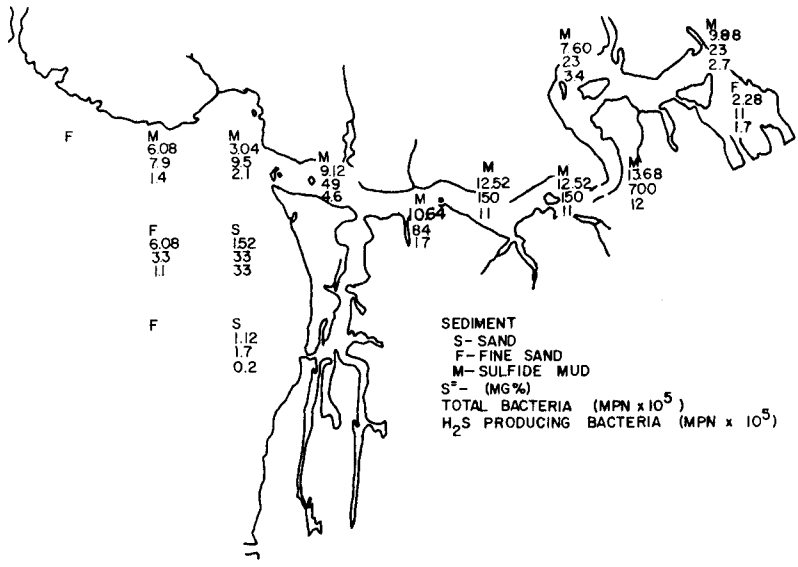

Figure 5. Distribution of haloduric bacteria (MPN) and H_2S producing bacteria (MPN) and sulfide levels in the sediments of Banana Creek and the sediment type at each site.

Upon relating sulfide ion and bacterial population in sediments from Banana Creek, it became evident that the highest concentration of sulfide ions, 13.68 mg. per cent, and the highest total bacterial count, 7.0×10^7 per gram, wet weight, in the sediment samples were encountered at a site located near the mouth of a waste-water outfall. Figure 5 demonstrates the decreasing sulfide ion concentrations and total bacterial MPN's with increasing distance in both directions from the above site. The MPN's of sulfide producing bacteria also generally decreased as the sites increased in distance from the site located near the mouth of the waste-water outfall. The highest MPN of sulfide producing bacteria, however, 1.7×10^6 per gram, wet weight, was obtained from the sample collected near the mouth of a large tributary which drained into Banana Creek downstream from the site located near the mouth of the waste-water outfall.

DISCUSSION

Banana Creek receives much of the natural water runoff from norther Merritt Island as well as waste water from the KSC. As a result, much terrestrial and waste materials are introduced into Banana Creek enriching the water and especially the sediments. The usual water movement is from east to west into the Indian River. The presence of copious amounts of hydrogen sulfide in the sediments located upstream from a few small islands in the creek suggests that these islands impede water movement, causing the deposition of the waste materials. Since these materials are enrichments, it should be possible to locate their probable sources by locating areas of increased populations of sulfide producing organisms. Should there be only one source of introduced materials into Banana Creek, the MPN's of sulfide producing bacteria should decrease with increasing distance from it due to dispersion. As is evident in Figure 5 this is generally the case in Banana Creek, but other sources of enrichment are also suggested. At the easternmost site, for example, the MPN of sulfide producing bacteria found in the sediment was relatively small. The sediments collected from the other three sites east of State Road 3 revealed increasing MPN's of sulfide producing bacteria with increasing distance downstream. The sample from a site located west of State Road 3 and downstream of the outfall had a lower MPN of sulfide producing bacteria than that from the site near the outfall. There is no apparent tributary, creek, outfall or other possible source of introduced materials located between these sites. The sediment sample collected from a site located at the mouth of a large tributary draining into Banana Creek, had the highest MPN of sulfide producing bacteria than any other sample collected, and reflects enrichment from both the waste water outfall and the tributary. A slightly higher MPN of sulfide producing bacteria occurred in the sediment at another site located at the mouth of a small creek.

The studies of the sediments collected near Vero Beach also suggested a correlation of sulfide ion concentrations (AKIMOTO, 1971) , with the MPN's of sulfide producing bacteria. Large populations of sulfide producing organisms also occurred in sediments collected from shallow water sites located near the mouth of drainage ditches and canals. At Vero Beach, the H_2S concentrations and the increased MPN's also related the increasing depths of water over the sites from which the samples were obtained. Since the sediments in the open lagoon under greater water depths, e.g. six feet or more, are probably not as greatly disturbed by water turbulence generated by wind, the accumulation therein of large amounts of sulfur containing materials would probably encourage the proliferation of anaerobic organisms and the development of anoxic conditions (HORNE, 1969).

The sediments, particularly those in areas of restricted water movement such as parts of Banana Creek and the mosquito control impoundment, were rich in H_2S. The predominant bacteria in these sediments were members of the genus Clostridium, many species of which are active H_2S producers. Most clostridia produce H_2S by degrading organic materials, but certain of them, such as C. nigrificans, may employ sulfates and the lesser oxides of sulfur as proton acceptors (FROBISHER, 1969).

Members of the genera Clostridium and Bacillus are gram-positive, rod shaped organisms which characteristically form endospores. These structures may be produced in one area and transported mechanically to another by air or water currents, remaining dormant until they reach an environment suitable for germination. There remains, then, some questions of their origin. They may, in fact, be indigenous to the sulfide sediments or may be adventitious transients.

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BIBLIOGRAPHY

AKIMOTO, D., "Survey of the Benthos at Two Sites in the Indian River for Sulfide Ions," M.S. Thesis, Florida Institute of Technology, June, 1971.
BETZ, J.V., Report submitted to the Environmental Information Center, Winter Park, Florida, November 14, 1971.
FROBISHER, M., Fundamentals of Microbiology. London: W.B. Saunders Co., 1969.
HORNE, R.A., Marine Chemistry. New York: Wiley-Interscience, 1969.
MANUAL OF MICROBIOLOGICAL METHODS. Society of American Bacteriologists, New York: McGraw-Hill Book Co., 1957.
MARSHALL, R.S., STEENBERGEN, J.F. and McCLUNG, L.S., "Rapid Technique for the Enumeration of Clostridium perfringens," Applied Microbiology, vol. 13, no. 4, July, 1965, p. 559.
PORTER, J.R., Bacterial Chemistry and Physiology. New York: John Wiley and Sons, 1950.
SCHNEIDER, W.K., DUBBELDAY, P.S. and NEVIN, T.A., "Measurement of Wind Driven Currents in a Lagoon," submitted to Quat. J. Fla. Acad. of Sci. 1973.
SHERMAN, J., "A Study of the Interactions of Organophosphate Pesticides and the Microorganisms involved in the Sulfur Cycle," M.S. Thesis, Florida Institute of Technology, August, 1972.
STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER. 13th ed., New York: American Public Health Association, 1971.