N85-32746

Microbial Communities and Microprofiles of Sulfide and Oxygen of Alum Rock Sulfur Springs

U. Fischer

Introduction

Microbial Community of Alum Rock Spring Field Site

The microbial community of Alum Rock sulfur spring site 3 was studied along one branch of the main stream and between the two branches, 150 cm distant from the source. The community at the source (sample J) was dominated by green sulfur photosynthetic bacteria of the genus *Chlorobium*. At 15-35 cm from the source (samples I and H) dominance in the community shifted to the genus *Flexibacter* at the surface of the mat and purple sulfur bacteria of the genus *Chromatium* underneath. At 50-80 cm (samples G and F) colorless sulfur-oxidizing bacteria of the genus *Thiothrix* began to appear. At 100 to 150 cm (samples D and E), the surface of the mat was still dominated by *Flexibacter* but underneath dominance shifted to purple sulfur bacteria as above, as well as cyanobacteria of the genus *Oscillatoria* and *Pseudonabaena*. The measurements of temperature along the stream showed no significant gradient. We believe that community variations are controlled more by sulfide than temperature. The temperature along the stream was 29°C at positions J and I, and 28°C at positions E and D. At position L, which was shaded, the temperature decreased to 19°C.

Ten ml of the overlying water were taken at position G and fixed immediately with 20 ml of 2 percent Zn-acetate to determine the sulfide concentration by the methylene blue method. A sulfide concentration of 106 uM was calculated for the overlying water.

Isolation of Cyanobacteria

Samples from positions E and L of the Alum Rock sulfur spring site 3 were taken to isolate cyanobacteria. The samples were placed on agar plates (containing the standard mineral medium BG 11 and 2 percent agar) and incubated at 27°C at a light intensity of 15-20 $uE\ m^{-2}\ sec^{-1}$. After 3 days single cyanobacterial colonies were transferred onto fresh plates and the microorganisms were studied under the microscope. This procedure was repeated several times, until only a single cyanobactrium species was detected by microscopic observations. From position E an *Oscillatoria* species, 5 um in width, was isolated (with some heterotrophic bacteria).

The *Oscillatoria* sp. was transferred from the plates to 10 ml of liquid BG 11 medium and grown for 5 days under the same conditions as described above for the plates. Then 5 ml of this preculture were transferred to 100 ml of liquid BG 11 medium and allowed to grow for 5 days. Some drops of this culture suspension were mounted on an agar slide as a preparation for photmicrographs using a Zeiss

Photomicroscope II. The filamentous cyanobacterium *Oscillatoria* is illustrated in Figure IV-19a. To determine whether the organism contains phycocyanin, an epifluorescence microscope with a 550 nm interference filter for the excitation light and a cut-off filter at 680 nm for the emitted light was used. The red color in the cyanobacterium is due primarily to excitation of phycocyanin. *Synechocystis* did not grow in a liquid medium. For photomicrographs, some colonies of *Synechocystis* were taken directly from the agar plate and mounted on an agar slide. The morphology is shown in Figure IV-19b. The presence of phycocyanin in *Synechocystis* as detected by its red color is also demonstrated by the use of an epifluorescence microscope (Figure IV-19d).

Materials and Methods

Culture Medium for Cyanobacteria

For the cultivation of cyanobacteria, the mineral medium, BG 11, described by Rippka et al., (1979) was used. Distilled water was replaced by Alum Rock spring water.

BG 11 medium (1 liter)

$NaNO_3$: 1.5 g
$K_2HPO_4.3H_2O$: 0.04 g
$MgSO_4.7H_2O$: 0.075 g
$CaCl_2.2H_2O$: 0.036 g
Citric acid: 0.006 g
Ferric ammonium citrate: 0.006 g
EDTA (disodium magnesium salt): 0.001 g
Na_2CO_3: 0.02 g
Trace element solution: 1 ml
Alum Rock Spring water: 1000 ml

After autoclaving and cooling, the medium had a pH of 7.4. For aerobic growth of cyanobacteria a 250 ml Erlenmeyer flask containing 100 ml of medium was used.

For the enrichment and isolation of cyanobacteria on solid media, 20 g of agar were added to the BG 11 medium.

Composition of Trace Element Solution
(Rippka et al., 1979)

Ingredient	Amount (g/l distilled water)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.390
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.0494

Figure IV-19. (A) *Oscillatoria sp.* isolated from Alum Rock spring, 7 mm = 10 μm; (B) *Synechocystis sp.* isolated from Alum Rock spring. 4 mm = 10μm.

Anaerobic Growth of Cyanobacteria in the Presence of Sulfide

Anaerobic growth in the presence of sulfide of isolated cyanobacterial strains from Alum Rock sulfur springs was achieved in 8 ml screw-cap test tubes. The tubes contained the standard BG 11 medium described above and, in addition, different concentrations of sulfide. Each of the tubes was inoculated with 1.5 ml (about 20 percent) of an anaerobically grown liquid culture.

Preparation of a Sulfide Stock Solution (6.25 mM)
(Modified After Pfennig and Trueper, 1981)

$Na_2S.9H_2O$	0.75 g
Na_2CO_3	0.5 g
Distilled water	50 ml

The solution was autoclaved and after cooling was partially neutralized with 2 ml of a sterile 2M H_2SO_4 solution.
(The carbonate was added to increase the growth yield of the cyanobacteria.)

