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Title THE RESPIRATION CHARACTERISTICS OF OCEAN BAY SEDIMENTS AND SELECTED MARINE ISOLATES

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The respiration characteristics of Pacific bay sediments reveal valuable information regarding the activity of the indigenous marine flora. It is the primary aim of the present study to clarify the respiration characteristics of such sediments in terms of their capacity to use molecular oxygen and nitrate oxygen to effect oxidation of substrate carbon. The aerobic systems made use of an electrolytic respirometer to measure oxygen uptake whereas, with nitrate respiration, nitrate accumulation, and nitrogen gas production were used as the primary indices of respiration.

The electrolytic respirometer allowed experiments to be conducted both aerobically and anaerobically. This apparatus also permitted the simultaneous determination of oxygen uptake, carbon dioxide evolution, and the evolution of nitrogen or oxides of nitrogen from helium flushed flasks by gas chromatography and infrared spectroscopy. Experiments were conducted at temperatures of 15, 20,

and 28 C.

Sediment samples were collected aseptically from Yaquina Bay at Newport, Oregon, at different locations and various depths using a 1 1/2 inch Phlager core. To insure adequate gas exchange, a one to six sediment suspension in 100 ml volume was employed and stirred with electromagnetic stirrers. Three percent Rila salts and 0.2 molar phosphate buffer served as the diluent. Each flask was flushed for 25 minutes with the desired gas mixture.

The respiration rate of test sediments gave a QO_2 g of 86 µl with the inclusion of 1 mm glucose and 37,500 µg of NO_3^--N when incubated at 28 C. Upon the exclusion of glucose and nitrate a very low QO_2 g value of 7.5 µl was obtained. Interestingly enough, the observed temperature quotient (Q_{10}) of 1.45, was something less than the theoretical value of 2.0. It should be pointed out that aerobic sediments amended with nitrate and incubated under aerobic conditions showed no evolution of nitrogen gas or oxides of nitrogen. Evidently, a major part of the added nitrate was used in an assimilatory manner rather than via the dissimilatory route.

Anaerobic sediments produced 1,850 µg of nitrogen in 110 hours at 28 C, an equivalent of 1.3 µg of N_2 per hour per gram of dry weight sediment. It appeared that these sediments contained a high flora level capable of using nitrate as the terminal acceptor of hydrogen.

Although a majority of the sediments tested gave the above type

of respiration picture, one core area showed a decrease in respiration rate at the higher temperature and a corresponding increase in respiration at the lower temperature. It might be postulated that this core area contained a much higher psychrophile population.

The following denitrifiers were isolated from bay sediments: *Pseudomonas* sp., two *Achromobacter* sp., a *Brevibacterium* sp., a *Bacillus* sp., a *Vibrio* sp., a *Flavobacterium* sp., and a *Nocardia* sp. The *Achromobacter* isolate number 62 demanded extra attention in that it could not use nitrate. However, in this case nitrite served as the sole hydrogen acceptor. In other words, this organism possessed a nitrite reductase while void of a nitrate reductase. The use of infrared spectroscopy proved to be a valuable supporting tool in establishing the genus designation of these marine denitrifiers.

For nitrite reduction, the *Achromobacter* culture required a component present in beef extract. The nitrite reductase of the *Achromobacter* species showed a cytochrome c like component associated with the electron transport system. No copper moity was evident from purified cytochrome absorption spectra.

THE RESPIRATION CHARACTERISTICS OF OCEAN BAY SEDIMENTS AND SELECTED MARINE ISOLATES

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TABLE OF CONTENTS

Page

INTRODUCTION 1

REVIEW OF LITERATURE 2
Microbial Population of Marine Sediments 2
Organic Content of Marine Sediments 6
Respiratory Characteristics of Marine Bacteria . . 7
Aerobic Respiration 7
Nitrate Fermentation 8
Nitrate Respiration with E. Coli 10
Nitrate Respiration with Denitrifiers 14

EXPERIMENTAL MATERIALS AND METHODS 19
Collection of Sediment Core Samples 19
Respiration Apparatus and Procedure 21
Solutions and Media 23
Solutions 23
Media 24

Analytical Procedures 26
Total Carbon 26
Total Nitrogen. 27
Nitrate Analysis 27
Nitrite-Analysis 27
Carbon Dioxide Titration 27
Gas Analysis 28

Cytochrome Purification 28
Infrared Spectroscopy of Bacteria 29

EXPERIMENTAL RESULTS AND DISCUSSION 31
Bacterial Population 31
Carbon and Nitrogen Content of Marine Bay Sediments 34
Respiration of Unstirred Bay Sediments 37
Anaerobic Nitrate Respiration of Unstirred Bay Sediments 40
Respiration Characteristics of Stirred Bay Sediments 40
Denitrifying Isolates from Bay Sediments 57
Studies on Achromobacter 62 74

Page

SUMMARY 91

BIBLIOGRAPHY 96

APPENDIX 101

LIST OF FIGURES

Figure		Page
1	Location map of core areas in Yaquina Bay . . .	20
2	Aerobic respiration of bay sediments - core area 1	43
3	Aerobic respiration of bay sediment - core area 2.	51
4	Aerobic respiration of bay sediment - core area 4.	52
5	Aerobic respiration of bay sediment - core area 3.	54
6	Infrared scale spectra of three Gram positive bacilli	63
7	Infrared scale spectra of the three *Pseudomonas* strains	64
8	Infrared scale spectra of three Gram negative bacilli	66
9	Comparative infrared scale spectra of Gram positive and Gram negative bacilli	67
10	Infrared scale spectra of three Gram negative bacilli	72
11	Utilization of nitrogen sources by *Achromobacter* 62 and *Nocardia* 50-2	79
12	Spectra of oxidized and reduced cytochrome purified from *Achromobacter* 62	89

LIST OF TABLES

Table		Page
I	BACTERIAL PLATE COUNTS OF MARINE BAY SEDIMENTS	32
II	TOTAL CARBON AND NITROGEN OF MARINE SEDIMENTS FROM YAQUINA BAY, NEWPORT, OREGON	35
III	AEROBIC RESPIRATION OF UNSTIRRED BAY SEDIMENTS AFTER 20 DAYS INCUBATION	39
IV	ANAEROBIC RESPIRATION OF UNSTIRRED BAY SEDIMENTS AFTER 20 DAYS INCUBATION . . .	41
V	AEROBIC RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION	44
VI	ANAEROBIC NITRATE RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION	46
VII	AEROBIC RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION	47
VIII	ANAEROBIC NITRATE RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION	48
IX	AEROBIC RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION	49
X	ANAEROBIC NITRATE RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION	50
XI	OBSERVED TEMPERATURE EFFECT OF AEROBIC AND STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION	55
XII	OBSERVED TEMPERATURE EFFECT OF ANAEROBIC NITRATE RESPIRATION AND STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION . .	56
XIII	AEROBIC RESPIRATION OF DEEP BAY SEDIMENTS AFTER 110 HOURS INCUBATION	58

Table		Page
XIV	ANAEROBIC RESPIRATION OF DEEP BAY SEDIMENTS AFTER 110 HOURS INCUBATION	59
XV	DESCRIPTION OF DENITRIFYING MARINE SEDIMENT ISOLATES	60
XVI	THE EFFECT OF VARIOUS HYDROGEN ACCEPTORS ON N_2 PRODUCTION AND ON CELLULAR GROWTH OF *ACHROMOBACTER* 62	76
XVII	THE EFFECT OF VARIOUS HYDROGEN ACCEPTORS ON N_2 PRODUCTION AND ON CELLULAR GROWTH OF *NOCARDIA* 50-2	77
XVIII	ANAEROBIC NITROGEN SOURCES TESTED WITH *ACHROMOBACTER* 62	78
XIX	TIME STUDY OF NITROGEN GAS PRODUCTION BY *ACHROMOBACTER* 62	80
XX	THE CO-FACTOR REQUIREMENTS OF *ACHROMOBACTER* 62 FOR NITROGEN GAS PRODUCTION .	82
XXI	THE EFFECT OF YEAST EXTRACT ON NITROGEN GAS PRODUCTION AND CELLULAR GROWTH OF *NOCARDIA* 50-2	84
XXII	THE EFFECT OF YEAST EXTRACT ON NITROGEN GAS PRODUCTION AND CELLULAR GROWTH OF *ACHROMOBACTER* 62	85
XXIII	ADAPTIVE OR CONSTITUTIVE NATURE OF NITRITE REDUCTASE SYSTEM OF *ACHROMOBACTER* 62 .	86

LIST OF DESCRIPTIVE CHARTS

Chart		Page
1	Morphological and Cultural Characteristics of 50-2 (Nocardia)	101
2	Physiological Reactions of 50-2 (Nocardia) . . .	102
3	Morphological and Cultural Characteristics of 51 (Pseudomonas)	103
4	Physiological Reactions of 51 (Pseudomonas) . .	104
5	Morphological and Cultural Characteristics of 53 (Breviabacterium)	105
6	Physiological Reactions of 53 (Brevibacterium). .	106
7	Morphological and Cultural Characteristics of 51-7B (unknown)	107
8	Physiological Reactions of 51-7B (Unknown) . . .	108
9	Morphological and Cultures Characteristics of 17 (Achromobacter)	109
10	Physiological Reactions of 17 (Achromobacter) . .	110
11	Morphological and Cultural Characteristics of 50-7 (Bacillus)	111
12	Physiological Reactions of 50-7 (Bacillus). . . .	112
13	Morphological and Cultural Characteristics of 51-1A (Pseudomonas)	113
14	Physiological Reactions of 51-1A (Pseudomonas) .	114
15	Morphological and Cultural Characteristics of 59 (Vibrio)	115
16	Physiological Reactions of 59 (Vibrio)	116

Chart | Page

17 Morphological and Cultural Characteristics of 36 (*Flavobacterium*) 117

18 Physiological Reactions of 36 (*Flavobacterium*) . 118

19 Morphological and Cultural Characteristics of 50-4 (*Pseudomonas*) 119

20 Physiological Reactions of 50-4 (*Pseudomonas*) . 120

21 Morphological and Cultural Characteristics of 62 (*Achromobacter*) 121

22 Physiological Reactions of 62 (*Achromobacter*) . 122

THE RESPIRATION CHARACTERISTICS OF OCEAN BAY SEDIMENTS AND SELECTED MARINE ISOLATES

INTRODUCTION

Little information on the over-all microbial activity of offshore marine sediments is available. A comparison of terrestrial and marine environments shows that 72 percent of the earth's surface is covered with water and 98 percent of the world's water occurs in the oceans of the world. The tremendous reservoir of organic and inorganic substrates and associated microbial activity in this nearly infinitive environment stands as one of the important links in the marine food chain. It is recognized that similar biological processes found in soil probably play an integral part in the nutritional cycle of the marine system. In this regard, the total respiration characteristics of bay sediments should give valuable insight into the activity of the marine population.

More specifically, in such marine environments one might conjecture that molecular oxygen would become a limiting respiration factor and other hydrogen acceptors such as nitrate, sulfate, and organic compounds might be used. It was the primary aim of this study to clarify the respiration characteristics of such sediments in terms of their capacity to use molecular oxygen and a second acceptor, nitrogen-oxygen.

REVIEW OF LITERATURE

By employing numerous basic techniques previously applied in the soils field, Cretes, Fischer and Russell demonstrated in the late 1880's the existence and activity of a general heterotrophic flora in sea water and marine sediments. These heterotrophs were found to be similar to the soil bacteria described by Winogradsky, Beijerinck and other early investigators. Subsequently, the importance of bacteria in the life cycle of the marine environment was acknowledged by such outstanding marine sediment pioneers as Waksman, Carey, and ZoBell.

Microbial Population of Marine Sediments

Drew (1912) reported an average of 1.6×10^7 bacteria per milliliter in mud obtained near the island of Andros in the West Indies. Lloyd (1932) demonstrated 3.0×10^5 bacteria per gram of dry sediment from the Clyde Sea. A year later Bavendaman (1933) from sediment deposits in the Bahama Islands calculated 1.7×10^7 bacteria per gram of wet mud. ZoBell and Anderson (1936) working off the coast of Southern California reported values of 3.8×10^6 bacteria per gram of wet sediment. Also off the California coast basin, Emery and Rittenberg (1952) demonstrated viable counts ranging from 3.5×10^4 to 1.8×10^7 bacteria per gram of wet sediment near the surface, and from 1×10^2 to 3.5×10^3 at the four foot core depth.

In several mid-Pacific depths of 1,700 to 5,900 meters, Morita and ZoBell (1955) reported 10 to 10^4 bacteria per gram in the topmost layer of pelagic sediments. Isatchenko (1937), employing the direct microscope technique, found 11 x 10^9 bacteria per gram of wet sediment in the Kara Sea. From the Gulf of Manaar, Velankar (1955) obtained values of 2.8 x 10^5 to 6.8 x 10^6 per gram weight of sediment. In the Okhotsk Sea adjacent to the Pacific Ocean, Kriss (1959) reported 10 x 10^2 to 4 x 10^4 heterotrophic bacteria per gram of wet sediment from the surface layer at depths of 2000 to 8000 meters. Oppenheimer (1960) observed aerobic counts of 1 x 10^3 to 5 x 10^6 per gram of sediment. In various sample areas, both Woods (1953) and Oppenheimer (1960) generally reported somewhat lower anaerobic counts.

The most biologically active area of marine sediments is the mud-water interface. The majority of investigators vividly demonstrated a sharp decrease in bacteria count with increase in core depth. With increased depth of mud, bacteria capable of growing aerobically decrease, and facultative anaerobes generally predominate. As a group, the sediment microflora are physiologically quite versatile, being able to catalyse the transformation of a large variety of organic and inorganic compounds. The predominant bacteria (80 percent) found by ZoBell and Upham (1944) were Gram negative bacilli. There seems to be a characteristically dominant marine bacterial flora with the

majority of species falling in decreasing order into the following genera: Pseudomonas, Vibrio, Flavobacterium, Achromobacter, and Bacillus.

Disregarding all the numerous factors responsible for variation in the distribution of microbial populations, denitrifiers have been reported in all areas of the marine environment. However, judging from various ecological studies, the majority of the denitrifiers are located in the water-sediment interface and the bottom deposits. Approximately half of the bacteria indigenous to the marine environment are capable of reducing nitrate to nitrite, while a substantially smaller number (5 to 10 percent) reduce nitrite to nitrogen or oxides of nitrogen.

Drew (1912) in his early work attributed the precipitation of $CaCO_3$ to the action of denitrifying bacteria. The two organisms involved were classified as Bacterium calcis and Pseudomonas calcis. Brandt (1929) attributed the lack of phytoplankton to the depletion of nitrate by denitrifiers. On the other hand, Waksman et al. (1933) concluded that limitation of available energy sources curtailed marine denitrifying activity. In discussing the mechanism of denitrification, Lloyd (1931) described B. Vibrio costatus as an active marine denitrifier. Kadota (1951) showed a symbiotic denitrification when Vibrio purpureus oxidized agar.

In a general study of marine denitrifiers located off the South

India coast, Screenivasan and Venkataraman (1956) isolated 32 organisms capable of reducing nitrate to nitrogen or oxides of nitrogen. The overall reactions of 20 of these strains indicated they could be classified into five large groups. The five groups, A to E, were based on general nutritional requirements and included all 20 isolated *Pseudomonas* species. In one area, 10 miles off Tuticorin, among 95 bacterial isolates 13 were denitrifiers. Interestingly enough, in the sea water where the denitrifiers were isolated, the dissolved oxygen content measured in the range of 4.0 to 6.3 mg per liter. Brisou and Vargues (1963) studied 131 strains isolated from sea water, estaurine muds, and fishes. Of these 131 strains, 77 were proteolytic, 77 were nitrate reducers, 51 had both proteolytic and nitrate reducing activity, and 26 were inactive. Although actual numbers were not cited, the denitrifiers belonged in decreasing numbers to the genera: *Erwinia*, *Pseudomonas*, *Vibrio*, and *Achromobacter*. Other genera mentioned by several workers included *Flavobacterium*, *Spirillum*, *Desulfovibrio*, and *Micrococcus*. None of these marine denitrifiers except *Pseudomonas calcis* and possibly a few other organisms have been completely studied or described. Bergey's Manual lists 13 species in the genus *Pseudomonas* which are sea water to brine inhabitants. However, the manual does not mention gas production from nitrate.

Organic Content of Marine Sediments

According to Reuszer (1933) the distribution of bacteria in marine sediment is directly correlated with the organic content. An average of four percent organic matter in a shallow bay sediment near Rockport, Texas, was reported by Shepard and Moore (1955). Volkmann and Oppenheimer (1959) found some sediments to contain an average of five percent organic matter. Emery and Rittenberg (1952) observed in California basin sediments that certain locations showed greater than ten percent organic matter on a dry weight basis. However, the majority of investigators feel that these values of Emery and Rittenberg are the exceptions, rather than representative values.

Variations in total organic matter are considered to reflect the general nutrient content of sediments. The ratio of total organic matter to organic carbon varies from 1.6 to 1.9. Emery and Rittenberg (1952), in discussing this problem, pointed out that the true ratio depends on the nature of the source material and on the state of oxidation of the organic matter. They also pointed out that differences as high as two percent organic carbon may occur when comparing results between wet combustion and empirical titration methods. Sediments taken by Orr and Emery (1956a) off the Southern California coast averaged 4.23 percent carbon.

Trask (1934), using the Kjeldahl procedure for total nitrogen and a titration method for organic carbon, obtained C:N values of 8.3 to 13.0. Vinogradov (1953) showed that the elemental composition of total organic matter from the Santa Catalina Basin was: carbon 77.6 percent, oxygen 9.5 percent, hydrogen 9.6 percent, nitrogen 2.6 percent, and sulfur 0.7 percent. Emery (1960) obtained total nitrogen data from seven basins off the Californian coast and reported values between 0.21 and 0.68 percent nitrogen for surface sediments.

Respiratory Characteristics of Marine Bacteria

Aerobic Respiration

Literature reports on the respiratory characteristics of marine sediment are conspicuous by their absence. Merket *et al.* (1957) compared the respiratory activity of a marine bacterium (A-11-3B 1) from the Fort Johnson collection with *Escherichia coli*. Data were obtained using a Warburg respirometer in the absence and presence of sea water. The isolate, with glucose as the substrate in the presence of sea water, consumed 212.4 µl oxygen per hour at 28 C. The same isolate in phosphate buffer with glucose consumed 83.5 µl oxygen per hour at 28 C. The endogenous value was 48.0 µl of oxygen per hour at 28 C, while with Casamino acids (0.17 percent) and glucose (0.03 M) the values were 191.8 µl and 21.3 µl, respectively. With

E. coli conditions were reversed. Both the endogenous and substrate respiration were greater in phosphate buffer than in sea water.

Johnson (1936) studied oxygen uptake for 25 pure cultures freshly isolated from sea water. The rate of oxygen uptake varied between 2×10^{-9} and 132×10^{-9} mm^3 per cell per hour, with an average for all species of 21.3×10^{-9} mm^3. In relation to temperature and oxidation of glucose, the Q_{10} average was 3.8 at 5 to 15 C and 2.4 at 15 to 25 C.

