SOME RELATIONSHIPS BETWEEN PHYSIOLOGY, MORPHOLOGY, AND DISTRIBUTION IN SULFUR-OXIDIZING BACTERIA

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ABSTRACT

SOME RELATIONSHIPS BETWEEN PHYSIOLOGY, MORPHOLOGY, AND DISTRIBUTION IN SULFUR-OXIDIZING BACTERIA

By

Douglas Edgar Caldwell

Twelve sulfide-containing aquatic environments were examined using phase microscopy, electron microscopy, gradient enrichments, plate counts and physicochemical determinations. In anaerobic hypolimnia, flagellate or gas-vacuolate photosynthetic bacteria were found. As light decreased and sulfide increased (with depth), there was an increase in the surface to volume ratio of the cells, a transition from internal to peripheral chromaphores, a decrease in the carotenoid content of the cells, an increase in bacteriochlorophyll d, and a decrease in oxygen tolerance. These changes were due to variations in community structure rather than the phenotypic adaptation of individual populations. The correlation of these transitions with gradients of sulfide, oxygen, and light can be explained on the basis of light requirements and sulfide and oxygen toxicity.

Bacterial populations occurred in sequential layers within hypolimnia. In Burke Lake the microstratification exhibited the following sequence (from the thermocline to the sediments): *Thiospirillum, Chromatium, Thiocystis,* and *Chlorochromatium.* In

Wintergreen Lake *Thiopedia, Thiocystis,* and *Clathrochloris* occurred in sequential layers while *Prosthecochloris* was randomly distributed within the layer of *Clathrochloris*. The first series of layers was slightly turbid and red, contained purple sulfur bacteria and is referred to as community A. The next series of layers appeared green, contained green sulfur bacteria and is referred to as community B. The lowest layer occurred within 1 m of the sediment, was turbid and white, contained colorless bacteria, and is referred to as community C. The bacterial populations found within this layer have not been reported previously. The two ponds studied contained only community A.

In littoral zones and in a sulfur spring, the sulfur-oxidizing bacteria were attached to surfaces either by a holdfast or by mucilage which encased microcolonies. Although both photosynthetic and heterotrophic bacteria were found, the chemoautotrophic thiobacilli were not. *In situ,* thiosulfate gradient enrichments selected fluorescent pseudomonads rather than thiobacilli and resulted in a three-fold increase in growth-rate as the thiosulfate concentration increased from 55 µg/l to 1 g/l $S_2O_3^=$. Isolate TBT - H and *Pseudomonas aeruginosa* were found to produce fluorescent sulfide-binding exudates which resulted in formation of globules of elemental sulfur from sulfide. This reaction did not consume oxygen.

Previous reports on the fluorescent properties of *P. aeruginosa* exudates were confirmed and additional observations were made on the effect of pH on fluorescence. From a pH of 2.0 to 10.0 the exudate had an absorption peak at 405 nm. From a pH of 6.0 to 12.9 the exudate had a fluorescence peak at 460 nm (excitation, 400 nm) which shifted to 430 nm at a pH of 1.0. Fluorescence at 460 nm was most intense at a pH of 7.0. Lake samples (pH 7.0) were found to fluoresce at 440 nm although the intensity was nearly three orders of magnitude below that of the culture exudate.

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"We should not be limited by what we believe is possible."

Robert Ornstein

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INTRODUCTION

The last review article on the ecology of the sulfur-oxidizing bacteria, "Studies on the Sulphur Bacteria", was written in 1925 by Bass-Becking. Since that time, very little has been added to our knowledge of the sulfur-oxidizing bacteria which participate in decomposition processes. Bass-Becking referred to this community of organisms as the "sulphuretum" community. He believed it was restricted to the surface of black muds, although it has since been found in anaerobic hypolimnia and sulfur springs.

The difficulty in studying the ecology of the sulfur bacteria or any group of bacteria was long ago recognized by both Bass-Becking (1925) and van Niel (1955), who stated:

> "Pure culture of an organism gives us certainty about a great many facts. It fails, however, to account for the particular place of the organism in an inorganic cycle, inasmuch as its occurrence is influenced and restricted by competition."
>
> Baas-Becking

> "It is therefore clear that we cannot draw sound conclusions concerning the natural role of microbes from experience gained with elective cultures unless we restrict our inferences to apply only to rigorously comparable conditions."
>
> van Niel

The bacteriologist was thus limited to chemical determinations, light microscopy, and plate counts in his study of bacterial ecology, despite the advances that occurred in physiology, biochemistry, and molecular genetics. In the present study, two new tools, the electron microscope and diffusion gradients, were used to minimize the difficulties cited by van Niel and Baas-Becking.

Chapters I and II concern the development and application of gradient methodology to enrich for sulfur-oxidizing bacteria *in situ*. Chapter III concerns laboratory studies of sulfide oxidation by *Pseudomonas aeruginosa*, a fluorescent pseudomonad similar to those obtained using *in situ* thiosulfate gradients. Chapter IV concerns the direct observation of sulfur-oxidizing communities and Chapter V correlates the results from the preceding chapters with distributional information.

CHAPTER I

AN *IN SITU* GRADIENT ENRICHMENT METHOD FOR THE ISOLATION AND STUDY OF FREE-LIVING BACTERIA

BACKGROUND

Natural habitats often abound with unknown bacteria. The use of direct observation techniques has revealed a remarkable diversity of form, size, and arrangement (7,9,10,12). The fraction of unknown bacteria which have been isolated is small compared with the total observed. This may be due, in part, to a shortage of effective techniques for isolating and studying the ecology of organisms from natural habitats.

The methods of Winogradsky (16), Beijerinck (2), and van Niel (14) have strongly influenced the design of enrichment procedures. In general, an attempt is made to reproduce the natural microenvironment after observing and measuring as many parameters as possible. Success normally depends on the precision of environmental measurements, though in some cases even an accurate measurement is insufficient. For example, conditions measured at the peak of blooms are often suboptimal since optimal conditions precede the stationary phase of population growth. Because of the changing environmental conditions in enrichments, the growth of more tolerant bacteria may lead to the suppression of more fastidious forms. Therefore, one usually obtains tolerant organisms, while isolation of those more susceptible to stress remains accidental.

The observation of nutrient concentration gradients in natural habitats has led to suggestions that these be used for enrichment procedures. The Winogradsky columns (16), Perfil'ev capillaries (10), or the use of redox gradients created by decaying algae (8) have demonstrated the success of the gradient approach.

The *in situ* gradient enrichment method described in this chapter was thus devised for isolating free-living bacteria and studying their response to changing environmental conditions. The concept of exposing organisms to a continuous gradient of test substances was originally used by Beijerinck (1). However, the extension of the gradient concept to more than one dimension, the replacement of time-dependent gradients with steady-state gradients, and the equilibration of these gradients with natural physicochemical conditions has resulted in a new tool for ecological studies.

Although many ecologists prefer to study the distribution and activity of organisms along natural gradients (3,13,15), the relationship between environmental variation and microbial response is difficult to demonstrate. An example of a natural gradient is the distribution of bacteria in the anaerobic zone of eutrophic lakes. Although the distribution of these bacteria has been quantitatively related to gradients of light quality, redox potential, H_2S concentration, and other factors (6,11), it has been impossible to determine the relative importance of each since all vary together as functions of a single dimension, in this case depth.

Artificial gradients allow the effects of environmental factors to be observed independently or in a limited number of combinations. The number of test variables that can be used is limited to the number of dimensions available. The gradient plate is a two-dimensional test surface in which a maximum of two artificial concentration gradients can be established while other environmental factors remain in equilibrium with *in situ* conditions. Thus, the effect of all combinations of concentration of two compounds on the test population can be evaluated. To do the same experiment with test tubes would require a forbidding number of tubes.

MATERIALS AND METHODS

The diffusion gradient plates used for laboratory enrichment studies were originally developed by Caldwell and Hirsch (4). These were later modified (5) and used for quantitative and *in situ* enrichment studies. The procedure below applies to the modified diffusion gradient plate (DGP-300, Uni-Lab Products, 419 E. La Salle, Royal Oak, MI 48073).

The gradient plate consisted of four units, the cover, window, diffusion plate, and base. These were assembled as shown in Figure 1 and held in place by six stainless steel bolts. The diffusion plate contained a square depression 25 mm on each side as well as two rectangular slots. After the slots were covered with masking tape, both the slots and the square depression were filled with molten agarose (1.0%, 60-100 C) using a 5 ml hypodermic syringe. The amount of agar in the square depression was adjusted until the surface of the agar (viewed by reflection of a light source across

Figure 1. Illustration of the diffusion gradient plate showing the cover, window, diffusion plate, and base (from top to bottom). The path of the solution with which the gradient is equilibrated is shown by the dark arrows. The path of the two test substances (which form the two-dimensional gradient) through the agarose layer within the diffusion plate is shown by the thin arrows.

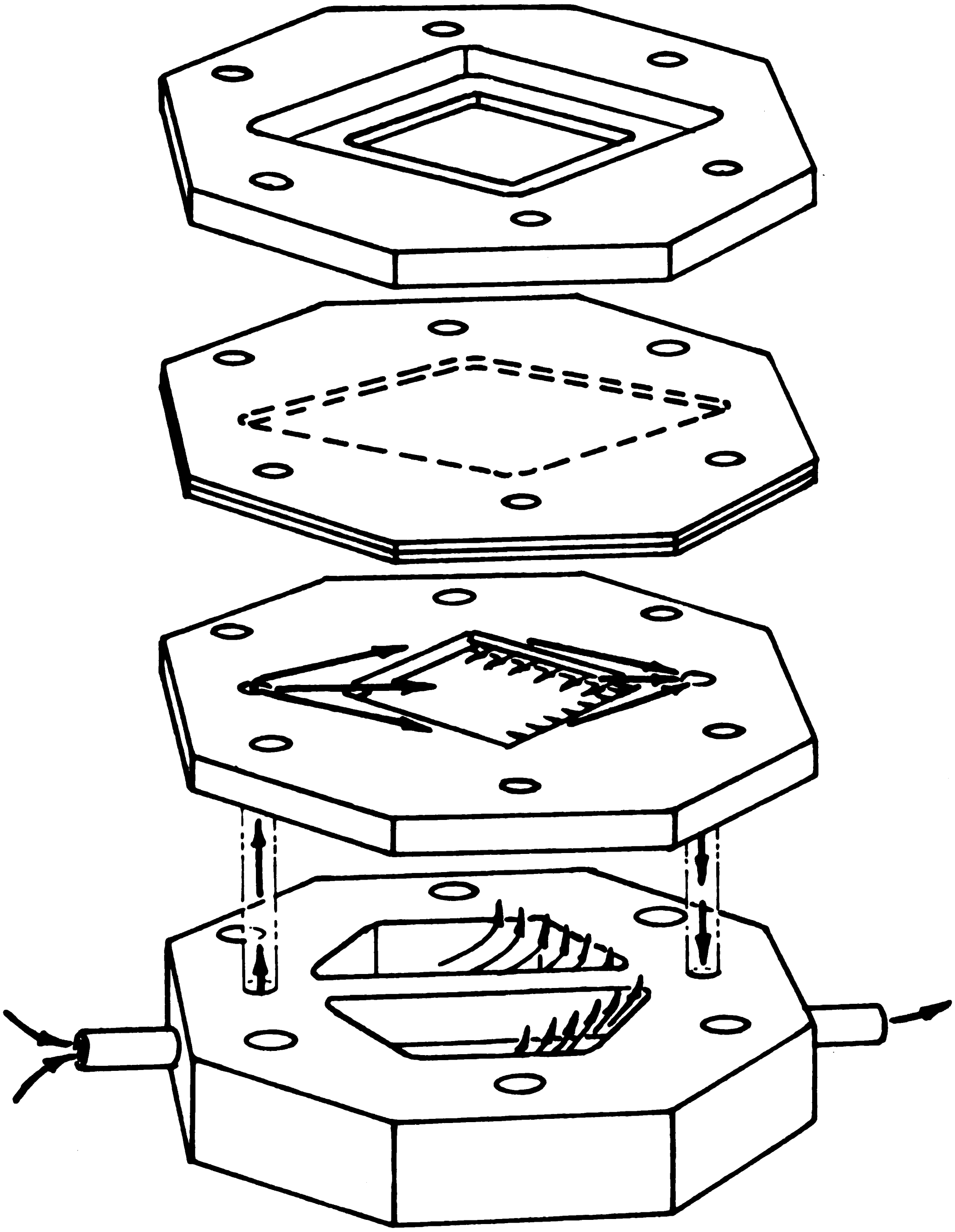


Figure 1

the room) was slightly depressed (allowing space for the addition of the inoculum later).

At all times during this procedure the agarose remained molten so that the agar in the slots and square depression was united by a continuous agar bridge through the slits on two sides of the square depression. Bubbles of air sometimes formed and these were removed using a syringe. The diffusion plate was passed over a burner to warm the plastic before and during this procedure. After the diffusion plate was filled with agar and all air bubbles were removed, the agar was allowed to cool.

Two saturated solutions of the test substances were added to the base plate. Crystals of the test substance were then added in addition to the saturated solutions, the diffusion gradient plate was assembled, inverted, and a flow of 10 ml/h was begun. The flow was across the plate toward the two slits which supply the diffusion plate with the test substances. After 24-48 h a steady-state gradient was established. The plate was then dismantled and the inoculum added in agarose (1.0%, 40 C). The depression was either filled until the surface of the agarose was even with the plastic surface (as viewed by reflected light) or filled to excess and a glass slide used to shape the agar. The diffusion plate was then returned to the gradient plate assembly and incubated.

All components of the gradient plate were sterilized under 15 psi of steam for 20 minutes except the diffusion plate, which was boiled, exposed to ultraviolet light or treated with ethylene oxide.

The most common problem in using the gradient plate was the rupture of the agarose bridge between the plate and the reservoir. This may have been caused by a pressure difference between the reservoirs and diffusion plate or by improperly pouring the agarose initially. The problem was avoided by using a short out-flow tube maintained at the same height as the gradient assembly. The equilibration solution was pumped in and allowed to flow out.

Concentrations were determined directly from Table 1, which summarizes the numerical solution of the gradient (5). The table has an estimated error of less than 5%; however, variations in the thickness of the plastics used to construct the plates result in an error of ± 50%. The values listed in the table represent the fraction of the saturating concentration of the test substances at various locations in the diffusion plate. The fraction found in the table is multiplied by the solubility of the test substance and added to the concentration of the test substance in the equilibration solution to obtain the concentration (g/liter) at any location in the diffusion plate. The first two digits of the table value represent the coefficient. The remaining digits represent the negative exponent. The table is valid only if the concentration of test substance in the equilibration solution is small (less than 0.1%) compared to the saturating concentration and if the flow rate is several orders of magnitude above the diffusion rate (10 ml/h). For a table value of 312, a solubility of 100 g/liter and a concentration of 1.0×10^{-6} g/liter in the equilibration solution, the concentration would be 3.1×10^{-2} multiplied by 100 g/liter plus 1.0×10^{-6} or 3.1 g/liter.

Table 1. Fraction of saturating concentration

	Distance (mm from edge of diffusion plate opposite reservoir)									
Depth (mm)	0.0	0.4	0.8	1.2	1.6	2.0	2.4	2.8	3.2	3.6
0.2	1710	1710	1910	2410	3110	4210	5710	7810	119	159
0.4	3310	3410	3910	4710	6110	8210	119	159	219	309
0.6	4810	5010	5710	6910	9010	129	169	239	319	439
0.8	6210	6410	7310	8910	129	169	219	299	409	569
1.0	7410	7610	8710	119	149	199	259	359	489	679
1.2	8510	8710	9910	129	169	219	299	409	559	769
1.4	9310	9510	119	139	179	239	329	449	609	849
1.6	9810	109	119	149	199	249	339	469	649	889
1.8	109	109	129	149	189	259	349	479	669	919
2.0	109	109	129	149	189	259	349	479	669	919

	Distance (mm from edge of diffusion plate opposite reservoir)									
Depth (mm)	4.0	4.4	4.8	5.2	5.6	6.0	6.4	6.7	7.1	7.5
0.2	219	299	409	569	789	118	158	218	298	418
0.4	419	579	809	118	158	218	308	418	588	808
0.6	609	849	128	168	228	318	448	618	848	127
0.8	789	118	158	218	298	408	568	788	117	157
1.0	939	138	188	258	358	488	678	948	137	187
1.2	118	158	208	288	408	558	778	117	157	217
1.4	128	168	228	318	438	608	848	127	167	237
1.6	128	178	248	338	468	648	898	127	177	247
1.8	138	188	248	348	478	668	918	137	187	247
2.0	138	188	248	348	470	668	918	137	187	247

Table 1 (continued)

Depth (mm)	Distance (mm from edge of diffusion plate opposite reservoir) 7.9	8.3	8.7	9.1	9.5	9.9	10.3	10.7	11.1	11.5
0.2	568	798	117	157	217	297	417	577	797	116
0.4	117	167	227	307	427	587	817	116	166	226
0.6	167	237	327	447	617	857	126	166	236	326
0.8	217	297	417	577	797	116	156	216	306	416
1.0	257	357	497	687	947	136	186	256	356	496
1.2	297	407	567	777	116	156	216	296	406	566
1.4	317	447	617	857	126	166	236	326	446	616
1.6	337	467	647	907	126	176	246	346	476	656
1.8	347	487	667	927	136	186	256	346	486	676
2.0	347	487	667	927	136	186	256	346	486	676

Depth (mm)	Distance (mm from edge of diffusion plate opposite reservoir) 11.9	12.3	12.7	13.1	13.4	13.9	14.3	14.7	15.1	15.5
0.2	156	216	306	416	576	806	115	155	215	305
0.4	306	426	586	816	115	165	225	305	425	595
0.6	446	626	866	125	175	235	325	455	625	865
0.8	576	796	115	155	215	305	415	585	805	114
1.0	686	956	135	185	265	356	505	695	965	134
1.2	786	115	155	215	295	405	565	785	114	154
1.4	856	125	165	235	325	445	625	865	124	174
1.6	906	125	175	245	345	475	655	915	134	184
1.8	936	135	185	255	355	485	675	935	134	184
2.0	936	135	185	255	355	485	675	935	134	184

Table 1 (continued)

	Distance (mm from edge of diffusion plate opposite reservoir)									
Depth (mm)	15.9	16.3	16.7	17.1	17.5	17.8	18.3	18.6	19.0	19.4
0.2	415	585	805	114	164	224	304	424	584	814
0.4	825	114	164	224	314	434	594	824	113	163
0.6	124	174	234	324	454	624	874	123	173	233
0.8	154	224	304	424	584	814	113	163	223	303
1.0	184	264	364	504	694	964	133	193	263	363
1.2	214	294	414	574	794	113	153	213	303	413
1.4	234	324	454	624	864	123	173	233	323	453
1.6	244	344	474	664	914	133	183	253	343	483
1.8	254	354	494	684	944	133	183	253	353	493
2.0	254	354	494	684	944	133	183	253	353	493

	Distance (mm from edge of diffusion plate opposite reservoir)									
Depth (mm)	19.8	20.2	20.6	21.0	21.4	21.8	22.2	22.6	23.0	23.4
0.2	113	163	223	303	423	583	813	112	152	212
0.4	223	313	433	603	833	122	162	212	302	422
0.6	323	453	633	873	122	172	232	322	452	622
0.8	423	583	813	122	162	222	302	422	582	802
1.0	503	703	973	142	192	262	362	502	702	972
1.2	573	803	112	152	212	302	412	582	802	111
1.4	623	873	122	172	232	322	452	632	882	121
1.6	663	923	132	182	252	342	482	672	932	131
1.8	683	953	132	182	262	362	492	692	962	131
2.0	683	953	132	182	262	362	492	692	962	131

Table 1 (continued)

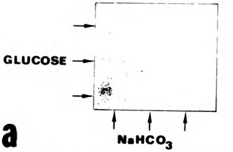
	Distance (mm from edge of diffusion plate opposite reservoir)				
Depth (mm)	23.8	24.2	24.6	25.0	25.4
0.2	282	372	462	532	552
0.4	562	742	932	111	111
0.6	842	111	141	161	171
0.8	111	151	191	231	241
1.0	131	181	241	301	311
1.2	151	211	291	371	401
1.4	171	241	341	461	531
1.6	181	261	371	561	100
1.8	191	271	391	611	100
2.0	191	271	391	611	100

RESULTS

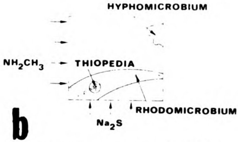
To demonstrate a differential growth pattern, *E. coli* K-12 was grown in a glucose vs. bicarbonate gradient (Figure 2a). Large colonies appeared where growth conditions were most favorable, while other cells of the inoculum remained static throughout the rest of the plate. Thus, there was an optimum growth range for both the pH and substrate. The inoculum (10 cells/ml) was purposely kept small to avoid cross-feeding effects between colonies.

