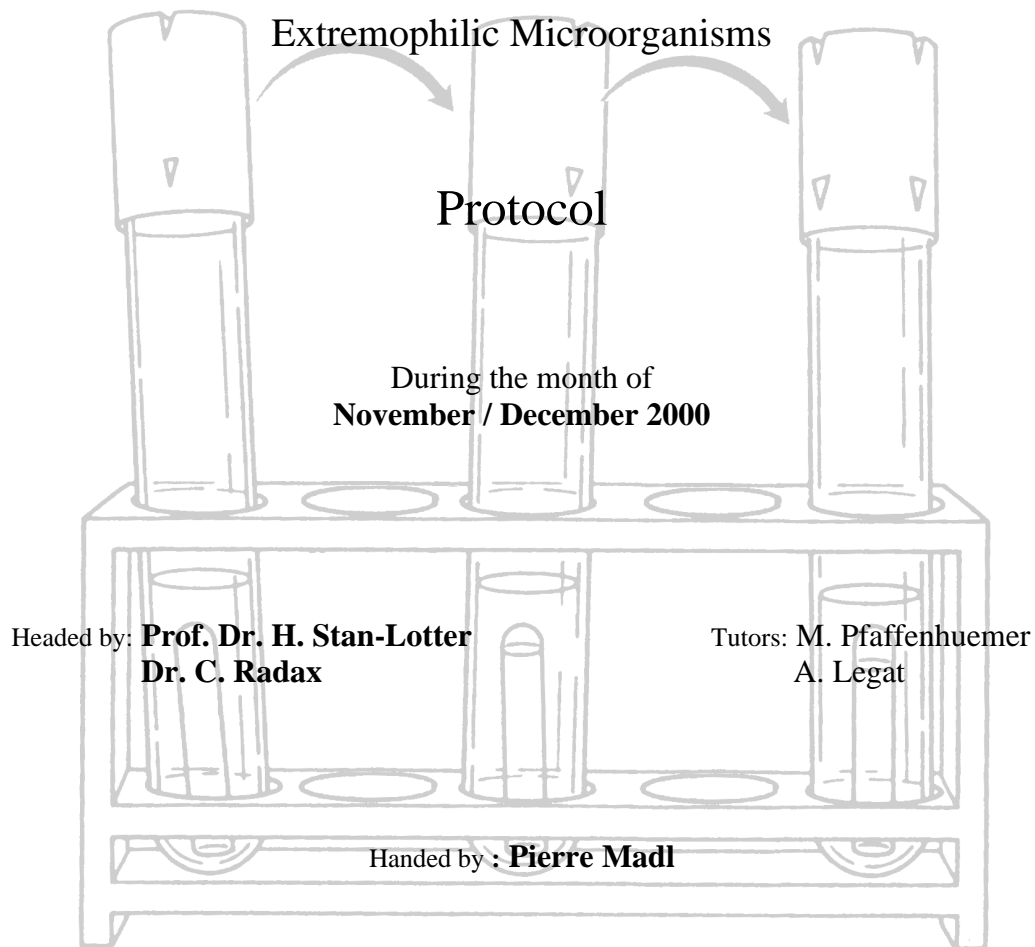


Microbiology III

Nov. 6th

Nov. 20th

Nov. 27th



Salzburg, February 23rd, 2001

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Introduction

In the course of this practical, we carried out several experiments with archaea strains from different sources such as from natural biotopes; i.e. open ocean, anaerobic habitats, and acidophilic samples. The bacteria used were also subjected to several treatments; for example, analyzing them under the phase-contrast microscope or an fluorescence microcope, monitoring the buildup of gas as a metabolic waste product, and controlling their productivity by evaluating both pH and metal-concentration of metallogenic strains. Ultimately, the results of each working group have been compared with each other and further evaluated.