ZoBell (1940) estimated that marine bacteria actively respiring in sea water consumed an average of 20.9×10^{-12} mg of oxygen per cell per hour at 22 C. Finally, ZoBell and Feltham (1942) estimated that the top centimeter of each square meter of Mission Bay sediment consumed 8.4 to 25.2 mg of oxygen per day at 20 C.

Nitrate Fermentation

On the basis of metabolic function, Taniguchi (1961) divided nitrate reduction into three areas: nitrate fermentation, nitrate assimilation, and nitrate respiration. Egami *et al.* (1957) used the term nitrate fermentation to represent the nitrate reduced by two-day old bean seed embryos. The homogenate of the young seedlings aerobically reduced nitrate in the presence of sucrose beyond the nitrite stage. The aerobic system was thought to represent the nitrate-assimilating system. However, using the same seedling homogenate,

a nitrite-accumulating reduction was evident under anaerobic conditions. The enzyme catalyzing this reaction was a DPNH-linked nitrate reductase containing a sulfhydryl and metaloflavoprotein component along with a terminal cytochrome b particulate fraction. Preliminary evidence indicated a tendency for the nitrate to be anaerobically reduced to nitrite and then transported to the seedlings where nitrite was aerobically assimilated. The physiological interpretation of a non-assimilative anaerobic reduction without cytochrome participation was called nitrate fermentation.

The organism *Clostridium welchii* showed nitrate reductase activity in the absence of free oxygen. Katsura *et al.* (1954) and Koyama (1961) demonstrated nitrite accumulation with intact cells and cell-free preparations. Bard and Gunsalus (1950) showed, via the *Clostridium* DPN-linked triose phosphate dehydrogenase and DPN, that fructose 1-6 diphosphate can be enzymatically converted into two molecules of triose phosphate. Also supporting this type of nitrate fermentation was a DPN-linked system employing ethanol as an electron donor. Koyama (1961) proved that the latter anaerobe can carry out a non-phosphoroclastic decomposition of pyruvate to acetate with molecular hydrogen serving as the physiological donor of hydrogen for nitrate reduction. Employing the soluble *Clostridium* system of DPN-linked or hydrogen-nitrate reductase, no effect was observed with externally added flavin derivatives.

Because of the ubiquitous distribution of nitrates in the marine environment, nitrate fermenters would probably occur in all areas, particularly in marine sediment. Gram positive spore formers have been reported by several workers, although direct nitrate fermentation studies are not available. Bavaendamn (1932) observed various kinds of cellulose fermenters, although nitrate fermenters were not specifically mentioned.

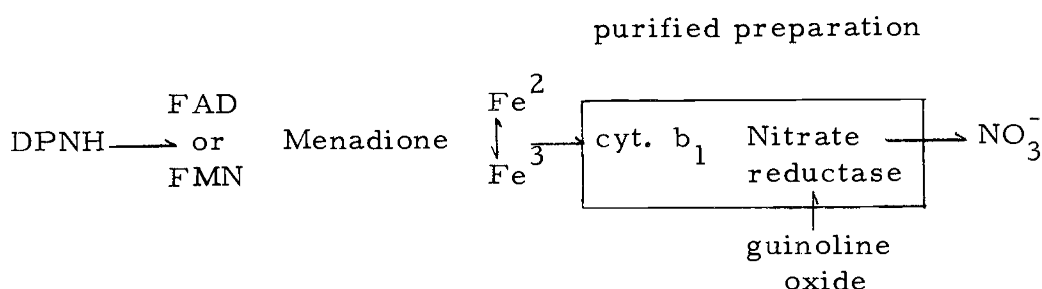
Nitrate Respiration with *E. coli*

Using a non-denitrifying bacterium *E. coli*, Quastrel *et al.* (1925) demonstrated nitrate reductase activity when the cells were grown anaerobically on lactate as the sole carbon source. It was observed that several other facultative anaerobes performed a similar process of replacing molecular oxygen with nitrate oxygen. In the growth of such obligate nitrate-reducing organisms nitrate was not reduced beyond the nitrite level. Collectively, facultative anaerobes capable of using nitrate oxygen are designated as nitrite-accumulating and nitrate-respiring bacteria.

With aerobically grown *E. coli* cells, Yamagata (1938) obtained cell-free extracts capable of reducing nitrate. In reconstructing the system five essential components were used: the electron donor (ethanol), alcohol dehydrogenase, intermediary electron carrier methlene blue, and cell-free extract containing the nitrate reductase,

and the acceptor nitrate. Sato and Egami (1949), employing a partially purified preparation, demonstrated the participation of cytochrome b as an anaerobic carrier of electrons from formate to nitrate. The participation of cytochrome b in the particulate system was subsequently shown by marked inhibition of the DPNH-nitrate reductase and by inhibition of cytochrome b oxidation.

Nicholas and Nason (1955), using *E. coli* strain B grown anaerobically, extracted and purified a soluble DPNH-nitrate reductase of a metalloflavoprotein nature and having molybdenum as a metal constituent. This soluble nitrate reductase is entirely different from the particulate reductase. No participation of a cytochrome b was observed. For simplicity, a schematic drawing is shown to summarize some of the data accumulated for the *E. coli* soluble system (Tanguchi 1961):



Comparing the nitrate reductase systems of *E. coli* (86 NCTC Oxford), Heredia *et al.* (1960) used cells grown aerobically on both synthetic and complex media. These cells were capable of growing

by assimilating nitrate. In addition to the flavin and cytochrome b_1 systems a second non-flavin, a non-cytochrome and a non-oxygen sensitive system were present. This latter transport sequence was responsible for the majority of the cells activity.

It is worth noting the work of Taniguchi and Itagaki (1960) where *E. coli* grown anaerobically in a nutrient-rich complex was stimulated by the addition of nitrate. Simultaneously, the addition of nitrate stimulated the cellular level of cytochrome b_1 and formate dehydrogenase. The specific activity of formate-nitrate reductase in *E. coli* grown under these anaerobic conditions was twenty times greater than with cells grown aerobically. Although the organism appeared to grow anaerobically by respiring nitrate, the induced synthesis of nitrate reductase was observed to proceed closely in parallel with both bacterial growth and nitrite accumulation. Of particular interest was the observation that nitrate reductase activity of the young cells was localized in the soluble supernatant solution obtained by centrifugation at 105,000 g for one hour. Conversely, the nitrate reductase activity of cells harvested at the end of the logarithmic growth phase was localized exclusively in the large particles (5,000 to 20,000 g-fraction) together with most of the photohaem and formate dehydrogenase activity. It was thus concluded that the induced synthesis of the nitrate-respiring system might be implicated in a special process in which functional subcellular structures were evolved from soluble materials.

Solubilized nitrate reductase activity was purified 1000 fold over the initial cell paste. Contaminating flavoproteins, simultaneously solubilized were shown to have no nitrate reductase activity and were therefore removed. The molecular weight of the enzyme protein was calculated to be approximately one million. The enzyme protein contained in non-dialzable form one atom of molybdenum and 40 atoms of non-heme iron per molecule. The amber colored homogenate exhibited a broad peak at 445-450 mμ which disappeared rapidly upon addition of nitrate. Neither the oxidized nor reduced spectra showed any similarity to the cytochromes. The data indicated that the E. coli nitrate reductase should be characterized as a metallo protein with two constituents, molybdenum and iron.

The lactose fermentation is not considered indigenous to the marine environment. Nevertheless, marine sediments harbor many organisms possessing a nitrate reductase similar to E. coli. The facultative organism Vibrio algosus rapidly reduced nitrate to nitrite and produced acid without gas from a variety of sugars. Micrococcus sedentarius isolated from bay sediments ferments glucose and maltose while reducing nitrate to nitrite. Another organism, Pseudomonas sessilis, reduced nitrate to nitrite and fermented glucose and xylose with acid but no gas. Likewise, Achromobacter aquamarinus rapidly accumulated nitrite from nitrate while producing only acid from glucose and maltose. Many other genera have been found to contain the

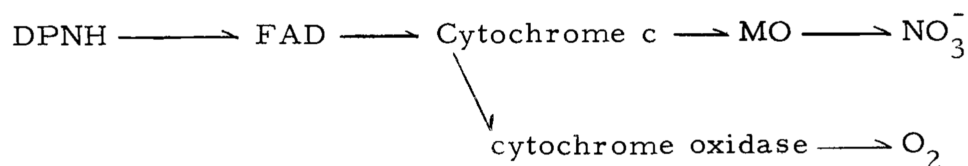
nitrite-accumulating system. However, it should be emphasized that in each case investigations regarding the nitrate reductase of these marine organisms are absent from the literature.

Nitrate Respiration with Denitrifiers

The term denitrification can be applied to nitrate respiration when the microorganisms using nitrate-oxygen as a terminal electron acceptor reduce the nitrate beyond nitrite. Depending upon the organism and the chemical and physical conditions of the environment, the reduced product may include nitrite, nitric oxide, nitrous oxide, molecular nitrogen, and possibly other oxidation states of nitrogen.

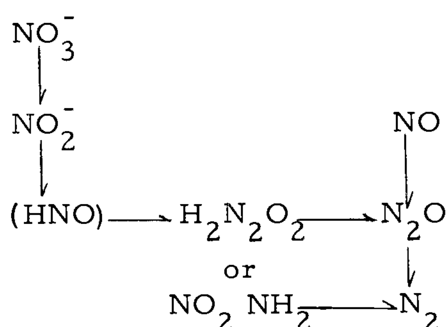
An adequate demonstration of cell-free preparations showing coupling of phosphorylation to nitrate respiration has not yet been reported. However, obvious physiological and enzymological similarities have indicated an energy-yielding reaction.

Fewson and Nicholas (1961), employing a denitrifying bacterium, *Pseudomonas aeruginosa*, showed close similarity to the nitrate-reductase of *E. coli*. The purified DPNH-specific sulhydryl flavenzyme contained cytochrome c and a molybdenum component. They suggested the following electron transport sequences:



Several workers, such as Kluyver (1953), Delwiche (1956), Nason and Takahashi (1958), demonstrated the adaptive nature of the nitrate-reducing enzyme. This was generally determined by the lag period in the reduction of nitrate by cells not grown in a nitrate-containing medium. Wainwright and Nevill (1956) showed that auxotrophic mutants of _E. coli_ that required specific amino acids did not form nitrate reductase. These mutants required ribonucleic acid for the synthesis of the nitrite reductase enzyme. Although the majority of the experiments illustrated the adaptive nature of the respiratory nitrate reductase, a few reports failed to support this viewpoint (Straughn, 1955; Farkas-Hinsley and Artman, 1957).

The scheme outlined below has been postulated for the dissimilation of nitrate. However, the participation of all components have not been ascertained.



Obviously, the first step involves the conversion of nitrate to nitrite mediated by a respiratory nitrate reductase. The next step

in denitrification involves a second two-electron change resulting in the formation of a hypothetical intermediate nitroxyl or perhaps a nitroxyl-enzyme derivative. With cell-free preparations of Pseudomonas stutzeri, Najjar and Chung (1956) reported a spontaneous dimerization of nitroxyl to hyponitrite or nitramide. This might then be followed by an enzymatic or spontaneous reaction with the removal of water to yield nitrous oxide (N_2O). Allen and Van Niel's (1952) original claim that Pseudomonas stutzeri hydrogenated nitramide to nitrogen gas was subsequently rejected by Kluyver and Verhoeven (1954). Later, they showed that neither Micrococcus denitrificans nor Pseudomonas aeruginosa evolved nitrogen gas from sodium hyponitrite.

Noteworthy is the work of Sacks and Barker (1952) regarding the over-all denitrification pathway. They concluded that nitrous oxide (N_2O) was not an obligatory intermediate in the formation of molecular nitrogen. Confirming this report was Allen and Van Niel's (1952) observations that the conversion in Pseudomonas stutzeri of nitrous oxide to N_2 was inhibited by cyanide, whereas the reduction of nitrate was unaffected.

Denitrification experiments of Najjar and Chung (1956) with crude cell-free extracts (P. stutzeri) showed reduction of nitrite to nitric oxide (NO) when TPNH and DPNH served as electron donors. In addition, a two-fold stimulation with FAD or FMN was observed. The crude ammonium sulfate fraction which contained cytochrome c with

copper and iron was inhibited by metal-binding agents. These data were subsequently confirmed by Walker and Nicholas (1961), employing a 600-fold purified nitrite reductase from P. aeruginosa. However, neither TPNH nor DPNH could serve as electron donors in the latter system. Fewson and Nicholas (1960) characterized a nitric oxide reductase also from P. aeruginosa, as a non DPN linked flavoprotein containing iron but not copper. Yamanaka and Okunuki (1960) obtained from P. aeruginosa a cytochrome oxidase which also catalysed the reduction of nitrite by reducing a cytochrome c like component.

Working with cell-free extracts of Pseudomonas denitrificans, Iwasaki and Mori (1958) indicated that, in the presence of lactate nitrate was reduced only to nitrogen gas; whereas, in the absence of lactate, nitrous oxide was almost exclusively evolved. They also reported that hyponitrite could not be utilized, although hydroxylamine had a stimulatory effect on the evolution of nitrogen gas. From these data, one could surmise that a portion of the nitrite was reduced to an intermediate such as hydroxylamine, which in turn reacted with the remainder of nitrite. Two years later, Iwaski (1960) purified two inactive fractions from his extract which, upon recombination, served as a denitrifying system. The first fraction had a typical cytochrome c-type absorption spectrum, while the other fraction was considered

the denitrifying enzyme. The denitrifying enzyme appeared red, due to the so called "crypotochtochrome c".

EXPERIMENTAL MATERIALS AND METHODS

Collection of Sediment Core Samples

The sediment samples and initial material for cultural isolation employed throughout this study were collected from four areas in Yaquina Bay at Newport, Oregon. The sample areas were chosen so that at least four or five feet of water covered the bay floor during all tidal changes. Likewise, consideration was given to the choice of location so as to minimize terrestrial and other contaminating sources. When additional samples were required,landmarks were used to locate as closely as possible the original core locations. Each core area was referred to by number as indicated on the map of Yaquina Bay (Figure 1).

The sediments were obtained aseptically using a 1 1/2 inch Phleger core. Core liners, core nose, and core catcher were chemically sterilized and stored in a 250 ppm hypochlorite solution. Immediately after collection the samples were transported to the laboratory in an ice chest, stored overnight at 5 C and used in subsequent experiments within 24 hours. This procedure not only provided standard core samples but also insured against loss of activity due to prolonged storage.

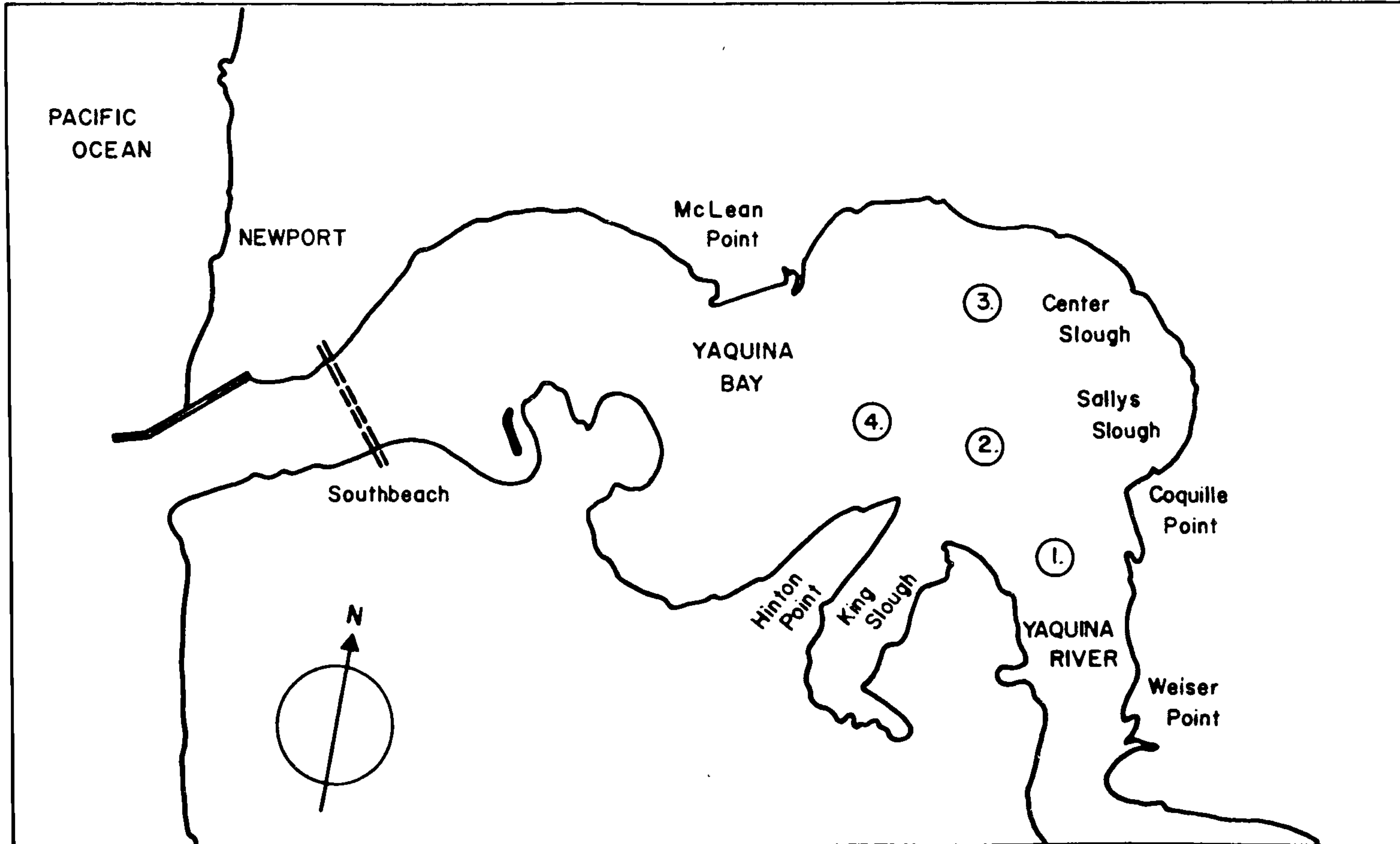


Figure I. Map of Yaquina Bay showing core locations numbers 1, 2, 3 and 4.

Respiration Apparatus and Procedure

Respiration experiments were conducted using the electrolytic respirometer described by McGarity *et al.* (1958) which was subsequently modified by various workers. The complete apparatus system was housed in an insulated cabinet in which was fixed a cooling and heating system permitting temperature controlled experiments in the 10 to 40 C range. In aerobic experiments the electrolytic respirometer enabled measurement of oxygen consumption, carbon dioxide evolution, and utilization of a specific substrate. The evolution of nitrogen and oxides of nitrogen was checked by gas chromatography and infrared spectroscopy. The use of electromagnetic stirrers insured adequate gas exchange. After the desired media and nutritional adjustments were made, the carbon dioxide traps containing 10 ml of a 10 percent sodium hydroxide solution, were suspended for carbon dioxide absorption. Finally, the manifolds were attached and the aerobic flasks flushed for 30 minutes with a 20 percent oxygen and 80 percent helium gas mixture. In the anaerobic system for nitrate respiration, nitrate accumulation and nitrogen or its oxides were the primary indices of nitrate-oxygen being used as the final hydrogen acceptor. Evolution of nitrogen and oxides of nitrogen were calculated from gas chromatography analysis. Anaerobic flasks were prepared similarly, except the flushing gas used was 100 percent helium. The

300 ml respirometer flasks, manifolds, carbon dioxide traps, and magnetic stirring bars were autoclaved for 30 minutes at 121 C.