A mixed community consisting of *Hyphomicrobium* sp. B-522, *Thiopedia* sp., and *Rhodomicrobium vannielii* was also used as an inoculum, with gradients of Na_2S and $CH_3NH_2 \cdot HCl$. Continuous illumination at an intensity of 30 ft-c was provided for the photosynthetic organisms. Each organism formed a characteristic pattern of colonies despite the random distribution of the inoculum (Figure 2b). Resting cells of *R. vannielii* were almost spherical, while those observed on the edges of large, fast-growing colonies were spindle shaped. These morphological changes were also observed in pure cultures.

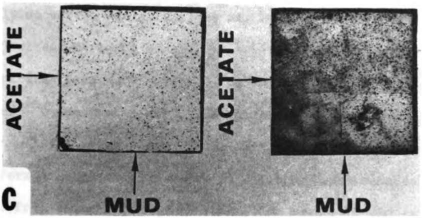
The effect of two factors on the natural surface community of a forest pond (Michigan State University Experimental Forest, Augusta, Michigan) was observed qualitatively. Pond surface water served as the inoculum while pond mud supplied naturally occurring growth factors. Acetate and pond mud were placed in the troughs. The plate was illuminated with 12 ft-c at a temperature of 24 C and the other parameters were as described above. After 81 h, many different colony types had developed and were densely distributed over the plate. While the large colonies consisted of bacteria with terminal



A diagrammatic representation of the distribution pattern of colonies formed by *E. coli* growing in a two-dimensional gradient of glucose vs. bicarbonate. Note that the bicarbonate gradient provides an optimal pH range and the substrate gradient also has an optimum.



The pattern of microcolonies formed by *Hyphomicrobium*, *Rhodomicrobium*, and *Thiopedia* growing in a two-dimensional gradient of Na_2S vs. $CH_3NH_2 \cdot HCl$. (Diagram.)



Response of the planktonic microflora of a forest pond to a gradient of acetate vs. mud. The same plate is shown in both A and B, but printed with different light intensities. (Left), large colonies enhanced. (Right), small colonies enhanced.

Figure 2

endospores, many of the smaller ones were isolated and identified as *Bacillus* sp. The background of small colonies could be eliminated by selecting a long exposure time and a less sensitive paper during the photographic printing process (Figure 2c [left]). Smaller colonies (Figure 2c [right]) appeared mainly in the area of high mud extract and high acetate concentration.

The biological utility of gradient plates depends on the successful cultivation of naturally occurring microorganisms. Table 2 shows a variety of microorganisms that have been successfully cultivated using two-dimensional gradients. The cultivation of *Haematococcus*, which has delicate cell walls, suggests that many naturally occurring microorganisms may be able to tolerate the physical stress of being heated and embedded in agarose.

Empirical data were collected by sampling gradient plates which contained ^{14}C-acetate by using 100 capillaries to withdraw plugs from the agarose layer (Figure 3). These empirical data were summarized in the form of a regression equation relating concentration and the distance from the reservoir containing the test compound (r^2=0.997) (Figure 4). A theoretical model was developed by Dr. Sung Ho Lai (5) to predict the gradient pattern at steady-state for any test compound used in the plate. The theoretical equation fell within the 95 percent confidence interval of the empirical regression equation.

When a gradient of nonradioactive phosphate or acetate was imposed at a right angle to a gradient of radioactive acetate, the empirical results remained within the 95 percent confidence interval

Table 2 Microorganisms that have been successfully grown in the gradient plate

	Test compounds		
	1st dimension	2nd dimension	Incubation site
Bacteria:			
Escherichia sp.	acetate	---	laboratory
Thiopedia sp.	$CH_3NH_2 \cdot HCl$	Na_2S	laboratory
Hyphomicrobium sp.	$CH_3NH_2 \cdot HCl$	Na_2S	laboratory
Rhodomicrobium sp.	$CH_3NH_2 \cdot HCl$	Na_2S	laboratory
Thiothrix sp.	Na_2CO_3	Na_2S	spring
Algae:			
Pediastrum sp.	$NaHCO_3$	K_2HPO_4	laboratory
Scenedesmus sp.	$NaHCO_3$	K_2HPO_4	laboratory
Haematococcus sp.	$NaHCO_3$	K_2HPO_4	laboratory
Cosmarium sp.	$NaHCO_3$	K_2HPO_4	laboratory
Fungus:			
Alternaria sp.	K_2HPO_4	NH_4NO_3	spring

of the regression expression for acetate alone (Figure 5). This showed that gradients can be perpendicularly superimposed without affecting their individual gradient patterns (Figure 6).

Thus, it is possible to calculate the concentration of test substance at any location in the agarose layer. All that is required is a knowledge of the physical dimensions of the plate, the solubility, and the *in situ* concentration of the test substance(s).

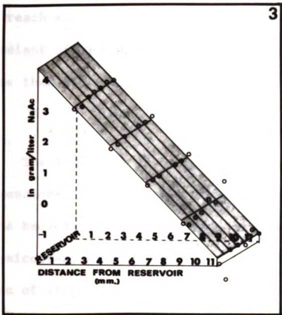
It should also be noted that the gradient pattern is not a function of the coefficient of diffusion for the test substance, the nature of the diffusion medium, or the flow rate. However, the flow

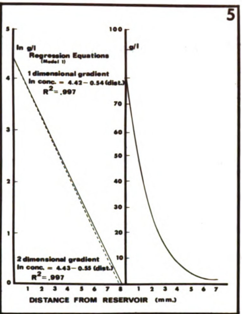
Figure 3. Concentration of ^{14}C-acetate as a function of distance for a one-dimensional gradient of ^{14}C-acetate.

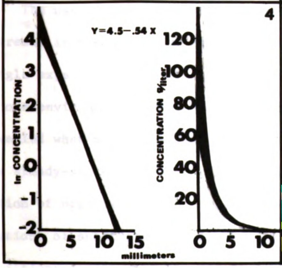
Figure 4. 95% confidence interval for the concentration of ^{14}C-acetate (based on 6 duplicate experiments) as a function of distance for a one-dimensional gradient of ^{14}C-acetate.

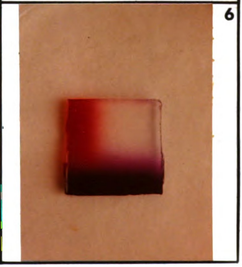
Figure 5. Concentration of ^{14}C-acetate as a function of distance in a two-dimensional gradient of acetate vs. ^{14}C-acetate. Also shown is the concentration of ^{14}C-acetate as a function of distance for a one-dimensional gradient of ^{14}C-acetate on a ln and linear scale.

Figure 6. Color photograph of a two-dimensional gradient of safranin vs. crystal violet.









Figures 3, 4, 5 and 6

rate must exceed the diffusion rate by several orders of magnitude, and the concentration of test compounds in the flowing solution must be less than 0.1% of their solubility. Although the time required to reach steady-state does depend on the diffusion coefficient, a gradient of most monomolecular substances reaches steady state in less than two days.

DISCUSSION

The selection of a two-dimensional plate rather than a three-dimensional cube (capable of including three variables at once) was made because of the difficulty of equilibrating a cube with *in situ* physicochemical conditions. A thin plate allows the rapid equilibration of steady-state gradients with aquatic environments.

The use of multi-dimensional gradients results in a logarithmic increase in the amount of information that can be obtained in a single experiment. Interaction effects, which occur if the response to one environmental variable is a function of another variable, are revealed when microorganisms are exposed to a two-dimensional gradient. The steady-state nature of the gradients allows the study and cultivation of organisms which compete most effectively when low concentrations of substrate are continually resupplied. Steady-state conditions also permit the use of longer incubation periods increasing the differential response of slow-growing microorganisms to test substances.

There are several limitations to the method. Even using artificial gradients, it is difficult to restrict the test variables to one per dimension due to the nature of most test substances. For example, if Na_2S is used as a gradient it will be accompanied by gradients of pH, osmolarity, and redox. This effect may be discounted in many cases since each of these factors would normally be determined by sulfide concentration in natural environments just as in the artificial gradient plates. There can be exceptions and, for that reason, one should always consider this limitation.

In some cases the test compound will be precipitated by some chemical factor in the environment. In this case the precipitating compound can be used in the reservoir in place of the original test substance. The fact that the original substance precipitates in the environment eliminates it as a factor at concentrations above its solubility. It is obvious that gradients cannot be extended below the environmental concentration for *in situ* studies (5). For this reason, it is only possible to use increases in concentration as test stimuli.

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CHAPTER II

ISOLATION OF A HETEROTROPHIC SULFUR-OXIDIZING BACTERIUM USING GRADIENT ENRICHMENTS

BACKGROUND

Sulfide, one of the products of bacterial decomposition, is slowly oxidized inorganically by O_2 to S^o, $S_2O_3^=$, or $SO_4^=$ depending on the reaction conditions (5). Chen and Morris (5) identified a pH dependent induction period for HS^- oxidation of from 0.2 to 6.0 h before abiologic O_2 uptake begins. Thus HS^- and O_2 can apparently exist simultaneously. In the littoral zone, HS^- and O_2 may be supplied together by decomposition processes and wave action. Thus reduced sulfur compounds can be available for aerobic bacterial oxidation despite simultaneous inorganic oxidation.

There are several groups of bacteria which might be involved in biologic HS^- oxidation. These include the *Chromatiaceae*, *Chlorobiaceae*, colorless sulfur bacteria, and heterotrophic sulfur-oxidizing bacteria. Our knowledge of the role of these bacteria in the transformation of inorganic sulfur compounds in nature is based primarily upon selective enrichment as described by van Niel (9) and also upon the subsequent study of pure cultures. However, the elegance and convenience of this approach have sometimes overshadowed the fact that minor environmental variations between the enrichment (elective) culture and the natural habitat result in a completely different bacterial flora. As stated by van Niel:

> "It is therefore clear that we cannot draw sound conclusions concerning the natural role of microbes from the experience gained with elective cultures unless we restrict our inferences to apply only to rigorously comparable conditions."

For this reason, an *in situ* enrichment method (3,4) was used to investigate sulfur transformations which occur in the littoral zone of the lake described here. This procedure insured that the conditions of enrichment were comparable to natural conditions. Direct observations of decomposing plant surfaces also served to confirm the conclusion that the organisms studied using gradients were actually members of the natural community of sulfur-oxidizing bacteria.

MATERIALS AND METHODS

Gradient enrichment culture

Gradient enrichment culture was carried out as described by Caldwell *et al.* (4) using diffusion gradient plates (Uni-Lab Products, 419 E. La Salle, Royal Oak, MI 48073 U.S.A.). The growth rate was determined from the number of cells per colony and the incubation period. Concentrations were determined using the numerical solution of the diffusion gradient. The inoculum was prepared by slicing decomposing plant materials (washed in sterile distilled water) into small sections and grinding with a ground glass homogenizer. The plant materials were taken from the littoral zone of Burke Lake and included decomposing aspen leaves and grasses. These were observed using phase and electron microscopy. They contained *Thiothrix* and

Thiocystis (Chapter IV, Figures 6 and 7) as well as unidentified rod-shaped organisms. Precautions to preserve sterility were taken throughout this procedure. The diffusion gradient plate was incubated with the base up on a plastic coated test tube rack. The cover was removed to expose the agarose directly to the water currents.

Media

Thiobacillus Broth (Difco Laboratories, Detroit, MI U.S.A.) was used with 1.5% Nobel Agar (Difco) for plate counts of thiobacilli (pH 4.8). To determine the presence or absence of thiobacilli in $S_2O_3^{=}$ enrichments the S, R, and F thiobacillus media recommended by Aaronson (1) were used in addition to the Thiobacillus Broth.

The TS (thiosulfate) medium for maximum pigment production of fluorescent pseudomonads is a modification of the media of Frank and DeMoss (6) and Sands and Rovira (8). It contained: 2% glycerol (w/v), 1% $Na_2S_2O_3 \cdot 7\ H_2O$, 1% (w/v) case amino acids, 0.01 M K_2HPO_4, 0.01 M $MgCl_2$, and 1.5% Nobel Agar (for solid media). In some cases antibiotics were added, including: 45 mg/liter Penicillin G, 45 mg/liter novobiocin (Upjohn, Albamycin), 75 mg/liter cycloheximide (Upjohn, Acti-Dione), and 5 mg/l chloramphenicol.

Fluorescent pseudomonads were detected by laying decomposing aspen leaves on the TS (thiosulfate) agar medium supplemented with antibiotics as suggested by Sands and Rovira (8). This resulted in the formation of microscopic fluorescent colonies in 24 to 48 h. The number of colonies per unit area of leaf surface was determined by counting fluorescent colonies using epifluorescence microscopy. By homogenizing a known leaf area and diluting the homogenate, it was possible to estimate the number of viable cells per colony.

Sulfide oxidizing activity

A substrate solution of 0.5 M $Na_2S \cdot 9\ H_2O$ in 0.5 M K_2HPO_4 was adjusted to pH 7.6 using HCl. This was added to filtered (0.45 µm filter, Millipore Corp., Bedford, MA U.S.A.) bacterial exudates to a final concentration of 0.1 mM HS^-. In active samples, a colloidal precipitate of elemental sulfur formed in 0.1 to 5 min. The pH remained at 7.6 during the reaction. Sulfur was determined after collecting the S^o precipitate on a 0.45 µm Millipore solvinert filter, washing five times with distilled water, drying, and dissolving overnight in petroleum ether on a wrist action shaker. Sulfur was then determined using the procedure of Bartlett and Skoog (2).

The presence or absence of S^o forming activity was determined for 8 isolates and 8 known genera: *Pseudomonas aeruginosa, Micrococcus* sp., *Proteus vulgaris, Escherichia coli, Sarcina lutea, Corynebacterium* sp., *Bacillus megatarium,* and *Enterobacter aerogenes.* Each organism was streaked on solid TS Media containing 0, 1, 5, 10, and 20% $S_2O_3 \cdot 7\ H_2O$. After a 48 h incubation period a crystal of $Na_2S \cdot 9\ H_2O$ was placed near the streak. A precipitate of S^o in the form of a white ring around the crystal was considered a positive test for S^o forming activity.

Habitats

Burke Lake is a spring fed lake 9.7 m in depth. Some of the physical, chemical and biological characteristics have been described in Chapter IV (Table 1). The gradients were equilibrated in Buttercup Spring (pH 7.5) which feeds Burke Lake.

RESULTS

Gradients of HS^-, $S_2O_3^=$, and HS^- versus $NaHCO_3$-bicarbonate were used to enrich for sulfur-oxidizing bacteria found on decomposing plant surfaces. Over a period of several weeks, no colony development occurred in HS^- gradients because a constant supply of HS^- could not be maintained due to the formation of a precipitate at higher HS^- concentrations. However, the one-dimensional $S_2O_3^=$ gradient (Figure 1) permitted the formation of S^o encrusted colonies of rod-shaped bacterial cells. *Thiothrix* also formed colonies within the agar and on the agar surface. The gradient conditions under which *Thiothrix* deposited sulfur intracellularly coincided with the conditions resulting in sulfur deposition by the rod-shaped organisms. This range was 0.067 to 13.5 g $S_2O_3^=$ per liter at a temperature of 8 C. The maximum growth rate of 19 h per generation was at a $S_2O_3^=$ concentration of 1-5 g/liter (Figure 1), which also corresponded to the maximum concentration for S^o deposition. The lowest growth rate occurred at 55 μg/liter, the lowest concentration tested, and no S^o was deposited. A S^o encrusted colony of rod-shaped organisms is shown in Figure 2. The S^o was not deposited intercellularly or intracellularly but contiguous to the colony surface and in the agarose adjacent to the colony.