Table 1 Schedule of experiments

	MON - Nov. 6 th	MON - Nov. 13 th	MON - Nov. 20 th	MON - Nov. 27 th
Experiment 1	Filtration and incubation		Visual census of 5% agar dishes	Visual census of 20% agar dishes
Experiment 2		Inoculation and Incubation		Evaluation (done on Jan. 24 th 2001)
Experiment 3	Inoculation	pH, and ion concentration	pH, and ion concentration	pH, and ion concentration
Experiment 4		Inoculation and incubation	Measurement of pH & gasforming reaction	Measurement of pH & gasforming reaction

Archaea bacteria:

16SrRNA sequencing and other studies have defined eight genera among the archeal Domain. They are referred as Halobacteria, they lack peptidoglycan in their cell walls and contain ether-linked lipids and archean type RNA polymerases: they are also insensitive to most antibiotics and posses the other general attributes of representatives of this domain. They all are GRAM^{neg}, reproduce by binary fission, and usually do not form resting stages or spores. Most are non-motile, but few strains are weakly motile by lophotrichous flagella. They are also chemo-organotrophs, and most species are obligate anaerobes. Most use amino acids or organic acids as energy sources and require a number of growth factors (mainly vitamins) for optimal growth. All of them require large amounts of sodium for growth. They are highly adapted for both internally and externally, to life in a highly ionic environment. Cellular components exposed to the external environments require high Na⁺ for stability, whereas internal components require high K⁺. In no other group of prokaryotes do we find this unique requirements for specific cations in such high amounts.

These aerobic and facultative anaerobic, chemo-organotrophic organisms are distinctly pleomorphic, ranging from rods through cocci to pleomorphic cells forming rectangles, triangles, and disks, but also they form a relatively tightly phylogenetic group.

1. Halophilic, Nanobacteria of the Indo-Pacific (Pt. Douglas - Australia)

Extreme halophilic Archaeobacteria are present in any body of seawater. They are easily found in areas where water loss due to evaporation exceeds water influx of seawater. Under certain conditions, when salinity reaches beyond 10%, halophilic archaea can easily proliferate as the ambient, abiotic conditions that reach the growth optimum for these bacteria. *Halobacteria* and *Halococcus*-strains can occur in such huge numbers, that the hypersaline body of water eventually turns reddish. Some extreme halophiles like these ones, overcome unsuitable environmental conditions, by developing a latent stage (not a resting stage or spore) which are far smaller than their normal reproductive stage; Archeobacteria in such stages are known as “Nano- or Ultramicrobacteria”.

1.1 Filtration and incubation (Nov. 6th)

Purpose: With this experiment it is attempted to isolate halophile macrobacteria (greater than 0.2µm) of open ocean water samples taken off the coast of Australia (Pt. Douglas). A sample of 20mL is passed through a 200nm membrane filter. The filtrate, along with the membrane is then placed onto a full medium agar dish. Incubation at 37-39°C for several weeks allow the bacteria to catch up with their normal reproductive cycle by forming easily visible colonies. Examination under a phase contrast microscope should further unveil their morphological characteristics.

Procedure:

- Sterilize the stainless-steel filter holder by sweeping it several times through the flame of the Bunsen burner;
- attach the filter holder to the rubber stopper, insert into the vacuum flask (for security reasons, wrap flask with adhesive tape), and connect the flask to the aspirator line (water hose);
- sterilize forceps by sweeping it through the flame and transfer the sterile membrane filter to the platform base of the filter with ruled side up (make sure to remove the yellow protective disc before assembling the filter holder);
- place the matched funnel unit over the filter disc (make sure that it is clamped in place firmly);
- pipette about 20mL from the storage container with the ocean water sample;
- turn on the vacuum source and gradually drip the 20mL of ocean water sample over the suction flask through the filter;

Note: use eyewear and work behind a security slide bar; don't flood the funnel with water as both the membrane filter with 0.2µm and the water itself pose a high resistance to the air flow; thus increasing the stress to the glassware flask;

- once all the water has passed through, with sterilized forceps, gently remove the filter membrane and place it with the ruled side up onto the 1st 5% agar dish; mark dish properly;
- repeat procedure with a new filter and place on 2nd 5% petri-dish;
- repeat procedure with a set of new filter membranes which are then placed onto the 20% agar dishes;
- incubate all four dishes for several weeks at 37-39°C;
- after incubation, macroscopically evaluate colony shape, coloration and surface texture;
- extract a tiny sample of a representative one colony with a sterile toothpick and transfer it to an object slide; add a droplet of sterile seawater substitute, place cover slide, and drip a single drop of immersion oil on top of it before evaluating sample under the phase contrast microscope;