Upon termination of the experiment, the flasks were attached to a manometer which enabled appropriate gas samples to be drawn for analysis. Gas chromatography calibration curves were used throughout for carbon dioxide, nitrogen, and oxides of nitrogen, whereas infrared analysis provided verification of the presence of these gases. Gas analysis also provided an efficient check on anaerobic flasks for possible leakage. Aliquots of media were pipetted for chemical tests following completion of gas analysis. The remaining media were acidified to drive out any trapped carbon dioxide.

Respiration experiments were conducted with undiluted sediments, with a 1-6 suspension of sediment, and with pure cultures isolated from these sediments. Various nutritional amendments were involved. In the case of the undiluted samples, 100 grams (wet basis) of sediment were aseptically transferred to the respiration flasks. However, the actual data were corrected for sediment water content which was obtained by the 24 hour oven-dry method. Rila salt (synthetic sea water 3.5 percent) served as the diluient for the 1-6 sediment-water suspension. For pure culture experiments a 1.0 ml inoculum (OD = 0.5) was used for growth studies and a final OD of 0.5 for mass cell experiments. The cells were grown under appropriate conditions upon desired media, harvested by centrifugation, and washed twice with 0.2 molar

phosphate buffer. Experiments were conducted at three temperatures 15, 20, and 28 C.

Solutions and Media

The test solutions were made just prior to each respiration experiment and sterilized by autoclaving. If the solutions were heat labile sterilization was accomplished by filtration through a 0.22 μ millipore filter. Where required, the diluent used was distilled CO_2-free water. Listed below are the concentrations for the solutions and media employed throughout this study.

Solutions

Ammonium phosphate; $(NH_3)_2 HPO_4$; 1mM, 2mM

Nitrate (potassium salt); 37,500 μg. NO_3^--N (750 ppm)

Nitrite (potassium salt); 21,000 μg. NO_3^--N (140 ppm)

Rila salt synthetic sea water; 3.5 percent, 5 percent, 7.5 percent, 10 percent, 12 percent (Rila products, Teaneck, N. J.)

Peptone; 0.5 percent solution

Protein hydrolysate;

Yeast extract 1 mg per ml

Beef extract (Difco); 1 mg per ml

Glucose; 1 mM, 2 mM

Phosphate buffer; 0.2 molar, pH's 6.8, 7.0 and 7.3

Media

Unless otherwise stated, all chemicals used were reagent grade and all ingredients were added in grams per liter of water. Rila salt (3.5 percent) synthetic sea water served as the diluient unless otherwise stated. With the exception of aerobic growth, NO_3^- served as the ultimate hydrogen acceptor for nitrate respiration.

Carbohydrate Broth Base

Tryptose	10.0 g
Beef extract	3.0 g
Bromocresol purple	0.016 g
Rila salt	35.0 g

Dimmick Synthetic Medium (1947)

Potassium phosphage (dibasic)	0.5 g
Sodium chloride	0.5 g
Magnesium sulfate	0.2 g
Potassium nitrate	2.0 g
Glucose	10.0 g
Yeast extract	1.0 g
pH adjusted 7.2	

Glucose Peptone Medium

Peptone (Bacto)	7.0 g
Glucose	5.0 g

Hugh-Liefson Medium (1953)

Trypticase	0.2%
Sodium chloride	0.5%
Potassium phosphate (dibase)	0.03%
Carbohydrate	1.0%
Bromothymol blue	0.003%
Agar	0.3%

Koser's Citrate Medium (Difco Manual p. 55); Lead Acetate Agar

(Difco Manual p. 169); Litmus Milk (Difco Manual p. 192); Nitrate Broth (Difco Manual p. 184); Nitrite Broth (Difco Manual p. 184); Nutrient Agar and Broth (Difco Manual p. 32); Nutrient Gelatin (Difco Manual p. 32); Thioglycollate Broth (Difco Manual p. 199); Tryptophan Broth (Difco Manual p. 269); Urea Broth (Difco Manual p. 170).

Nitrate Reducing Plating Medium

Potassium nitrate	0.5%
Trypticase extract	1.0%
Potassium phosphate (dibasic)	0.05%
Yeast extract	0.1%
Glucose	0.1%
pH adjusted 7.3	

Nitrate Reducing Solution

Potassium sodium tartrate	1.0%
Sodium tartatte	1.0%
Potassium nitrate	2.0%
Potassium phosphate (dibasic)	0.05%
pH adjusted 7.3	

Nitrate Reducing Testing Medium (NRT)

Potassium nitrate	1.0 g
Potassium phosphate (dibasic and monobasic)	1.0 g
Beef extract	3.0 g
Peptone	5.0 g
Yeast extract	1.0 g
Glucose	10.0 g
Magnesium sulfate	0.5 g
Ammonium nitrate	1.0 g

Reuszer Medium

Peptone	1.0 g
Glucose	1.0 g
Potassium phosphate	0.05 g
Yeast extract	1.0 g
pH adjusted 7.6	

Rila Salts Marine Mix

Synthetic Sea Water	3.5%
Rila Products, Teaneck, N. J.	

Starch Agar

Soluble starch	10.0 g
Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g

Analytical Procedures

Total Carbon

Total organic carbon was determined by dry combustion of 0.5 grams of sediment at 1200 C after Bollen[1] and Moodie. This determination depends on complete oxidation of all organic carbon to carbon dioxide present in the sediment. The carbon is oxidized in a stream of commercially compressed oxygen which has been scrubbed free of impurities by passing through various trains of sulfuric acid, carbon dioxide, absorbent Ascarite, and anhydrous magnesium perclorate. After carbon oxidation the stream of gas leaves the furnace and passes through both a dust trap and a manganese dioxide activated trap which filter out nitrogen oxides, sulfur oxides, and halogen gases. Most of the remaining water is removed by absorption in concentrated sulfuric acid and anhydrous magnesium perchlorate. The carbon dioxide

[1] Personal communication with Dr. W. B. Bollen.

is absorbed by Ascarite and the amount calculated by the net increase in column weight.

Total Nitrogen

Total nitrogen or Kjeldahl analysis was conducted as described in the Official Methods of Analysis of the Association of Official Agricultural Chemists, Ninth Edition, 1960.

Nitrate Analysis

Nitrate-nitrogen determinations were conducted according to the procedure of Eastoe and Pollard (1950). Due to periodical ion interference rigid controls were required.

Nitrite-Analysis

Saltzman's (1954) modified technique using N-(1-napthyl)-thylene-diamine dihydrochloride was employed for nitrite-nitrogen determination.

Carbon Dioxide Titration

Absorbed carbon dioxide was measured by titration with standardized acid using a Beckman Model K automatic Titrator.

For all standard cell suspension and color intensity measurements a Bausch and Lomb "Spectronic 20" was used.

Gas Analysis

Gas analyses were performed by the use of a Beckman GC-2 Gas Chromatograph. Determinations were conducted at 40 C with helium as the carrier gas. The analysis for nitrogen, oxygen, and nitrogen dioxide made use of a 12 foot molecular sieve column, whereas, for carbon dioxide and nitric oxide, a 1.5 foot activated charcoal column was employed. Trace amounts of other gases, carbon dioxide, and oxides of nitrogen were verified by using a 10 cm gas cell on a Beckman IR5 Infrared Spectrophotometer.

Cytochrome Purification

For the cytochrome studies the *Achromobacter* 62 and *P. stutzeri* nitrate-reducing test media were used. The cells were collected by centrifugation and washed twice with chilled 0.2 molar phosphate buffer. The cells were lysed by the freeze-press method, and the debris was removed by centrifugation. The remaining cell-free extract was slightly turbid and fairly viscous. The extract was precipitated by adding to previously chilled -5 C acetone. The addition was made slowly while constantly stirring the acetone. The precipitate was washed with chilled acetone and dried over calcium chloride in a vacuum desicator. The precipitate was then solubilized in 0.2 molar phosphate buffer (pH 7.0) and

separated from the debris by centrifugation. The presence of the cytochromes was confirmed by measurement of the absorption spectrum with a Cary number 11 Recording Spectrophotometer.

Infrared Spectroscopy of Bacteria

After growth on appropriate media, the cells were collected by centrifugation and then washed in 0.2 molar phosphate buffer (pH 7.0). All cultures were incubated at 20 C for 18 hours. After the bacteria were washed, a dense aqueous homogenous suspension was carefully prepared. From this suspension 0.25 ml were pipetted on silver chloride discs. The cells were then worked back and forth several times to obtain a more uniform film. The sample was air-dried in a tilted position to obtain a gradient in the film. Since the silver chloride discs are light sensitive, the films were air-dried in a dark box. The discs were mounted in special holders for recording.

The instrument used in this study was a Beckman IR5 Double Beam Recording Infrared Spectrophotometer. Proper functioning and standardization of the instrument was assured by recording the infrared spectrum of a polystyrene film standard. The instrument was advanced to 8.00 microns and then stopped. The dried sample of variable film thickness was manipulated until the transmission through the sample film registered exactly 50 percent transmission. The disc

was then anchored in this position and the instrument reversed to two microns and scanned through 2-16 microns. The disc was readjusted to 50 percent transmission at 6.00 microns and rescanned.

EXPERIMENTAL RESULTS AND DISCUSSION

Bacterial Population

The primary aim of this thesis is to investigate the overall aerobic and nitrate respiration characteristics of the marine sediment microflora. However, general information relating to microbial population levels and associated physiological activity is quite scarce. As a consequence, it became necessary to first study specific aspects of the sediment population.

The first problem that required attention appertained to the total bacterial population of bay sediments. Since the majority of bacteria become absorbed or attached to particles in the sediment, adequate separation of bacteria from these particles must be obtained. Thus, the sediment samples were shaken for 30 minutes at 250 rpm on a New Brunswick rotary shaker with 0. 2 molar phosphate buffer and 0. 1 percent peptone serving as the diluents. The sediment suspension was allowed to settle out for five minutes and plated in triplicate on the ZoBell and Reuszer medium. Samples for population determinations were obtained in summer and winter as well as at the one inch and ten inch core depths. The plates were incubated at two temperatures, 15 and 25 C.

As shown in Table I, the maximum count of 2.3×10^8 per gram of dry weight sediment was obtained from a one inch core depth using

TABLE I. BACTERIAL PLATE COUNTS OF MARINE BAY SEDIMENTS.

Season	Depth	Temperature C	ZoBell*	Reuszer*
			Cells/g Dry Sediment	
Winter	1"	15	3.6×10^6	1.0×10^7
		25	1.6×10^7	2.3×10^8
Winter	10"	15	6.1×10^4	3.0×10^4
		25	4.7×10^5	2.8×10^5
Summer	1"	15	2.8×10^7	1.1×10^6
		25	5.1×10^7	6.5×10^7
Summer	10"	15	3.3×10^6	1.7×10^6
		25	1.2×10^5	2.5×10^5

*Counts average of triplicate plates.

the Reuszer medium and incubation at 25 C. On the other hand, the lowest count was obtained at the ten inch depth with incubation at 15 C. The data indicate an abundant microbial population (over a million cells) during both seasons at the one inch core level. However, with an increase in core depth, the microbial population decreased to 3×10^4 - 2.8×10^5 cells per gram of sediment. As expected, the highest counts resulted at the 25 C incubation temperature, particularly when calculations were made at the three day and five day period.

In core area one, 47 percent of the bacterial isolates tested were capable of reducing nitrate to nitrite. Still more interesting was the observation that 16 of the 47 isolates reduced nitrite to nitrogen gas and oxides of nitrogen. Although only one core area was tested, it is apparent that this sediment contains a relatively high (16 percent) denitrifying flora. Approximately 16 percent of the total sediment flora was capable of converting nitrate to the gaseous state and 47 percent capable of accumulating nitrite from nitrate.

A rather common property of marine bacteria from these sediments is the utilization of carbohydrates without production of much acid and gas.

A general visual observation worth noting is the unusually high bacterial population exhibiting phleomorphism. The isolates also exhibit a higher proportion of Gram variable cells than observed in the soil environment. In preparation of the Gram stain a common

fault encountered in the procedure is over-decolorization. After careful time checks against non-marine forms, it may be concluded that the marine bacteria were more vulnerable to the loss of their Gram positivity.

Carbon and Nitrogen Content of Marine Bay Sediments

The organic matter in marine sediments cannot be attributed to a single source, therefore, variation in chemical composition might be expected. As indicated in Table II, the total organic carbon of the one inch sediments varies between 1.40 to 3.03 percent. Sampling areas one to three however, are more closely grouped with values of 1.93, 1.56, and 1.40 percent respectively. Large pieces of organic matter may be added accidentally to the sediments creating an abnormally high localized concentration which would explain the 3.03 percent obtained in sample area number four. Total percent organic carbon at the ten inch level generally was higher and exhibited somewhat less variation. The literature listed values of one percent total organic dry weight of sediments on the mainland shelfs with basin and trough areas between five and ten percent. Shepard and Moore (1955) found an average of four percent organic matter in shallow bays near Rockport, Texas.

The lower organic content average of 1.98 percent as found in the four test areas indicated could be attributed to high microbial

TABLE II. TOTAL CARBON AND NITROGEN OF MARINE SEDIMENTS FROM YAQUINA BAY, NEWPORT, OREGON.

Location	Depth	% Organic C	% CO_3^--C	% Total C	% Nitrogen	C/N Ratio
1	1"	1.93	12.52	14.45	0.85	2.2
	10"	3.09	11.53	14.62	0.85	3.5
2	1"	1.56	22.05	23.63	1.39	1.1
	10"	1.81	22.50	24.31	1.43	1.2
3	1"	1.40	15.79	18.19	1.07	1.3
	10"	1.86	9.19	11.05	0.65	2.8
4	1"	3.03	-	-	0	-
	10"	2.11	3.67	5.78	0.34	6.1

degradation and a low input of original organic matter from the bay waters. The derivation of total carbonate carbon is not a real experimental value. This percent was derived from multiplying Emery and Rittenberg's (1952) factor of 17 by total nitrogen. Although these values are not necessarily applicable, an idea of the average percent carbonate can be realized. Interestingly enough, carbonate carbon comprised a large portion of the total carbon which could supply ample carbon for autotrophic microbial growth.

Total nitrogen, as derived by the Microkjeldahl method, portrayed a wide range of values from zero to 1.39 percent for surface sediments. As in the case of organic carbon, the large range of nitrogen values may be explained by variable decomposition rates and abnormal organic matter additions. Core area number four, which showed other pecularities, did not contain any detectable nitrogen at the one inch level and a contained substantially lower average value of 0.34 percent at the ten inch level. A slightly higher nitrogen content was obtained with increase in sampling depth.

It is evident that among other factors, the kind of native material, the oxidation state, and the rate and kind of decomposition will dictate the C/N ratio. Thus, no precise conclusion can be ascertained from the C/N ratio. However, it is safe to conclude that C/N ratios of 1.2 to 3.5 would not indicate a nutritional deficiency in nitrogen. On the other hand, the data implies a carbon nutritional limitation in

these particular bay sediments. In this regard, the literature reveals that much of the carbon in the sediment cannot be readily assimilated by the majority of the bacterial population.

Respiration of Unstirred Bay Sediments

The respiration of marine sediments reflects the overall physiological activity of the flora and gives information relating to nutrient levels and degree of organic matter stabilization. Limitations of this experimental approach primarily involves technical difficulties.

In sampling, the one and one half inch Phlager core represented only a small portion of sediment compared to the total volume of the bay. Likewise, only a limited amount of sediment from a particular area was available for each experiment. Actual experiments have shown a loss of activity of the sediment after 24 hours storage. Therefore, all respiration experiments have to be conducted within 24 hours of sample collection. This limited the number and kinds of experiments that could be conducted at a particular time from any one core area sampled. Terrestial contamination was limited by selecting sites which were well away from pollution sources and were covered by four to five feet of water at all tidal changes.

Variations in the physical and chemical nature of the sediments can be detected via differences in the respiration data. This in turn reflects the kind and amount of flora activity present in a given

period in a particular area. A 100 gram wet weight sample was used in each flask for aerobic respiration of solid unstirred top sediment. Adjustments for water content were made in the calculations of results as displayed in Table III. The five nutritional adjustments were run in duplicate at 20 C for a period of 20 days. The endogenous sediment respiration gave 56.5 ml of oxygen, an equivalent QO_2 g of 2.3×10^{-3} ml of O_2 per gram of dry weight sediment per hour. This rate is somewhat lower than generally experienced in unamended soils. A small amount of endogenous CO_2 (13.2 milligrams) was evolved reflecting the low RQ of 0.48.

When a readily assimilated carbon source such as glucose was added to the sediment, a 2.2 fold increase in oxygen uptake resulted. However, even though sufficient oxygen was regenerated in the system, poor diffusion into the unstirred sediment accounted for the rather low oxygen uptake rate. The addition of glucose also stimulated a major increase in CO_2 evolution. Of 144 mg of C added as glucose, 79.8 mg was recovered as CO_2, indicating that 56 percent of the carbon was evolved as CO_2 and 44 percent assimilated as cellular material. The pH of the sediment drastically increased from 7.38 to 8.7, probably demonstrating residual ion effects. The resulting RQ of 1.3 indicated more or less complete oxidation of added carbon with the surface sediment.

The addition of 750 ppm NO_3^- effected no significant increase in

TABLE III. AEROBIC RESPIRATION OF UNSTIRRED BAY SEDIMENTS AFTER 20 DAYS INCUBATION.

Sediment Treatment*	Temperature C	O_2 ml	Mg C as CO_2	Final pH	RQ	QO_2 g*
2 mM glucose	20	125.7	79.8	8.7	1.3	5.2×10^{-3}
2 mM glucose 37,500 µg NO_3^--N	20	111.0	77.2	8.1	1.47	3.9×10^{-3}
2 mM glucose 75,000 µg NO_3^--N	20	93.5	53.2	9.2	1.42	3.4×10^{-3}
75,000 µg NO_3^--N	20	37.3	16.2	7.5	0.89	1.5×10^{-3}
None	20	56.5	13.2	7.38	0.48	2.3×10^{-3}

*Core area one.

**QO_2 g = ml O_2/g/hr

respiration rate as compared to the previously described glucose treated sediment. These results would seem to indicate that the sediments lack a carbon rather than a nitrate-nitrogen source.

The sediment as shown in Table III, containing glucose and 1400 ppm NO_3^--N, gave a slight inhibition of oxidation due to excess of nitrate added to the sediment. The nitrate inhibition of oxidation has been demonstrated in the soil system by various investigators. Nitrate, particularly at 1000 ppm, retards the aerobic oxidation of carbohydrates. The last treatment of 1400 ppm NO_3-N likewise demonstrated nitrate inhibition of oxidation of endogenous carbon.