The rod-shaped, S^o depositing organism was subcultured every two weeks *in situ* within $S_2O_3^=$ gradients for a period of six weeks. This resulted in a lawn of microcolonies. From this enrichment the

Figure 1. Oikography, the *in situ* response of a littoral bacterial community to a one-dimensional gradient of $S_2O_3^{=}$. Decomposing plant debris was homogenized and used to inoculate an *in situ* enrichment culture. The gradient cultures were equilibrated (11/5/72 - 11/17/72) with Buttercup Spring (pH 6.9, 8 C). The number of cells in the microcolonies which formed (Figure 2) was used to determine the growth rate which was plotted versus the concentration of thiosulfate. The range of thiosulfate concentration resulting in the formation of elemental sulfur falls between the two dashed lines. The organism (TBT-H) was identified as a fluorescent pseudomonad.

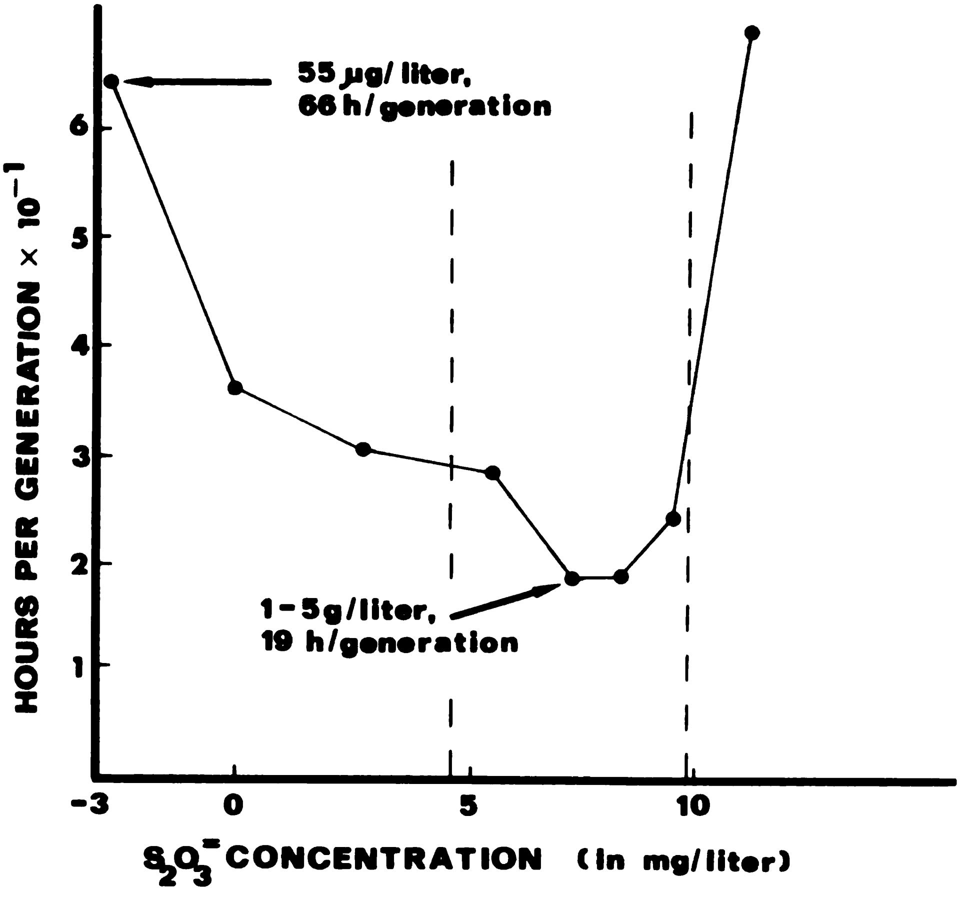
55 µg/ liter,
66 h/generation
1-5g/liter,
19 h/generation
HOURS PER GENERATION × 10^{-1}
1
2
3
4
5
6
-3
0
5
10
$S_2O_3^=$ CONCENTRATION (in mg/liter)

Figure 1

Figure 2. A S^o encrusted colony from the gradient described in Figure 1. a. Colony of fluorescent pseudomonads in the $S_2O_3^=$ gradient (2). Elemental sulfur was deposited at the colony surface (1) and in the agar surrounding the colony (3). Bar represents 100 µm. b. Higher magnification of the colony. The crust of S^o globules (4) encasing the cells (5) can be seen. Bar represents 10 µm (phase micrograph). c. The colony shown above has been crushed to show that the refractile crust encasing the cells is composed of individual S^o globules. The characteristic diffraction pattern of S^o globules can also be seen (6). Bar represents 10 µm (phase micrograph).

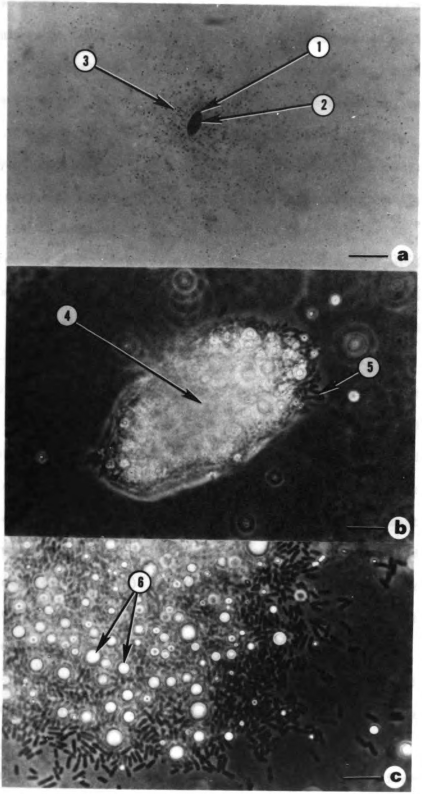

Figure 2

rod-shaped organism was isolated in pure culture using the TS medium. The organism was identified as a fluorescent pseudomonad. The isolate, TBT-H, utilized glucose, lactose, mannitol, and citrate as carbon sources. It was nonfermentative, Gram negative, motile, catalase positive, microaerophilic, and hydrolized gelatin. Sulfide was not produced and no growth occurred on thiobacillus media.

The organism excreted a number of fluorescent pigments when grown in the TS medium and in the TS medium when $SO_3^=$ or $SO_4^=$ were substituted for $S_2O_3^=$. No pigments were produced when inorganic sulfur compounds were deleted from the medium although growth still occurred. When the isolate was grown in liquid TS medium (pH 7.6), the addition of HS^- to cell free exudates (1 mM) resulted in the formation of 0.49 ± 0.13 mM (95% confidence interval) of elemental sulfur (final pH 7.6). No S^o was formed when the HS^- was added to uninoculated medium or to boiled (20 min) exudates. As an additional control, eight heterotrophic organisms isolated from the littoral zone, which did not produce extracellular fluorescent pigments, together with eight pure cultures of known genera were tested for S^o forming activity (Table 1). Only the exudates from the fluorescent isolate and *Pseudomonas aeruginosa* (both of which produced fluorescent pigments under these conditions) were active. Both of these organisms failed to produce either the pigment or activity on plates which lacked $S_2O_3^=$. No S^o was formed on uninoculated control plates containing only the TS medium.

All attempts to isolate *Thiothrix* in the TS medium, from either natural samples or gradient enrichments, yielded pure cultures of

Table 1. Growth, extracellular pigment production, and extracellular deposition of elemental sulfur by eight isolates and eight known genera of bacteria

	% $Na_2S_2O_3 \cdot 7H_2O$														
	Growth					Extracellular pigment production					Extracellular deposition of elemental sulfur				
% Thiosulfate:	0	1	5	10	20	0	1	5	10	20	0	1	5	10	20
Organism:															
H	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-
O	+	+	+	+	-	-	-	-	-		-	-	-	-	
I	+	+	+	+	-	-	-	-	-		-	-	-	-	
E	+	+	+	-	-	-	-	-			-	-	-		
L	+	+	-	-	-	-	-				-	-			
M	+	+	-	-	-	-	-				-	-			
R	+	+	+	-	-	-	-	-			-	-	-		
T	+	+	+	+	-	-	-	-	-		-	-	-	-	
Pseudomonas aeruginosa	+	+	+	+	-	+	+	+	+		-	+	+	+	-
Micrococcus sp.	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Proteus vulgaris	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Escherichia coli	+	+	+	+	-	-	-	-	-		-	-	-	-	
Sarcina lutea	+	+	+	-	-	-	-	-			-	-	-		
Corynebacterium sp.	+	+	+	+	-	-	-	-	-		-	-	-	-	
Bacillus megatarium	+	+	+	+	-	-	-	-	-		-	-	-	-	
Enterobacter aerogenes	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-

Sphaerotilus. The use of a variety of liquid media also failed to yield a culture of *Thiobacillus* from the enrichments. The presence of *Thiothrix* at the leaf surface was confirmed by direct observation of the holdfast, sheath, cellular filaments, and intracellular sulfur globules which are characteristic of this genus. The problem of detecting fluorescent pseudomonads, which have no distinguishing morphological features, was overcome by laying plant leaves on the TS agar medium supplemented with antibiotics as suggested by Sands and Rovira (8). This resulted in the formation of microscopic fluorescent colonies in 24 to 48 h. The number of colonies per unit area of leaf surface was determined by counting fluorescent colonies using epifluorescence microscopy. By homogenizing a known leaf area and doing dilution plate counts, it was possible to also estimate the number of viable cells per colony. We found the method most effective with decaying aspen leaves. There were approximately 200 colonies/mm^2 and 10 cells/colony. These values serve to demonstrate the presence of fluorescent pseudomonads in these aquatic environments but do not represent an extensive quantitative survey.

DISCUSSION

The presence of fluorescent pseudomonads in HS^- containing waters among autotrophic sulfur-oxidizing bacteria is anomalous due to the toxicity of HS^-, which binds the ferric iron of cytochrome oxidase and prevents the reoxidation of cytochromes, thus preventing aerobic respiration. In addition, S^o forming activity as well as the threefold increase in growth rate caused by thiosulfate gradients are inconsistent with the role of pseudomonads as oxidative heterotrophs.

Further studies to confirm the excretion of S^o forming compounds by fluorescent pseudomonads as well as studies on the effect of reduced sulfur compounds on metabolism are thus required. It is possible that fluorescent pseudomonads occupy the ecological niche previously attributed to the thiobacilli since thiosulfate gradients selected pseudomonads *in situ* while inorganic thiosulfate laboratory enrichments commonly select thiobacilli.

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CHAPTER III

FLUORESCENT SULFUR-OXIDIZING EXUDATES FROM *PSEUDOMONAS AERUGINOSA*

BACKGROUND

Pseudomonas aeruginosa excretes several water soluble pigments. One of these, pyocyanin, has been isolated and studied previously (2,6,7,9). The fluorescent pigments have not been isolated (5,13,15) but have been studied spectrophotometrically and spectrophotofluorometrically (5,11,16). The effect of culture conditions on fluorescence has also been investigated (8,13). Despite the possibility that more than one fluorescent compound may be excreted (5,13), the fluorescent exudates have been referred to as "fluorescein" (12) or "pyoverdine" (5).

Sulfate, sulfite (13), or thiosulfate (Chapter II) is required for the production of both pyocyanin and the fluorescent pigments, despite the fact that these pigments are not known to contain S (13). Inorganic sulfur compounds also induce the formation of a protein-carbohydrate(1) slime (13). Although pyocyanin is known to serve as an H donor for nitrite reductase (4), no chemical or biological activity has been found for the fluorescent pigments. The results of preliminary experiments, discussed in Chapter II, suggested that fluorescent exudates from pseudomonads might be involved in the formation of S^o globules from HS^-. This would partially explain the occurrence of fluorescent pseudomonads among sulfur-oxidizing bacteria in nature (Chapter V) as well as the induction of fluorescent pigments by inorganic sulfur compounds. For these reasons, the effect of fluorescent exudates from *P. aeruginosa* on the formation of S^o from HS^- was investigated. This organism is well known as are the spectral characteristics of its exudates. The spectral characteristics of the exudates could thus be studied and compared to previous reports (5,10,11).

MATERIALS AND METHODS

An inoculum of *Pseudomonas aeruginosa* was taken from an agar slant of the TS medium (Chapter II), transferred to 50 ml of TS medium in a 250-300 ml Erlenmeyer flask and incubated at 25 C on a rotary shaker at 200-250 rpm. After 20-24 h the cultures became blue-green and 1 ml was subcultured to a 1,000 ml Erlenmeyer flask containing 250 ml of the TS medium with $MgCl_2$ and glycerol deleted. In 15-20 h the culture appeared slightly yellow and turbid. The walls of the flask were encrusted with cells and the medium fluoresced blue. The liquid was decanted and centrifuged at 8,000 g for 20 min (4 C) in a refrigerated centrifuge (Sorvol Inc., Norwalk, CO). The fluid was decanted and filtered (0.45 µm membrane filter, HAWG-047-00, Millipore Corp., Bedford, MA) at 4 C. The filtrate was then sterile filtered (0.45 µm Nalgene Filter Unit, 120-0045, Nalge Sybron Corp., Rochester, NY) and stored at 4 C before use.

An Aminco-Bowman spectrofluorometer and recorder were used to obtain excitation and emission spectra. Three volumes of 0.2 M phosphate buffer (pH 7.6) were added to exudates and the pH adjusted before spectra were recorded.

Consumption of HS^- by exudate was determined in a reaction mixture containing an equal volume of 0.2 M phosphate buffer (pH 7.6) and exudate. Sulfide was added to a final concentration of 1 mM and the sample incubated in a sealed reaction vessel; the final pH was 7.6. The incubation was ended by acidifying the sample and flushing with N_2 for 10 min through a trap of ZnAcetate. Sulfide was assayed by a modification of the method of Johnson and Nishita (8) as described by Roy and Trudinger (14). Resazurin was used to titrate the sample to an orange end-point (pH 3.8) before flushing with N_2. In a preliminary experiment, the addition of H_2SO_4 to a final concentration of 1.7 M resulted in a recovery of 0.95 ± 0.13[a] mM HS^- from a 1 mM solution containing phosphate buffer alone. However, when HS^- was added to the buffered TS medium, the addition of H_2SO_4 to 1.7 M resulted in recovery of only 0.06 ± 0.03[b] mM HS^-. If the buffered medium was instead titrated to a resazurin end-point (pH 3.8) with 0.2 M H_2SO_4, then 0.92 ± 0.10[a] mM HS^- were recovered. Thus the pH indicator was used in all subsequent HS^- determinations. Sulfide dependent gas uptake or production by buffered exudates was measured using a Warburg respirometer. Exudates were buffered by adding an equal volume of 0.2 M phosphate buffer (pH 7.6). Samples

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[a] 95% confidence interval from 5 duplicates.

[b] 95% confidence interval from 3 duplicates.

(50 ml) were then added to 100 ml Warburg flasks. Sulfide was added from the side arm of the flasks to a final concentration of 1.0 mM. The pH was determined after all experiments and found to be 7.6.

Elemental sulfur was determined as described in Chapter II.

RESULTS AND DISCUSSION

Some of the fluorescent characteristics of exudates from *Pseudomonas aeruginosa* are shown in Figures 1 and 2. From a pH of 2.0 to 10.0 the exudate had an absorption peak at 405 nm. From a pH of 6.0 to 12.9 the exudates had a fluorescence peak at 460 nm (excitation, 400 nm). However, below a pH of 6.0 the fluorescence peak shifted to 430 nm at a pH of 1.0. Fluorescence at 460 nm was most intense at a pH of 7.0. The emission spectrum (excitation, 400 nm) of a water sample taken from a bed of *Chara* within the littoral zone of Burke Lake, which had been filtered (0.45 µm membrane filter) and adjusted to a pH of 7.0, is also shown in Figure 1. The relative intensity of fluorescence of the culture exudates was 840 times as great as the fluorescence of the lake sample. The lake sample had a fluorescence maximum at 440 nm. Since the bed of *Chara* contained large numbers of fluorescent pseudomonads (Chapter V), there is a possibility that naturally occurring fluorescent pigments were partially responsible.

The addition of sulfide (1 mM, pH 7.6) to a 24 h culture of *P. aeruginosa* resulted in the formation of refractile globules 0.1 to 5.0 µm in diameter, which produced a diffraction pattern of colored concentric rings in a light microscope and adsorbed to the

Figure 1. Emission spectrum of *Pseudomonas aeruginosa* exudate and a lake sample at an excitation of 400 nm. The exudate was diluted with 3 volumes of buffer and scanned at a multiplier setting of 0.03 (460 nm peak). The lake sample was undiluted and scanned at a setting of 0.001 (440 nm peak).

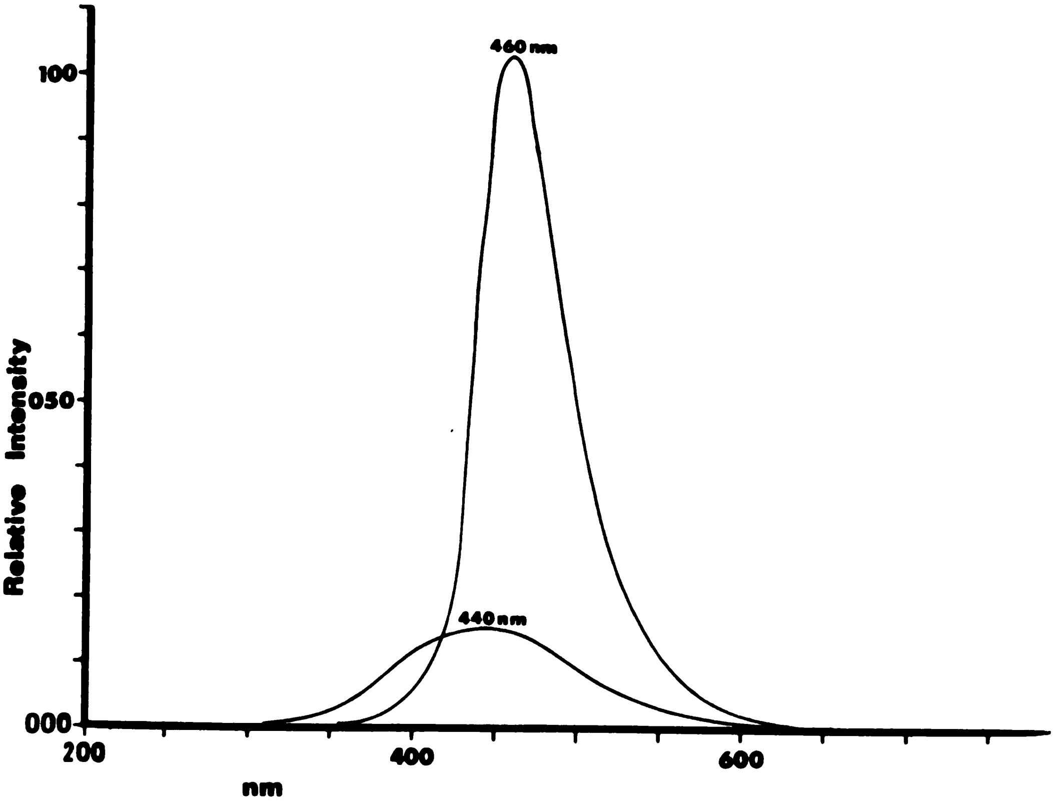

Figure 1

Figure 2. The wavelength of the absorption peak (▲), fluorescence peak (■), and the fluorescence intensity (●) vs. pH for *Pseudomonas aeruginosa* exudates.