Culture Medium for *Thiothrix*

For the enrichment and isolation of *Thiothrix* species of Alum Rock spring on agar plates, I used the following medium (Wiessner, 1981):

Per 1 liter:

NH_4Cl	50 mg
K_2HPO_4	100 mg
$CaSO_4.2H_2O$	2 mg
$MgSO_4.7H_2O$	10 mg
$ZnSO_4.7H_2O$	0.1 mg
$MnSO_4.4H_2O$	0.02 mg
H_3BO_3	0.1 mg
$Co(NO_3)_2$	0.01 mg
$NaMoO_4.2H_2O$	0.01 mg
$CuSO_4.5H_2O$	0.0005 mg
$FeSO_4.7H_2O$	7 mg
EDTA (Na_2-salt)	9.2 mg
Na-acetate	10 mg
$Na_2S.9H_2O$	300 mg
agar	12.5 g

Adjust the pH of the medium to about 7.0
Important: $FeSO_4$ and EDTA must be mixed separately and added to the medium after a short period of boiling.

Culture Medium for *Flexibacterium*.

For the enrichment and isolation of *Flexibacteria* species of Alum Rock sulfur springs only agar plates were prepared. The medium used was Number 27; Vy/2 agar medium (Reichenbach and Dworkin, 1981).

The medium contained per 100 ml:

Baker's yeast	0.5 g
$CaCl_2$	0.1 g
Agar	1.5 g
Cyanocobalamine	50 ug

pH is adjusted to 7.2

Absorption Spectra of Cyanobacterial Chlorophyll

Absorption spectra were determined in a Varian Techtron double beam spectrophotometer model 635, connected to a recorder.

In vivo spectra were obtained by suspending cell material in 50 percent sucrose to avoid settling of whole cells in the cuvette. *In vitro* spectra of chlorophylls were obtained by extracting wet cell material either in 100 per cent methanol or acetone. The cell material was harvested by centrifugation (12,000 rpm for 10 minutes) in a Sorvall RC2-B. To the pellet, 5 ml of methanol or acetone were added and then allowed to stand in the dark at 4°C for 10 minutes before the suspension was centrifuged the same way a second time. The supernatant was used to determine the absorption spectra of extracted pigments. All spectra were recorded in the range of 750 nm to 350 nm.

Field Experiments with Oxygen and Sulfide Microelectrodes

To study the microprofile of oxygen and sulfide in an Alum Rock sulfur spring (see profiles of salt ponds and marsh site), handmade microelectrodes (Fig. IV-2) were attached to a micromanipulator which was held in place on a stand. Profiles were obtained with microelectrodes from the overlying water and from the microbial mats, with measurements taken at 100 um or 250 um increments. In addition, in the overlying water, sulfide concentration was determined by the methylene blue method (below) and oxygen was determined using the method of Winkler (below).

Sulfide Determination by the Methylene Blue Method

Determination of sulfide used the method of Pachmayr (1960) as modified by Trueper and Schlegel (1964).

The assay was done in 100 ml volumetric flasks which contained:

10 ml Alum Rock spring water
20 ml 2 per cent Zn-acetate[a]
10 ml DMPD-solution[b]
0.5 ml FAS-solution[c]

[a,b,c]: for preparation, see below

This reaction mixture was shaken vigorously and allowed to stand for 10 minutes at room temperature. The flask was then filled up to 100 ml with distilled water. The absorption was measured at 670 nm against a blank without Alum Rock spring water.

Preparation of (a) Zn-acetate, (b) DMPD and (c) FAS solutions:

a) Zn-acetate: 20 g Zn-acetate were dissolved in 1000 ml distilled water and 1-2 drops of acetic acid were added.

b) DMPD: 2 g dimethyl-p-phenylene-diamine chloride were suspended in 200 ml distilled water. Then 200 ml of concentrated H_2SO_4 were carefully added. Distilled water was added to make a 1 liter solution which was stored in a 1000 ml volumetric flask wrapped with aluminum foil.

c) FAS: 50 g $NH_4Fe(SO_4)_2.12H_2O$ were dissolved in 100 ml distilled water by adding 10 ml concentrated H_2SO_4. The solution was then filled up to 500 ml with distilled water and kept in a 500 ml volumetric flask wrapped with aluminum foil.

Results

Isolation of *Thiothrix* and *Flexibacter*

No attempt to enrich or isolate *Thiothrix* or *Flexibacter* from Alum Roc sulfur spring on agar plates was successful.

Absorbance Spectra of Isolated Cyanobacteria

To determine the composition of the pigments from the isolated cyanobacteria strains, absorbance spectra were taken from whole cells or extracted pigment preparations. Three major groups of pigments are normally present in cyanobacteria: chlorophyll a, biliproteins, and carotenoids. The *in vivo* spectrum (Fig. IV-20) of the isolated *Oscillatoria* strain shows maxima at 680 nm and 435 nm, indicating chlorophyll a (for isolated *Synechocystis*: 685 nm and 445 nm, Fig. IV-21), at 632 nm indicating phycocyanin (for *Synechocystis* at 632 nm), and at 488 nm indicating carotenoids (for *Synechocystis* at 500 nm).