Anaerobic Nitrate Respiration of Unstirred Bay Sediments

Anaerobic nitrate respiration data presented in Table IV revealed a substantially lower CO_2 evolution than the previously described aerobic system. Nevertheless, when nitrate was added, an increase in both CO_2 and N_2 evolution occurred as compared to non-nitrate containing sediment. Thus, the data indicated the capacity of the sediment to use nitrate-oxygen as a terminal hydrogen acceptor in the oxidation of a carbon substrate.

Respiration Characteristics of Stirred Bay Sediments

As observed from the presented data on unstirred sediments, diffusion of oxygen to the site of biological activity became a serious

TABLE IV. ANAEROBIC RESPIRATION OF UNSTIRRED BAY SEDIMENTS AFTER 20 DAYS INCUBATION.

Sediment Treatment*	Temperature C	µg NO_2	Mg C as CO_2	µg N_2
None	20	Trace	3.8	93
2 mM glucose	20	Trace	11.2	207
2 mM glucose 37,500 µg NO_3^--N	20	3,800	42.7	785

*Core area one.

limiting step governing sediment respiration. Therefore, all subsequent sediment experiments were stirred at 150 rpm with electromagnetic stirrers to insure adequate gas exchange (Figure 2). The stirring reduced incubation time to 110 hours with the major portion of the substrate carbon oxidized in 48 hours. The highest QO_2 g with unstirred sediment was 5.2 μl, whereas with the stirred sediment a QO_2 g of 86 μl was obtained. As might be expected, the latter QO_2 g value was obtained with the inclusion of 1 mM of glucose plus 37,500 μg (750 ppm) of nitrate-nitrogen and incubation at the higher temperature of 28 C. Complete oxidation of the substrate is indicated in Table V by a RQ value of 0.98. A total of 69 percent carbon was evolved as CO_2 and the remaining 31 percent presumably was assimilated by the marine flora. The temperature quotient (Q_{10}) for the 25 C versus 15 C was calculated to be 1.45, something less than the theoretical value of 2.0. The reason for not obtaining a theoretical Q_{10} of 2.0, as has been demonstrated in soil systems, may possibly be explained by the fact that the sediments harbor a flora which tends to be somewhat psychrophilic. That is, at incubation temperatures of 28 C, optimum activity of many sediment organism may be slightly retarded.

As shown in Figure 2, with a reduction of the temperature to 15 C, the QO_2 g rate drops to 51.8 ul. The RQ index of 1.04 again indicated complete oxidation of the substrate (Table V). Total percent carbon evolved as CO_2 decreased slightly to 60 percent, with 40 percent being

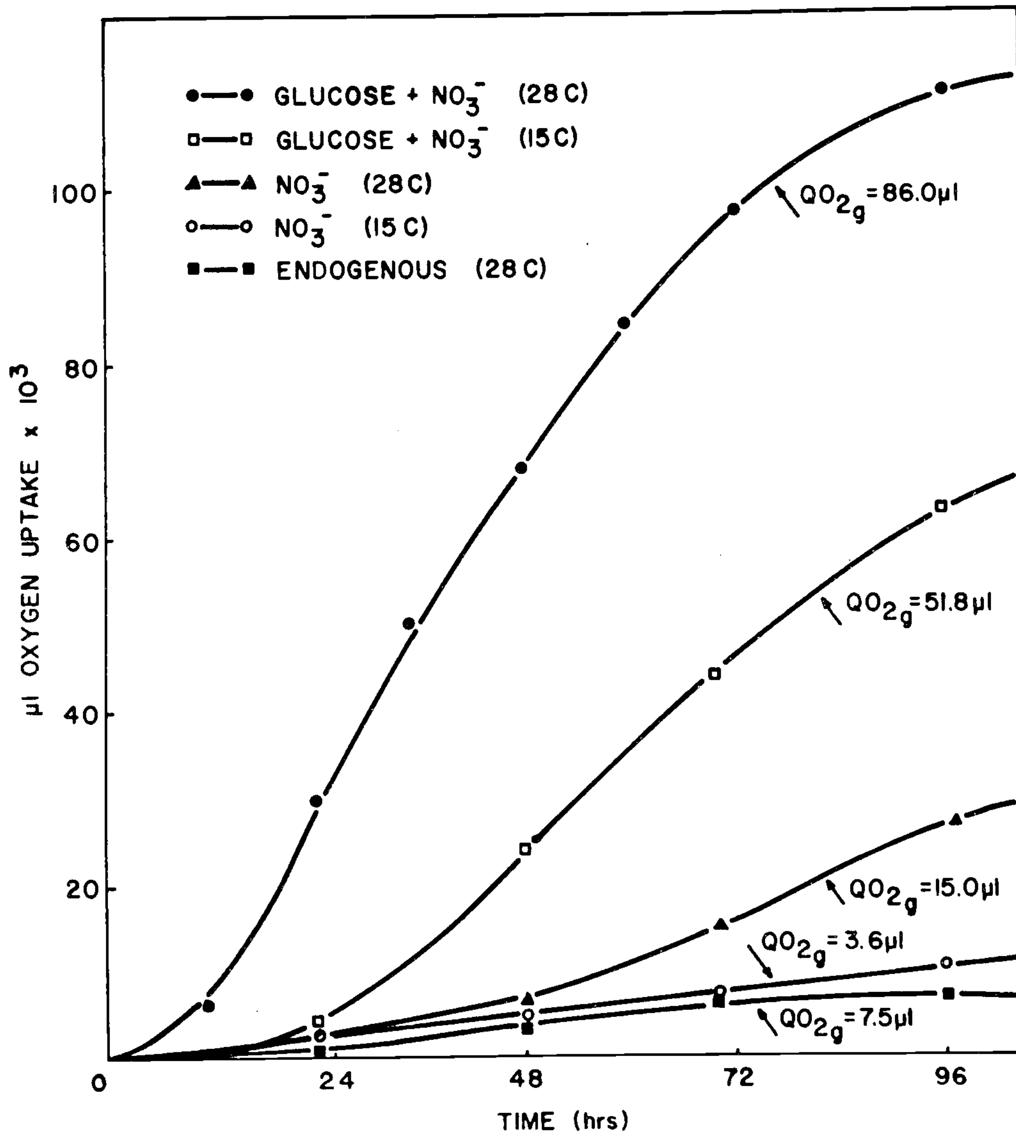


Figure 2. Aerobic respiration of bay sediments - core area 1.

TABLE V. AEROBIC RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment*	Temperature C	ml O_2	Mg C as CO_2	RQ
37, 500 µg NO_3^--N	15	12.8	4.8	0.52
37, 500 µg NO_3^--N	28	32.9	4.9	0.30
1 mM glucose 37, 500 µg NO_3^--N	15	97.8	42.9	1.04
1 mM glucose 37, 500 µg NO_3^--N	28	124.0	50.5	0.98

*Core area one.

assimilated. Upon exclusion of glucose and nitrate from the sediments very low QO_2 g were obtained. It should be pointed out that with sediment plus nitrate and incubation under aerobic conditions no nitrogen gas or oxides of nitrogen were detected. Evidently, a major part of the added nitrate was used in an assimilatory manner rather than via the dissimilatory route.

Anaerobically, as illustrated in Table VI, 1,600 μg of nitrogen gas were produced in 110 hours at 15 C; an equivalent of 1.12 μg of nitrogen per hour per gram of dry weight sediment at 28 C. Therefore, it became clear that these sediments contain a high flora density capable of using nitrate as the terminal acceptor of hydrogen. Presumably, in such a heterogeneous population nitrate was also used in an assimilatory manner. As with aerobic respiration only a very slight increase was observed at the higher incubation temperature of 28 C. This is again indicative of a more psychrophile population in marine sediments than usually encountered in soils.

The data in Tables VII, VIII, IX, and X, along with Figures 2 and 4, show the respiration characteristics of core areas two and four, respectively. Although variations were encountered, these respiration data closely resembled core area one which was previously portrayed in Figure 2. Collectively, these data represented the general aerobic and nitrate respiration characteristics of marine bay sediment. It becomes interesting to note that these results are comparable

TABLE VI. ANAEROBIC NITRATE RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment*	Temperature C	Mg C as CO_2	µg N_2
1 mM glucose 37,500 µg NO_3^--N	15	21.8	1,600
1 mM glucose 37,500 µg NO_3^--N	28	25.3	1,850

*Core area one.

TABLE VII. AEROBIC RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment*	Temperature C	ml O_2	Mg C as CO_2	RQ
37, 500 µg NO_3^--N	15	10.2	2.4	.48
37, 500 µg NO_3^--N	28	14.8	4.7	.65
1 mM glucose 37, 500 µg NO_3^--N	15	83.1	36.3	.89
1 mM glucose 37, 500 µg NO_3^--N	28	106.5	44.9	.93

*Core area two.

TABLE VIII. ANAEROBIC NITRATE RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment*	Temperature C	Mg C as CO_2	µg N_2
1 mM glucose 37,500 µg NO_3^--N	15	18.7	1,400
1 mM glucose 37,500 µg NO_3^--N	28	22.4	1,690

*Core area two.

TABLE IX. AEROBIC RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment*	Temperature C	ml O_2	Mg C as CO_2	RQ
37,500 μg NO_3^--N	15	11.6	3.5	.61
37,500 μg NO_3^--N	28	18.1	4.9	.54
1 mM glucose 37,500 μg NO_3^--N	15	86.4	39.8	.94
1 mM glucose 37,500 μg NO_3^--N	28	112.1	47.7	.87

*Core area four.

TABLE X. ANAEROBIC NITRATE RESPIRATION OF STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment*	Temperature C	Mg C as CO_2	μg N_2
1 mM glucose 37,500 μg NO_3^--N	15	11.8	750
1 mM glucose 37,500 μg NO_3^--N	28	14.3	870

*Core area four.

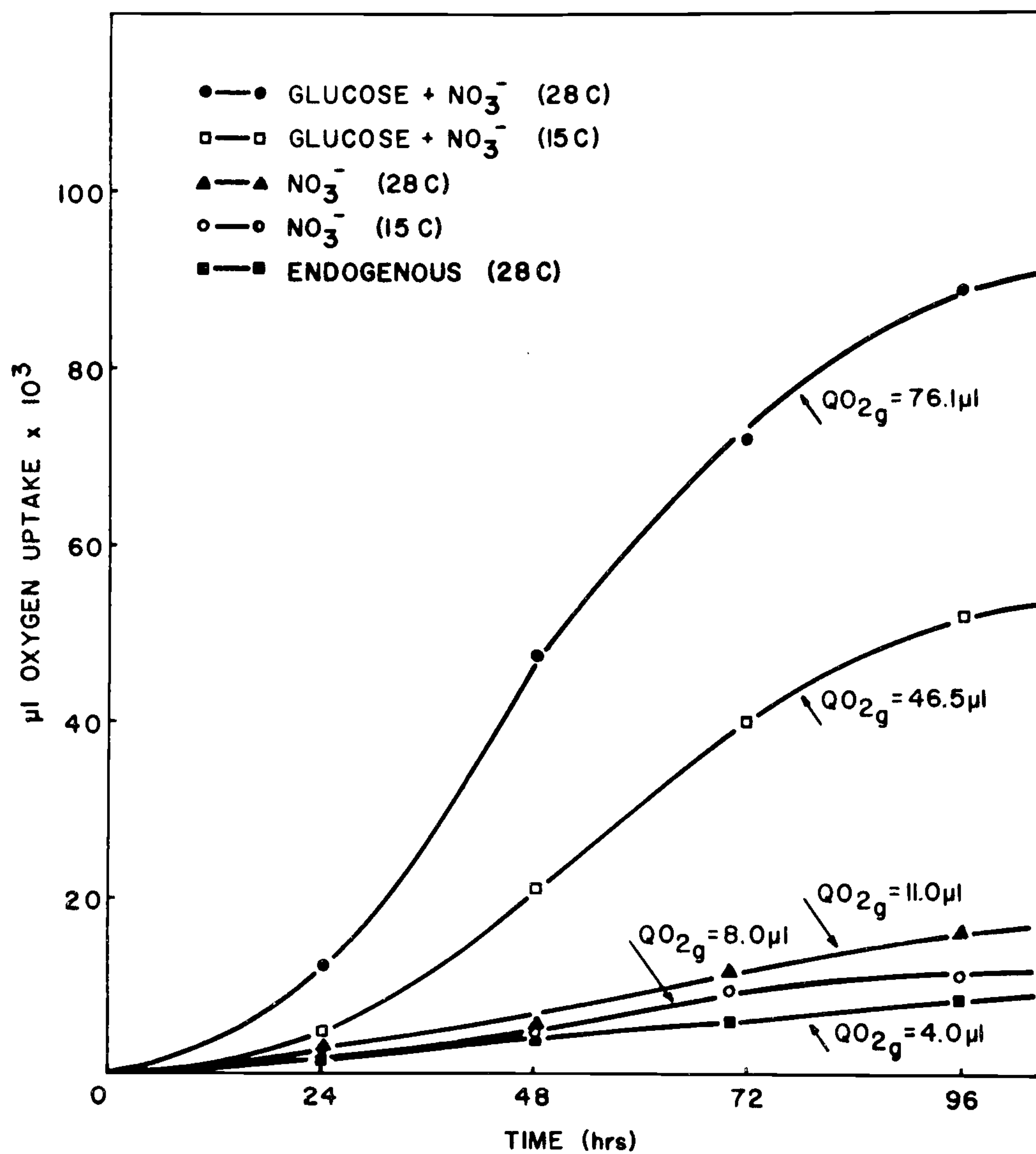


Figure 3. Aerobic respiration of bay sediments – core area 2.

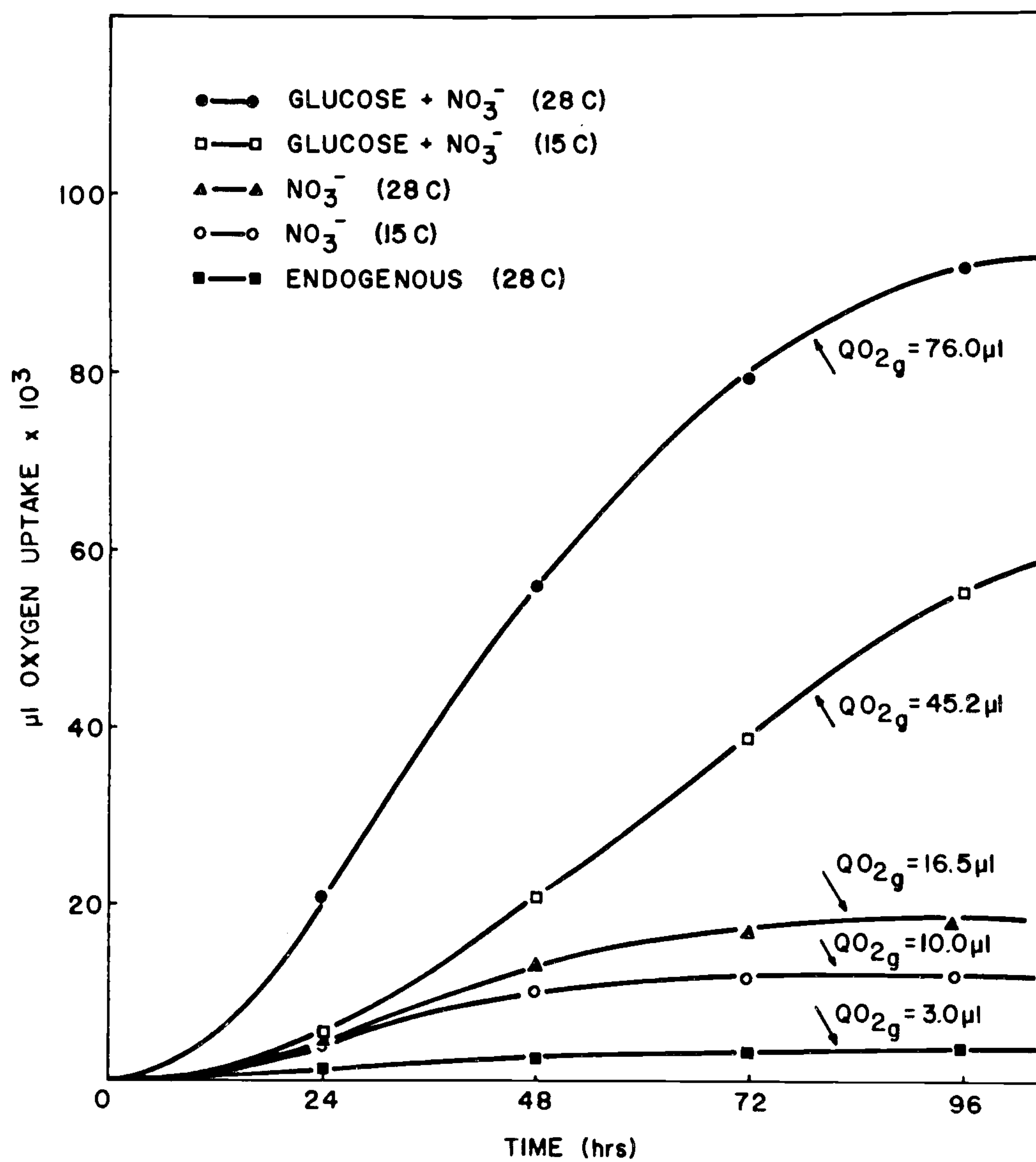


Figure 4. Aerobic respiration of bay sediments – core area 4.

to soil except that sediment respiration rates are slightly lower. The maximum QO_2 g obtained with glucose as the substrate was 86 µl in core area one, while the minimum was 11.6 µl in core area three at 28 C. At the lower temperature of 15 C the maximum was 103.9 µl in core area three, whereas a minimum of 10.2 µl was obtained in core area two. However, core area three was sampled in April, while core area four was sampled in May. It was not possible to establish any seasonal trend corresponding to the aforementioned respiration results. Similarly, no correlation was evident between respiration rate and the native carbon and nitrogen content of the sediment. Noteworthy is core area four, where no Kjeldahl nitrogen was detected, it resulted in the lowest endogenous respiration. In subsequent experiments with core areas three and four, after storage for one week at 5 C, a two fold decrease in respiration rate resulted.

