CULTIVATION EXPERIMENTS WITH ANAEROBIC ANOXYGENIC PHOTOTROPHIC IRON OXIDIZING BACTERIA

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ABSTRACT

Fresh environmental samples from an iron-rich area of School St, Woods Hole, MA, USA were cultured in growth media specific to anoxygenic photoautotrophic iron oxidizing bacteria. After 1-3 weeks aliquots were transferred to fresh media with different pHs (6, 7, 8), salinities (0, 4, 10, 30 ppt) and with an organic carbon source (malate). Samples were examined microscopically and the composition of bacteriochlorophylls was investigated spectrophotometrically in pigment extracts. A biofilm experiment with environmental samples was set up under anoxic conditions to examine effects of substratum heterogeneity on cell densities and compare growth on artificial medium to growth on filtrate from School St.

Cultivation of anoxygenic photoautotrophic iron oxidizers succeeded as indicated by massive production of oxidized iron after few weeks and presence of high concentrations of bacteriochlorophyll *a* in certain enrichments. Phototrophic iron oxidation was sensitive to pH and salinity changes. Growth only occurred at pH 7 and a salinity of 0 ppt. The most abundant microbe closely resembled the iron-oxidizing *Thiodictyon* strain previously isolated from School St. Malate addition resulted in black precipitates, dark purple coloration of the medium and less precipitation of oxidized iron. High concentrations of bacteriochlorophyll *a* were present. The dominant microbe morphologically resembled known *Chromatium* spp. The biofilm experiments revealed increasing microbial densities with increasing substratum heterogeneity, as well as much higher densities of microbes when grown on 'natural' medium compared to artificial growth medium.

INTRODUCTION

Anaerobic anoxygenic iron oxidation by phototrophic bacteria was discovered by Widdel et al. (1993) in enrichments of freshwater pond sediments. The existence of these microbes and their importance in the early Earth had been speculated previously (Hartman 1984) due to the existence of massive iron deposits in Archaean rock formations dating as far back as ~3.8 Ga (Walker et al. 1983). The fact that iron occurs in both reduced and oxidized form in these deposits, also known as 'banded iron formations' (BIF's), had provided a puzzle to geochemists: what caused the presence of oxidized iron species in rocks from a geological period before the advent of molecular oxygen on Earth? While abiotic photo-oxidation of iron by UV radiation had been demonstrated under controlled laboratory settings (Braterman et al. 1983, François 1986) there had been no demonstration of this process in nature. Isotopically depleted organic carbon from BIF's were evidence in support of a biologically driven process (Walker 1986, House et al. 2000). Microfossils from Archaean rock formations morphologically resembled present-day cyanobacteria (Schopf 1992). Photosynthetic production of free oxygen could have fueled abiotic iron oxidaton, or facilitated biotic oxidation by aerobic iron oxidizing bacteria. However, there is little evidence for the presence of free oxygen in the early atmosphere (Kasting 1993). The discovery of anaerobic anoxygenic phototrophs capable of iron oxidation provided an alternative pathway that may have led to the formation of the BIF's.

The ability to oxidize iron in the absence of molecular oxygen has since been recognized in several groups: purple sulfur bacteria (i.e. *Thiodictyon* spp., Widdel et al. 1993 and Croal et al., submitted; *Rhodovulum* spp., Straub et al. 1999), purple non-sulfur bacteria (i.e. *Rhodobacter ferrooxidans* strain SW2, Ehrenreich and Widdel 1994) and green sulfur bacteria (*Chlorobium ferrooxidans* sp., Heising et al. 1999). All known strains were isolated from freshwater and marine sediments. The following net reaction has been proposed (Widdel et al. 1993):

$$HCO_3^- + 4\,Fe^{2+} + 4\,H_2O \rightarrow (CH_2O) + 4\,Fe^{3+} + 7\,H^+$$

In the process of anaerobic iron oxidation these bacteria rely on free Fe^{2+} rather than solid iron minerals and hence require a minimum solubility of their ferrous substrates (Kappler and Newman, submitted). Sufficient solubility for growth was demonstrated for ferrous sulfide (FeS) and siderite ($FeCO_3$), but not for vivianite ($Fe_3(PO_4)_2$), magnetite (Fe_3O_4) or pyrite (FeS) (Kappler and Newman, submitted).

Only four strains of anoxygenic phototrophic iron oxidizing bacteria have been isolated to date. The specific conditions required for growth among these microbes are still poorly understood. Experiments with isolates have indicated strong pH-sensitivity of different strains (Widdel et al. 1993, Ehrenreich and Widdel 1994, Straub et al. 1999). In this project I cultured anoxygenic photoautotrophic iron oxidizing bacteria from School St, Woods Hole, MA, USA and examined pH-sensitivity and salinity tolerance in transfers from initial enrichment cultures. Furthermore, I conducted enrichment experiments to select for photoheterotrophic iron oxidizing purple non-sulfur bacteria by addition of an organic carbon source. I attempted to isolate bacterial strains using agar plates and agar shakes and searched for the presence of *Thiodictyon* sp. strain F4, a strain previously isolated from School St (Croal et al., submitted), by means of FISH (fluorescent *in-situ* hybridization) with an oligonucleotide probe specific to this strain. Complementary to traditional culture techniques, I attempted to culture phototrophic iron oxidizing bacteria in biofilm experiments conducted in anaerobic flow chambers. By using different substrata, I examined effects of surface heterogeneity on microbial populations.