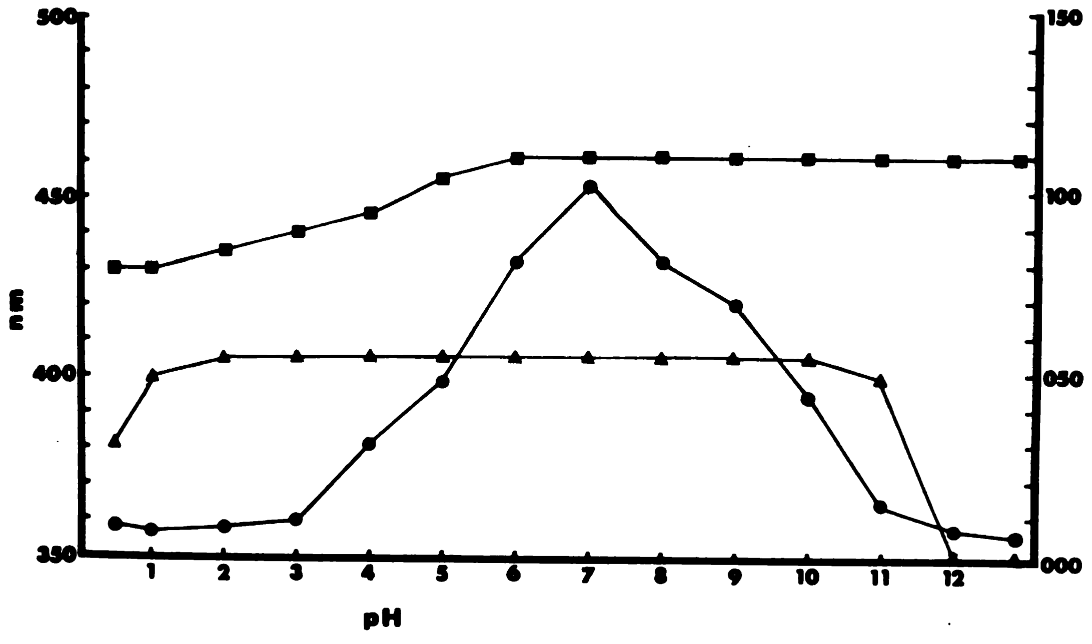
500
450
400
350
nm
150
100
050
000
Relative Intensity
1
2
3
4
5
6
7
8
9
10
11
12
pH

Figure 2

cells. The globules were produced only upon addition of sulfide and were soluble in CCl_4, petroleum ether, or pyridine but not in water or 0.1 M HCl. This was considered presumptive evidence that the globules were composed of elemental sulfur.

Although the addition of HS^- to phosphate buffer (pH) resulted in the release of H_2S, the addition of HS^- to the exudates produced no H_2S odor. When the reaction was carried out in the Warburg respirometer (Figure 3), an initial volume increase occurred in the buffer flask but not in the exudate flask. Thus the volume changes corresponded to the qualitative difference in the presence of H_2S odor. After an induction period of 50 min the volume of the buffer flask decreased at a constant rate. This was probably due to the consumption of O_2 resulting from abiologic HS^- oxidation (3). Oxygen uptake apparently did not occur in the case of the exudate, since the vessel remained at a constant volume after 50 min. Thus, the inorganic oxidation of HS^- by O_2 appears to have been prevented by the exudate. In addition, HS^- must have been rapidly bound upon addition to the exudate. If it had not, the initial pH decrease would have immediately released H_2S as it did with buffer alone. The rate of globule formation was followed spectrophotometrically as the change in OD_{340} per min (Figure 3). Sulfur globule formation occurred during the initial period of apparent binding (Figure 3); no globules were formed in vessels without exudate.

The consumption of HS^- by buffered exudate was compared to the consumption of HS^- by buffered medium (Figure 4). The concentration of HS^- remained constant in the buffer but decreased to 0.5 mM in

Figure 3. Volume change following addition of HS^- to phosphate buffer (○) and to *Pseudomonas aeruginosa* exudate in phosphate buffer (●). Formation of S^o globules (▲) by later incubation mixture is shown as change in $O.D._{340}$ per min.

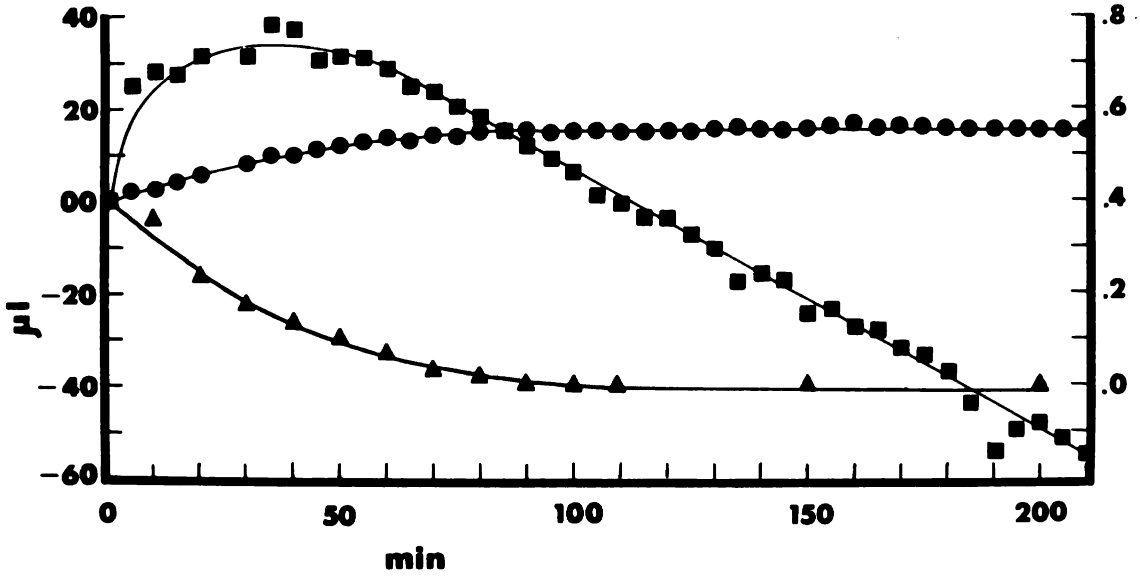

Figure 3

Figure 4. Sulfide consumption by *Pseudomonas aeruginosa* exudate in phosphate buffer (●) and by buffered medium alone (■).

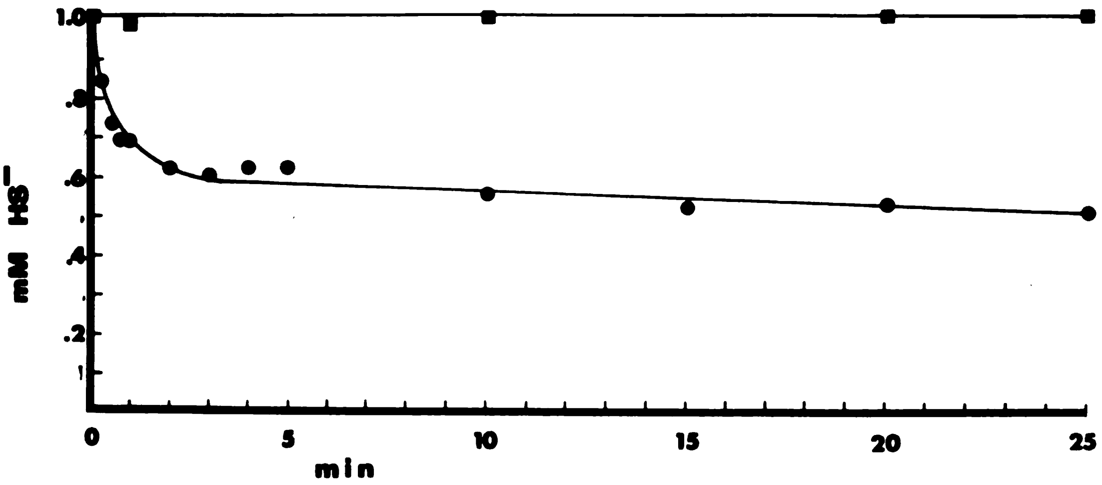

Figure 4

the presence of exudate. After 3 min both the formation of S^o globules and the uptake of sulfide ended. Thus, when HS^- was added to buffered exudates there was a decrease in the level of HS^- (Figure 4), S^o was formed (Figure 3), and no measurable O_2 uptake occurred (Figure 3). Since the only HS^- oxidation product which does not require O_2 is S^o, it appears likely that the exudates of *Pseudomonas aeruginosa* result in the formation of S^o from HS^-.

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CHAPTER IV

THE CELLULAR MORPHOLOGY OF SULFUR-OXIDIZING BACTERIAL COMMUNITIES

BACKGROUND

The range of *in situ* conditions leading to the numerical dominance of a few species is often narrow and definable (6,7). As a result, the morphology of the dominant bacteria seen under selective *in situ* conditions may be remarkably uniform (20,28). In contrast, transfer to laboratory media may result in pleomorphism because of unusual and variable culture conditions in which the organism would not normally be able to successfully compete (27,29). There are many instances in which the natural microflora cannot be successfully or reproducibly cultivated using artificial conditions (7,19,28,29). In particular, the gas vacuolate sulfur bacteria of the hypolimnion are difficult to cultivate while transient *Rhodospirillaceae* grow readily (29,32). Despite these difficulties, differential and selective media are commonly used for the enumeration of aquatic bacteria (32). Direct observation, however, allows one to observe all of the dominant morphological types in a community, provides information on the population density and biomass of each type (5), and allows one to observe physical relationships among the components of the community. For these reasons, direct observation was used here preferentially for studying the bacteria of the anaerobic hypolimnion, the littoral zone, and a sulfur spring.

Previous *in situ* descriptions of these bacteria have been limited to the light microscope and photographs have often been of poor resolution and contrast. This has resulted in questionable identification of some of the bacteria and uncertainty of the existence of certain bacterial groups (1). In this study, the *in situ* morphology of common bacterial forms from the hypolimnia and littoral zones of two southern Michigan lakes has been documented by phase and electron microscopy. The morphology of isolated genera has been compared to that found in nature.

MATERIALS AND METHODS

Lake samples from the hypolimnion were obtained as described elsewhere (5) so as to minimize exposure of the samples to O_2, light, and high temperature. After collection, samples were stored on ice and observed within 8 h. All photographs shown were taken of samples obtained from Wintergreen Lake, T1S:R9W:S8, Kalamazoo County, Michigan, and Burke Lake, T5N:R1W:S23, Clinton County, Michigan. The characteristics of these lakes have been described elsewhere (5).

Light microscopy

A Zeiss photomicroscope was used for phase microscopy. All cells were mounted on slides coated with a thin dried layer of Nobel Agar (Difco, Detroit, MI). The coated slides were prepared by adding a thin layer of sterile melted agar (0.75%) to a cleaned slide and allowing the agar to dry in a laminar flow hood under ultraviolet light. A small drop of lake water (0.02 ml) was placed on the dry agar and cover slip was immediately pressed over the drop. The resulting film of water was absorbed by the agar which pressed the organisms against the coverslip in a monolayer. The deformation of the agar by the cells increased the contrast between the cells and the background. Many of the morphological characteristics described could not be clearly observed unless this procedure was used.

Refractile inclusions which could be removed either by centrifugation or by pressing the cover slip of prepared slides were considered to be gas vacuoles. The remaining inclusions which produced a diffraction pattern of colored concentric rings in the phase microscope were considered to be sulfur granules.

Electron microscopy

One hundred milliliter lake samples were collected in suction flasks containing 100 ml of glutaraldehyde fixative (6% in 0.1 M phosphate buffer at pH 7.2). The cells were collected on a Millipore filter (UHW P04700 Millipore Corp., Bedford, MA) with a pore size of 0.22 μm, and a thin layer of 1% Nobel Agar (Difco, Detroit, MI) was then added to the filter under a slight vacuum. The filter was placed in 3% glutaraldehyde fixative (in 0.1 M phosphate buffered at pH 7.2) overnight. The filter was then washed in 3 to 5 changes of 0.1 M phosphate buffer (pH 7.2) and the cells fixed overnight with OsO_4 according to the Ryter-Kellenberger procedure (22). The filters were subsequently treated for 2 h with 0.5% uranyl acetate dissolved in the Ryter-Kellenberger buffer and dehydrated (5 min in 50%, 70%, 95%, 3 changes of 100% ethanol, 3 changes in propylene oxide). The filters were infiltrated with plastic by submerging them for 30 min in 2 parts propylene oxide and 1 part Epon 812 (Polysciences, Warrington, PA), 1 h in 1 part propylene oxide and 2 parts Epon 812, and finally in undiluted Epon 812 overnight. The specimens were polymerized for 36 h at 40 C and then for 24 h at 60 C. Sections were prepared using glass knives and an LKB Ultotome. The thin sections were post stained 10 min in 2% uranyl acetate (11) and 5 min in 2% lead citrate (3,41). The sections were observed using a Hitachi 11 electron microscope. To assist in identification of the thin-sections, glutaraldehyde fixed samples and 1 μm thick sections were examined by light microscopy.

Whole cell specimens were prepared by allowing the cells to clump after a brief exposure to oxygen (29) and transferring a clump to a formvar coated electron microscope grid. Excess moisture was drawn from the grid using a tissue. Cells at the edge of a clump were observed after shadowing at an angle of 15 degrees using a platinum-carbon pellet.

RESULTS AND DISCUSSION

The organisms were grouped according to genus in the case of previously described forms and according to morphological similarity in the case of unreported forms. The morphological groups were assigned a type number to facilitate their discussion. All of the organisms described are either sulfur-oxidizing bacteria themselves or associated with sulfur-oxidizing communities.

Since the samples were of natural communities made up of several populations, it is not possible to be absolutely certain that we have correctly related the organisms observed by electron microscopy (particularly for thin-sections) to the same organism observed by phase microscopy. In the subsequent descriptions where the morphological relationship between organisms shown by both methods is not obvious, we have indicated the reasons for our assignment. In many cases the presence of chlorobium vesicles (a cortical array of photosynthetic vesicles), which occur only in the green bacteria, was used to identify members of this group and to distinguish them from the purple bacteria which contain either intracytoplasmic vesicles or lamellae (31).

Pelodictyon clathratiforme

Pelodictyon, a green sulfur bacterium, was described by Szafler (39) and Lauterborn (25) as a rod-shaped organism which forms a net-like aggregate (Figure 1a). Closer examination has revealed that the rod-shaped cells (0.5 to 1.5 by 1.2 to 4 μm) are not only attached to each other end to end but may form truncated prosthecae (Figure 1a,b,c). This characteristic is retained in enrichments (30) and appears more reliable for identification purposes than the net-like configuration commonly observed for the aggregate in nature. Thin sections reveal the cortical array of vesicles that extend into the truncated prosthecae (30) (Figure 1b). The gas vacuoles are usually arranged parallel to the longitudinal axis of the cell (Figure 1b,c). The cell wall is bounded by a layer of striations with an amplitude of 150 Å (Figure 1d). Strains 1831

Figure 1. *Pelodictyon clathratiforme* (bars represent 1 μm)

a. The net-like configuration, gas vacuoles, and truncated prosthecae characteristic of the genus *Pelodictyon* (phase micrograph).

b. A longitudinal cross section showing the cortical vesicles (2), longitudinal gas vacuoles (3), truncated prosthecae (4) and surface striations (5) (electron micrograph).

c. A shadowed preparation showing the above features and the characteristic longitudinal gas vacuoles (1) (electron micrograph).

d. An oblique section showing the surface striations (6) which have an amplitude of 150 Å (electron micrograph).

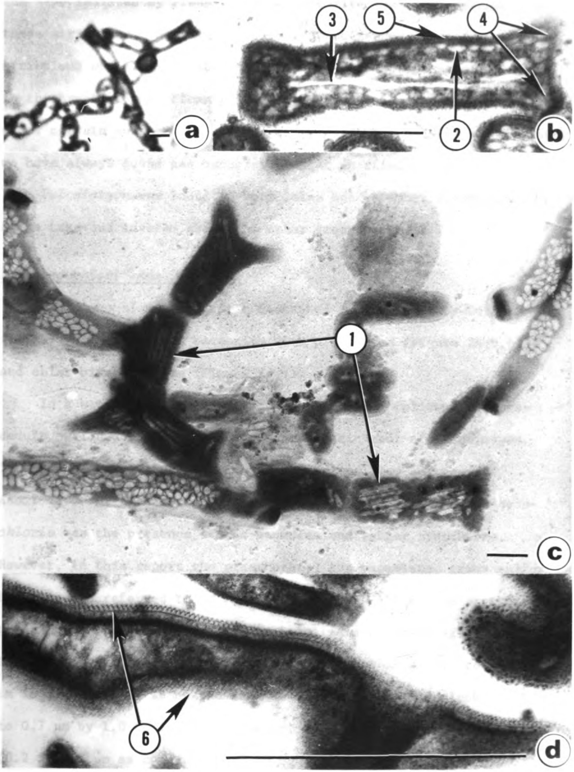

Figure 1

and 2730 isolated by Pfennig and Cohen-Bazire (30) also possess these striations. Pfennig's strain 2730 possessed two layers of striations while strain 1831 had only one, as did the strain found in the hypolimnion. Pfennig and Cohen-Bazire also found that under certain culture conditions gas vacuoles are not formed, although we have always found gas vacuoles present *in situ*.