Of special interest was core area three which, unlike the other samples, exhibited a lower oxygen uptake of 47.3 ml at the higher temperature of 28 C as compared to 103.9 ml oxygen at 15 C. Carbon evolution did not show such a dramatic decrease, with 12.4 mg evolved at 28 C and 16.6 mg of carbon dioxide at 15 C. Anaerobically via nitrate respiration the decrease was more visable where 1,150 µg of nitrogen gas was evolved at 28 C, whereas 2,200 µg nitrogen was evolved at 15 C after 110 hours incubation. It may be postulated that this core contained a much higher psycrophile population. In fact,

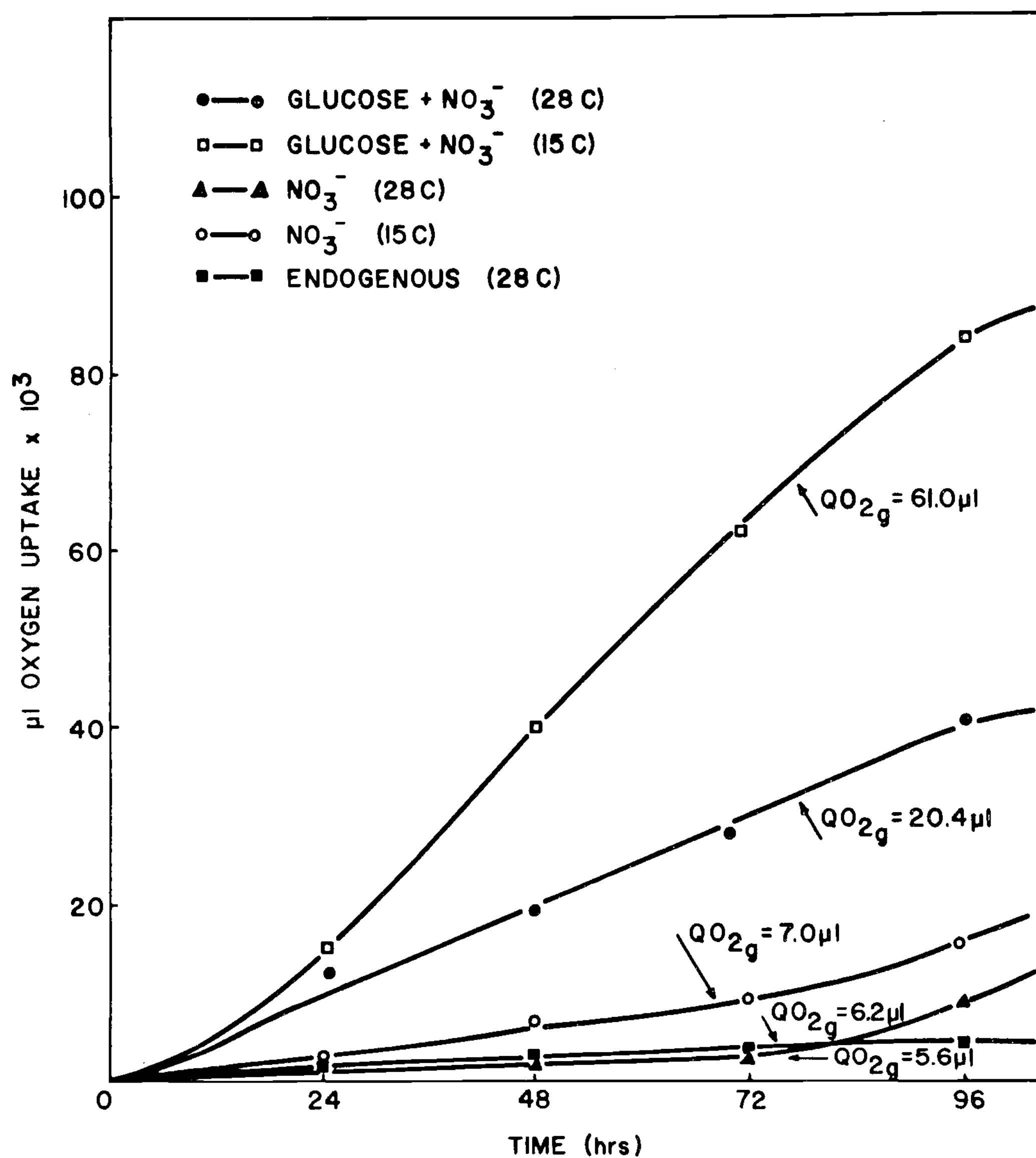


Figure 5. Aerobic respiration of bay sediments - core area 3.

TABLE XI. OBSERVED TEMPERATURE EFFECT OF AEROBIC AND STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment*	Temperature C	ml O_2	Mg C as CO_2	RQ
37,500 µg NO_3^--N	15	17.6	2.9	0.33
37,500 µg NO_3^--N	28	11.6	2.5	0.47
1 mM glucose 37,500 µg NO_3^--N	15	103.9	16.6	0.24
1 mM glucose 37,500 µg NO_3^--N	28	47.3	12.4	0.71

*Core area three.

TABLE XII. OBSERVED TEMPERATURE EFFECT OF ANAEROBIC NITRATE RESPIRATION AND STIRRED BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment *	Temperature C	Mg C as CO_2	µg N_2
1 mM glucose 37,500 µg NO_3-N	15	26.7	2,200
1 mM glucose 37,500 µg NO_3-N	28	21.2	1,150

*Core area three.

sediments in general may contain pockets or areas where psychrophile activity dominates.

Respiration of the test sediments at the ten inch depth was similar to sediments from the one inch depth except somewhat slower rates were encountered at the lower depths. Typical aerobic and anaerobic nitrate respiration data are shown in Tables XIII and XIV, respectively.

Denitrifying Isolates from Bay Sediments

Following the marine sediment respiration experiments, some 60 denitrifying cultures were obtained from core areas previously studied. Eleven of these shown in Table XV have been given a preliminary genus designation. Among the genera represented are three *Pseudomonas* sp., two *Achromobacter* sp., a *Brevibacterium* sp., a *Bacillus* sp., a *Vibrio* sp., a *Flavobacterium* sp., and a *Nocardia* sp. One of the *Achromobacter* species, given the laboratory strain number 62 demands extra attention in that it cannot reduce nitrate. However, nitrate will serve as its sole hydrogen acceptor. This nitrite reducing organism will be evaluated in greater detail in the latter part of this dissertation.

The first organism shown in Table XV (*Nocardia* sp.) is a facultative anaerobe that reduces nitrate to nitrogen gas in an anaerobic environment. Bergey's Manual of Determinative Bacteriology (1957)

TABLE XIII. AEROBIC RESPIRATION OF DEEP BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment*	Temperature C	ml O_2	Mg C as CO_2	RQ
37, 500 µg NO_3-N	15	15. 5	10. 0	1. 33
37, 500 µg NO_3-N	28	20. 3	11. 3	1. 14
1 mM glucose 37, 500 µg NO_3-N	15	48. 1	24. 1	1. 02
1 mM glucose 37, 500 µg NO_3-N	28	107. 4	61. 0	1. 16

*Core area one and depth ten inches.

TABLE XIV. ANAEROBIC RESPIRATION OF DEEP BAY SEDIMENTS AFTER 110 HOURS INCUBATION.

Sediment Treatment	Temperature C	Mg C as CO_2	µg N_2
1 mM glucose 37,500 µg NO_3-N	15	16.1	1,170
1 mM glucose 37,500 µg NO_3-N	28	20.3	1,450

Core area one and depth 10 inches.

TABLE XV. DESCRIPTION OF DENITRIFYING MARINE SEDIMENT ISOLATES.

Organism Number	Cell Morphology	Gram Strain	Motility	H Acceptor	Gas µg* N_2	Preliminary Genus Designation
50-2	Filaments	G+	Nonmotile	NO_3^-	5,700	Nocardia
51	Rods	G-	Polar	NO_3^-	4,310	Pseudomonas
53	Rods (Chains)	G+	Peritrichous	NO_3^-	13,949	Brevibacterium
51-7B	Filaments	G Var	Nonmotile	NO_3^-	3,270	Unknown
17	Rods	G-	Peritrichous	NO_3^-	980	Achromobacter
50-7	Rods	G+	Motile	NO_3^-	1,160	Bacillus
51-1A	Rods	G-	Polar	NO_3^-	1,780	Pseudomonas
59	Rods	G-	Polar	NO_3^-	1,450	Vibrio
36	Rods	G-	Nonmotile	NO_3^-	2,210	Flavobacterium
50-4	Rods	G-	Motile	NO_3^-	9,700	Pseudomonas
62	Rods	G-	Nonmotile	NO_2^-	12,317	Achromobacter

*Nitrogen gas per 60 hours in growing system at 20 C with 37,500 µg NO_3-N.

describes 45 species of Nocardia, none of which can use nitrate-nitrogen as a final hydrogen acceptor. However, the manual does list two aerobic marine forms: Nocardia marina, which does not reduce nitrate, and Nocardia atlantica, which does reduce nitrate with the accumulation of nitrites. Culture number 50-2 preliminarily designated as a Nocardia species (Charts 1 and 2, appendix) has similar cultural and physiological characteristics to the aforementioned marine Nocardia. The Nocardia sp. optimum growth temperature is 20 C in NRT media at 3. 5 percent salt concentration. Under these optimum growth conditions, large amounts of gaseous nitrogen were produced after a 16 hour incubation. Although the culture grew in ten percent sodium chloride, only moderate turbidity and a small amount of nitrogen gas were produced in 48 hours. Likewise, an increase in temperature above 25 C retarded nitrate reduction and cellular growth. In addition, the Nocardia isolate did show growth with various carbohydrates. However, very little acid and no gas were produced aerobically after prolonged incubation. Either the absence of fermentation products or the accumulation of fermentation products was substantiated by the Hugh-Liefson carbohydrate metabolism test. The cells produced an antibiotic which showed sensitivity to Staphlococcus aureus FAD 209 and Bacillus substilis ATCC 6633. On the other hand, no sensitivity was observed with several of the Gram negatives organisms that were tested. The infrared spectra of the presumed Nocardia species is shown in Figure

6. A preliminary species designation of Nocardia denitrificans (species nova) has been proposed.

The second organism in Table XV was considered to be a Pseudomonas species. The isolate produced 4,310 µg of nitrogen gas in ten hours from the terminal hydrogen acceptor nitrate. Generally, as typical of most marine Pseudomonas isolates, carbohydrates were not rapidly fermented. However, slow utilization of glucose and mannitol without gas evolution was demonstrated. This facultative Gram negative rod is described in Charts 3 and 4 (appendix). The key to the species of the genus Pseudomonas in Bergey's Manual of Determinative Bacteriology (1957) includes a subdivision of sea-water and brine forms. In this subdivision, eight species are listed. However, none of these are indicated as marine denitrifiers. The isolate does resemble the marine forms Pseudomonas calcis and the non-marine soil form P. stutzeri listed in the key. The similarity of the infrared spectra of this marine isolate to other valid Pseudomonas species is given in Figure 7.

When interpreting the infrared spectra of bacteria, three primary and three secondary points are most critically involved. The bands of 6.05 and 6.45 microns are largely derived from peptide linkages of the bacterial protein. The bands between 8.0 and 8.1 microns are due to absorption of the bacterial nucleic acids. The last primary

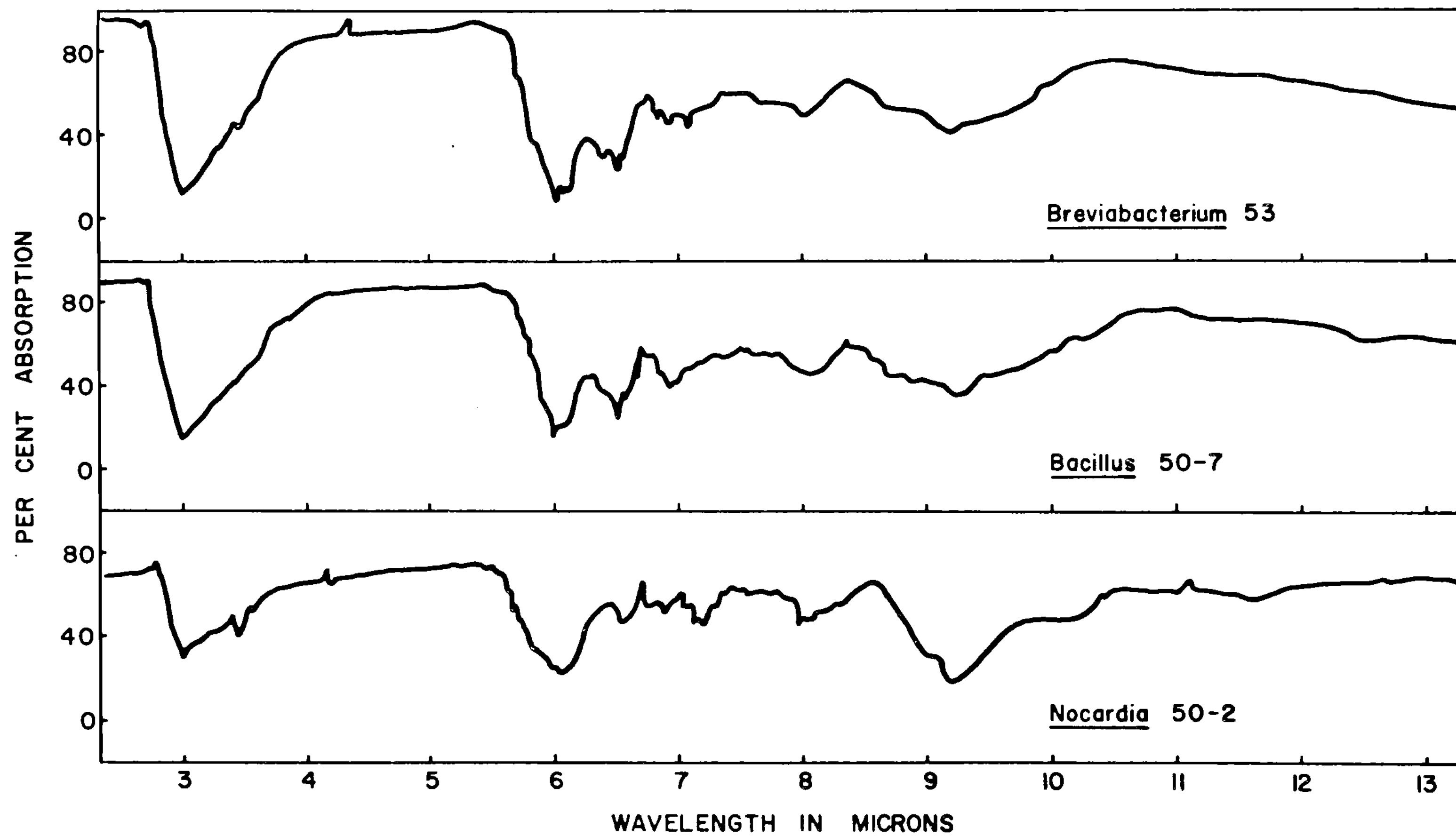


Figure 6. Infrared scale spectra of three Gram positive marine bacilli.

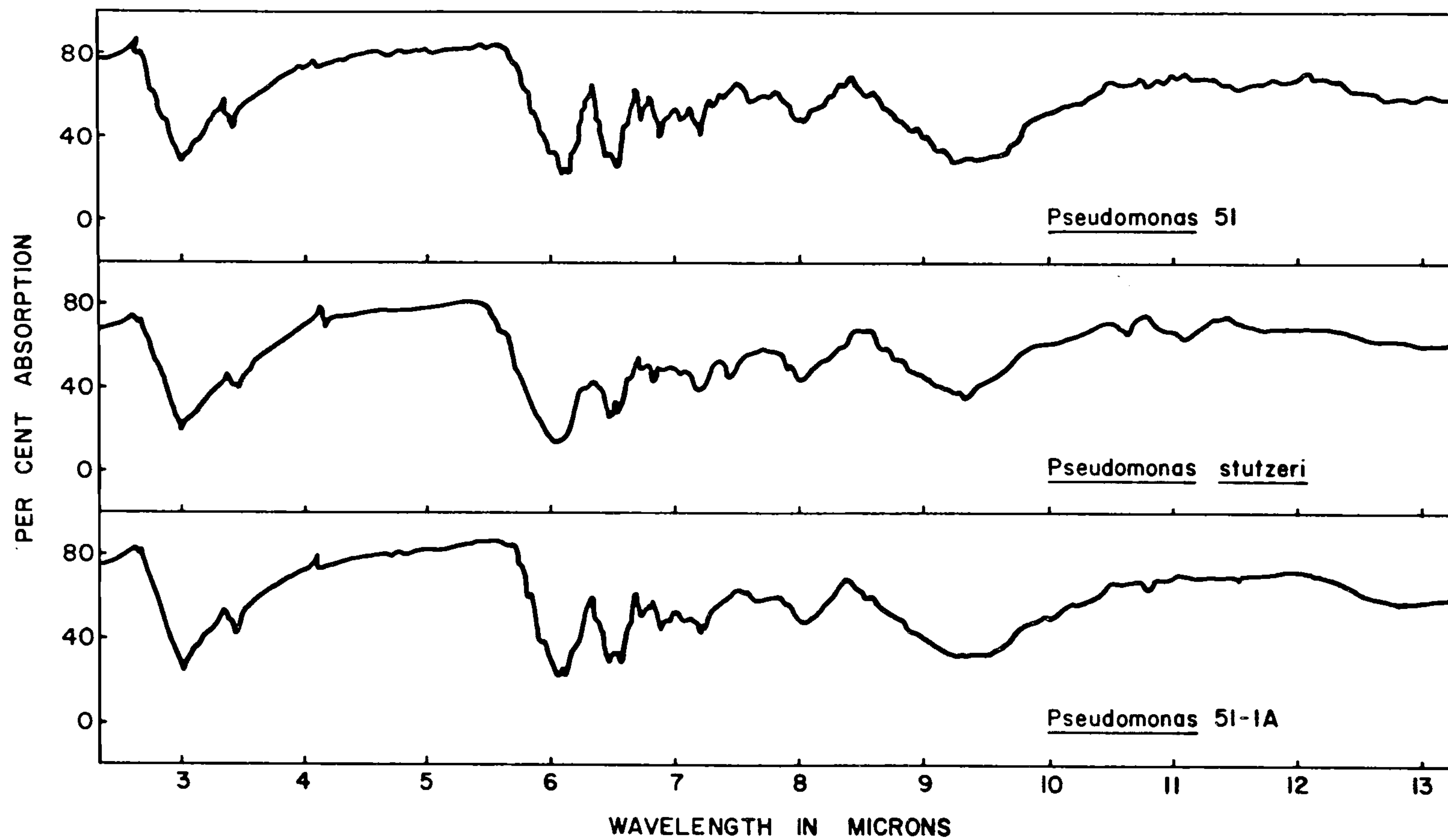


Figure 7. Infrared scale spectra of three *Pseudomonas* strains.

band with absorption between 8.6 to 10 microns signifies carbohydrates and some nucleic acids. There are several secondary bands of minor importance; 3.0 microns signify the nitrogen-hydrogen vibration, 5.85 microns to the absorption of the carbon-oxygen linkage, and 7.0 microns relating to N-H band of amino sugars. Because bacteria have generally similar spectra, the apparent dissimilarities observed among the absorption bands of microorganisms are due to small differences in the percentages of infrared transmission. Differences less than two percent are not considered applicable. The infrared spectra of the isolates used in this dissertation are used as a supporting tool rather than the sole criterion for classification. Because of the biochemical inactivity of most of the Gram negative marine bacteria, the spectra proved to be an excellent aid in ascertaining genus designation. It is important to realize that the Figures shown are scale drawings of the original 7 1/2 by 24 inch infrared spectra.