Material: Water perm. marker
 Adhesive tape
 Bunsen burner w/ igniter
 Sterile membrane filter apparatus w/ sterile 0.2µm pore cellulose-nitrate filters
 Tweezers
 Sterile toothpicks
 Microscope (1000x) Leitz used for Phase Microscopy
 Immersion oil
 5 Object slides w/ cover glass
 Protective Eyewear
 Incubator (37-39°C)
 25mL Measuring Pipette
 some sterile Q-tips
 2 M2.5-Agar dishes w/ 5% NaCl*
 2 M2-Agar dishes w/ 20% NaCl*

Samples:
 20mL Ocean water sample off the coast of Australia - Pt. Douglas
 Sterile seawater substitute

(*) provided by the tutors; see also appendix - Agar preparation



Fig 1.1 Membrane filter apparatus

1.2 Results and Discussion (Nov. 20th):

Macroscopic valuation of the 5% NaCl dish revealed to be very difficult, as too many colonies aggregated within the area (fig.1.3). The morphological characteristics of the few isolated colonies are depicted in fig. 1.2.

Several colonies were found to have smooth edges, pulvinate elevation, smooth surface, and white-yellow in color;

Colony diameter: 1-3mm

Fig. 1.2 sketch of colony morphology

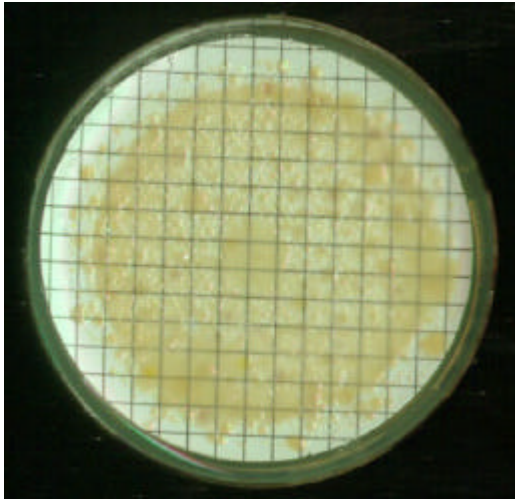


Fig.1.3 visual census of 5% NaCL agar (natural size)

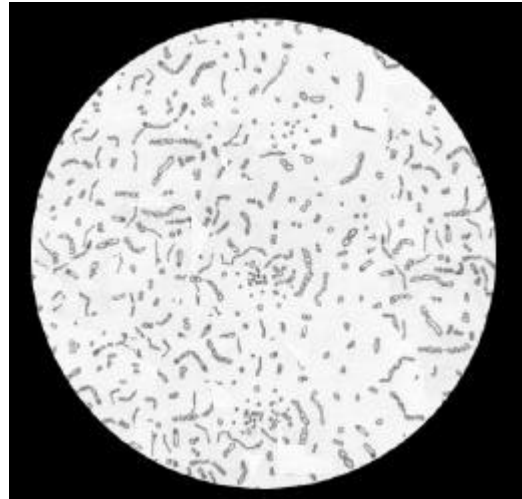


Fig.1.4 visual census of 5% NaCL agar (1000x)

The evaluation of the 20% NaCl sample clearly simplified the procedure, as far less colonies were present even after extending the incubation period for an extra fortnight. Both macroscopic colony morphology and pigmentation did not change. The microscopic observation revealed a slightly altered picture, in that more stalked forms than in the previous 5% NaCl sample were found (fig.1.6).