When the pigments of *Oscillatoria* and *Synechocystis* were extracted by methanol or acetone, the absorption maxima were more distinct and were shifted towards shorter wavelengths. This is

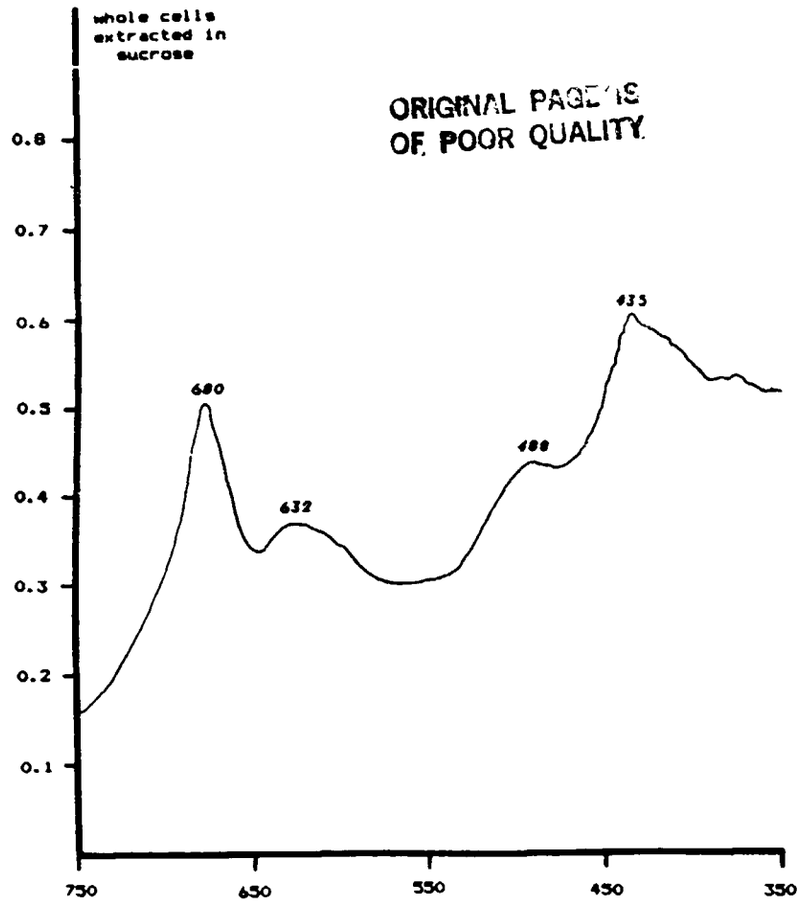

Figure IV-20. Absorbance spectrum of *Oscillatoria*.

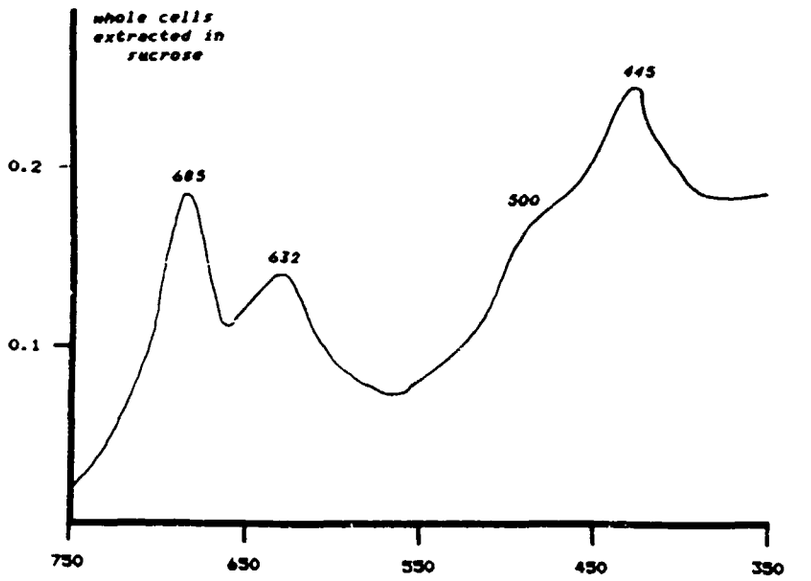

Figure IV-21. Absorbance spectrum of *Synechocystis*.

illustrated in Figures IV-22 and 24 for *Oscillatoria* and for *Synechocystis* in Figures IV-23 and 25.

Anaerobic Growth of *Oscillatoria* in the Presence of Sulfide

Some cyanobacteria perform oxygenic or anoxygenic photosynthesis in the presence of sulfide. Cyanobacteria of Alum Rock sulfur springs may be able to carry out photosynthesis when sulfide is present. Attempts were made to determine how isolated *Oscillatoria* grow under anaerobic conditions with different sulfide concentrations. The anaerobic growth experiment was carried out as described in the Methods section. The sulfide concentration ranged from 0 to 4 mM. The control contained no sulfide. For aerobic growth conditions, one screw-cap test tube contained only 1.5 ml of the cell suspension and 5 ml of the BG 11 medium. The inoculated 8 ml screw cap test tubes were incubated for 5 days at 27°C and 15-20 $\mu E\ m^{-2}\ sec^{-1}$. The result is shown in Table IV-7 (see end of preceding subchapter).

During the 5 days after the inoculation period the generation of a gas, probably oxygen, was observed in the culture tubes containing 0 to 0.05 mM sulfide. Under these growth conditions, *Oscillatoria* had a dark green color and showed very good growth at the bottom of the culture tubes. At higher sulfide concentrations (from 0.1 to 1 mM) the color of the culture was more or less light green and the organisms formed a thin layer from the bottom to the surface. The culture exposed to 4 mM sulfide did not grow, sank down to the bottom of the tube, and showed a yellow-brownish color two days after inoculation. Whether this *Oscillatoria* strain shows the same behaviour concerning photosynthesis found for *Oscillatoria limnetica* from Solar Lake remains to be studied.