Pelodictyon was found in both lakes but was most conspicuous in Burke Lake and layered above the other green bacteria.

Prosthecochloris-Ancalochloris

Prosthecochloris was first described and isolated by Gorlenko (14,15). It is a green bacterium with prosthecae (Figure 2a,b,c) and chlorobium vesicles (Figure 2a,c).

In a second isolation attempt Gorlenko (16) followed the procedure outlined by Pfennig (29) for the enrichment of *Pelodictyon*. In this case a gas vacuolate organism was enriched and named *Ancalochloris*. The difference between this organism and *Prosthecochloris* was the presence of gas vacuoles and longer prosthecae. However, in this report the prosthecate, gas vacuolate, green sulfur bacteria are referred to as *Prosthecochloris*. All of the prosthecate green sulfur bacteria found in the hypolimnia possessed gas vacuoles. The size of the free-living form of *Prosthecochloris* (0.5 to 1.0 μm in diameter) is about the same as the isolates of Gorlenko (0.5 to 0.7 μm by 1.0 to 1.2 μm); however, the prosthecae are larger (0.2 by 0.7 μm as compared to 0.10 by 0.17 μm for the isolate).

Figure 2. *Prosthecochloris* (bars represent 1 µm).

a. Shadowed preparation of *Prosthecochloris*. The cells are prosthecate (1) and contain gas vacuoles as well as intracellular granules (electron micrograph).

b. Phase micrograph of *Prosthecochloris* with gas vacuoles removed by centrifugation. The cells are clearly prosthecate (2). No sulfur granules are present.

c. A thin section of two cells which shows all of the features above plus the presence of a cortical array of vesicles (3). Both the cytoplasm and vesicles extend into the prosthecae. The granules do not have the refractile appearance of sulfur.

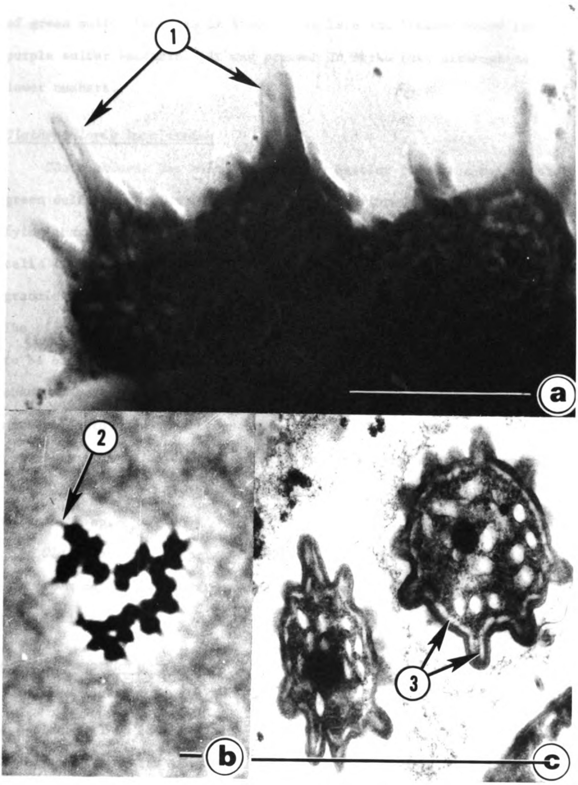

Figure 2

Prosthecochloris accounted for 5 to 20% of the total community of green sulfur bacteria in Wintergreen Lake and layered below the purple sulfur bacteria. It was present in Burke Lake although in lower numbers.

Clathrochloris hypolimnica

Clatrochloris has been described by Geitler (13) as a unicellular green sulfur bacterium which deposits sulfur internally. The identifying characteristic is the trellis-like aggregation of gas vacuolate cells (Figure 3a). Each of the cells was found to contain storage granules although sulfur granules were not apparent (Figure 3b,c). The pigment in samples containing primarily *Clathrochloris* was found to be bacteriochlorophyll d (chlorobium chlorophyll 650). The observed purity ratio (optical density 650/optical density 500) in methanol saturated with hydrogen sulfide was 23.5, which compared favorably to a value of 24.7 for the chromatically purified preparations of Kaplan and Sieberman (21) obtained from pure cultures of *Chlorobium* sp.

Clathrochloris comprised 80-90% of the bacterial community in the 4.0 m zone of Wintergreen Lake; it layered immediately below the purple bacteria.

Chlorochromatium aggregatum

Although recognition of the genus *Chlorochromatium* has been questioned (37), the fine structure of coenobia taken from the hypolimnion supports its validity. The coenobium consisted of a central colorless cell with peripheral green cells which gave the

Figure 3. *Clathrochloris hypolimnica* (bars represent 1 μm).

a. Phase micrograph of a typical aggregate. Gas vacuoles cause the high refractility of the cells.

b. Phase micrograph after the gas vacuoles were removed by centrifugation.

c. A shadowed preparation showing intracellular granules (1), gas vacuoles (2) and the trellis-like arrangement for which the aggregate was named (electron micrograph).

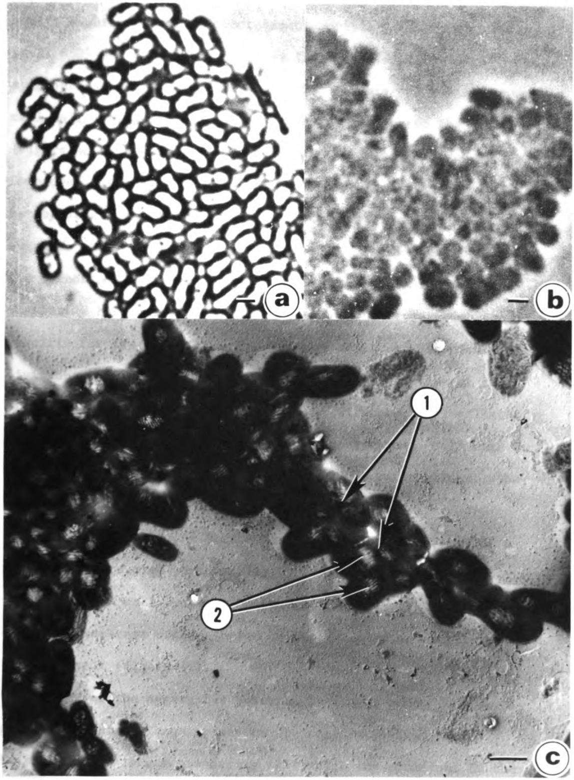

Figure 3

aggregate a barrel-like appearance (Figure 4a,b). The photosynthetic symbiont contained bacteriochlorophyll d (chlorobium chlorophyll 650) and chlorobium vesicles (Figure 4c,d). The heterotrophic symbiont lacked photosynthetic vesicles (Figure 4c) and its outer cell wall consisted of a layer of cups which covered the surface (Figure 4c,e). This structural feature has been observed in *Lampropedia* (8) and *Thiopedia* (Hirsch, unpublished data), which are metabolically dissimilar but which both form plates of cells. This suggests that the cups may play a role in maintaining the shape of the coenobium. Possibly, the heterotrophic symbiont of *Chlorochromatium* has evolved an adhesive surface structure which is specific for its symbiont. Its fusiform shape and one-dimensional division plane would prevent cohesion and the formation of cell plates as in *Thiopedia* and *Lampropedia*. Without the flagellated heterotrophic symbiont, the photosynthetic symbiont would lack a mechanism for maintaining its position in the water column. The symbiosis may thus be obligatory in the hypolimnion.

The envelope of the photosynthetic symbiont consisted of a membrane interrupted periodically by mesosomal invaginations (Figure 4c) and a cell wall composed of four electron dense layers (Figure 4d). The membrane often adhered to the vesicles and separated from the cell wall (Figure 4c).

A few of the aggregates observed possessed brown instead of green symbionts. These appear to fit the description of the *Pelochromatium roseum* consortium (25) which was abandoned in the seventh edition of Bergey's Manual (4). However, this genus is still cited due to the common occurrence of the aggregate in hypolimnia (17,35) and will be reinstated in the eighth edition of Bergey's Manual (Pfennig, personal communication). No differences other than pigmentation were noted between the two types.

Chlorochromatium was observed in both of the lakes studied, but it was most dense during the winter stratification of Burke Lake and gave the water a bright green color. The high density of *Chlorochromatium* in samples used for electron microscopy made identification of thin-sections of this organism simple.

Type 1 procaryotes (T1)

T1 consists of an aggregation of elliptical cells 1.2 to 1.5 μm by 1.5 to 2.5 μm (Figure 5b,c). The cells are joined at the base forming a rosette. Each cell has an optically dense area near its outer tip which is most clearly seen when the gas vacuoles are removed (Figure 5c). This dense area contains both lamellae and gas vacuoles but not photosynthetic vesicles (Figure 5a,b). These lamellae suggest that T1 may be either a blue-green alga or a purple bacterium since both of these groups may possess photosynthetic lamellae. However, polar stacks of membranes, like those shown in Figure 5a, occur only in the budding purple bacteria (31). Buds observed on the cell surface (Figure 5b) were also seen in thin-sections. Protruding from the surface of each cell are 10 to 50 filaments approximately 100 Å in diameter and 3 μm in length. T1 was distributed among the purple and the green bacteria but was never found in great abundance.

Figure 4. *Chlorochromatium aggregatum.*

a. The aggregation after being mounted on an agar slide (bar represents 1 μm). Both the colorless heterotroph (1) and the outer photosynthetic cells can be seen (2) (phase micrograph).

b. The aggregation as it appears in a wet mount before being mounted on an agar slide (bar represents 1 μm). Only the green photosynthetic cells are visible (phase micrograph).

c. A cross section through the transverse axis of *Chlorochromatium aggregatum* showing the peripheral vesicles of the photosynthetic symbionts (4) and the heterotrophic cell (5) with external surface structures (6) (bar represents 1 μm).

d. Enlargement of the peripheral vesicles (7), cell wall (8) and membrane (9). The membrane often separates from the wall and adheres to the vesicles (bar represents 0.1 μm).

e. An oblique section of the central heterotroph showing the external surface structures which appear cup-shaped (10) (bar represents 1 μm).

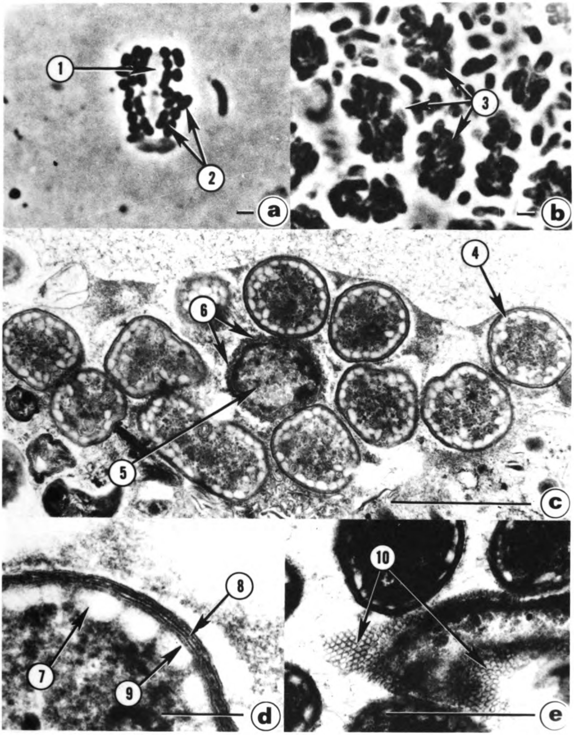

Figure 4

Figure 5. The Type 1 procaryotes (T1) (bar represents 1 μm).

a. A thin section of T1 showing the polar stack of lamellae and gas vacuoles.

b. Phase micrograph of the aggregate (3) after centrifugation to remove gas vacuoles. The cytoplasm toward the outside of the aggregation is dense and well defined (4).

c. A shadowed preparation showing the filaments which protrude from the surface of each cell (1). The upper portion of each cell contains a cluster of gas vacuoles (2) (electron micrograph).

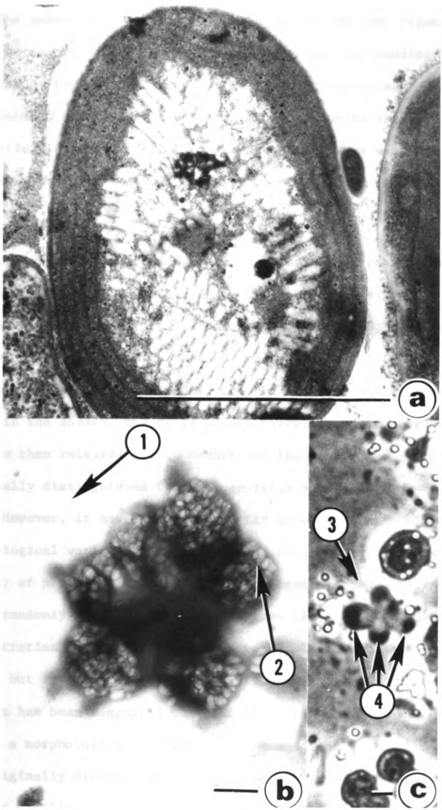

Figure 5

The assumption that Figure 5a,b,c is of the same organism is based on the increased density of the cytoplasm surrounding the gas vacuoles at the ends of the cells (Figure 5b), which suggests that the photosynthetic apparatus surrounds the vacuoles as shown in Figure 5a. The size of the section also correlates with the size of the organism in light micrographs.

Thiothrix

It has been suggested by several authors that the bacterial genera *Cladothrix* (2,33,38), *Sphaerotilus*, and *Thiothrix* (36) may represent morphological variations of a single genus, *Sphaerotilus*. *Sphaerotilus* consists of a chain of rod-shaped cells enclosed in a sheath with a holdfast attaching the chain to the substratum (24). While in the sheath, the cells become flagellated (lophotrichously) and are then released (36). Members of the genus *Cladothrix* were originally distinguished from *Sphaerotilus* by dichotomous branching (9). However, it has been subsequently shown that branching is a morphological variation of *Sphaerotilus* induced by low concentration (<0.1%) of peptone (34). At high concentrations (>0.25%), cells occur randomly within a mucilaginous mass (12). Changes in the concentration of phosphate buffer have little effect on filamentous growth but levels about 0.05 M inhibit growth (12).

It has been suggested that *Thiothrix*, like *Cladothrix*, is simply a morphological variation of *Sphaerotilus* (18). *Thiothrix* was originally distinguished from *Sphaerotilus* on the basis of the gliding motility of conidia, intracellular deposition of sulfur, formation of a true trichome and obligate chemoautotrophic metabolism

(18,36). Although Skerman has shown that *Sphaerotilus* is capable of depositing sulfur internally (in the presence of sulfide), he still maintained the differentiation between genera on the basis of true trichomes and gliding motility (the autotrophic metabolism of *Thiothrix* has never been shown directly since no pure cultures have been isolated) (33). However, the gliding motility of conidia observed by Winogradsky in 1888 (18) has not yet been confirmed despite the efforts of Keil (23) and Pringsheim (33).

The forms of *Thiothrix* observed in the littoral zone (Figures 6 and 7) on decaying plants deposited elemental sulfur, contained peripheral lamellae, were 2 to 5 μm in diameter, and attached to the plants using a holdfast (Figure 7). However, the lamellae were not observed by Drawert and Metzner-Kuster (10).

Thiocystis, *Chromatium*, *Thiospirillum*, and *Thiopedia*

These organisms were prevalent in many samples but are well known and for that reason are only briefly mentioned. *Chromatium* and *Thiocystis* generally deposited sulfur. *Thiocystis* attached to decomposing plant surfaces in the littoral zone (Figure 6) using its mucilage as a holdfast. Figure 8a shows a plate of *Thiopedia* cells containing sulfur granules. Contrary to most keys, *Thiopedia* (Figure 8a,b) and *Thiospirillum* were usually found without sulfur granules when growing in the hypolimnion in the presence of measurable sulfide.

Figure 6. The microenvironment at the surface of decomposing plant material taken from the littoral zone of Burke Lake (electron micrograph of a thin section). A microcolony of *Thiocystis* fills the upper portion of the figure. The large inclusions (2,3) are sulfur granules. The microcolony is embedded in mucilage (1) which serves to anchor the colony to the plant wall (6). A transverse section through a *Thiothrix* filament shows the sheath (5) and peripheral membranes (4). Also seen are organisms with a scalloped cell wall and nucleophilic granules (7) which are characteristic of thiobacilli. Bar equals 1 µm. Micrograph by Sarah Caldwell.

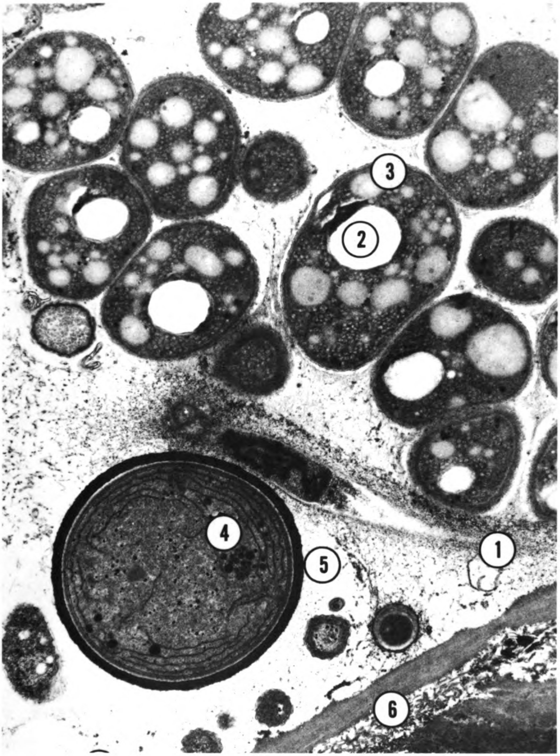

Figure 6

Figure 7. Bacterial attachment to the surface of decomposing plant material taken from Burke Lake (electron micrograph of thin section). A holdfast characteristic of the genus *Thiothrix* is shown (1). The debris at the base (2) generally contains iron hydroxide (soluble in dilute acid) and as a result is unusually electron dense. A bacterial microcolony is also shown which attaches to the plant surface using the mucilage which encases the colony (4) and which contains centrally located membrane stacks (5). *Nitrocystis* also contains structures resembling carboxysomes (6) and is coccus shaped. *Thiocystis* is also present in the section (3). Micrograph by Sarah Caldwell.