The similarities between the two marine pseudomonads and *Pseudomonas stutzeri* based on general gross tracing are clearly evident. One can therefore visualize a typical spectrum for all *Pseudomonas* species. However, on closer examination it is obvious that the spectra of the two marine *Pseudomonas* isolates, 51 and 51-1A, are more closely related when compared to the soil isolate *P. stutzeri* form. The most important points considered in the determination are: (1) With absorption at 5.75 to 6.45 microns, the soil form *P. stutzeri*

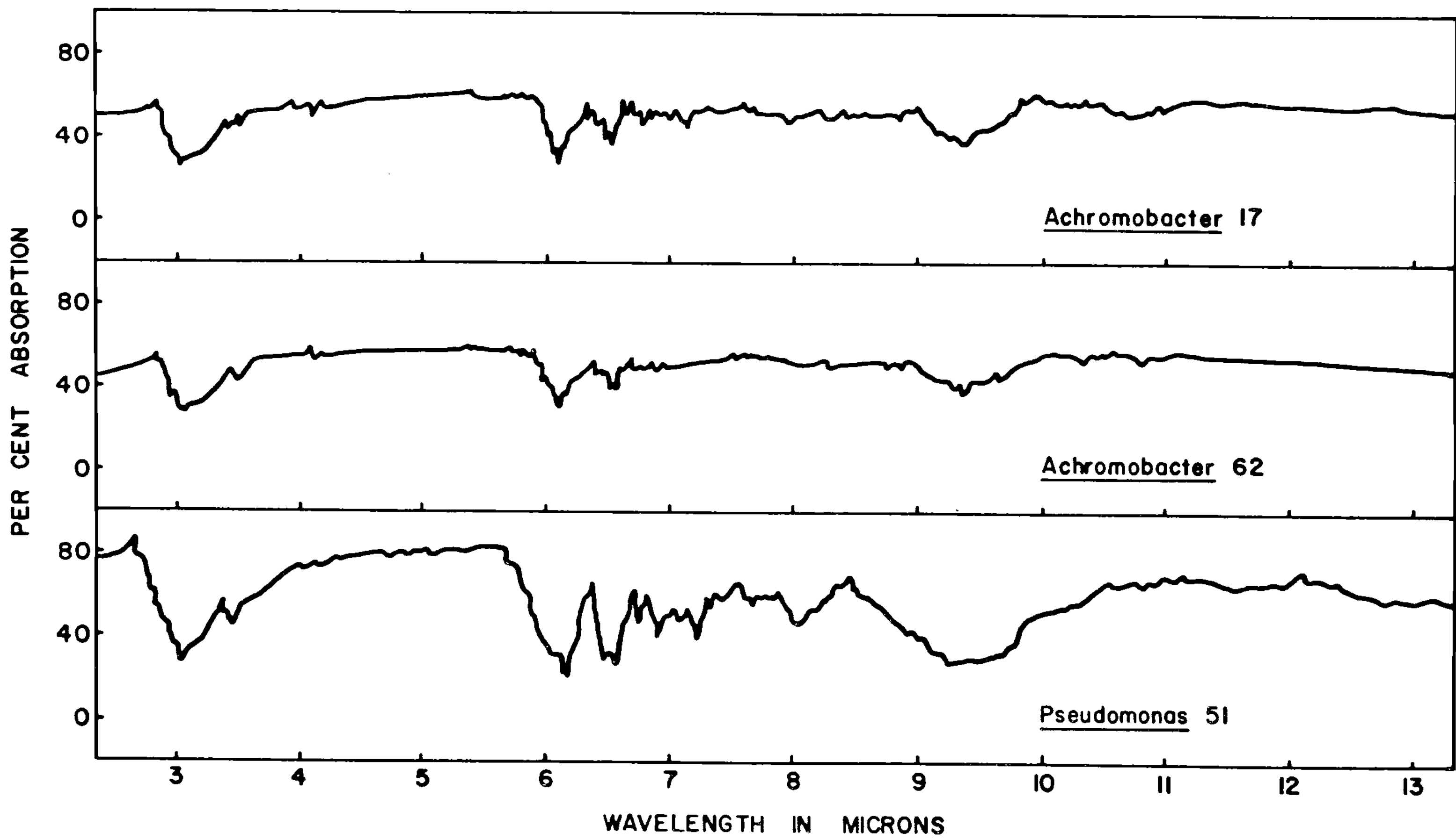


Figure 8. Infrared scale spectra of three Gram negative marine bacilli.

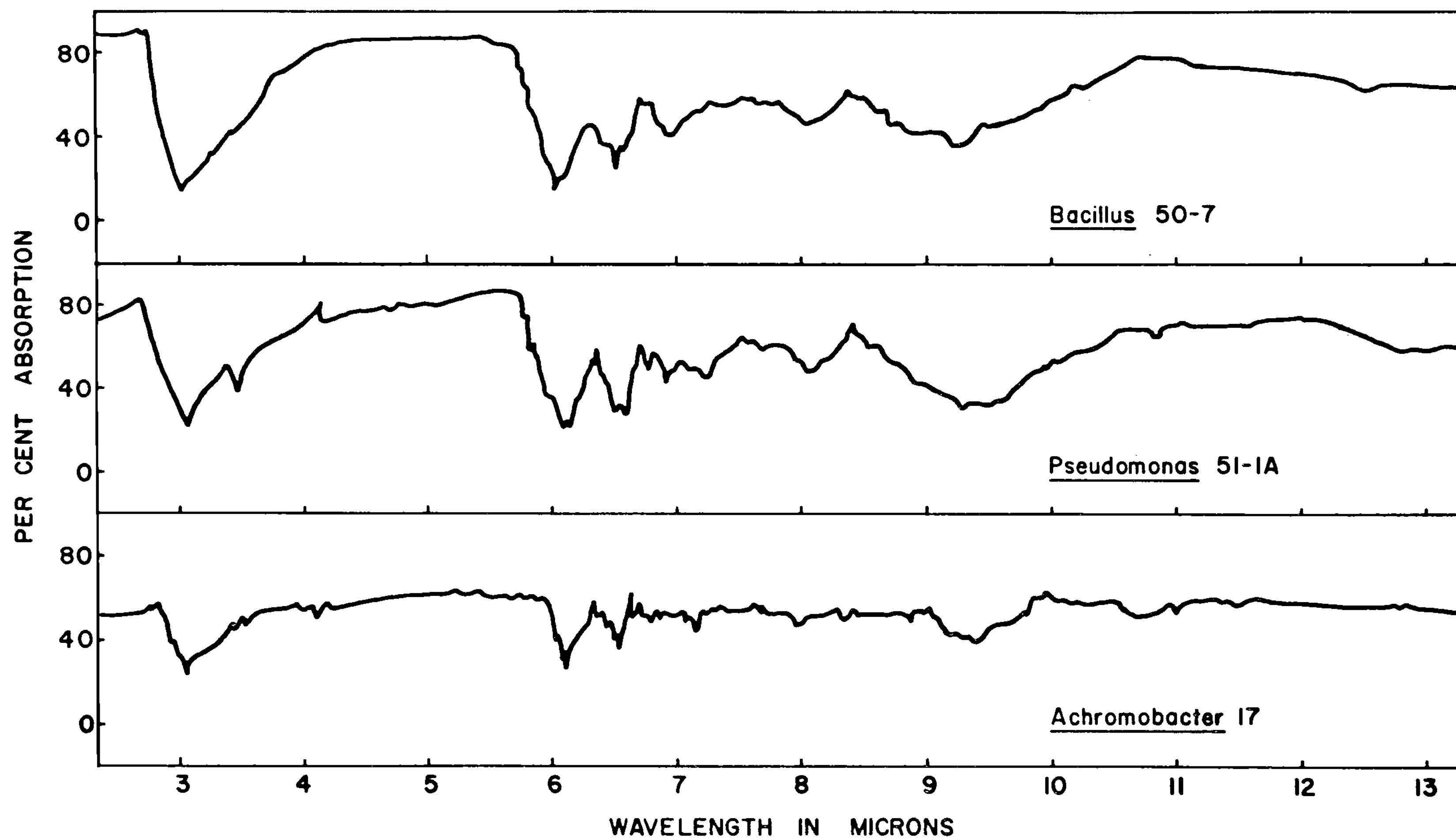


Figure 9. Comparative infrared spectra of Gram positive and Gram negative marine bacilli.

is broader and has a maximum absorption of 15 percent, whereas the two marine forms have a narrow valley and a maximum absorption somewhat less than 20 percent. (2) The front part of the valley at 5.75 microns in *P. stutzeri* is smooth, while the two marine forms exhibit several shoulders. (3) In the nucleic acid peak of 8.0 microns, *P. stutzeri* showed a sharp valley, compared to a broad valley exhibited by both marine forms. (4) The next major absorption considered is between 8.6 and 10 microns where the terrestrial form showed a sharp valley, while the marine form revealed wide absorption. (5) Several other minor absorption points useful in differentiation are located at 4.0 microns, 6.5 to 7.3 microns, and 10.75 microns.

When closely scrutinizing the spectra of the two marine *Pseudomonas* species, 51 and 51-1A, several differences can be observed. The same three major points, mainly 5.75 to 6.45 microns, 8.0 microns and 8.6 to 10.0 microns, are more critically compared. At 6.0 microns note the two smaller sub-peaks. Here the *Pseudomonas* 51 isolate shows two sharper points, as compared to two broader points in organism 51-1A. Other differences between the two marine *Pseudomonas* cultures may be observed at many points along the entire spectra.

The organism that produced the largest amount of nitrogen gas (13,949 µg in 60 hours) was designated as a *Brevibacterium* species. This Gram positive organism exhibited no inhibition of growth or

decreased rate of nitrate reduction at concentrations of ten percent Rila salt. On the other hand, this culture gave substantially slower biological activity at the three percent Rila salt concentration. Small amounts of acid but no gas were produced from glucose. The organism showed extensive pleomorphism even when young actively growing cells were microscopically examined. Similarly, the young cells readily lost their Gram positive property. A more detailed description of the isolate is given in Charts 5 and 6 (appendix). The infrared spectra of the organism is shown in Figure 6. Of special significance is the difference between Gram positive and Gram negative spectra.

The data in Charts 7 and 8 (appendix) give the morphological, cultural, and physiological characteristics of a marine denitrifier that was not assigned to any particular genus.

The culture numbered 17 is thought to be an Achromobacter species, as described in Charts 9 and 10 (appendix). This isolate is a Gram negative rod which is motile by means of peritrichous flagella. Carbohydrate reactions again were typically inactive as in most Gram negative marine bacteria. The infrared spectra of Pseudomonas strain 51, Achromobacter strain 17, and Achromobacter strain 62 are compared in Figure 8. Differences in the spectra between the two Achromobacter cultures and the Pseudomonas species are obvious. Also evident are the similarities between the two Achromobacter

isolates. However, the latter two cultures still exhibit sufficient differences to be interpreted as distinct species.

The marine isolate 50-7 proved to be a Gram positive Bacillus culture. As evident from the data in Charts 11 and 12 (appendix), this organism is biochemically more active than the Gram negative marine forms. In Figure 9, the infrared spectra of the Bacillus strain is compared with two Gram negative marine denitrifiers, Pseudomonas species 51 and Achromobacter species 17. Differences between the Gram positive and Gram negative bacilli are clearly evident at numerous points along the spectra.

The morphological, cultural, and physiological characteristics of Pseudomonas strain 51-1A are portrayed in Charts 13 and 14 (appendix). The infrared spectra are shown in Figure 6.

Another Gram negative denitrifier isolated from bay sediments has been designated a Vibrio species as shown in Charts 15 and 16 (appendix). The Vibrio culture is not as biochemically inactive as the Pseudomonas isolates, in that starch hydrolysis and gelatin liquefaction are evident. Also, acid without gas is slowly produced from a majority of the carbohydrates tested. Salt concentrations above 3.5 percent sharply curtail the organism's biochemical activities. The culture Lloyd (1931) describes as Bacterium costatum is probably a Vibrio species which reduces nitrate to nitrogen gas. According to Brison and Vargues (1963) the Vibrio genus contains the third most

numerous marine denitrifier population encountered in sediments. However, Bergey's Manual of Determinative Bacteriology lists 34 species but does not include a marine denitrifier.

The next organism shown in Table XV has been identified as a Flavobacterium species number 36 which produced 2,210 μg of nitrogen gas from nitrate. This organism is generally more biochemically active than other Gram negative sediment isolates, as shown by the data in Chart 18 (appendix). The morphological and cultural characteristics shown in Chart 17 closely resemble the Flavobacterium strain of Zobell and Upham (1944). In their classification they described six Flavobacterium strains. However, none were denitrifiers.

The morphological and physiological characteristics of the last Pseudomonas isolate (50-4) are shown in Charts 19 and 20 (appendix), respectively. The infrared spectra are portrayed in Figure 10. Although it possesses all the morphological and physiological characteristics of a Pseudomonas species, yet, its spectrum do not indicate the isolate to be a member of the genus Pseudomonas.

The final organism listed in Table XV, number 62, is considered to be an Achromobacter species. The organism is of particular interest because it does not reduce nitrate. However, it will reduce nitrite to nitrogen gas. In other words, this culture possesses a nitrite reductase while being void of a nitrate reductase. The organism produced 12,317 μg of nitrogen gas from nitrite when incubated

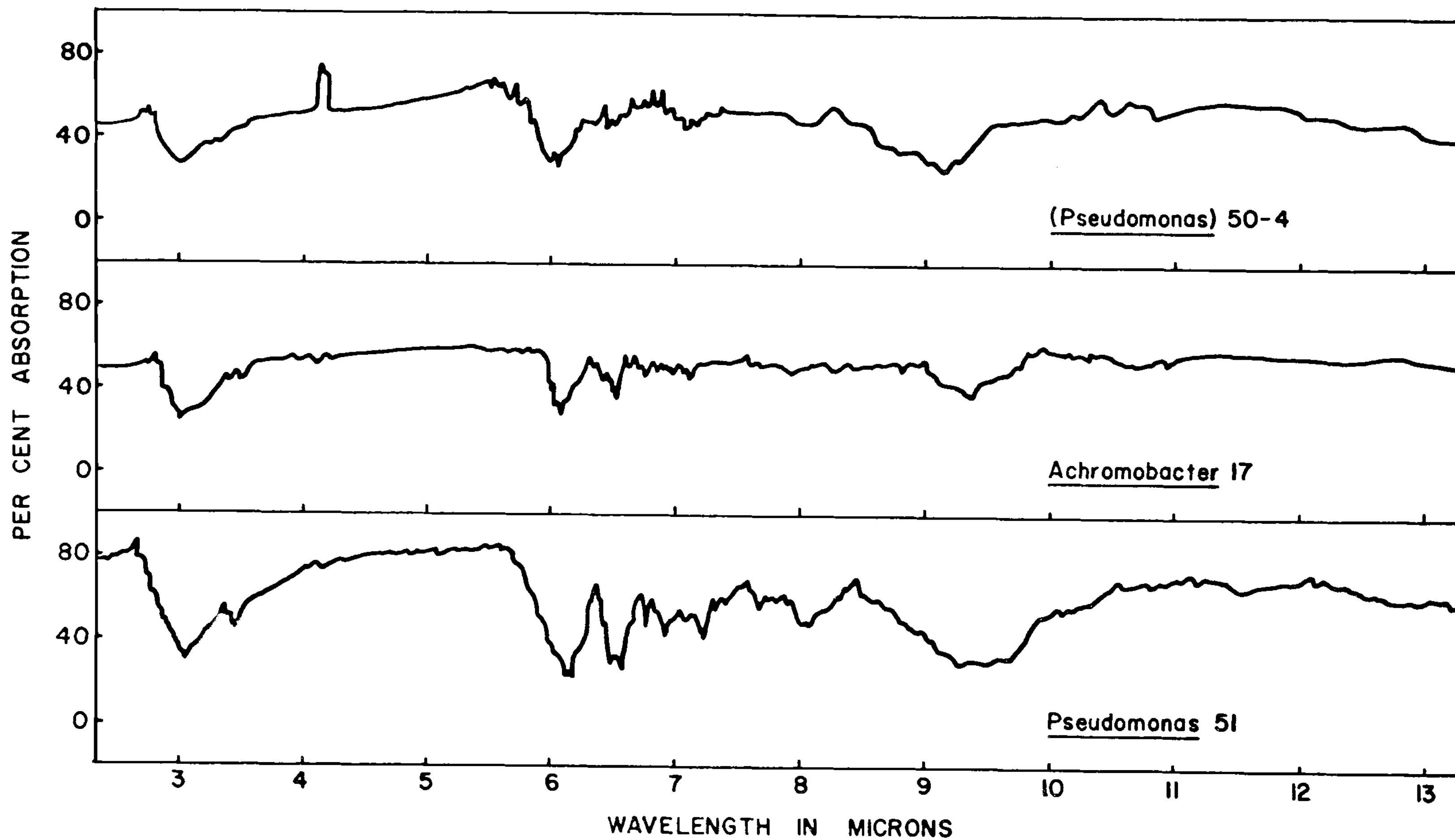


Figure 10. Infrared spectra of three Gram negative marine bacilli.

for 60 hours at 20 C.

This marine bay sediment isolate is a short Gram negative rod occurring singly or in pairs. Motility cannot be demonstrated by employing either dark field, hanging-drops, semi-solid agar, or flagella staining. Pleomorphism, as with many other marine bacteria, is quite commonly encountered. The *Achromobacter* culture's morphological, cultural, and physiological characteristics are shown in Charts 21 and 22 (appendix). As evident from the physiological reactions, this organism is quite biochemically inactive. Glucose and sucrose are slowly utilized aerobically with little acid and no gas produced. However, with glucose and nitrite serving as the hydrogen acceptor, the activity increases concomitantly with the reduction of nitrite to nitrogen gas.

The differentiation of the marine *Achromobacter* group does pose certain difficulties due to numerous negative physiological tests and to their similarity to the marine pseudomonads. Both motile and non-motile species are found in the sediment environment according to Zobell and Upham (1944). However, Shewan *et al.* (1961) have never encountered a motile *Achromobacter* species corresponding to *Achromobacter liquefaciens* as described in Bergey's Manual of Determinative Bacteriology (1957). However, Payne (1961) has isolated an organism which possesses morphological and biochemical properties typical of an *Achromobacter* culture, yet is motile by peritrichous flagella. In this dissertation the main criteria in placing the nitrate

reducing isolate in the Achromobacter genus rather than the Pseudomonas genus are based on the following points: (1) non-motile, (2) sensitive to pencillin, and (3) the infrared spectra pattern shown in Figure 10. The organism, although giving similar morphological and physiological characteristics to the Pseudomonas genus is obviously, by virtue of its infrared spectra, not a Pseudomonas species. There are several points along the spectra that are substantially different as is evident in Figure 10. A preliminary species designation of Achromobacter denitrificans is proposed pending future investigation and a continued literature search. After a lengthy search of the literature no marine Achromobacter culture possessing only a nitrite reductase, while being void of nitrate reductase, was found. However, Yourtt (1954) has used, in a study on the effect of oxygen on denitrification, an Achromobacter strain isolated from soil which reduces only nitrite.

Studies on Achromobacter 62

Preliminary data indicated that the Achromobacter 62 isolate possesses an electron transport system void of the ability to reduce nitrate, while being able to use nitrite-oxygen as the terminal hydrogen acceptor. This unique characteristic of this isolate warranted further study. The marine Nocardia culture 50-2 and P. stutzeri, both of which reduce nitrate to nitrogen gas, were used as controls.

As is evident by the nitrogen gas evolved by Achromobacter 62

(Table XVI), only nitrite would serve as the final acceptor of hydrogen. However, being typically facultative in nature, molecular oxygen is preferentially used over nitrite, but no gas is evolved.