Microprofile of an Alum Rock Sulfur Spring

The distribution of sulfide and oxygen in the overlying water and microbial mat at Alum Rock spring site 2 was measured with handmade microelectrodes. This sulfur spring was choosen for measurements because it was easy to place the tripod with the micromanioulator and microelectrodes directly in front of the spring. Since the mat of the spring was growing on a vertical rock substrate, it was necessary to insert the microelectrodes more or less horizontally into the mat. Three profiles of oxygen and sulfide were taken across the spring, 5-10 cm down from the top of the source. The main stream in the middle of the spring had a white color and the community was comparable to that of spring site 3 at positions H and J. The borders on both sides of the main stream had a dark green color and the community was nearly the same as that described for positions C and D of the spring site 3. In the overlying water the sulfide concentration was determined by the methylene blue method (see Methods section above) along the stream. From Figure IV-26 it can be seen that there is a decrease in sulfide concentration from the top, at the source, to the bottom. Light intensity decreases from 60 $\mu E\ m^{-2}\ sec^{-1}$ at the top to 50 $\mu E\ m^{-2}\ sec^{-1}$ at the bottom. From the top to the ground there is a

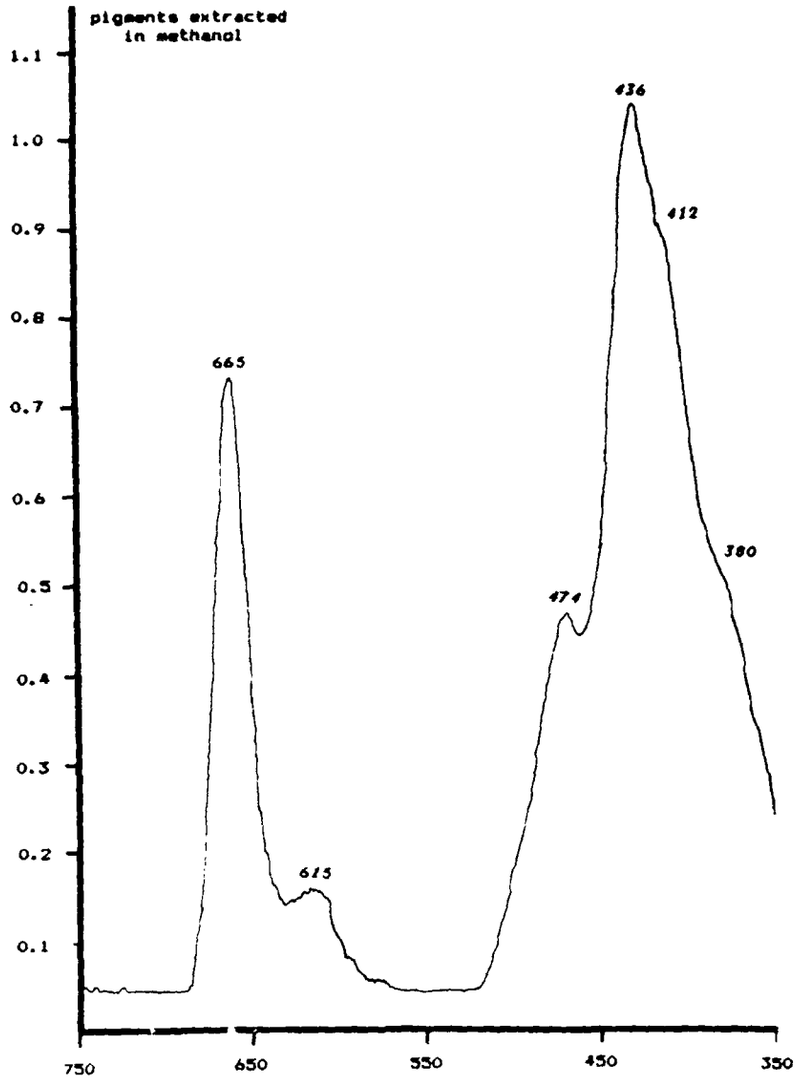

Figure IV-22. Absorbance spectrum of *Oscillatoria*.