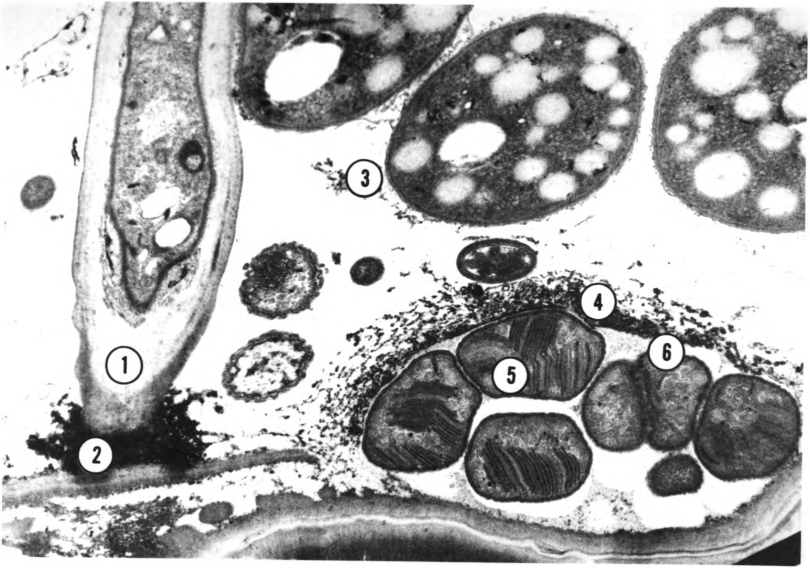

Figure 7

Figure 8. *Thiopedia* and the Type 2 procaryotes (T2) (bar represents 1 μm).

a. *Thiopedia* with gas vacuoles removed by centrifugation revealing several sulfur granules (1).

b. Plate of *Thiopedia* cells with gas vacuoles (phase micrograph).

c. T2 as it appears with phase microscopy (2).

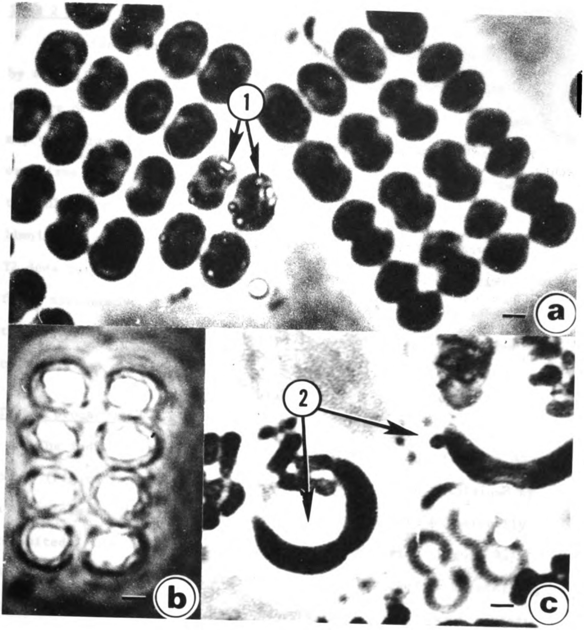

Figure 8

Type 2 procaryotes (T2)

The size of the cells of type 2 procaryotes is 1.2 to 1.5 μm by 4 to 12 μm (Figure 8c). The curved fusiform characteristic (Figure 8c) is generally present although the degree of curvature may vary (in some cases the cells are simply fusiform). These organisms do not contain gas vacuoles or sulfur granules. This was the only large, curved, and fusiform organism found in the hypolimnion. As in the case of *Thiospirillum* and *Prosthecochloris,* T2 does not appear to be colored when viewed by phase or bright field microscopy. T2 has a distribution pattern which is identical to that of the green bacteria though it was often only 1% of the community.

Type 3 procaryotes (T3)

The individual cells of T3 occur in plates of 2,4,8,16, or 32 cells (Figure 9a,b,c). The arrangement appears to be maintained by a colorless mucilage in which refractile granules are occasionally deposited. The cells are separated by a clear region (Figure 9a). No surface structures that might possibly be involved in maintaining the plate arrangement have been observed. The cells always contain gas vacuoles. Reproduction is by binary fission which occurs when the ends of the curved cells touch. The cells are 0.5 to 0.6 μm by 2.0 to 6.0 μm in size. There is a strong resemblance between T3 and the gas-vacuolated strains of *Microcyclus aquaticus* described by Van Ert and Staley (40). However, T3 is from the anaerobic zone, occurs in plates, and forms complete circles (Figure 9a), each cell being of uniform thickness. The gas vacuolated forms of *Microcyclus*

Figure 9. The Type 3 procaryotes (T3) (bar represents 1 μm).

a. A shadowed preparation showing gas vacuoles (1) and binary fission (2). Plates of 2, 4, 8 or 16 cells are typical. Although they may be broken into two cell units, they rarely occur unicellularly (electron micrograph).

b. T3 before removal of gas vacuoles (arrow) with cell plates disrupted (phase micrograph).

c. T3 after the removal of gas vacuoles by centrifugation (phase micrograph).

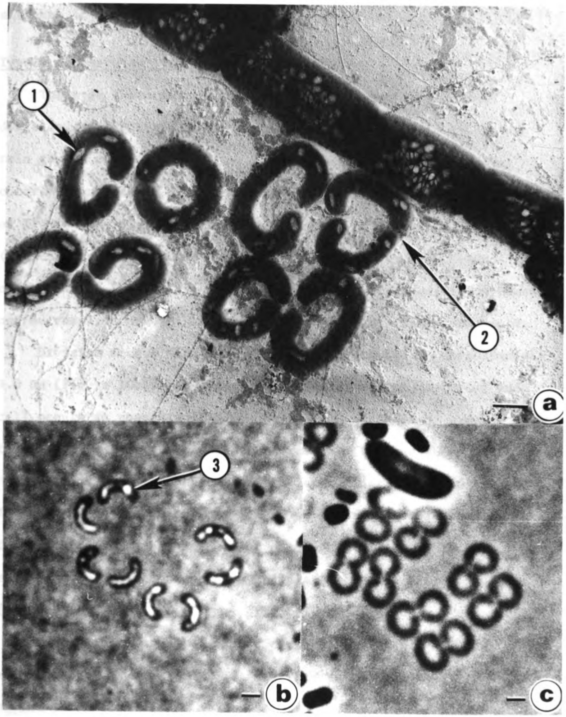

Figure 9

aquaticus are aerobic, unicellular, and vibriod (40). T3 is most abundant immediately above the sediments (20 to 40% of the community) although it appears in low numbers throughout the hypolimnion.

Type 4 procaryotes (T4)

The type 4 procaryotes are distinguished by their mode of division and the shape of their coenobia. The fusiform cells contain gas vacuoles and occur in a pallisade arrangement within a colorless mucilage. The structure of the coenobia, as in the case of T3, is very distinct from that of *Lampropedia* (8) and *Thiopedia* (Hirsch, unpublished data) and does not require contact between cells (Figure 10a,b). T4 always appears very transparent and could not be resolved unless phase microscopy and agar slides were used.

Division occurs after the cells have elongated to approximately 9.0 μm (Figure 10b). An apparent thickening at the center of each cell occurs in coenobia (Figure 10c). Thin sections of samples containing T4 frequently reveal structures like the one shown in Figure 10d. Based on phase microscopic examination of the sample, T4 is the organism which most likely could produce this structure. The thickening may be a result of an unusual division process resulting in the formation of a cellular extension after fission (Figure 10a,d).

T4 is distributed throughout the hypolimnion but is most abundant at the water-sediment interface reaching a density of 20% of the total community.

Figure 10. The Type 4 procaryotes (T4) (bar represents 1 μm).

a. Note the palisade arrangement, gas vacuoles (1) and cellular extension (2) (electron micrograph of a shadowed preparation).

b. T4 after the division process (gas vacuoles removed by centrifugation, phase micrograph).

c. T4 during the division process. Note the thickening (3) at the center of the plate (gas vacuoles removed by centrifugation, phase micrograph).

d. A thin section showing the site of possible division (4).

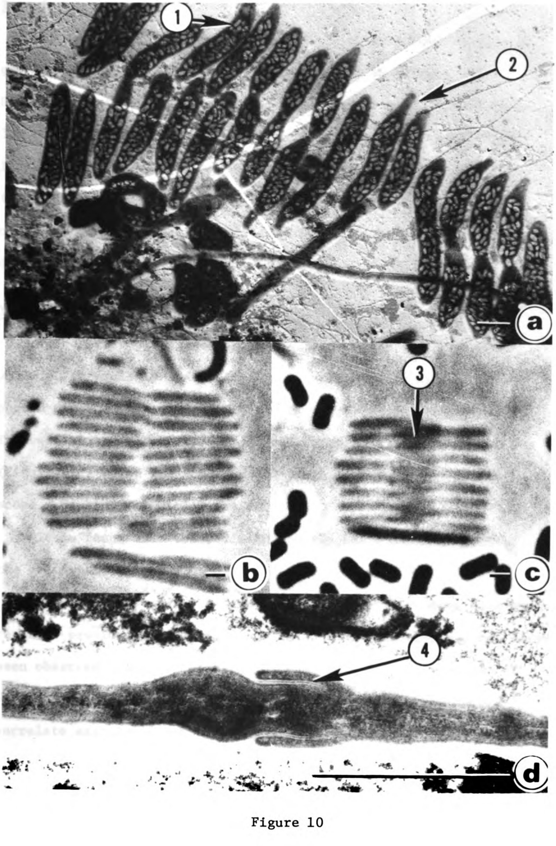

Figure 10

Type 5 procaryotes (T5)

T5 is fusiform in shape (Figure 11a,b,c) with two clusters of gas vacuoles on either side of the division site (Figure 11b). Division occurs by binary fission. The cells are 0.5 to 0.8 μm by 3.0 to 15 μm in length. The poles of the cells are square (Figure 11c). The cells may be either straight or curved. The curvature is often confined to the ends of the cell (Figure 11a). These organisms are confined to the sediment-water interface reaching a density of 50-60% of the total community.

Type 6 procaryotes (T6)

T6 consists of filamentous cells (1.0 to 1.5 μm by 10 to 100 μm) with irregularly spaced cross walls (Figure 11c,d,e) which are photon dense (Figure 11d,e) and electron transparent (Figure 11c). The wall of the filament is covered .by longitudinal striations (Figure 11c) which are occasionally visible in the light microscope. Each cell contains gas vacuoles.

T6 is found near the sediment and reaches a density of 5% of the bacterial community.

Most of the purple and green bacteria described above have been reported previously (29), although their *in situ* morphology has never been observed using the electron microscope. Only in the case of *Chromatium* and *Thiocystis* did the description of pure cultures correlate exactly with morphology of these genera *in situ*. The gas-vacuolate achlorophyllous bacteria found at the sediment-water interface have never been isolated or reported previously.

Figure 11. The Type 5 and 6 procaryotes (T5 and T6) (bar represents 1 μm).

a. T5 with gas vacuoles removed by centrifugation. The cells may be curved at the tips or rod shaped (phase micrograph).

b. T4 with gas vacuoles which are separated into two clusters (1,2) by the division site (phase micrograph).

c. T5 and T6 showing the square shape and dense cytoplasm (3) of the ends of T5 and the longitudinal striations (4) of T6 (electron micrograph of a shadowed preparation).

d. T6 with gas vacuoles removed (5) by centrifugation (phase micrograph).

e. T6 with gas vacuoles (6) (phase micrograph).

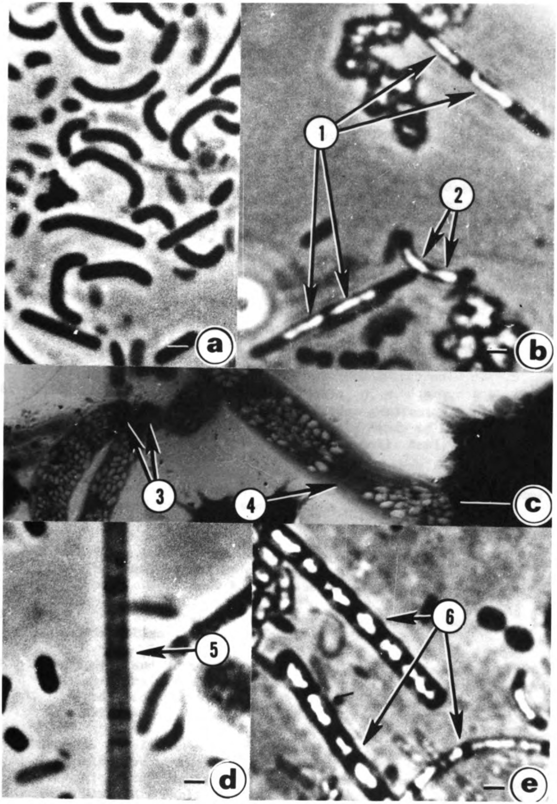

Figure 11

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CHAPTER V

THE DISTRIBUTION OF SULFUR-OXIDIZING BACTERIA

BACKGROUND

In anaerobic hypolimnia, sulfide may be produced and oxidized biologically in the absence of oxygen. However, in the upper region of an anaerobic hypolimnion, in the littoral zone, and in sulfur springs, sulfide and oxygen containing waters can be mixed. When this occurs, sulfide oxidation proceeds both biologically and abiologically. Abiologically sulfide is oxidized to sulfur, thiosulfate, and/or sulfate depending on the reaction conditions (7). Biologically sulfide may be oxidized by the *Chromatiaceae*, *Chlorobiaceae*, colorless sulfur bacteria and heterotrophic sulfur-oxidizing bacteria. In this chapter the distribution of these organisms in sulfide containing environments is discussed and interpreted using the information from preceding chapters on physiology, morphology, and *in situ* gradient enrichment.

Although fluorescent pseudomonads and thiobacilli were enumerated using plating media, the failure of cultural methods to support many photosynthetic bacteria and *Thiothrix* sp. required the use of direct microscopy. The distinctive morphology of sulfur-oxidizing bacteria has allowed several previous studies on the distribution of these bacteria as a function of physicochemical factors (2,3,8,11,12,13).

The present study improves on these by providing more detailed information on morphological variation in freshly collected samples, by collection of samples from smaller depth increments (0.1 m for our study of hypolimnia), by attempting to correlate structural information with determinative environmental parameters, and by including heterotrophic sulfur-oxidizing bacteria.

MATERIALS AND METHODS

Media

Thiobacillus Broth (Difco Laboratories, Detroit, MI U.S.A.) was used with 1.5% Nobel Agar (Difco) for plate counts of thiobacilli (pH 4.8). Plates were observed after 48 h, 7 days, and 30 days.

The TS (thiosulfate) medium (pH 7.5) was used to induce pigment production by fluorescent pseudomonads. It is a modification of the media of Frank and DeMoss (10), Sands and Rovira (21), and Rovira and Ridge (20). It contained: 2% glycerol (w/v), 1% $Na_2S_2O_3 \cdot 7H_2O$, 1% case amino acids, 0.01 M K_2HPO_4, 0.01 M $MgCl_2$, and 1.5% Nobel agar (for solid media). In some cases antibiotics were added, including: 45 mg/liter Penicillin G, 45 mg/liter novobiocin (Upjohn, Albamycin), 75 mg/liter cycloheximide (Upjohn, Acti-Dione), 5 mg/liter chloramphenicol.

Sampling

For hypolimnetic bacterial counts and chemical determinations, the following sample collection procedure was used. Amber serum bottles (37 ml) filled with nitrogen and sealed with silicone rubber septa were used as collection bottles. The bottles were evacuated at the sampling site by introducing a 27 gauge hypodermic needle which was connected to a vacuum source. The sample was drawn from the lake through a latex hose which was connected to a second hypodermic needle. To initiate collection this needle was inserted through one wall of an evacuated rubber tube allowing the sampling hose to be flushed with several volumes of sample. The evacuated hose was then pressed against the septum of the collection bottle (which was still connected to the vacuum source) and the needle was inserted through both the second wall of the vacuum hose and the septum of the evacuated bottle. Once the bottle had filled, the vacuum and collection needles were removed. At the end of the collection period the samples were placed in a bucket of ice water.

Samples were taken at 0.1 m increments of increasing depth. The sampling hose was connected to a rigid sample inlet and a lead weight which was lowered by a brass chain to the desired depth. Sampling was done from 3 to 5 am to avoid surface disturbances and direct sunlight. For less intensive samplings, a Van Dorn sampler was used. Samples from the littoral zone and springs were collected using sterile 50 ml syringes to replace the sampling hose.

Bacterial enumeration

For estimating fluorescent pseudomonads in samples taken either from springs or the littoral zone, syringes were used to collect and dispense aliquots on sterile, 0.45 μm black, membrane filters (HABG, Millipore, Bedford, MA U.S.A.) and these were incubated on agar media. Thiobacilli were estimated directly on agar spread plates.

In the case of plant materials, samples were collected in sterile, 500 ml, wide-mouthed bottles. The plant materials were ground in a Waring Blender (previously rinsed with 70% ethanol) and then homogenized (using a sterile ground-glass homogenizer). The dry weight of plant material per milliliter was determined by drying 5 ml aliquots in aluminum containers at 110 C. Bacterial numbers were then determined as described above.

Direct counts of the total number of bacterial cells found in the littoral zone were obtained by drying a 1.0 μl aliquot from each sample on a formvar coated 300 mesh electron microscope grid. Each grid was counted using a transmission electron microscope. The number of cells per milliliter was calculated from the area of the drop, the area of open spaces within each grid, and the number of cells per space.

Enumeration of morphological types

Within 5 h after collection the samples were centrifuged and the pellet transferred to agar coated slides. These slides were examined by phase microscopy and photographed. One slide was prepared for each sample and ten microscopic fields were photographed for each slide. Each field contained 10 to 500 cells with an average of approximately 100 cells per field. The population data are reported for each morphological type as percent of total cells observed; this has been referred to as congruity by Terborgh (24). Sulfur granules in *Thiopedia* cells were easily observed by microscopy after removing the gas vacuoles by pressing on the cover slip or by centrifuging. The use of photography was necessary for more accurate size determination and allowed the data to be recorded immediately after sampling before morphological and numerical changes could occur.