METHODS

Site description. All samples were collected from School St, Woods Hole, MA, USA. The sampling sites were on the road and in puddles in an area downstream to the seepage face of groundwater laden in dissolved iron species derived from a corroding staircase made of iron. The groundwater travels across the street into a creek in the fresh, upper reaches of School St Marsh (Fig. 1). The high concentrations of oxidized iron are indicated by the bright red coloration of sediments and associated particles in the area of groundwater flow.

Enrichment cultures. Particle-rich sites with bright red coloration were selected for sampling. Samples consisted of flocculent organic matter suspended in water. They were collected on June 20, 2003 and July 1, 2003 in sterile 50 mL centrifuge tubes and immediately transferred to sterile, anoxic media. To select for anaerobic anoxygenic photoautotrophic iron oxidizing bacteria I prepared a freshwater mineral medium after Ehrenreich and Widdel (1994) to which I added 30 mL of 1 M sodium bicarbonate buffer to a final concentration of 30 mM HCO_3. Medium was filled into 50 mL Pfennig bottles. To each bottle I added 0.5 mL of 1 M $FeSO_4$ solution for an initial concentration of 10

mM. The slightly alkaline pH of the resulting solution was adjusted to ~7.0 by addition of 1 M HCl. From each Pfennig bottle 5 mL of medium were removed and replaced by 5 mL of fresh, resuspended sample. When necessary the remaining head space was filled with medium to avoid or minimize entrapment of bubbles.

Samples were incubated at room temperature (~20-24°C) approximately 40 cm away from a light source. After 24 hours of incubation a greenish-gray precipitate had accumulated at the bottom of bottles which was attributed to the abiotic formation of siderite ($FeCO_3$). This reaction typically removes 4-6 mM of free Fe^{2+} leaving 4-6 mM in solution (Croal et al., submitted) and hence available to phototrophic iron oxidizers. Dominant microbes in enrichment cultures were documented via light and epifluorescence microscopy using DAPI.

Transfers. After 7-17 days three successful enrichments were selected for transfers. Five mL of initial enrichments were transferred to 50 mL Pfennig bottles containing fresh medium. We examined pH sensitivity of the microbes by including treatments with pH 6, 7 and 8. Salinity tolerance was examined in transfers to bottles (pH 7) with 0, 4, 10, and 30 ppt of NaCl.

The effects of the presence of organic carbon were investigated in treatments to which malate had been added to a 5 mM concentration. Malate is conventionally used to select for purple non-sulfur bacteria due to the ability of this group to grow photoheterotrophically. Other purple bacteria can utilize organic carbon sources, but generally only if the redox state is equally or more reduced than intracellular carbon. Purple non-sulfur bacteria are capable of reducing more oxidized species and using them as carbon sources. Due to rapid microbial growth in malate treatments I started second transfers from one of the bottles 17 days after the first transfer. There were four treatments: 1) medium, $FeSO_4$, malate, light (as in malate transfers), 2) basic medium, $FeSO_4$, light (as in non-malate treatments), 23) medium, $FeSO_4$, malate, no light, and 4) medium, $FeSO_4$, malate, thiosulfate, light. These treatments were chosen as positive controls for photoheterotrophy (1), to test for photoautotrophic capability (2) and chemoheterotrophy (3), and determine the ability to use thiosulfate as an electron donor (4).

Pigment analyses. Aliquots of 3 mL were taken from Pfennig bottles at the end of the experiment. Particles and associated cells were centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and replaced by 3 mL of a mixture 7:2 acetone:methanol. Pigments were extracted overnight in the dark at 4°C. Absorption spectra were measured after ~12 hours.

In an attempt to isolate iron oxidizers, I transferred 1 mL samples from all treatments to 9 mL agar shakes (containing 3 mL medium as described in "Enrichments" section and 6 mL standard fresh water agar) in hungate tubes flushed with N_2-CO_2 (80:20) and closed with butyl rubber stoppers. After 1 week there were no colonies. Moreover I attempted isolation of purple non-sulfur bacteria from malate treatments on agar plates provided with succinate and without iron, and incubated in an anoxic chamber. In this case there were many colonies after 1 week, but none of the microbes in colonies resembled the dominant microbes in enrichments suggesting that they were not iron oxidizers.

Biofilm experiments. Four flow chambers were constructed from petri dishes. In- and outflow were on opposite sides to each other and consisted of holes in the sides through

which tubing had been inserted and sealed on the sides to prevent leakage (Fig. 2). Lids were placed on flow chambers to prevent contamination. Flow was channeled by insertion of "walls" made of plexiglass (Fig. 2). A peristaltic pump moved medium into each flow chamber at a rate of ~4 mL/hr. Since the total volume of each chamber was 20-25 mL this resulted in a turnover time of ~6 hrs.

Two types of media, basic ('artificial') medium (see "Enrichments" section of "Methods") and 'natural', were used. 'Natural' medium consisted of water from the sampling sites that had been filtered through 0.2 μm nucleopore filters to remove all microbes and to which subsequently $FeSO_4$ had been added to a concentration of 10 mM. While there was one treatment with natural medium, there were four treatments with artificial medium designed to examine the effect of surface heterogeneity on abundance and composition of attached microbes: 1) petri dish without additional substratum, 2) with a mono-layer of fine quartz sand, 3) flat granite rock that had been polished, and 4) flat granite rock that had not been polished. The latter two treatments were in the same flow chambers. I avoided cross comparisons of different rocks with potentially different surfaces and chemical composition by polishing one half of the upward facing side of a rock while leaving the other side unpolished. I then compared microbial growth on each side.