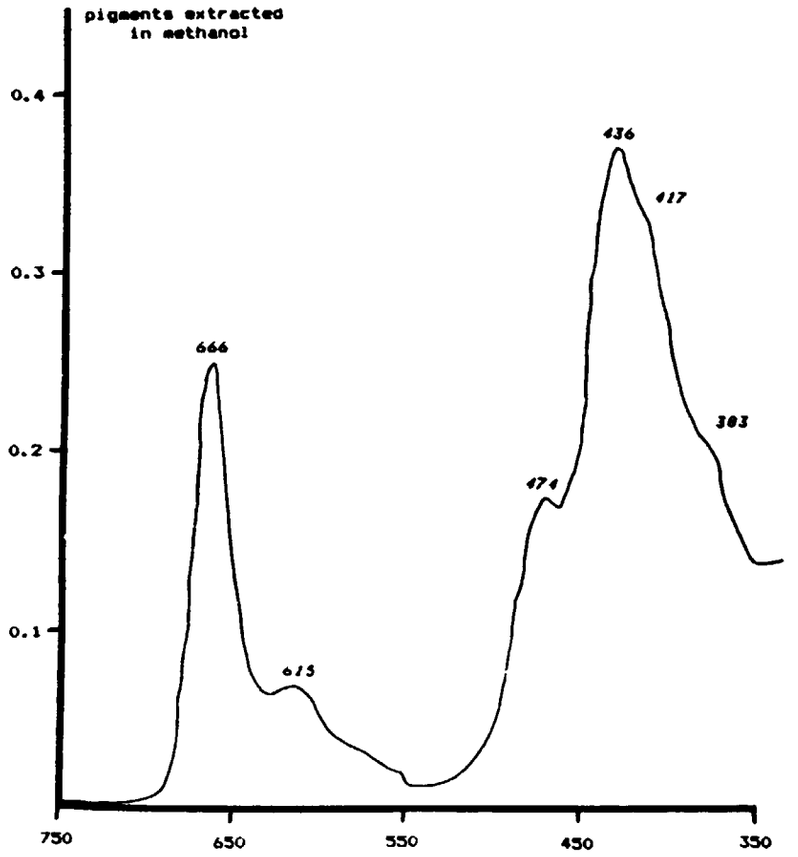

Figure IV-23. Absorbance spectrum of *Synechocystis*.

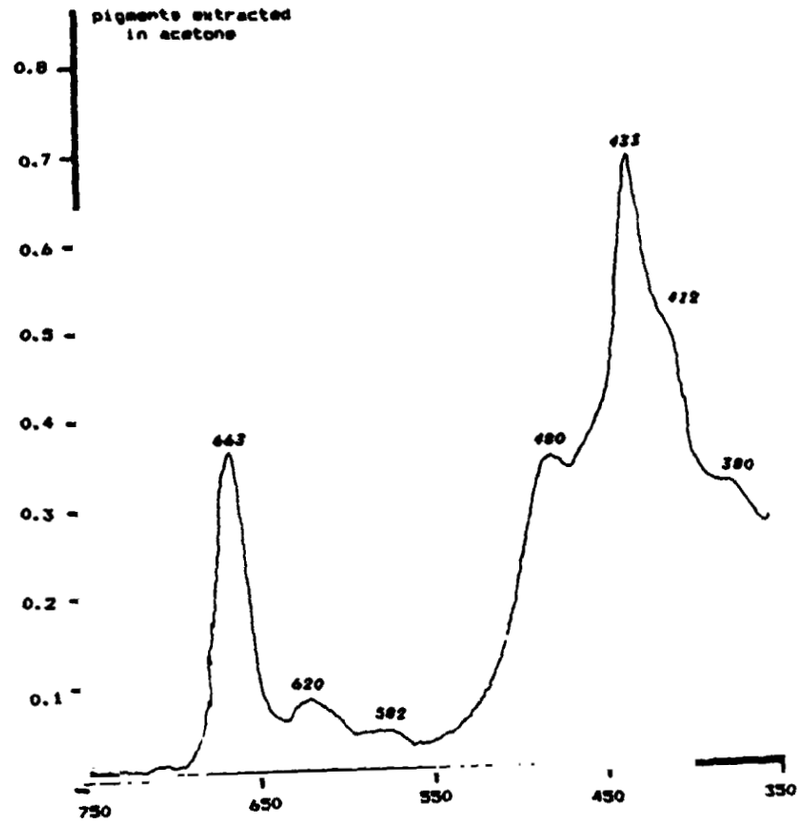

Figure IV-24. Absorbance spectrum of *Oscillatoria*.

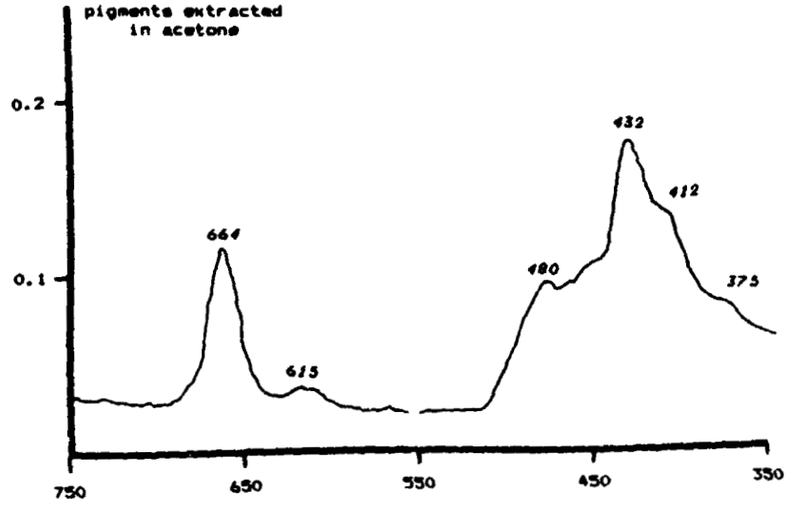

Figure IV-25. Absorbance spectrum of *Synechocystis*.