Chemical assays

Sulfide was determined by the method of Pachmayr as described by Brock *et al.* (4). Serum bottles containing 2 ml of 0.2% zinc acetate in 0.2% acetic acid were used to collect samples. One tenth milliliter of phenylenediamine reagent was added to 10 ml of sample. The tubes were mixed, 0.1 ml of ferric reagent was added, and the tubes again mixed. The absorbance was read at 670 nm against the appropriate blank using a Beckman DB-G spectrophotometer. A standard curve using $Na_2S \cdot 9H_2O$ was prepared for each assay. Known quantities of Na_2S added to hypolimnetic samples were accurately determined using this assay.

Oxygen was determined by a modification of the Winkler method as described by Mackereth (17).

Chlorophyll was determined on the pellet of cells obtained from centrifuging 37 ml of lake sample. The pellet was lysed by the addition of 5 ml of H_2S saturated acetone (15). The extract was then recentrifuged or passed through an acetone insoluble membrane filter with a pore size of 0.22 μ (Millipore UHW P04700, Bedford, MA) to remove particulate debris. Spectra from 400 to 700 nm were obtained using a Beckman DB-G scanning spectrophotometer. The concentration of bacteriochlorophyll was calculated using the method of Stanier and Smith (23).

Elemental sulfur was determined as described in Chapter II.

Habitats

Numerous lakes and ponds located within a 50 mile radius in southern Michigan were surveyed in this study as well as a sulfur spring in the Florida Keyes ("Flowing Well", John Pennekamp Coral Reef State Park). The location and characteristics of these lakes are shown in Tables 1 and 2. The reported data on community structure are from samplings done on 7/4/71 for Wintergreen Lake and 8/23/72 for Burke Lake. Similar studies were done on 6/26/71 and 8/21/72 for Wintergreen Lake and 7/16/72, 8/1/72 and 9/6/72 for Burke Lake. Qualitative observations were also made from 1969 to 1974.

RESULTS AND DISCUSSION

The distribution of sulfur-oxidizing bacteria in hypolimnia

Three distinct microbial communities were recognized in the hypolimnia of Wintergreen and Burke Lakes. The upper layer was slightly turbid and red, contained purple sulfur bacteria and is referred to as community A. The next lower layer appeared green, contained green sulfur bacteria and is referred to as community B. The lowest layer occurred within 1 m of the sediments, was turbid and white, contained colorless bacteria, and is referred to as community C. The two ponds studied contained only community A.

Microstratification of populations of bacteria occurred within the upper two communities of photosynthetic bacteria as predicted by van Niel (26) from studies on enrichment cultures. In Burke

Table 1. Location and characteristics of the aquatic habitats studied

Name	Area, ha[a]	Maximum depth, m[a]	Water source	Hypolimnion	Michigan county	Geographic location[a]
Cassidy Lake	10.1	7.0	spring	anaerobic	Barry	T1N:R7W:S17
Gull Lake	761.0	--	--	aerobic	Barry	T1N:R7W
Yankee Springs	--	--	--	--	Barry	T5N:R10W
Purdy Bog	1.4	--	--	--	Barry	T1N:R9W:S36
Lawrence Lake	1.2	--	--	aerobic	Barry	T1N:R9W:S27
Wintergreen Lake	17.8	6.3	--	anaerobic	Kalamazoo	T1S:R9W:S8
Duck Lake	11.5	4.2	--	anaerobic	Kalamazoo	T1S:R9W:S5
Forest Pond	0.05	1.0	creek	--	Kalamazoo	T1S:R9W:S22
EF Pond	0.02	1.0	creek	--	Kalamazoo	T1S:R9W:S22
Augusta Creek	--	--	--	--	Kalamazoo	T1S:R9W:S22
Three Lakes (middle)	21.8	--	--	--	Kalamazoo	T1S:R10W:S25
Burke Lake	0.72	11.7	spring	anaerobic	Clinton	T5N:R1W:S23

[a] Data supplied by the Department of Resource Development, Michigan State University (1962), and the Michigan Department of Conservation, Lake Survey Project (1964).

Table 2. Concentration of HS^-, S^o, thiobacilli, and total procaryotes in 13 water samples from lakes in southwestern Michigan. See text for explanation.

Sample	mg/l HS^-	mg/l S^o	Thiobacilli x 10^{-5}	Total procaryotes x 10^{-5}
Burke Lake	3.0	237	<1	3,800
Three Lakes	2.0	91	<1	100
Three Lakes	1.2	167	<1	30
Wintergreen Lake	0.4	83	<1	60
Duck Lake	0.2	108	<1	640
Augusta Creek	0.0	---	<1	16
Lawrence Lake	0.0	---	<1	880
Gull Lake	0.0	---	<1	160
Purdy Bog	0.0	48	<1	4,400
Purdy Bog	0.0	59	<1	8,400
Yankee Springs	0.0	56	<1	40
EF Pond (sediment surface)	9.0	224	<1	8,000
EF Pond (above sediment)	1.1	64	<1	410

Lake the microstratification of photosynthetic bacteria exhibited the following sequence (from the thermocline to the sediments): *Thiospirillum*, *Chromatium*, *Thiocystis*, *Pelodictyon* and *Chlorochromatium*. In Wintergreen Lake *Thiopedia*, *Thiocystis*, and *Clathrochloris* occurred in sequential layers while *Prosthecochloris* was randomly distributed within the layer of *Clathrochloris*.

The distributional pattern of the three major communities shown in Figures 1 and 2 corresponds to the ecotone model of Terborgh (24), which is a description of biological distribution along physical gradients. In biological systems possessing this characteristic the spread of populations is blocked by habitat discontinuities in contrast to the gradient and competition models in which distribution is controlled either directly by physical characteristics varying continuously and in parallel with the gradient or by competitive exclusion (24). This implies that the distribution of hypolimnetic bacteria may not be simply controlled by H_2S, O_2 or light as previously suggested (18,26), since they each varied continuously, but rather by a function, $F(H_2S, O_2, \text{light}, x\ldots)$, which is discontinuous through the hypolimnion. From Figures 1 and 2 it can be seen that a massive change from a purple sulfur flora to a green sulfur flora occurred in less than 0.1 m (Figure 1), while sulfide, oxygen, and light varied continuously.

The data shown in Figures 1 and 2 as well as the data from four similar studies suggest that the anaerobic bacteria from the hypolimnia of these lakes possess distinct characteristics held in common as well as unique characteristics which can be used to distinguish the three major communities.

Figure 1. Some physical, chemical and biological parameters of Wintergreen Lake on 7/4/71, and Burke Lake on 8/23/72 as a function of depth. The organisms shown are representatives of the A, B, and C communities which occur below the thermocline in sequential layers.

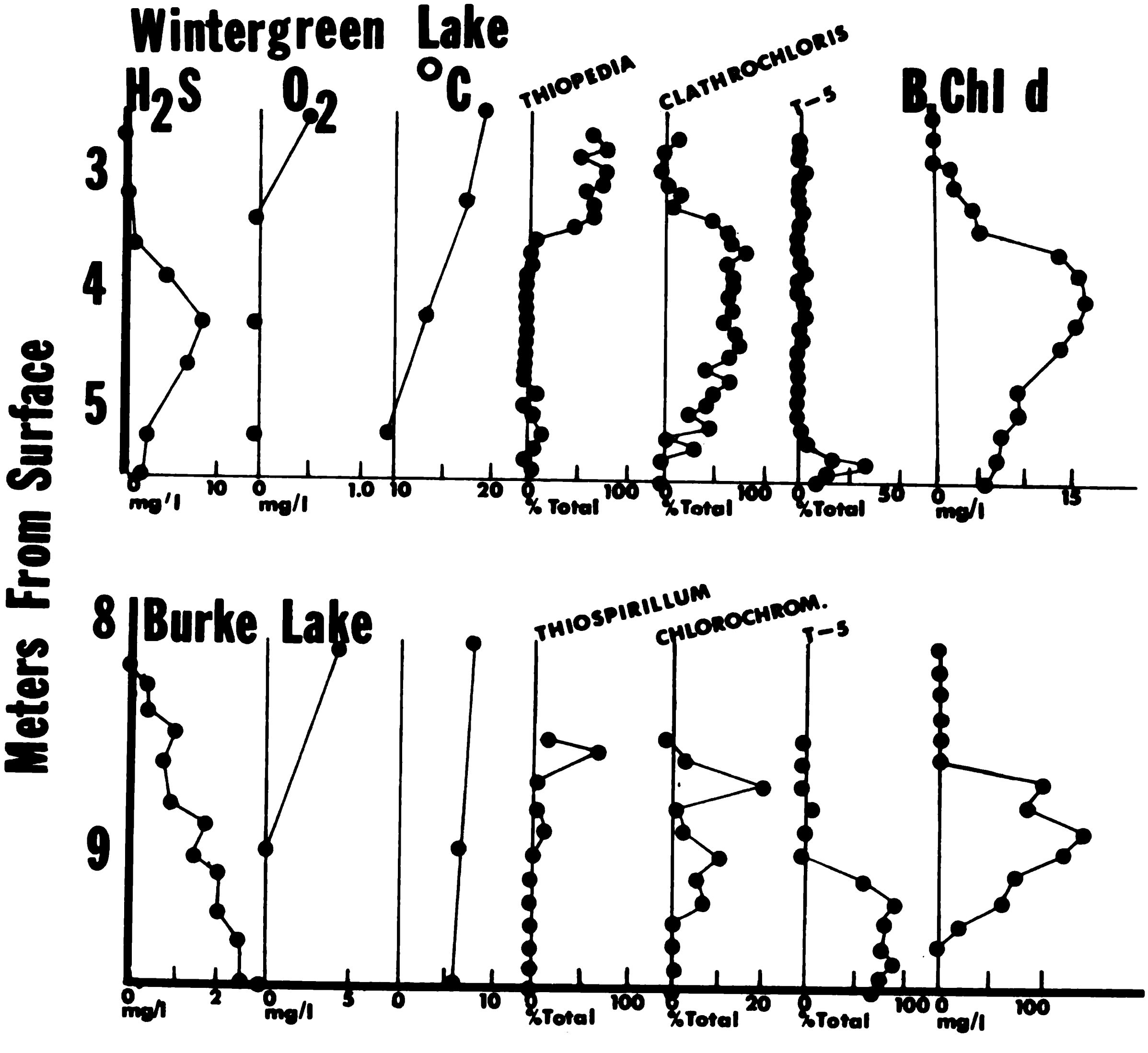
Wintergreen Lake
H_2S
O_2
°C
THIOPEDIA
CLATHROCHLORIS
T-5
B.Chl d
Meters From Surface
3
4
5
0 mg/l 10
0 mg/l 1.0
10 20
0 % Total 100
0 %Total 100
0 %Total 50
0 mg/l 15
8
Burke Lake
THIOSPIRILLUM
CHLOROCHROM.
T-5
9
0 mg/l 2
0 mg/l 5
0 10
0 %Total 100
0 %Total 20
0 %Total 100
0 mg/l 100

Figure 1

Figure 2. The community structure of Wintergreen Lake on 7/4/71, and Burke Lake on 8/23/72.

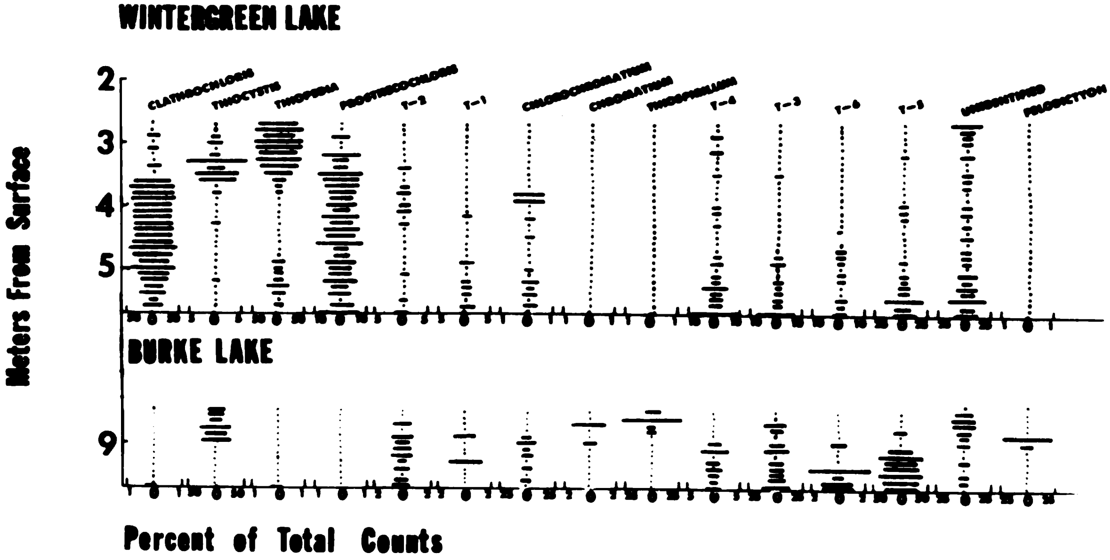
WINTERGREEN LAKE
BURKE LAKE
Meters From Surface
2
3
4
5
9
CLATHROCHLORIS
THIOCYSTIS
THIOPEDIA
PROSTHECOCHLORIS
T-2
T-1
CHLOROCHROMATIUM
CHROMATIUM
THIOSPIRILLUM
T-4
T-3
T-6
T-5
UNIDENTIFIED
PELODICTYON
Percent of Total Counts

Figure 2

The organisms possessed gas vacuoles or flagella (with the possible exception of T2 since flagellation was not determined). In all cases, samples taken from the hypolimnia clumped and migrated in an irregular fashion (bioconvection patterns) as observed by Pfennig (18). The clumping of the cells could be reversed by adding Na_2S to a concentration of 1 g/l. Since the organisms were capable of reversible (upward and downward) migrations, possessed either flagella or gas vacuoles (but not both), and occurred in discrete layers, the assumption that gas vacuoles are used to regulate bouyancy and hence position in the water column is reasonable. However, diurnal migrations did not occur in the hypolimnion as they do in the epilimnion (this did not include the movements of invertebrates [*Stentor* sp.] which were observed feeding on purple bacteria). The slow rate of movement of *Chromatium* and *Thiospirillum*, less than 10 mm/h (25), makes the possibility of daily bacterial migrations unlikely. The layers shown in Figures 2-9 were stable from day to day and week to week as evidenced by the qualitative observation of samples throughout a 24 h period and by the distributions shown in Figure 3 for samples collected one week apart. Over periods of weeks, the layers of bacteria expanded and contracted, but the relative position of the three major communities described below remained constant. The density of each community varied accordingly and reached a maximum of 10^8 cells/ml when the layers of green bacteria were compressed during the winter months in Burke Lake.

The three communities could be separated on the basis of density by centrifuging a concentrated sample at 7,000 g for 10 min. The gas

Figure 3. The fraction of the bacterial community in Wintergreen Lake composed of photosynthetic bacteria (▲), *Thiopedia* sp. (●) and *Thiopedia* sp. containing intracellular sulfur granules (○) versus depth. Data for graph A were obtained on 7/4/71, and for graph B on 6/26/71.

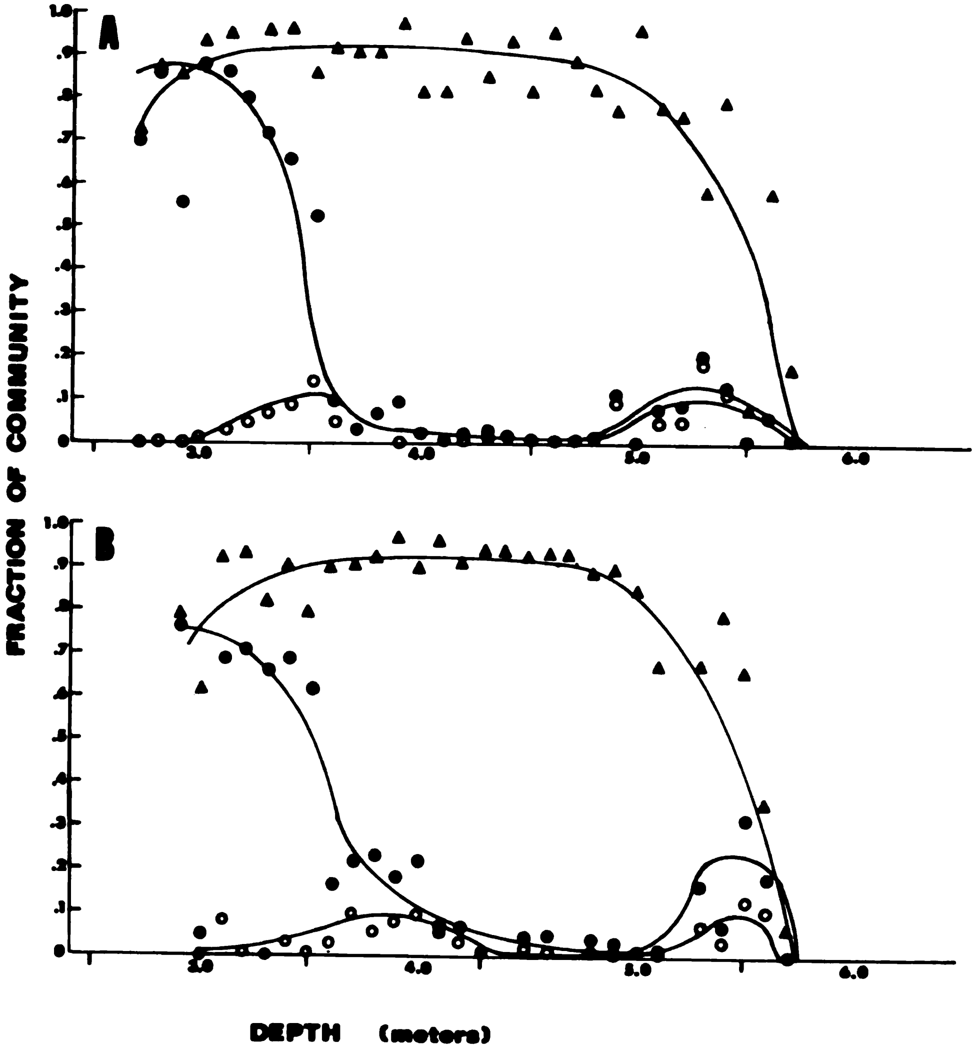
A
B
FRACTION OF COMMUNITY
DEPTH (meters)
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
3.0
4.0
5.0
6.0

Figure 3

Plate 1 Burke Lake hypolimnetic water samples (phase micrographs). All cells were centrifuged to remove gas vacuoles. Bars represent 1.0 μm.