Flow chambers were placed in an anaerobic chamber (glove box) with an H_2-N_2 mixture (10:90). Five mL of fresh samples from School St Marsh were placed in each flow chamber and inoculated to allow attachment of microbes to substrata. After 24 hrs the pumps were turned on and the experiment started. The experiment was conducted from July 18-24. On July 24 incubations were terminated, stained with CybrGreen and examined under a confocal microscope.

Fluorescent *In-Situ* Hybridization (FISH). Initially I had planned on using an oligonucleotide probe specific to *Thiodictyon* sp. strain F4 (initial isolation from School St) for identification and to examine numerical abundance of this purple sulfur bacterium in enrichment cultures as well as flow chambers. Despite several days of preliminary experiments, I did not succeed in getting this probe to work.

RESULTS

Enrichments. After 1-2 weeks reddish-orange precipitates began to form on the side of Pfennig bottles and the flocculent material at the bottom started to change color from greenish-gray to reddish-orange indicating anaerobic iron oxidation. After 2-3 weeks almost all of the flocculent had turned to rust color and there were massive ferric iron precipitates on the sides of bottles (Fig. 3).

Transfers. Approximately 1-2 weeks after the first transfer precipitates in most bottles kept at pH 7 had turned reddish-orange, once again indicating anaerobic iron oxidation. Enrichments at pH 6 and pH 8 showed no sign of iron oxidation even after 3 weeks (Fig. 4). The same was true for the salinity treatments: none of the samples inoculated with salinities of 4, 10 and 30 ppt showed noticeable signs of iron oxidation after 3 weeks (Fig. 5). Light microscopic examination of successful enrichments at pH 7 showed high abundances of apparently non-motile, rod-shaped bacteria with conspicuous gas vacuoles that closely resembled *Thiodictyon* sp. strain F4 (Croal et al., submitted) and *Thiodictyon* sp. strain L7 (Ehrenreich and Widdel 1994). Cells were observed in attachment to iron cristals as single cells and clusters, and as free-floating filaments (Figs. 6 and 7).

First transfers to bottles containing malate started showing conspicuous black precipitates on the sides of bottles after 1 week and the medium had developed a weak purple color (Fig. 8). After another week the medium had turned dark purple and there were orange streaks on the sides of bottles opposite to the light source suggesting anaerobic iron oxidation (Fig. 8). Light microscopic examination revealed high concentrations and numerical dominance of motile *Chromatium*-like rods (Fig. 9).

Second transfers showed conspicuous color changes after only three days. Bottles to which thiosulfate had been added had turned completely black due to precipitates on the sides. No purple coloration was visible. Treatments that contained the same medium as in the first transfer (with malate and $FeSO_4$) had light black precipitates and the medium had turned moderately purple. The treatment without malate was clear with no sign of black precipitates, while the dark treatment with malate was clear, but had light black precipitates on bottle walls. Two days later, the malate treatment with light had turned dark purple and developed orange streaks on the bottle side opposite to the light source. The treatment omitting malate had changed from clear to slightly turbid. The remaining two treatments had not changed noticeably in color (Fig. 9). As in the first transfer, *Chromatium*-like rods (Fig. 8) were very abundant and by far outnumbered other bacteria in the treatment with malate and $FeSO_4$. The treatment selecting for photoautotrophy had lower concentrations of *Chromatium*-like cells, but was also dominated by this morphotype. The dark and the thiosulfate treatments had very low counts of these cells (and of cells in general) suggesting little growth or no growth at all.

Pigment analyses revealed the presence of bacteriochlorophyll *a* in extracts from treatments with and without malate addition. There was no evidence for the presence of other bacteriochlorophylls. The highest values were found in cultures from initial enrichments and transfers with basic medium and $FeSO_4$, as well as in transfers containing basic medium, $FeSO_4$ and malate. In the second transfers of malate treatments the enrichment for photoautotrophs had a comparably low, but distinct absorption peak in the range of bchl *a* (~770 nm). Both the dark treatment and the treatment to which thiosulfate had been added had very low, barely detectable absorption in this range.

Biofilm Experiments. Examination of the three flow chambers incubated with the basic enrichment medium under a confocal microscope revealed increasing densities of microbes with increasing substratum heterogeneity (Fig. 10). The petri dish surface had the lowest cell density followed by polished rock surfaces. Densities were highest on the unpolished rock surface and on sand grains. Compared to the petri dish incubated with the basic enrichment medium, the petri dish incubated with iron-enriched filtrate from School St had vastly higher cell densities. In fact, cell densities in this treatment exceeded those in all flow chambers inoculated with artificial medium despite the lower surface heterogeneity compared to some of the other treatments.