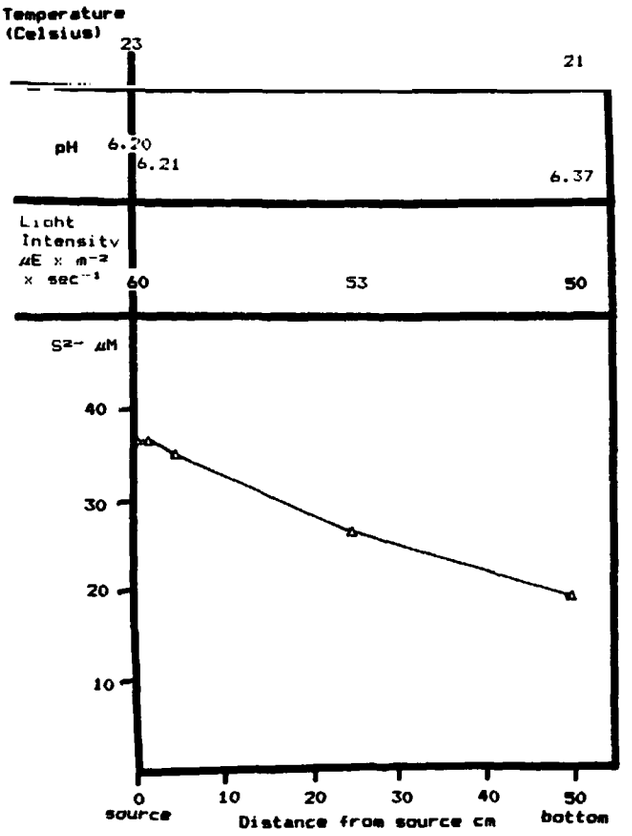

Figure IV-26. Sulfide, light intensity, pH, and temperature in the main stream of Alum Rock (at site 2). Air temperature 28° by the spring (in the shade).

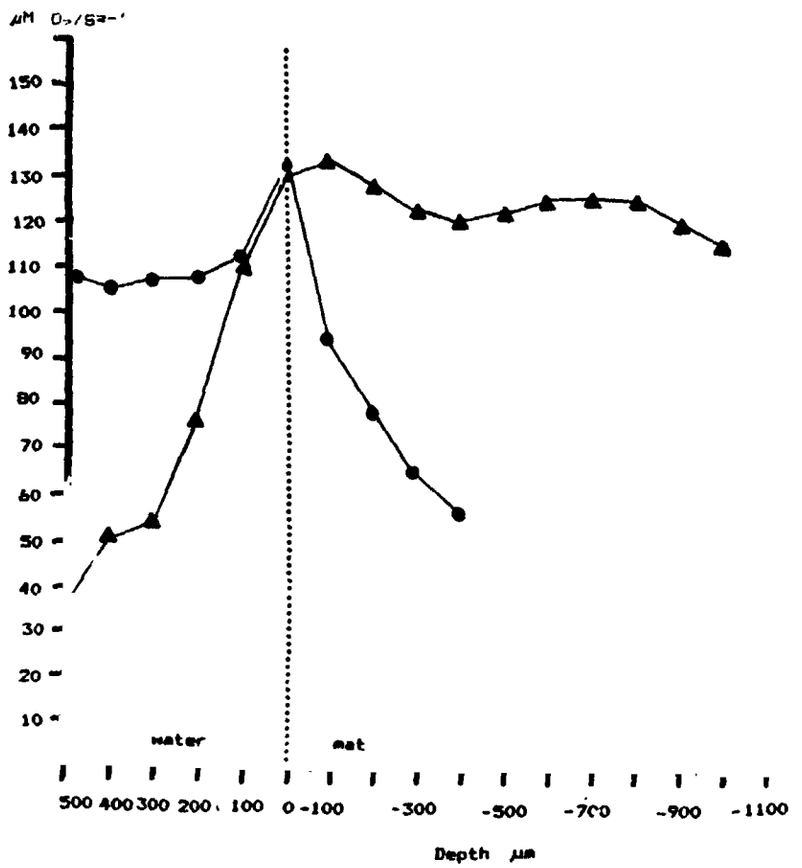

Figure IV-27. Oxygen and sulfide in the main stream and at microbial mat (white). (Alum Rock, site 2; 10 cm below top of spring).

▲ = S^{2-}; ● = O_2

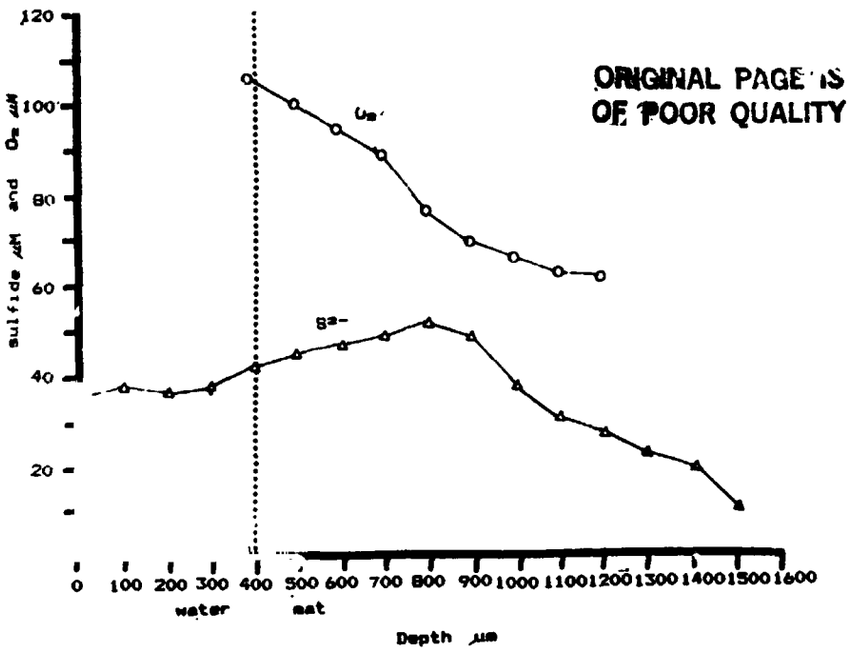

Figure IV-28. Oxygen and sulfide in the overlying water and microbial mat (border of the main stream, green). Alum Rock, site 2; 10 cm below top of spring.