Figure 4. 8.5 m, Community A. Cells exceed 2 μm in diameter, have either gas vacuoles or flagella (but not both) and are purple sulfur bacteria with intracytoplasmic vesicles or membranes. Shown are *Thiospirillum* (1), *Chromatium* (2), and *Thiocystis* (3).

Figure 5. 9.0 m, Community B. Cells are less than 2 μm in diameter, have either gas vacuoles or flagella (but not both) and are green sulfur bacteria with peripheral vesicles. Shown is *Chlorochromatium aggregatum* (4).

Figure 6. 9.5 m, Community C. Cells are less than 2 μm in diameter, have gas vacuoles and are colorless bacteria. Shown are morphological types T3(5), T5(6), and T6(7).

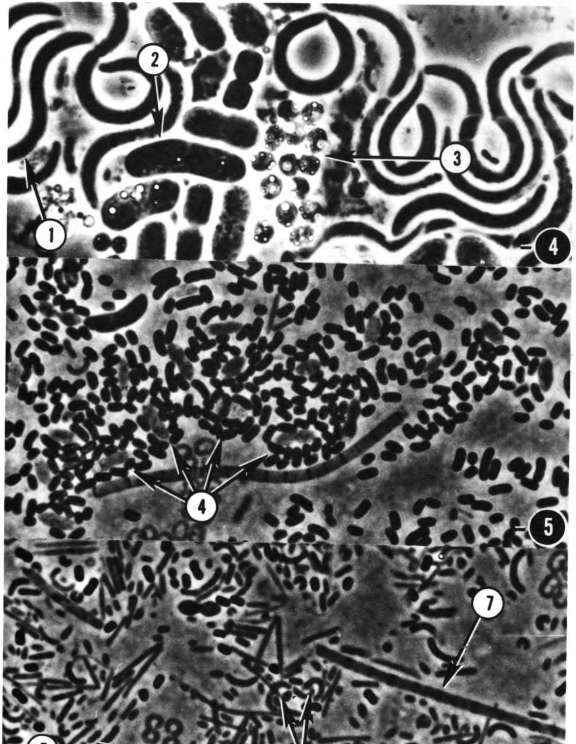

Plate 1

Plate 2. Wintergreen Lake hypolimnetic water samples (phase micrographs). All cells were centrifuged to remove gas vacuoles. Bars represent 1.0 μm.

Figure 7. 3.0 m, Community A. Cells exceed 2 μm in diameter, have either gas vacuoles or flagella (but not both) and are purple sulfur bacteria. Shown is *Thiopedia* (1).

Figure 8. 4.3 m, Community B. Cells are less than 2 μm in diameter, have either gas vacuoles or flagella (but not both) and are green sulfur bacteria. Shown is *Clathrochloris*.

Figure 9. 5.7 m, Community C. Cells are less than 2 μm in diameter, have gas vacuoles and are colorless bacteria. Shown are the morphological types T4(4), T5(3) and T6(2).

Plate 2

vacuoles collapsed and the organisms formed a pellet with green (community B), white (community C) and red (community A) layers occurring sequentially from top to bottom.

The occurrence of the first community below the thermocline (community A) has been thoroughly documented by Genovese (11), although he did not describe the organisms which caused the presence of the "red water" which he observed. These organisms were 2 µm in diameter or larger, purple sulfur bacteria, and possessed internal vesicles or lamellae. Although the A community may have been depressed well below the thermocline it was always the first community of anaerobic bacteria. In Burke Lake (Figures 2 and 4) this layer was numerically dominated by *Thiospirillum* (8.5 m, 50% of the population), *Chromatium* (8.6 m, 4%), and *Thiocystis* (8.7 to 9.0 m, 40%). In Wintergreen Lake (Figures 2 and 7), *Thiopedia* (2.6 to 3.5 m, 90%) was the dominant organism, while in Cassidy Lake both *Thiopedia* and *Thiocystis* were present. The fact that all of the organisms present in community A are anaerobes, have unusually low surface to volume ratios (compared to other bacteria), possess internal chromaphores, and are the prevalent organisms in shallow ponds, suggests that O_2 tolerance is a factor which gives the A community an advantage at high light intensities where traces of oxygen occur. Oxygen tolerance has also been demonstrated using pure cultures of *Chromatium* (14). In contrast, the characteristics of members of community B (described below) make them more susceptible to the oxidation of bacteriochlorophyll.

Genovese (11) observed that the A community occurred where quantities of HS^- ranged from 0.78 mg/l to 49.5 mg/l while Vetter (27) found *Thiopedia* within a HS^- range of 0.5-0.9 mg/l. In our study the A community occurred in habitats containing 0-2 mg/l HS^-. Schegg (22) found that O_2 concentrations of less than 1 mg/l were required for the formation of the layer of *Thiopedia* which occurs in the Rotsee while Vetter found that *Thiopedia* occurred if O_2 concentrations were less than 2.5 mg/l (28). Genovese (11) states that no detectable O_2 was present in the "red layer" of Lake Faro. We have similarly found that the A community occurred where there was less than 1 mg/l O_2.

In Wintergreen Lake the morphology of *Thiopedia* changed dramatically as a function of depth (Figure 3). At 3.0 m the bacterial community consisted of 80 to 90% *Thiopedia* sp. and all cells were virtually free of intracellular sulfur (S^o). However, at 3.5 m the photosynthetic portion of the community increased while the numbers of *Thiopedia* declined and the intracellular accumulations of sulfur began to appear until at 4 m 50% of the *Thiopedia* cells contained sulfur granules. At this depth 4-8 mg/l of sulfide was present and no oxygen was detected. Below this depth 80-100% of the cells of *Thiopedia* observed contained sulfur granules. At 5.0 m a second peak of *Thiopedia* occurred. This peak corresponded to a decrease in sulfide to 2-3 mg/l. Although this peak was much smaller than the one at 3 m, it was reproducible (Figure 3A,B). Possibly, sulfide toxicity is also an important factor in the natural distribution of *Thiopedia* along with oxygen and light. This effect of sulfide toxicity has been demonstrated previously in enrichments of purple and green sulfur bacteria (26). Thus, the accumulation of internal granules of sulfur in not a reliable taxonomic character but rather may be a response to excess sulfide.

The B community was contiguous with communities A and C. B community organisms were 2 µm or smaller, contained peripheral (chlorobium) vesicles, were green sulfur bacteria, contained bacteriochlorophyll d and did not deposit S^o internally. *Chlorochromatium* (8.8-9.0 m, 10% of the community as aggregates with 15-25 cells per aggregate) was the dominant representative of the B community in Burke Lake (Figures 2 and 5), while *Clathrochloris* (3.6-5.0 m, 95%) was dominant in Wintergreen Lake (Figures 2 and 8). The small cell size and peripheral location of vesicles characteristic of the B community could explain its apparently greater sensitivity to oxygen. On the other hand, the peripheral location of vesicles (in the case of *Prosthecochloris* the vesicles are also located in the prosthecae) and the high surface to volume ratio could result in more efficient light utilization. This would give community B a competitive advantage over community A in deeper zones with lower light intensities. Thus, community A occurs in shallow ponds and the upper hypolimnion where light is more abundant, traces of oxygen are present (less than 1 mg/l) and sulfide is low (less than 2 mg/l) while community B occurs in the lower portion of the hypolimnion where light is limiting, oxygen is undetectable, and sulfide is high (greater than 2 mg/l).

It should be recognized that the range of concentrations of sulfide, oxygen and light in which each community can be found is important only as a preliminary observation. In the hypolimnion the relative effect of each factor cannot be determined since light, oxygen and sulfide vary simultaneously. The actual relationship between light, oxygen, sulfide, growth and competition could be determined by using artificial gradients (5,6) which hold all but one (per dimension) of the independent variables constant.

The C community was contiguous with community B and the sediments. Cells were 2 μm in diameter or smaller, contained gas vacuoles, were colorless and did not deposite internal S^o. This community of bacteria (Figures 2, 6 and 9) has been overlooked previously because of its close proximity to the sediments. It occurs in a layer generally 0.1 to 0.7 m thick immediately above the sediments. The most notable characteristics are the absence of detectable concentrations of bacteriochlorophyll (except, in some cases, chlorophylls derived from sedimenting cells from upper layers) and the presence of gas vacuoles.

The distribution of sulfur-oxidizing bacteria in the littoral zone

In a study of Quarry Spring (6/21/74) which feeds Burke Lake, the number of fluorescent pseudomonads was determined at three locations between the source of the spring and the lake. The spring feeds a small pool containing *Chara*, flows across the gravel floor of the quarry, and passes into a second pool (also containing *Chara*) before entering the lake. The flow rate was 2 l/sec. Between the first and second pool, the sand and gravel on the bed of the stream was covered by macroscopic masses of periphytic diatoms (pennate and centric).

The spring and its stream were located entirely within the quarry, thus reducing the possibility of contamination by fecal and soil bacteria. The spring was sampled at its source, at the outlet of the first pool, and at the outlet of the second pool. Samples of diatoms between the pools and *Chara* from the second pool were also taken. At the mouth of the spring (9 C) there were 8.7 ± 1.7[a] fluorescent colony forming units per milliliter. At the outlet of the first pool (17 C) there were 86.5 ± 9.5,[a] and at the outlet of the second pool (18 C) there were 102 ± 17.[a] Virtually all of the colonies formed were fluorescent although the medium was differential rather than selective (Chapter II, Table 1). There were 50,800 ± 24,000[a] fluorescent colonies formed per gram dry weight in the sample of periphytic diatoms and 34,200 ± 19,000[a] per gram dry weight of *Chara*. The number of non-fluorescent colonies found in both the planktonic and the plant samples was insignificant (<2%). This suggests that fluorescent pseudomonads were indigenous, numerically dominant, and associated with aquatic plants and microalgae in this environment.

In a recent survey of 17 pond and lake samples from southwest Greenland (9), *Thiobacillus thioparus* and *Thiobacillus neapolitans* were either absent or present in low numbers. In the study of thirteen samples shown in Table 2, the same result was obtained despite the presence of sulfide and elemental sulfur in many of the samples.

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[a] 95% confidence interval based on 5 duplicate samples.

Electron microscopic observation of thin sections (Chapter IV, Figures 6 and 7) of littoral plant materials shows that heterotrophs, *Thiothrix*, and *Thiocystis* may be members of the littoral community. *Thiothrix* attached to plants using its holdfast and *Thiocystis* attached using its mucilage. The presence of fluorescent pseudomonads as microcolonies on plant surfaces was noted in Chapter II.

The distribution of sulfur-oxidizing bacteria in a sulfur-spring

A sulfur spring ("Flowing Well" John Pennekamp Coral Reef State Park) was observed qualitatively from 5/11/73 to 5/14/73. The source of the spring was covered with filaments of *Thiothrix*. However, filamentous algae were also found in abundance and may have served as a source of organic substrates. Examination of the sandy soil surrounding the spring revealed three layers of photosynthetic organisms including a filamentous alga beneath which two layers of *Thiocystis* were present. Although *Thiocystis* occurred among particles of silica, the overlying algae may have provided organic substrates.

A comparison of the sulfur spring, littoral zone, and hypolimnion

Comparison of the sulfur spring, the littoral zone, and the hypolimnion revealed that aquatic habitats containing reduced sulfur compounds, oxygen, and organic substrates favored the proliferation of fluorescent pseudomonads, *Thiothrix* or *Chromatiaceae*. The structure of the community depended on the proportion and concentration of each of these substrates, the form of reduced sulfur, the availability of a substratum for attachment and subsequent formation of a micro-environment modified by bacterial exudates.

In the hypolimnion, each bacterial population appeared to maintain its position along continuous gradients of increasing sulfide and decreasing oxygen concentration with increasing depth. Where sulfide and oxygen existed simultaneously, the large (greater than 2 μm) planktonic *Chromatiaceae* predominated to the exclusion of all other bacteria while at lower depths where oxygen was depleted the *Chlorobiaceae* were dominant.

In the littoral zone, wave action constantly replenished the supply of oxygen despite the decomposition of plant debris. As a result, even sequestered crevices on the plant surfaces failed to support the growth of *Chlorobiaceae* and, instead, the oxygen tolerant *Chromatiaceae* predominated as in the upper regions of the hypolimnion and the sulfur spring. However, in addition to the *Chromatiaceae* several other groups of bacteria were present in the littoral zone, including *Thiothrix* and *Pseudomonas*. These organisms are characteristic of aerobic sulfide containing environments.

The niche of several bacterial groups is shown in Figure 10. The niches shown represent that portion of the habitat hypervolume (29) in which each group of organisms successfully competed. The niches are tentative and incomplete in that only three physicochemical factors, sulfide concentration, oxygen concentration, and the product of substratum availability and washout rate, are considered. However, the figure summarizes the results discussed above and also provides a conceptual perspective for future studies of sulfur-oxidizing bacteria.

Figure 10. The relative position of sulfur-oxidizing bacterial communities within the HS^-, O_2, and substrata x washout hypervolume (29). Each of the three ranges (1,2,3) shown represents a range of physicochemical conditions affecting bacterial competition.

Range 1 represents the potential conditions found in anaerobic hypolimnia where no O_2 is present and HS^- varies. There is no substratum and no washout. The B and C communities are found here.

Range 2 represents the potential conditions found in the upper region of anaerobic hypolimnia where O_2 and HS^- vary but HS^- does not reach the high level sometimes found in sulfur springs or lower hypolimnia. There is no substratum and little washout. The A community is found here.

Range 3 represents the potential conditions found in sulfur springs, the littoral zone, and small ponds where HS^- varies widely, as do the washout and availability of substrata; however, O_2 is higher here than in the anaerobic hypolimnia. Fluorescent pseudomonads, *Thiothrix* sp. and *Chromatiaceae* are found here.

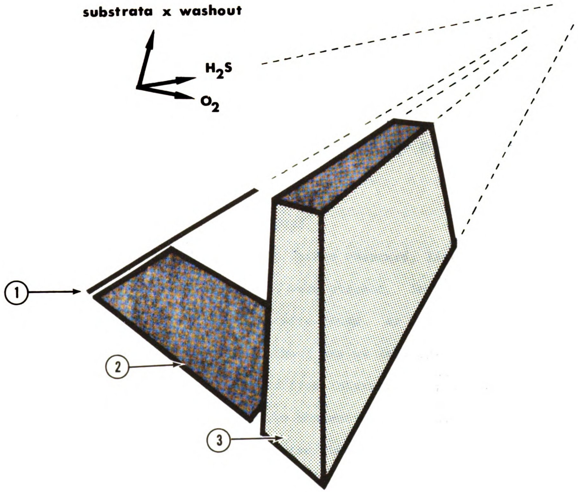

Figure 10

From qualitative observations, it appears that in stagnant waters (i.e., hypolimnia), the availability of substrata is not an important factor. However, in flowing waters substrata permit colonization by periphytic bacteria which would otherwise be washed from the system despite the existence of a favorable chemical environment. Qualitative observations show that periphytic sulfur-oxidizing bacteria occur in the littoral zone and springs while planktonic bacteria occur in the hypolimnion. For this reason the product of substratum availability and washout rate was included as a physicochemical factor although it was not measured nor the units defined.

CONCLUSIONS

In all of the sulfide containing waters observed, light was available and photosynthetic bacteria were present. In hypolimnia, flagellate or gas-vacuolate bacteria were found. As light decreased and sulfide increased (with depth), there was an increase in the surface to volume ratio, a transition from internal to peripheral vesicles, a decrease in the carotenoid content of the cells, and an increase in bacteriochlorophyll d. These changes in bacteria ecotype were due to changes in community structure rather than the phenotypic adaptation of individual populations. The correlation of these biochemical and morphological transitions with physicochemical gradients of sulfide, oxygen, and light is adequately explained on the basis of light requirements as well as sulfide and oxygen toxicity.

In the littoral zone and the sulfur spring, the sulfur-oxidizing bacteria were attached to surfaces either by a holdfast or by mucilage which encased microcolonies. Only the oxygen tolerant or aerobic sulfur-oxidizing bacteria were found in the littoral zone and sulfur spring. Photosynthetic and heterotrophic organisms were found but members of the genus *Thiobacillus* were not. *In situ* enrichments using thiosulfate gradients selected fluorescent pseudomonads rather than thiobacilli and resulted in a three-fold increase in growth rate. Isolate TBT-H and *Pseudomonas aeruginosa* were found to excrete a fluorescent sulfide binding compound which resulted in the formation of sulfur (S^o) globules. However, the metabolic role of sulfide in fluorescent pseudomonads was not determined.

The statements of Vishniac (28) and Rittenburg (19) suggesting the importance of heterotrophic and/or mixotrophic bacteria in sulfur oxidation are supported by the results obtained here. Although there have been numerous studies on the mixotrophic capabilities of autotrophic thiobacilli (28,19), the present study indicates that the mixotrophic capabilities of fluorescent pseudomonads should also be investigated. The excretion of a sulfide binding or oxidizing compound(s) would allow aerobic bacterial respiration in sulfide containing waters. Despite the extracellular oxidation of sulfide, energy could still be available for metabolism since the subsequent intracellular oxidation of sulfite or elemental sulfur to sulfate can result in the production of ADP from AMP (16). Thus sulfide might be detoxified by incomplete oxidation extracellularly and then further oxidized intracellularly. This would explain the excretion of a sulfide binding compound(s), the presence of elemental sulfur extracolonially but not intracolonially or intracellularly, and a three-fold increase in growth rate caused by gradients of thiosulfate. However, further physiological studies, both in the laboratory and *in situ*, will be required before the role of fluorescent pseudomonads in the biogeochemistry of sulfur compounds can be elucidated.

The concepts of habitat and niche as defined by Whittaker (29) rely on the gradient concept as discussed in Chapter I. The niche may be determined either by correlating physicochemical conditions with successful competition or by observing the distribution of organisms along environmental gradients. The distributional data summarized in Figure 10 were collected in this way. Figure 10 is thus a conceptual model of the niche hypervolume for various sulfur-oxidizing bacteria. However, using a soil inoculum and artificial two-dimensional gradients, the hypervolume could be determined experimentally. The application of gradient methods to correct and expand the results shown in Figure 10 may thus be the next step in the ecology of sulfur-oxidizing bacteria.

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