DISCUSSION

The presence of bacteriochlorophyll *a* and large quantities of oxidized iron combined with the dominance of a bacterial strain with close morphological resemblance to known anoxygenic iron-oxidizing photoautotrophs, strongly suggest that the enrichments for photoautotrophs were successful. The next step towards demonstrating that this is the case is isolation of the dominant microbe on the same medium. FISH using the oligonucleotide probe designed for *Thiodictyon* sp. strain F4 can be used to determine if

this microbe is in fact the same *Thiodictyon* strain isolated by Croal et al. in School St. Sequencing of 16S sequences from environmental samples, initial enrichments, transfers, and isolates could be used to scan for the presence of more than one iron-oxidizing *Thiodictyon* strain. Sequencing of environmental samples, initial enrichments and transfers might be of particular importance: the known F4 strain from School St was cultured with the same medium used in this study. It cannot be precluded that it is a superior competitor over other *Thiodictyon* strains with the specific medium used.

The enrichments with malate provide a more complex scenario: these cells contained bacteriochlorophyll *a* and, though in much lower quantity, oxidized iron was present in the enrichments. Growth also occurred in transfers to media without malate suggesting the ability for photoautotrophic growth. It cannot be precluded, however, that growth was fueled by remaining malate transferred along with cells to the inorganic medium. Nonetheless, the presence of photosynthetic pigments and the absence of growth in the dark strongly suggest that the *Chromatium*-like bacteria are phototrophs, and most likely, at least facultatively, photoheterotrophs. The dominance by a *Chromatium*-like bacterium is puzzling since 1) *Chromatium* spp. are purple sulfur and not purple non-sulfur bacteria and hence believed to be incapable of metabolizing organic carbon species that are more oxidized than their intracellular carbon and 2) most, if not all, known *Chromatium* spp. can use thiosulfate as an electron donor for photoautotrophic growth. They are frequently observed to form visible globules of elemental sulfur within cells under excess supply of reduced sulfur. This trait can generally be used to distinguish purple sulfur from purple non-sulfur bacteria. Neither did I observe intracellular storage of sulfur, nor did I find any evidence for an increase of these cells in the presence of thiosulfate. The presence of oxidized iron in malate enrichments with light and without thiosulfate suggests activity of anaerobic phototrophic iron-oxidizing microbes. To determine that free Fe^{2+} is essential to this organism's growth, the next step will be to determine its ability to grow in the absence of free ferrous iron. In approaching a better understanding it will also be necessary to have negative controls without bacteria to account for abiotic processes. Black precipitates were formed in all treatments with malate and in particular after addition of thiosulfate. Yet little to no growth was observed in the latter treatment. Abiotic reactions of thiosulfate with, i.e. free Fe^{2+} cannot be ruled out. Moreover, abiotic reactions of malate with free Fe^{2+} might be occurring.

The outcome of the biofilm experiment provided no surprises within the narrow analytical limits imposed by the brief experimental period and the absence of a functional FISH probe specific to phototrophic iron oxidizers. On more general terms, it showed that growth of attached bacteria is dependent on surface heterogeneity: as surface heterogeneity increased there was an increase in bacterial counts. Possible explanations are the increased surface area for bacterial settlement that may reduce density-dependent competitive interactions between cells, as well as the increase in niche diversity associated with greater diversity of microenvironments. The much greater cell densities in flow chambers inoculated with filtered water from School St than with artificial medium are not surprising: the high concentration of organic compounds in the filtrate may have stimulated growth of heterotrophs in general. Moreover, the diversity of organic compounds may have provided carbon substrates to a plethora of different heterotrophic bacteria in the absence of much competition.

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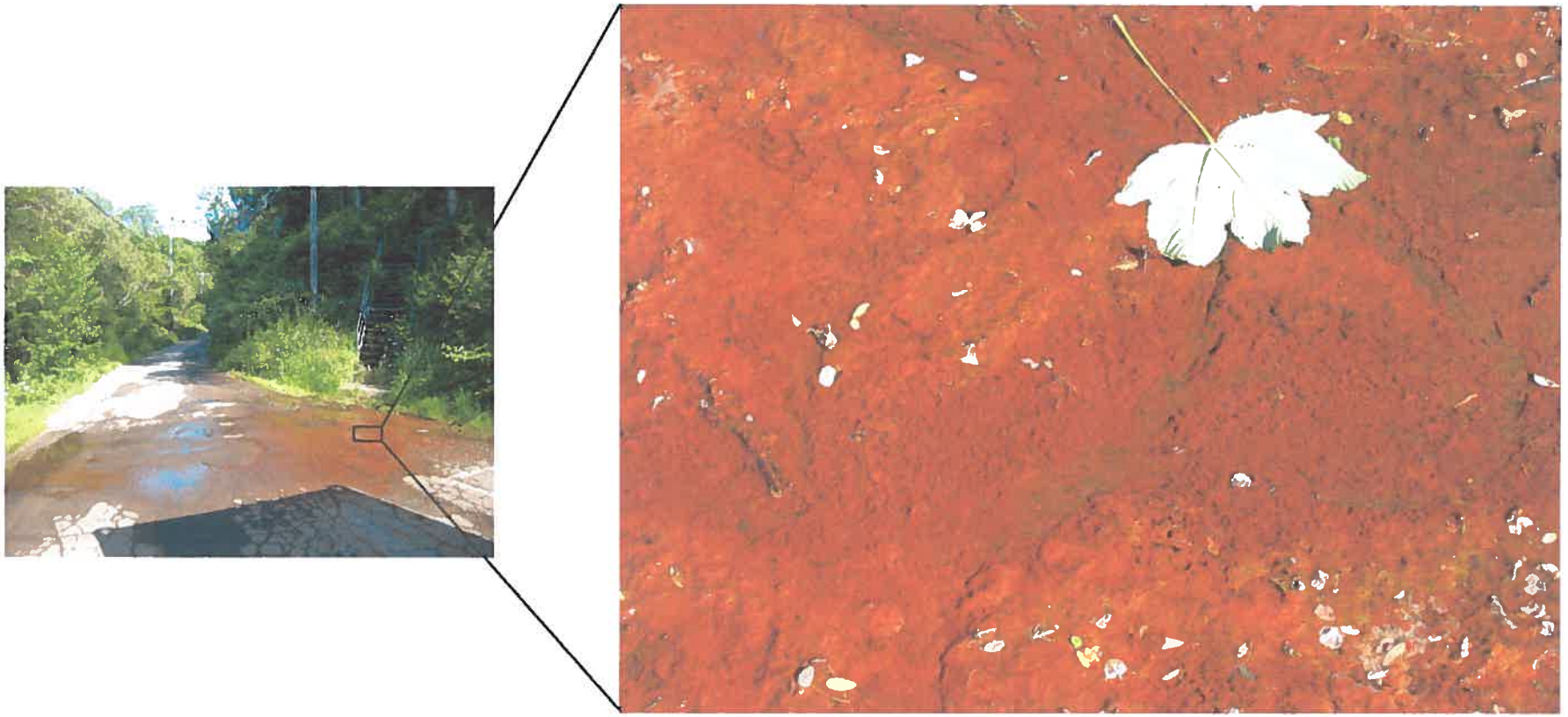