As is shown in Table XVII, the Nocardia isolate 50-2 produced 19, 250 μg of nitrogen gas in 48 hours when incubated at 20 C. Although less nitrogen gas was produced from nitrite (13, 600 μg), gas began to appear sooner than with nitrate. This was expected because only two oxygen atoms have to be removed from nitrite before nitrogen gas is released. Apparently, the Nocardia species is able to use a secondary acceptor, since some nitrogen is evolved in the complete medium (NRT) minus the inorganic acceptors. In no case is nitrogen gas found when sufficient aeration is provided, even though maximum growth is obtained when oxygen is the terminal hydrogen acceptor.

As is shown in Table XVIII, when employing synthetic media under anaerobic conditions, NH_4^+ and NO_2^- are required by Achromobacter 62 for any substantial increase in initial OD and nitrogen gas evolution. On the other hand, the Achromobacter 62 culture under aerobic conditions does not use NO_3^- as a nitrogen source, as is evident from Figure 11. Moreover, the Achromobacter 62 isolate cannot use nitrate by either the assimilatory or dissimilatory routes. In other words, this isolate does not possess any type of functional nitrate reductase enzyme system.

The data in Table XIX relating to mass culture experiments

TABLE XVI. THE EFFECT OF VARIOUS HYDROGEN ACCEPTORS ON N_2 PRODUCTION AND ON CELLULAR GROWTH OF _ACHROMOBACTER_ 62.

Nutrients and Hydrogen Acceptors*	OD	µg N_2
NRT	0	0
NRT, NO_2^- (140 ppm)	.58	9,500
NRT, NO_3^- (750 ppm)	.05	0
NRT, $SO_4^=$ (200 ppm)	.04	0
NRT, Oxygen	.67	0

*Anaerobic system incubated 48 hours at 20 C.

TABLE XVII. THE EFFECT OF VARIOUS HYDROGEN ACCEPTORS ON N_2 PRODUCTION AND ON CELLULAR GROWTH OF NOCARDIA 50-2.

Nutrients and Hydrogen Acceptor*	OD	µg N_2
NRT	0.12	580
NRT, NO_2^- (140 ppm)	0.59	14,600
NRT, NO_3^- (750 ppm)	0.68	19,250
NRT, $SO_4^=$ (200 ppm)	0.14	950
NRT, Oxygen	0.87	0

*Anaerobic system incubated 48 hours at 20 C.

TABLE XVIII. ANAEROBIC NITROGEN SOURCES TESTED WITH <u>ACHROMOBACTER</u> 62.

Nutritional Adjustment of Synthetic Media*	OD	µg N_2
None	0.02	0
NO_2^--N 21,000 µg	0.04	0
NO_3^--N 35,700 µg	0.03	0
NH_4^+-N 28,000 µg NO_2^--N 21,000 µg	0.39	8,080
NH_4^+-N 28,000 µg NO_3^--N 35,700 µg	0.06	0

*Incubation at 20 C for 6 hours (media supplemented with 1.0 g per liter of beef extract).

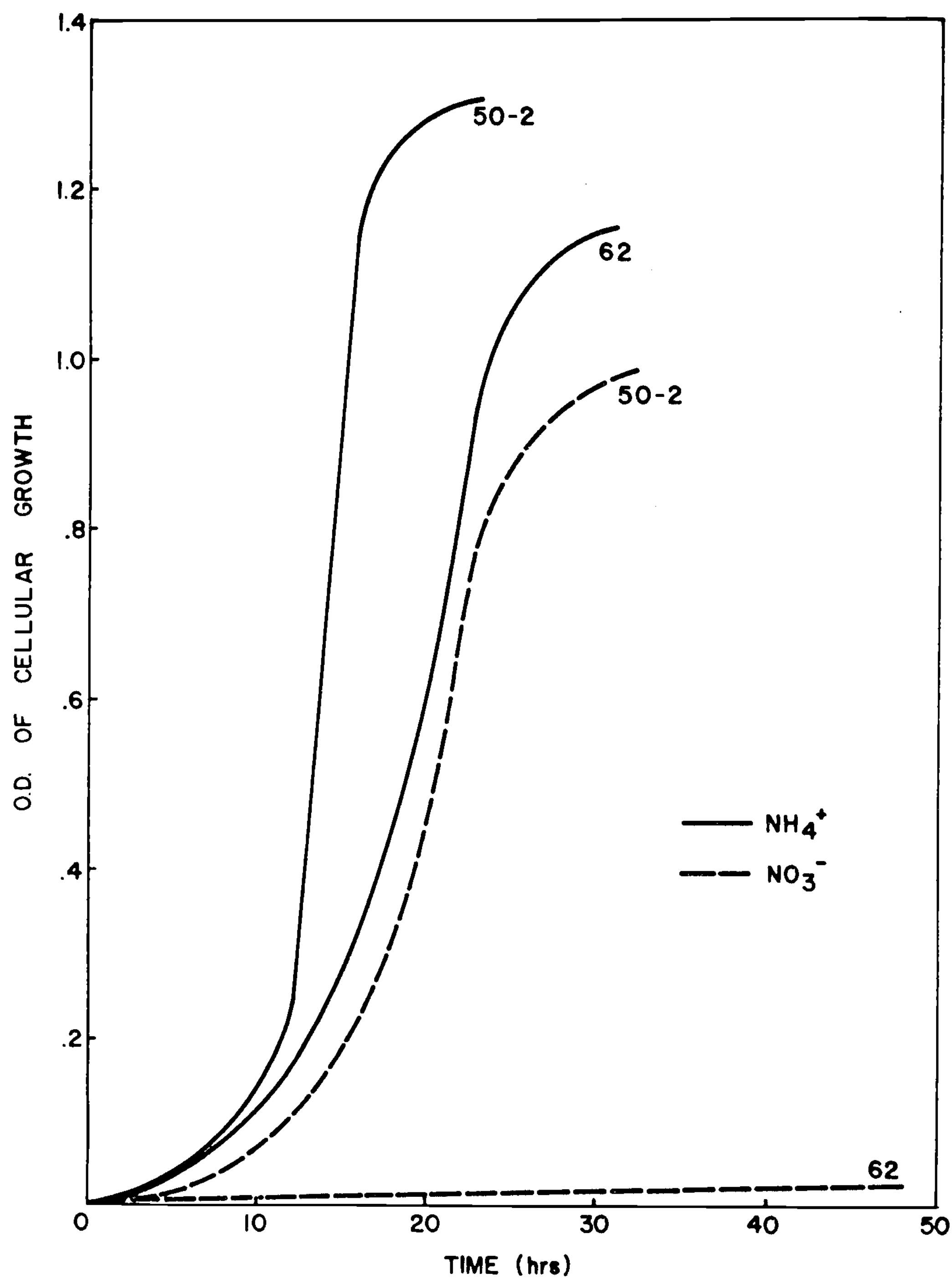


Figure 11. Utilization of nitrogen sources by Achromobacter 62 and Nocardia 50-2.

TABLE XIX. TIME STUDY OF NITROGEN GAS PRODUCTION BY *ACHROMOBACTER* 62. *

Hours	µg NO_2	µg N_2
0	15,000	0
1	9,000	2,234
2	3,500	3,085
3	0	8,260
4	0	9,820
5	0	9,187
6	0	12,317

*Mass culture in a complete media incubated at 20 C.

demonstrate isolate 62's capacity to reduce nitrite to nitrogen gas. At the end of two hours, all that the nitrite added (15,000 µg) disappears and in six hours 80 percent of the nitrite is converted to nitrogen gas. As may be observed, there is lag between the nitrite disappearance and the amount of nitrogen gas produced, suggesting the presence of one or more intermediates.

It was noticed that in unamended synthetic media, cellular growth and in particular nitrogen gas decreased in comparison to the complete media (NRT).

As the data in Table XX indicate, the Achromobacter culture requires a component in beef extract for the production of nitrogen gas. It was observed that nitrite is not utilized when beef extract is omitted from the media, suggesting that the beef extract requirement is associated with the nitrate reductase enzyme and not some intermediate in the reduction pathway. Protein hydrolysate, peptone, or yeast extract does not satisfy the beef extract requirements. It should be noted that some growth (OD 0.21) without nitrogen gas evolution resulted with nitrite and the protein hydrolysate. Similarly, a growth level of OD 0.28 is obtained with the protein hydrolysate, yeast extract, and nitrite. Likewise, with peptone, yeast extract, and the hydrogen acceptor nitrite growth without gas is obtained. Only with the inclusion of beef extract does reduction of nitrite to nitrogen gas (8,080 µg) and a nearly two fold growth increase (OD 0.46) result.

TABLE XX. THE CO-FACTOR REQUIREMENTS OF *ACHROMOBACTER* 62 FOR NITROGEN GAS PRODUCTION. *

Nutritional Adjustment of Synthetic Media	OD	µg of N_2
Protein hydrolysate	0.02	0
Protein hydrolysate NO_2^--N 21,000 µg	0.21	0
Protein hydrolysate Yeast Extract 1 mg per ml NO_2^--N 21,000 µg	0.28	0
Peptone Yeast extract 1 mg per ml NO_2^--N 21,000 µg	0.28	0
Beef extract 1.0 g per liter NO_2^--N 21,000 µg	0.46	8,080

*Growing cells incubated 48 hours at 20 C.

The data, therefore, clearly indicate that the beef extract contains a factor other than a growth nutrient, presumably some essential co-factor necessary for nitrite reduction.

The role of yeast extract associated with denitrification and particularly with nitrate reductase activity is well documented in the literature by numerous workers. The majority of the work on the requirement of yeast extract for nitrate reduction has been conducted with soil isolates. As seen from Table XXI, the marine isolate *Nocardia* 50-2 requires yeast extract for optimum growth and nitrogen gas production. The yeast extract concentration is not linear to nitrogen gas production as found with many soil studies. A minimum concentration seems to be required for growth and nitrogen gas evolution. It is not known whether this is solely a growth factor or the result of both increased growth and nitrate reductase activity.

In contrast the *Achromobacter* 62 (Table XXII) which is void of the nitrate reductase system, does not show any stimulation to the varied concentration of yeast extract. The nitrite reductase does not require any co-factor associated with yeast extract. The slight growth stimulation from OD 0.56 to 0.66 accounts for the increase in nitrogen gas production.

A mass culture experiment was conducted (Table XXIII) to determine the adaptive or constitutive nature of the nitrite reductase system of the *Achromobacter* number 62 isolate. The culture is first

TABLE XXI. THE EFFECT OF YEAST EXTRACT ON NITROGEN GAS PRODUCTION AND CELLULAR GROWTH OF *NOCARDIA* 50-2.

Yeast Extract* mg/ml	OD	μg of N_2
0.025	0.09	121
0.05	0.12	160
0.10	0.13	162
0.25	0.21	121
0.50	0.27	253
0.75	0.31	143
1.0	0.75	22,309

*Anaerobic system in synthetic media incubated for 96 hours at 20 C with 37,500 μg NO_3^--N.

TABLE XXII. THE EFFECT OF YEAST EXTRACT ON NITROGEN GAS PRODUCTION AND CELLULAR GROWTH OF ACHROMOBACTER 62.

Yeast Extract* mg/ml	OD	µg of N_2
0	0.56	9,650
0.025	0.56	9,540
0.05	0.55	9,780
0.10	0.56	9,500
0.25	0.57	10,200
0.50	0.58	10,460
0.75	0.61	11,090
1.0	0.66	12,870

*Anaerobic system in synthetic media incubated for 96 hours at 20 C with 21,000 µg of NO_2^--N.

TABLE XXIII. ADAPTIVE OR CONSTITUTIVE NATURE OF NITRITE REDUCTASE SYSTEM OF <u>ACHROMOBACTER</u> 62*.

Nutritional Adjustments of NRT Media	Terminal Acceptor	µg N_2
None	NO_2^-	22,377
None	NO_3^-	212
NO_2^--N 21,000 µg	NO_2^-	19,911
NO_2^--N 21,000 µg	NO_3^-	0
NO_3^--N 37,500 µg	NO_2^-	23,272
NO_3^--N 37,500 µg	NO_3^-	335
NO_2^--N 21,000 µg NO_3^--N 37,500 µg	NO_2^-	18,942
NO_2^--N 21,000 µg NO_3^--N 37,500 µg	NO_3^-	0

*Mass cells incubated 18 hours at 20 C.

grown aerobically for 24 hours, then harvested and washed twice in 0.2 M phosphate buffer. The cells are then inoculated into NRT media and exposed to either nitrate or nitrite for 18 hours at 20 C. From the results it is evident that no pre-induction is necessary for nitrite reductase activity. The reason for 212 µg of nitrogen gas from nitrite when incubated with nitrate is possibly due to residual nitrite reductase activity. Upon transfer to a fresh medium, actively growing cells give off some reduced by-products, in this case nitrogen gas. When the culture is grown in the presence of the inducer nitrite, no increase in gas production is observed. Similarly, when initially grown in the presence of nitrate no substantial increase in nitrogen gas production is evident.

Nitrite reductase is an important enzyme in the nitrate reduction process. In the case of the Achromobacter 62 isolate, under anaerobic conditions nitrite reductase is involved in the energy-yielding process similar to aerobic respiration. Yamanaka (1964) has purified and crystallized the cytochrome oxidase of Pseudomonas aeruginosa. This cytochrome oxidase not only possesses the general properties of cytochrome oxidase but also acts as a nitrate reductase. Pseudomonas cytochrome oxidase acts as a nitrate reductase in that it reduces nitrite to nitric oxide with reduced Pseudomonas cytochrome c-551 as the electron donor under anaerobic conditions. The spectrum of the Pseudomonas cytochromes have peaks at 280 mµ, 412 mµ, 525 mµ, and

635 mμ in the oxidized form and 420 mμ, 523 mμ, 549 mμ, and 554 mμ in the reduced form. In comparison, the cytochrome spectra of *Achromobacter* 62 species shown in Figure 12 have peaks at 350 mμ, 416 mμ, 520 mμ, and 555 mμ in the oxidized form and 355 mμ, 420 mμ, 530 mμ, and 560 mμ in the reduced form. Only one peak is common to both cytochrome systems; therefore, obviously different cytochrome are associated with respective nitrite reductases. Also the *Pseudomonas* cytochrome has a peak at 460 mμ which is the γ-band due to haem a_2. This band is not evident in the *Achromobacter* cytochrome system.

Other differences between the two nitrite reductases are reflected by the fact that the *Pseudomonas* reductase is adaptive in nature, whereas the *Achromobacter* reductase is constitutive. That is, with the *Pseudomonas* culture, nitrate reductase activity is present only in cells grown in the presence of nitrate, whereas, in the *Achromobacter* system, the nitrite reductase is present regardless of inducers. Although both cytochrome systems contain no copper, the *Pseudomonas* species is stimulated by copper sulfate, whereas no increase in density of the peak were observed upon the addition of copper sulfate. On the other hand, Walker and Nicholas (1961), also working with *P. aeruginosa*, reported that the enzyme contained an additional absorption between 630 and 635 mμ which is probably associated with copper. However, with the *Achromobacter* cytochrome no peak was found

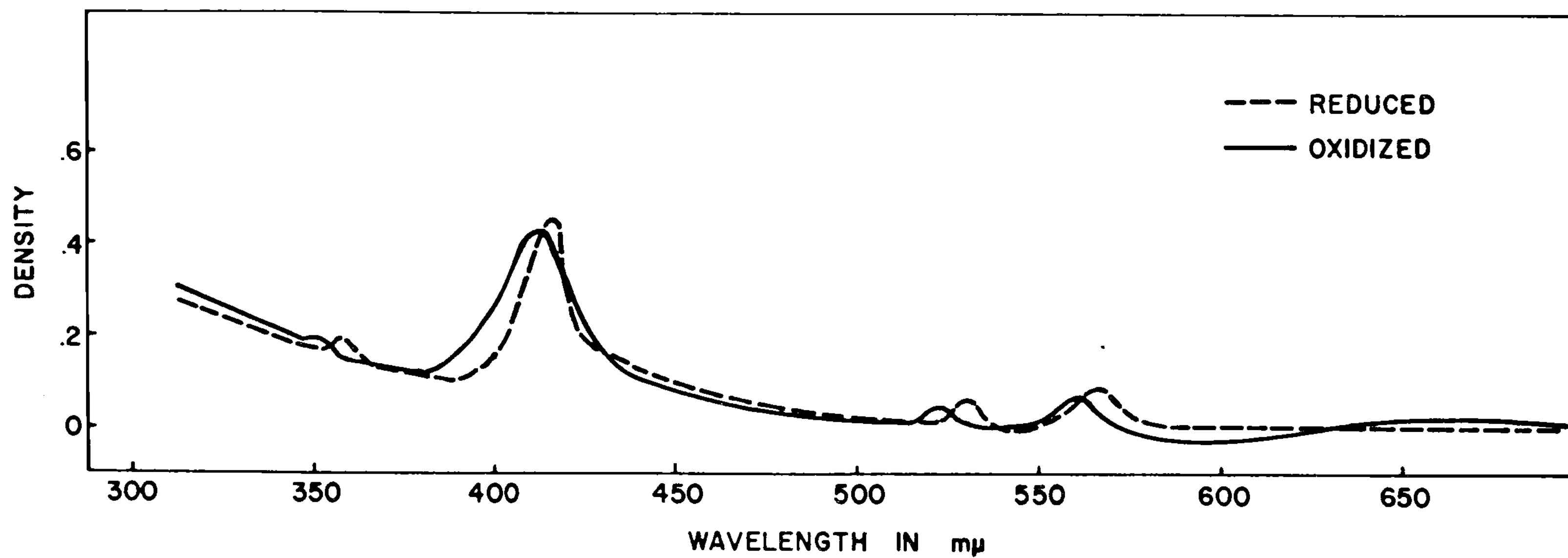


Figure 12. Spectra of oxidized and reduced cytochrome purified from *Achromobacter* 62.

between 630 and 635 mμ in the spectra pattern (Figure 12).

Realizing that further purifications are required it can be concluded that the nitrite reductase system of the Achromobacter, although possessing a typical cytochrome c, is substantially distinct from the nitrite reductase system found in nitrate reducing bacteria.

A preliminary species designation of Achromobacter denitrificans (species nova) is proposed.

SUMMARY

1. The determination of total bacteria population of marine bay sediment counts of over a million cells per gram of dry weight reflects an abundant microbial population. A maximum count of 2.3×10^8 per gram of dry weight sediment was obtained from the one inch core depth. On the other hand, the lowest count of 3.0×10^4 - 2.8×10^5 was obtained at the ten inch core depth. No seasonal variation in respect to total population was evident. Total bacterial counts were not as high as realized in many soil systems.

2. From a total of 100 bacterial isolates tested for nitrate reductase activity, 47 were capable of reducing nitrate to nitrite. More interesting was the fact that 16 of these bacterial isolates were able to reduce nitrite to nitrogen gas and oxides of nitrogen. Thus, it is apparent that marine bay sediments harbor a large indigenous flora capable of using nitrate and nitrate as the final hydrogen acceptor.

3. Total organic matter of marine sediment varied between 1.40 and 3.03 percent. Organic carbon at the ten inch level as compared to surface sediments, generally gave higher values.