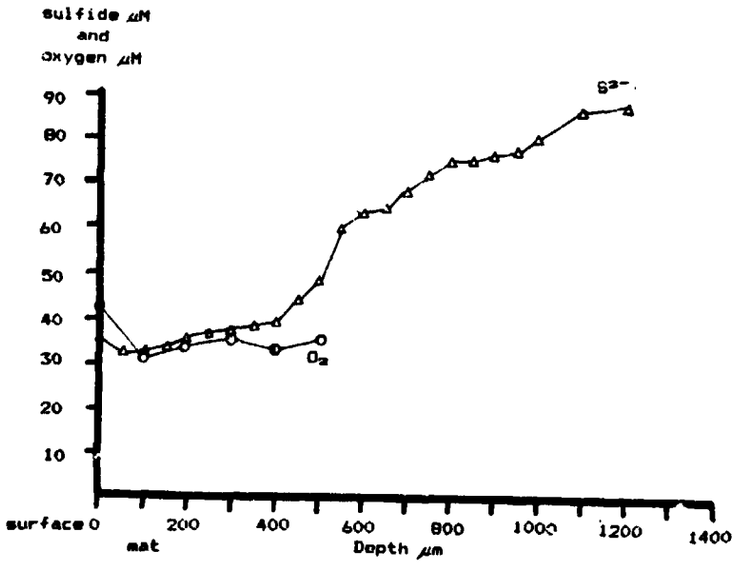

Figure IV-29. Oxygen and sulfide at a microbial mat (border of the main stream, green). Alum Rock, site 2; 5 cm below top of spring.

temperature difference of 2°C. The pH increases from 6.2 to 6.37. In the overlying water of the main stream (white color of the mat), an enormous increase in sulfide was measured with depth, while in the mat a low decrease was measurable (Figure IV-27). By contrast, the oxygen concentration decreased rapidly in the first 500 *um* of the mat. One explanation for this steep decrease in oxygen in this part of the mat is that a predominantly heterotrophic community is present, with only a small number of cyanobacteria producing oxygen during photosynthesis. The other profiles were taken at the border of the main stream, in very well developed (dark green) cyanobacterial mats (Fig. IV-28 and 29). The oxygen concentration decreases very slowly during the first 500 *um* of depth because of the oxygenic photosynthesis activity of cyanobacteria, the dominant organisms at this part of the spring. The sulfide concentration in the mat increases with depth when oxygen decreases.

References

Baumgartl H., and Lubbers, D.W., 1973. Platinum needle electrodes for polarographic measurement of oxygen and hydrogen. In *Oxygen Supply*, (M. Kessler, D.F. Bruley, L.C. Clark Jr., D.W. Lubbers, I.A. Silver, J. Strauss eds.), Urban and Schwarzenberg, Munich, pp. 130-136.

Cohen, Y., 1984. Oxygenic photosynthesis, anoxygenic photosynthesis and sulfate reduction in cyanobacterial mats. In *Perspective in Microbial Ecology*, (M. Klug, ed.), ASM publ.

Cohen, Y., Castenholz, R.W., and Halvorson, H.O. (eds.), 1984. *Microbial Mats: Stromatolites*, Alan R. Liss, N.Y.

Cohen, Y., Jørgensen, B.B., Padan, E., and Shilo, M., 1975. Sulphide-dependent anoxygenic photosynthesis in the cyanobacterium *Oscillatoria limnetica*. Nature 257:489-492.

Fahey, R.C., Brody, S., and Mikolajczyk, S.D., 1975. Changes in the glutathione thiol-disulfide status of *Neurospora crassa* conidia during germination and aging, J. Bacteriology, 12: 144-151.

Fahey, R.C., Brown, W.C., Adams, W.B., and Worsham, M.B., 1978. Occurrence of glutathione in bacteria, J. Bacteriology, 133:1126-1129.

C-4

Fahey, R.C. and Newton, G. L., 1983. Occurrence of low molecular weight thiols in biological systems. In *Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects*. (A. Larsson, et al., eds.), Raven Press, New York.

Fahey, R.C., Newton, G.L., Arrick, G., Overdank-Bogart, T., and Alev, S. B., 1984. *Entamoeba histolytica*: a eukaryote without glutathione metabolism, Science, 24:70-72.

Garlick, S., Oren, A., and Padan, E., 1977. Occurrence of facultative anoxygenic photosynthesis among filamentous and unicellular cyanobacteria, J. Bacteriology, 129:623-629.

Griffith, O.W. and Meister, A., 1979. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine), J. Biol. Chem., 254: 7558-7560.

Hartman, H. 1984. In *Microbial Mats: Stromatolites* (Y. Cohen, R.W. Castenholz, and H.O. Halvorson, eds.), Alan R. Liss, N.Y.

Ho, K K., and Krogmann, D.W., 1982. Photosynthesis in cyanobacteria. In *The Biology of Cyanobacteria*, Vol. 19, (N.G. Carr and B.G. Whitton, eds.), Blackwell Scientific Publications.