Fig. 1. Above to the left, a photograph of the sampling area on School St, Woods Hole, MA, USA. In the back right is an iron stair case from where corroded iron leaches into the groundwater which seeps on the street. Above to the right, a closeup view of a typical sampling site. The bright red coloration indicates precipitation of oxidized iron species.

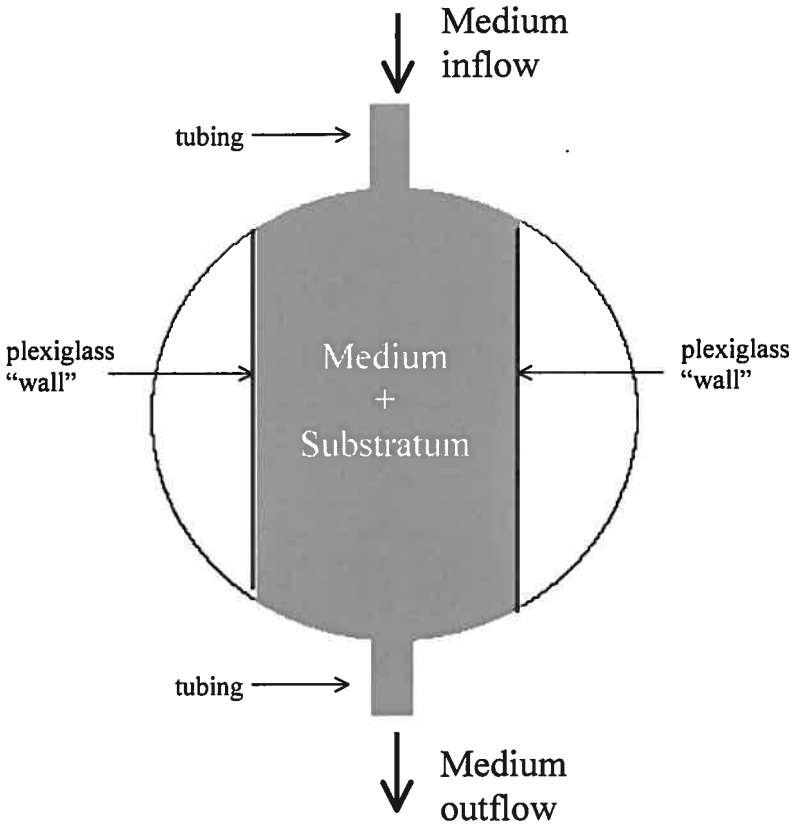

Fig. 2. Design of individual flow chambers.

Fig. 3. Above an arrangement of enrichment bottles in order of quantity of oxidized iron. The bottle on the far left has little to no oxidized iron whereas the bottle on the far right has the most oxidized iron.

Fig. 4. Enrichment transfers to media adjusted to different pH values after 3 weeks of incubation. Two of the bottles at pH 7 contain significant amounts of oxidized iron, whereas there is little to no oxidized iron in the other two pH treatments.

Fig. 5. Enrichment transfers to media adjusted to different salinity values after 3 weeks of incubation. Two of the bottles 0 salinity indicated oxidation of large quantities of iron. The other treatments contain little if any oxidized iron.