   Total nitrogen exhibited a wide range of values from zero to 1.39 percent for surface sediments. A slightly higher

nitrogen content was obtained with increase in core depth.

Although predictions as to nutritional supply drawn from C/N values are sometime misleading, sediment ratios between 1.2 to 3.5 do not indicate a nitrogen deficiency.

4. The general aerobic respiration characteristics of the four core areas studied indicate an active sediment flora comparable to many soil systems. In core area number one, amended with 1 mM glucose and 37,500 µg NO_3-N, a QO_2 g value of 86.0 µl was obtained when incubated at 28 C. Complete oxidation of substrate was indicated by the RQ value of 1.04. Upon the omission of substrate glucose, respiration levels were greatly reduced. Endogenous aerobic respiration varies from QO_2 g of 7.5 µl to as low as 3.0 µl. It should also be pointed out that with nitrate incubated under aerobic conditions no nitrogen gas or oxides of nitrogen were detected. Evidentally, a major part of the added nitrate was used in an assimilatory manner rather than via the dissimilation route.

Anaerobically 1,850 µg of nitrogen gas was produced in 110 hours, an equivalent of 1.3 µg of nitrogen gas per hour per gram of dry weight sediment. This indicates that the marine sediment contains a high flora level capable of using nitrate as the terminal acceptor of hydrogen.

Interestingly enough, one core area, unlike the other

sediment samples, exhibited a lower QO_2 g of 20.4 µl at 28 C, as compared to 61.0 µl at 15 C. Similarly, anaerobically when nitrate-oxygen served as the hydrogen acceptor 1,150 µg of nitrogen gas was produced as compared to 2,200 µg of nitrogen gas at the lower temperature of 15 C. Sediments probably contain pockets or areas where psychrophile activity dominates.

5. Some eleven sediment isolates showing varied capacity to denitrify have been given a preliminary genus designation. Among the genera represented are three *Pseudomonas* sp., two *Achromobacter* sp., a *Breviabacterium* sp., a *Bacillus* sp., a *Vibrio* sp., a *Flavobacterium* sp., and a *Nocardia* sp. A preliminary species designation of *Nocardia denitrificans* (species nova) is proposed pending a continued search of the literature. One of the *Achromobacter* isolates number 62 demands extra attention in that it cannot use nitrate; however, nitrite will serve as its sole hydrogen acceptor.

The capacity of these organisms to reduce nitrate varies between 13,949 µg to 980 µg of nitrogen gas in 60 hours when incubated at 20 C. This quantity of nitrogen gas evolved reflects the large denitrifying capacity of these marine isolates. However, being typically facultative in nature, oxygen is preferentially used over nitrate.

6. Realizing some of the problems involved in classification of the marine pseudomonads, infrared spectra of the whole cells proved to be a valuable supporting tool in ascertaining genera designations.

7. The *Achromobacter* isolate (62) displays the unique characteristics of using nitrite-oxygen as its final hydrogen. In other words, this Gram negative bacilli possesses a nitrite reductase while being void of a nitrate reductase. This *Achromobacter* culture requires a component, presumably some essential co-factor in beef extract for nitrite reduction. Interestingly enough, yeast extract does not satisfy its co-factor requirement. The organism's main nitrogen source is ammonia, although slow utilization of amino acids were observed. Neither nitrate nor nitrite satisfied the culture's nitrogen requirement.

The nitrite reductase of the marine *Achromobacter* 62 species proved constitutive in nature, whereas, in contrast, the nitrite reductase of the *Nocardia* species, which possesses a nitrate reductase, is inducible.

The nitrite reductase of the *Achromobacter* 62 indicates a cytochrome c-like component associated with the electron transport system. No copper moity is evident from purified cytochrome absorption spectra.

A preliminary species designation of *Achromobacter denitrificans* (species nova) is proposed pending a continued search of the literature.

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APPENDIX

Descriptive Chart 1

Morphological and Cultural Characteristics of 50-2 (Nocardia)

Morphology

Vegetative cells	. Filament in young cultures
. . . . .	. Medium to long rods
Gram stain	. Gram positive
Flagella stain	. No flagella
Endospore stain	. no endospore

Surface Colonies (Plate Count)

Form	. Irregular, and pilled as older
Elevation	. Raised
Surface	. Rough
Edge	. Undulated and irregular
Optical characters . . .	. Opaque and dull
. . . . .	. Yellowish as older
Consistency	. Dough-like

Streak (Agar Slant)

Form	. Effused to filiform
Surface	. Slightly raised
Optical characters . . .	. Dull and yellow
Consistency	. Dough-like

Gelatin Stab

Liquefaction	. Very slow, cratenform
. . . . .	. Growth along inoculation

Liquid Cultures (Nutrient broth)

Surface growth	. Very small film
Sediment	. Flaky and abundant

Descriptive Chart 2

Physiological Reactions of 50-2 (Nocardia)

	48 hours	96 hours
Ammonium Phosphate Utilization	4+	4+
Acetyl-Methyl-Carbinol	-	-
Catalase Production	4+	4+
Citrate Utilization	4+	4+
Gelatin Liquefaction	1+	1+
Hydrogen Sulfide Production	-	-
Indole Production	-	-
Litmus Milk Reaction	-	-
Nitrate Reduction	4+	4+
Sodium Chloride 7.5 percent	3+	3+
Sodium Chloride 12 percent	2+	2+
Starch Hydrolysis	1+	1+

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not tested

Descriptive Chart 3

Morphological and Cultural Characteristics of 51. (Pseudomonas)

Morphology

Vegetative cells	Small rods
Gram stain	Gram negative
Flagella stain	Polar flagella
Endospore stain	No endospores

Surface Colonies (Plate Culture)

Form	Circular, with some spreading
Elevation	Raised
Surface	Slightly granular
Edge	Irregular to entire
Optical characters	Grayish white
Consistency	Opaque, brownish as older

Streak (Agar Slant)

Form	Filiform
Surface	Smooth, flat
Optical characters	Opaque, whitish
Consistency	Soft

Gelatin Stab

Liquefaction	Slow, infundibule

Liquid Cultures (Nutrient broth)

Growth	Turbid
Sediment	Heavy, slightly viscid

Descriptive Chart 4

Physiological Reactions of 51, (Pseudomonas)

	48 hours	96 hours
Ammonium Phosphate Utilization	4+	4+
Acetyl-Methyl-Carbinol	-	-
Catalase Production	-	-
Citrate Utilization	NT	NT
Gelatin Liquefaction	-	2+
Hydrogen Sulfide Production	-	-
Indole Production	-	-
Litmus Reaction	-	1+
Nitrate Reduction	4+	4+
Sodium Chloride 7.5 percent	2+	2+
Sodium Chloride 12 percent	1+	1+
Starch Hydrolysis	-	1+

Reaction Scale:

-	No Reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested

Descriptive Chart 5

Morphological and Cultural Characteristics of 53 (*Brevibacterium*)

Morphology

Vegetative cells	Ovoid short rods
Gram stain	Gram positive
Flagella stain	Peritrichous
Endospore stain	No endospore

Surface Colonies (Plate Culture)

Form	Circular
Elevation	Pulvinate
Surface	Smooth and glistening
Edge	Entire
Optical characters	Clear to white
Consistency	Soft

Streak (Agar Slant)

Form	Effuse
Surface	Granular
Optical characters	White
Consistency	Butyrous

Gelatin Stab

Liquefaction	None

Liquid Cultures (Nutrient Broth)

Growth	Granular
Sediment	Scanty

Descriptive Chart 6

Physiological Reactions of 53 (*Brevibacterium*)

	48 hours	96 hours
Ammonium Phosphate Utilization	4+	4+
Acetyl-Methyl-Carbinol	-	-
Catalase Production	NT	NT
Citrate Utilization	NT	NT
Gelatin Liquefaction	-	-
Hydrogen Sulfide Production	-	-
Litmus Milk Reaction	-	1+
Nitrate Reduction	4+	4+
Sodium Chloride 7.5 percent	4+	4+
Sodium Chloride 12 percent	3+	4+
Starch Hydrolysis	-	-

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested

Descriptive Chart 7

Morphological and Cultural Characteristics of 51-7B (unknown)

Morphology

Vegetative cells . . . . . . Filaments, rods as older
Gram stain . . . . Gram variable
Flagella stain . . . . Non motile
Endospore stain . . . . . . No endospores

Surface Colonies (Plate Culture)

Form . . . . Circular
Elevation . . . . Convex
Surface . . . . Smooth and glistening
Edge . . . . Entire
Optical characters . . . . Clear
Consistency . . . . Soft

Streak (Agar Slant)

Form . . . . Effuse
Surface . . . . Granular
Optical Characters . . . . Opaque
Consistency . . . . Granular

Gelatin Stab

Liquefaction . . . . Slow, crateriform

Liquid Cultures (Nutrient Broth)

Surface Growth . . . . . . None
Sediment . . . . Turbid

Descriptive Chart 8

Physiological Reactions of 51-7B (Unknown)

	48 hours	96 hours
Ammonium Phosphate Utilization	3+	4+
Acetyl-Methyl-Carbinol	2+	2+
Catalase Production	-	-
Citrate Utilization	1+	2+
Gelatin Liquefaction	-	-
Hydrogen Sulfide Production	-	-
Indole Production	-	-
Litmus Milk Reaction	-	-
Nitrate Reduction	3+	4+
Sodium Chloride 7.5 percent	2+	3+
Sodium Chloride 12 percent	1+	2+
Starch Hydrolysis	-	-

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested

Descriptive Chart 9

Morphological and Cultures Characteristics of 17 (Achromobacter)

Morphology

Vegetative cells . . . . . . Rods, medium size
Gram stain . . . . Gram negative
Flagella stain . . . . Peritrichous
Endospore stain . . . . . . No endospores

Surface Colonies (Plate Culture)

Form . . . . Circular
Elevation . . . . Convex
Surface . . . . Smooth
Edge . . . . Entire
Optical characters . . . . White
Consistency . . . . Heavy

Streak (Agar Slant)

Form . . . . Beaded
Surface . . . . Waxy and glistening
Optical characters . . . . Opaque
Consistency . . . . Heavy and butyrous

Gelatin Stab

Liquefaction . . . . None

Liquid Cultures (Nutrient Broth)

Growth . . . . Turbid
Sediment . . . . Viscous and heavy

Descriptive Chart 10

Physiological Reactions of 17 (*Achromobacter*)

	48 hours	96 hours
Ammonium Phosphate Utilization	4+	4+
Acetyl-Methyl-Carbinol	-	-
Catalase Production	NT	NT
Citrate Utilization	NT	NT
Gelatin Liquefaction	-	-
Hydrogen Sulfide Production	-	-
Indole Production	-	-
Litmus Milk Reaction	-	-
Nitrate Reduction	4+	4+
Sodium Chloride 7. 5 percent	1+	3+
Sodium Chloride 12 percent	1+	1+
Starch Hydrolysis	-	-

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested

Descriptive Chart 11

Morphological and Cultural Characteristics of 50-7 (Bacillus)

Morphology

Vegetative cells	Rods, pairs and chains
Gram stain	Gram positive
Flagella stain	Motile
Endospore stain	Endospores

Surface Colonies (Plate Culture)

Form	Circular and large
Elevation	Convex
Surface	Smooth
Edge	Entire
Optical characters	Opaque
Consistency	Soft

Streak (Agar Slant)

Form	Beaded
Surface	Flat to rough
Optical characters	Opaque
Consistency	Mucoid

Gelatin Stab

Liquefaction	Moderate, crateriform

Liquid Cultures (Nutrient Broth)

Surface growth	Pellicle
Sediment	Scanty

Descriptive Chart 12

Physiological Reactions of 50-7 (*Bacillus*)

	48 hours	96 hours
Ammonium Phosphate Utilization	4+	4+
Acetyl-Methyl-Carbinol	2+	3+
Catalase Production	1+	2+
Citrate Utilization	2+	3+
Gelatin Liquefaction	2+	2+
Hydrogen Sulfide Production	-	-
Indole Production	-	-
Litmus Milk Reaction	-	2+
Nitrate Reduction	3+	4+
Sodium Chloride 7.5 percent	2+	4+
Sodium Chloride 12 percent	2+	3+
Starch Hydrolysis	2+	3+

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested

Descriptive Chart 13

Morphological and Cultural Characteristics of 51-1A (Pseudomonas)

Morphology

Vegetative cells . . . . . . Rods
Gram stain . . . . Gram negative
Flagella stain . . . . Polar
Endospore stain . . . . . . No endospore

Surface Colonies (Plate Culture)

Form . . . . Circular
Elevation . . . . Convex
Surface . . . . Smooth
Edge . . . . Diffuse
Optical characters . . . . Gray to white
Consistency . . . . Soft

Streak (Agar Slant)

Form . . . . Echinulate
Surface . . . . Smooth
Optical characters . . . . Glistening, off white
Consistency . . . . Butyrous

Gelatin Stab

Liquefaction . . . . Slow, crateriform

Liquid Cultures (Nutrient Broth)

Growth . . . . Turbid
Sediment . . . . Scanty

Descriptive Chart 14

Physiological Reactions of 51-1A (*Pseudomonas*)

	48 hours	96 hours
Ammonium Phosphate Utilization	4+	4+
Acetyl-Methyl-Carbinol	-	1+
Catalase Production	NT	NT
Citrate Utilization	1+	1+
Gelatin Liquefaction	1+	1+
Hydrogen Sulfide Production	1+	1+
Indole Production	-	-
Litmus Milk Reaction	1+	2+
Nitrate Reduction	3+	4+
Sodium Chloride 7.5 percent	2+	3+
Sodium Chloride 12 percent	1+	3+
Starch Hydrolysis	1+	1+

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested

Descriptive Chart 15

Morphological and Cultural Characteristics of 59 (Vibrio)

Morphology

Vegetative cells	Curved rods
Gram stain	Gram negative
Flagella stain	Polar
Endospore stain	No endospore

Surface Colonies (Plate Culture)

Form	Circular
Elevation	Pulvinate
Surface	Smooth
Edge	Entire, lobate as older
Optical characters	Opaque
Consistency	Hard, wrinkled

Streak (Agar Slant)

Form	Filiform and beaded
Surface	Flat and rough
Optical characters	Opaque
Consistency	Hard and glistening

Gelatin Stab

Liquefaction	Slow

Liquid Cultures (Nutrient Broth)

Growth	Turbid heavy
Sediment	Viscid

Descriptive Chart 16

Physiological Reactions of 59 (*Vibrio*)

	48 hours	96 hours
Ammonium Phosphate Utilization	4+	4+
Acetyl-Methyl-Carbinol	-	-
Catalase Production	-	-
Citrate Utilization	-	-
Gelatin Liquefaction	-	1+
Hydrogen Sulfide Production	-	-
Indole Production	-	-
Litmus Milk Reaction	-	-
Nitrate Reduction	4+	4+
Sodium Chloride 7.5 percent	1+	1+
Sodium Chloride 12 percent	-	1+
Starch Hydrolysis	1+	1+

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested

Descriptive Chart 17

Morphological and Cultural Characteristics of 36 (*Flavobacterium*)

Morphology

Vegetative cells	Rods, single
Gram stain	Gram negative
Flagella stain	No flagella
Endospore stain	No endospore

Surface Colonies (Plate Culture)

Form	Irregular to circular
Elevation	Flat
Surface	Dull
Edge	Slightly undulate
Optical characters	Light brownish-yellow
Consistency	Gummy

Streak (Agar Slant)

Form	Filiform
Surface	Glistening
Optical characters	Yellow
Consistency	Waxy to soft

Gelatin Stab

Liquefaction	Infundibule

Liquid Cultures (Nutrient Broth)

Growth	Pellicle, some turbidity
Sediment	Scanty sediment

Descriptive Chart 18

Physiological Reactions of 36 (*Flavobacterium*)

	48 hours	96 hours
Ammonium Phosphate Utilization	3+	4+
Acetyl-Methyl-Carbinol	NT	NT
Catalase Production	NT	NT
Citrate Utilization	-	1+
Gelatin Liquefaction	2+	2+
Hydrogen Sulfide Production	-	-
Indole Production	-	-
Litmus Milk Reaction	1+	2+
Nitrate Reduction	4+	4+
Sodium Chloride 7.5 percent	1+	2+
Sodium Chloride 12 percent	-	2+
Starch Hydrolysis	-	-

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested

Descriptive Chart 19

Morphological and Cultural Characteristics of 50-4 (*Pseudomonas*)

Morphology

Vegetative cells	Rods, small
Gram stain	Gram negative
Flagella stain	Motile
Endospore stain	No endospore

Surface Colonies (Plate Culture)

Form	Punctiform
Elevation	Convex
Surface	Smooth
Edge	Entire
Optical characters	Translucent
Consistency	Soft

Streak (Agar Slant)

Form	Echinulate
Surface	Smooth
Optical characters	Translucent
Consistency	Mucoid

Gelatin Stab

Liquefaction	None

Liquid Cultures (Nutrient Broth)

Growth	Turbid, small pellicle
Sediment	Slight

Descriptive Chart 20

Physiological Reactions of 50-4 (*Pseudomonas*)

	48 hours	96 hours
Ammonium Phosphate Utilization	4+	4+
Acetyl-Methyl-Carbinol	-	-
Catalase Production	NT	NT
Citrate Utilization	-	-
Gelatin Liquefaction	-	-
Hydrogen Sulfide Production	-	-
Indole Production	-	-
Litmus Milk Reaction	-	-
Nitrate Reduction	4+	4+
Sodium Chloride 7.5 percent	1+	3+
Sodium Chloride 12 percent	1+	2+
Starch Hydrolysis	-	-

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested

Descriptive Chart 21

Morphological and Cultural Characteristics of 62 (Achromobacter)

Morphology

Vegetative cells . . . . . . Rods, medium to small
Gram stain . . . . Gram negative
Flagella stain . . . . None
Endospore Stain . . . . . . No endospore

Surface Colonies (Plate Culture)

Form . . . . Circular
Elevation . . . . Convex
Surface . . . . Smooth
Edge . . . . Entire
Optical characters . . . . Translucent, white as older
Consistency . . . . Soft

Streak (Agar Slant)

Form . . . . Effuse
Surface . . . . Beaded to smooth
Optical characters . . . . Translucent
Consistency . . . . Soft to butyrous

Gelatin Stab

Liquefaction . . . . None

Liquid Cultures (Nutrient Broth)

Growth . . . . Scanty turbidity
Sediment . . . . Viscous

Descriptive Chart 22

Physiological Reactions of 62 (*Achromobacter*)

	48 hours	96 hours
Ammonium Phosphate Utilization	4+	4+
Acetyl-Methyl-Carbinol	-	-
Catalase Production	NT	NT
Citrate Utilization	-	1+
Gelatin Liquefaction	-	1+
Hydrogen Sulfide Production	-	-
Indole Production	-	-
Litmus Milk Reaction	-	-
Nitrate Reduction	-	-
Sodium Chloride 7.5 percent	2+	3+
Sodium Chloride 12 percent	1+	2+
Starch Hydrolysis	-	-

Reaction Scale:

-	No reaction
1+	Slight
2+	Moderate
3+	Abundant
4+	Heavy
NT	Not Tested