Howarth, R.W., and Marino, R., 1984. Sulfate reduction in salt marshes with some comparisons to sulfate reduction in microbial mats. In *Microbial Mats: Stromatolites*, (Y. Cohen, R.W. Castenholz and H.O. Halvorson, eds.), Alan R. Liss, N.Y., pp. 245-264.

Jørgensen, B.B., and Cohen, Y., 1977. Solar Lake (Sinai), The sulfur cycle of the benthic cyanobacterial mats, Limnol. and Oceanog. 22:657-666.

Jørgensen, B.B., Revsbech, N.P., Blackburn, T.H. and Cohen, Y., 1979. Diurnal cycle of oxygen and sulfide microgradients and microbial photosynthesis in a cyanobacterial mat sediment, Appl. Environ. Microbiol. 38:46-58.

Jørgensen, B.B., Revsbech, N.P., and Cohen, Y., 1983. Photosynthesis and structure of benthic microbial mats: microelectrode and SEM studies of four cyanobacterial communities, Limnol. Oceanog. 28:1075-1093.

Jørgensen, B.B., Revsbech, N.P., and Cohen, Y., 1985. Transition from anoxygenic to oxygenic photosynthesis in a *Microcoleus chthanoplastes* cyanobacterial mat, Limnol. Oceanogr., (in press)

Martens, C.S., and Berner, R.A., 1974. Methane production in the interstitial waters of sulfate depleted marine sediments, Science, 185:1167-1169.

Mills, G.C., 1957. Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown, J. Biol. Chem. 229:189-197.

Murray, J.W., and Gill, G., 1978. The geochemistry of iron in Puget Sound, Geochim. Cosmochim. Acta, 42:9-19

Newton, G.L. and Javor, B., 1985. Gamma-glutamylcysteine and thiosulfate are the major low molecular weight thiols in halobacteria, J. Bacteriology, (in preparation).

Pachmayr, F., 1960. Vorkommen und Bestimmung von Schwefelverbindungen in Mineralwasser. Doctoral thesis, Univ. Munich, West Germany.

Padan, E. and Cohen, Y., 1982. Anoxygenic photosynthesis. In *The Biology of Cyanobacteria*, (N. G. Carr and B. A. Whitton, eds.), Blackwell Scientific Publications, Oxford, 688 pp.

Parsons, T.R., Takahashi, M., and Hargrave, B., 1977. *Biological Oceanographic Processes*, Pergamon Press.

Pfennig, N. and Trueper, H.G., 1981. Isolation of members of the families Chromatiaceae and Chlorobiaceae. In *The Prokaryotes*. (M.P. Starr, H. Stolp, H.G. Trueper, A. Balows and H.G. Schlegel, eds.). Vol. 1, Springer Verlag, New York, pp. 279-289.

Read, L.K., Margulis, L., Stolz, J.F., Obar, R., and Sawyer, T., 1983. A new strain of *Paratetramitus jugosus* from Laguna Figueroa Baja California, Mexico, Biological Bulletin, 165:241-264.

Reichbach, H. and Dworkin, M., 1981. The order Cytophagales (with addenda on the genera *Herpetosiphon*, *Saprospira*, and *Flexithrix*). In *The Prokaryotes*. (M.P. Starr, H. Stolp, H.G. Trueper, A. Balows, and H.G. Schlegel, eds.). Vol. 1, Springer Verlag, New York, pp. 356-379.

Revsbech, N.P., Jørgensen, B., and Cohen, Y., 1983. Microelectrode studies of the photosynthesis and O_2, H_2S, and pH profiles of a microbial mat, Limnol. Oceanog., 28:1062-1074.

Revsbech, N.P., Jørgensen, B., and Brix, O., 1981. Primary production of microalgae in sediments measured by oxygen microprofile, $H^{14}CO_3^-$ fixation and oxygen exchange methods, Limnol. Oceanog. 6:717-730.

Revsbech, N.P. and Ward, D.M., 1984. Microprofiles of dissolved substances and photosynthesis in microbial mats measured with

microelectrodes. In *Microbial Mats: Stromatolites*, (Y. Cohen, R.W. Castenholz, and H.O. Halvorson, eds.), Alan R. Liss, N.Y.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., and Stanier, R.Y., 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria, J. Gen. Microbiol., 111:1-61.

Skyring, G.W., 1984. Sulfate reduction in marine sediments associated with cyanobacterial mats in Australia. In *Microbial Mats: Stromatolites*, (Y. Cohen, R.W. Castenholz, and H.O. Halvorson, eds.), Alan R. Liss, N.Y., pp. 265-276.

Stookey, L.L., 1970. Ferrozine: a new spectrophotometric reagent for iron, Anal. Chem., 42.

Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total oxidized glutathione: applications to mammalian blood and other tissues, Anal. Biochem. 27:502-522.

Trueper, H.G. and Schlegel, H.G., 1964. Sulphur metabolism in Thiorhodaceae. I. Quantitative measurements on growing cells of *Chromatium okenii*, Antonie van Leeuwenhoek J. Microbiol. Serol., 30:225-238.

Wiessner, W., 1981. The family Beggiatoaceae. In *The Prokaryotes*. (M.P. Starr, H. Stolp, H.G. Trueper, A. Balows, and H.G. Schlegel, eds.). Vol. 1, Springer Verlag, New York, pp. 380-389.