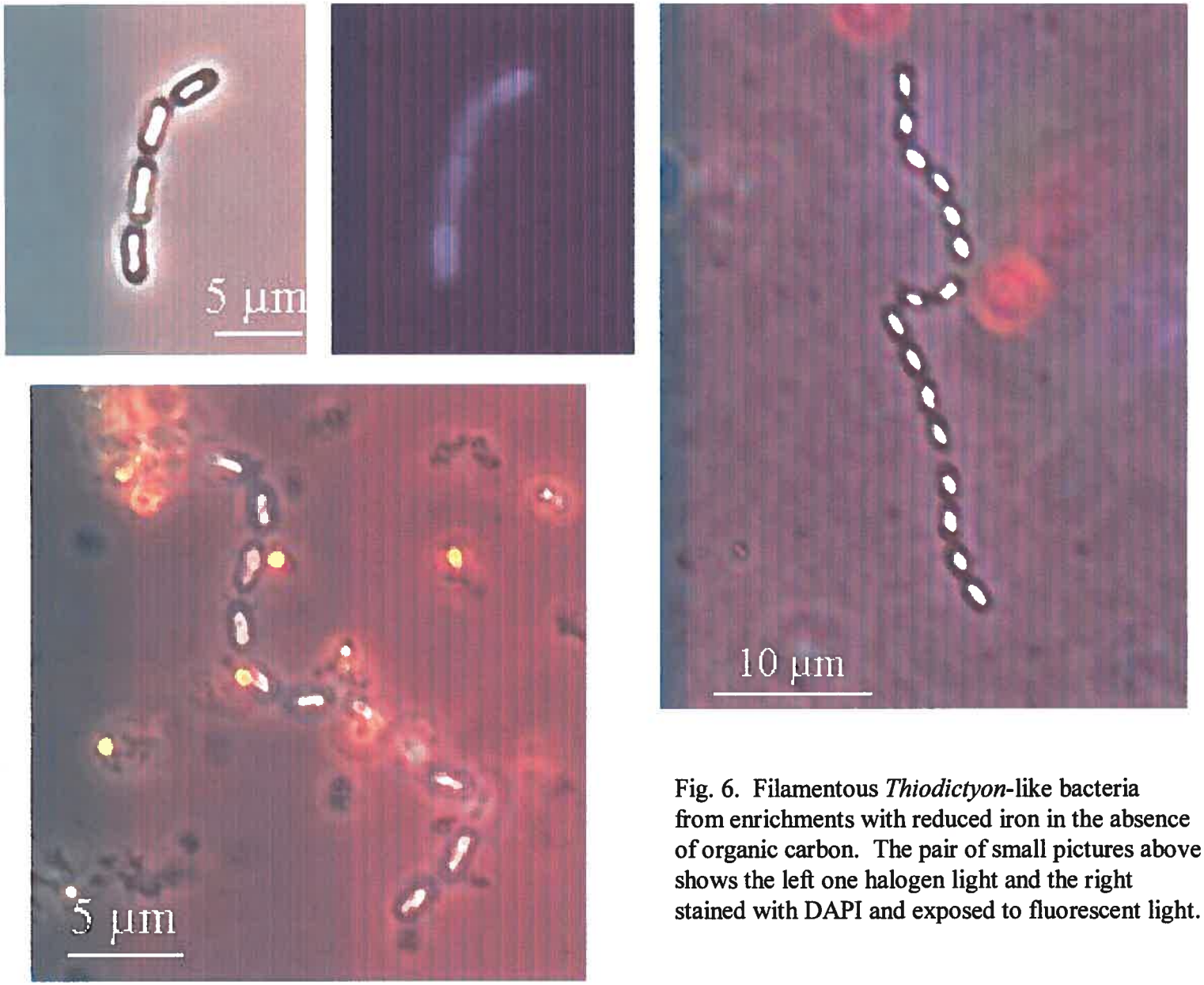

Fig. 6. Filamentous *Thiodictyon*-like bacteria from enrichments with reduced iron in the absence of organic carbon. The pair of small pictures above shows the left one halogen light and the right stained with DAPI and exposed to fluorescent light.

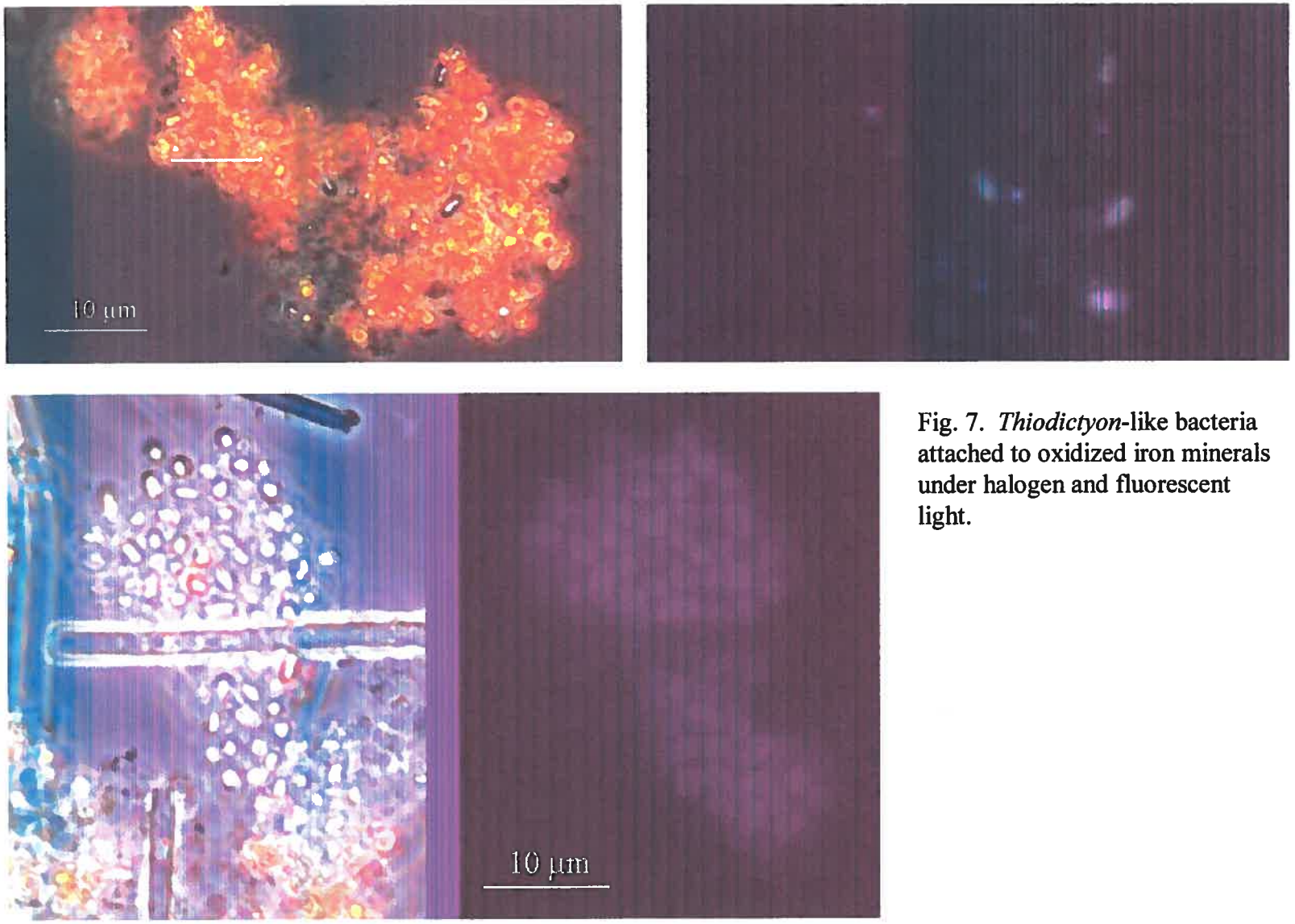

Fig. 7. *Thiodictyon*-like bacteria attached to oxidized iron minerals under halogen and fluorescent light.

Fig. 8. Above two enrichments to which malate had been added as an organic C source. The left bottle has black mineral precipitates on the bottle sides and the medium has a dark purple color as seen in the bottle neck. The pale orange streaks on the right picture indicate the presence of oxidized iron.

To the right, second transfers of malate treatments on day 1 and at day 6. Not visible on this photograph is a light turbid coloration of the second transfer without malate (far left) after five days.

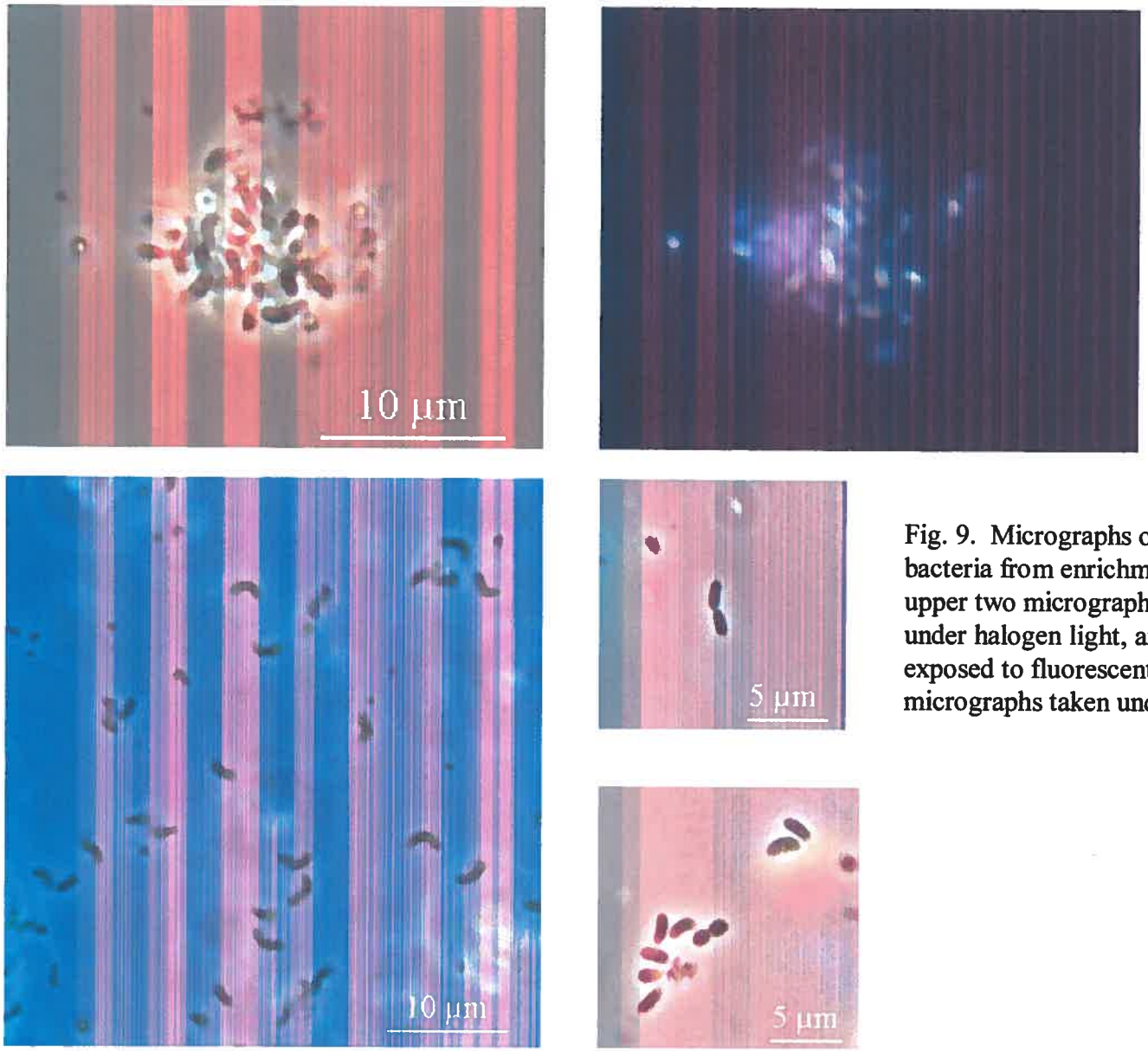

Fig. 9. Micrographs of Chromatium-like bacteria from enrichments with malate. The upper two micrographs show the same cluster under halogen light, and stained with DAPI and exposed to fluorescent light. To the left are micrographs taken under halogen light.

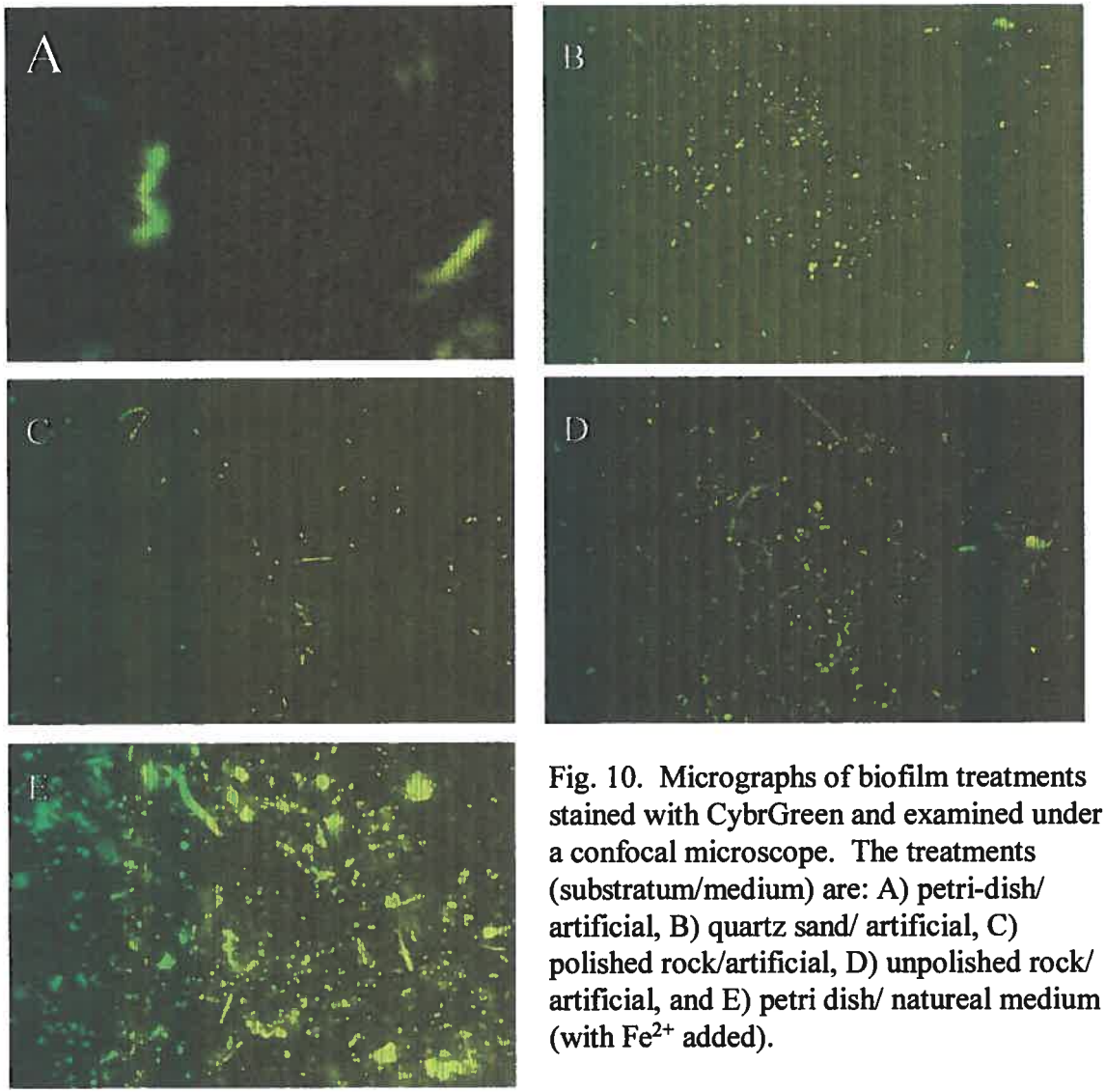

Fig. 10. Micrographs of biofilm treatments stained with CybrGreen and examined under a confocal microscope. The treatments (substratum/medium) are: A) petri-dish/ artificial, B) quartz sand/ artificial, C) polished rock/artificial, D) unpolished rock/ artificial, and E) petri dish/ natureal medium (with Fe^{2+} added).