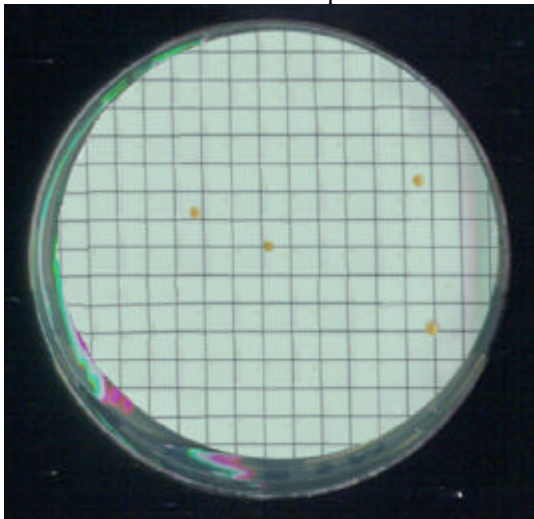


Fig.1.5 visual census of 20% NaCL agar (natural size)

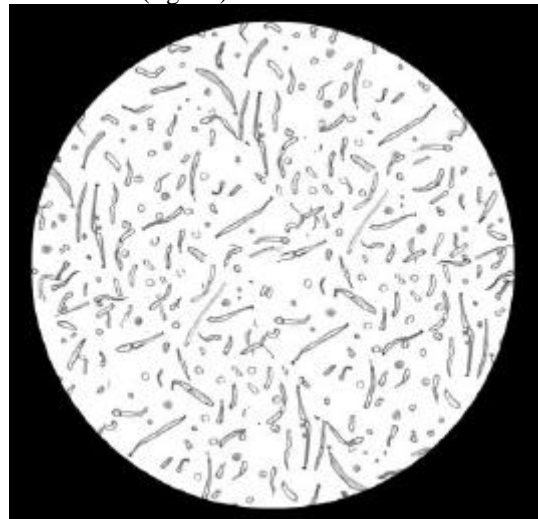


Fig.1.6 visual census of 20% NaCL agar (1000x)

2. Fluorescence Microscopy of Halobacteria

2.1 Inoculation & Incubation w/ Halobacterial strain (Nov. 6th)

Purpose: Coloration of the *Halococcus sp.* with DAPI (4,6-Diamidino-2-phenylindole); it can easily pass the cellular membrane it enables the visual localization of the bacterial DNA. Even though this procedure was developed for eukaryotes only, recent adaptations paved the way for the application with prokaryotes. DAPI which attaches into the minor-groove of the DNA-double helix can be excited at 358nm. After an **Internal Conversion** (a type of relaxation in excited DAPI-electrons, that involves the transfer of excess energy of a species in the lowest vibrational level to a lower electronic state), a very fast but non-irradiating transition from the excited state to a lower or ground state. This relaxation results in the emission of electromagnetic radiation (particularly in the visible region at about 461nm) from singlet state electrons as they fall back to their ground states.

Procedure: staining of extreme halophilic strains with DAPI and further evaluation under the fluorescence microscope;

- Pipette about 20mL of liquid M2 medium into the sterilized Erlenmeyer flask (**remember:** every time the stopper is removed from the flask, sweep the opening through the flame of the Bunsen burner);
- pipette about 1µL of stem broth containing the *Halococcus sp* H4 strain into the Erlenmeyer flask;
- place sealed flask in incubator and activate shaking mode (moderate level) at 37°C for at least a week;
- after incubation, extract 400µL from the broth and pipette it into a plastic bullet; to avoid an unbleached centrifugation;
Note: fill up another bullet with the same amount and place it into the opposite position of the turntable;
- centrifuge at 12000rpm's for at least 3mins;
- carefully extract the clear surface liquid without stirring up the condensed bottom pellet;
- add 30µl of double distilled water to the pellet, seal it, and shake well on Vortex;
- add 70µL of ethanol to the pellet, allow to settle for a minute or so before placing it again on the vortex;
- finally, add 20µL of TN-buffer (or if not available double distilled water) and shake well;
- transfer 3µL from the bullet along with 3µL of the DAPI-working solution onto an object slide, gently mix it, seal it with cover glass, and place one droplet of immersion oil on top of it;
- place it under the microscope, focus and take a picture with the built-in CCD-camera
Note: work quickly, as the fluorescence reaction quickly fades out as the hard UV-radiation gradually destroys the bacterial DNA;

2.2 Results and Discussion (Jan. 24th):

As can be seen in fig. 2.1, the cells tend to cluster in groups of 4; each group is further attached with other clusters to form an assemblage of loosely tied up packs; many of such packs can be seen on the slide, although the larger packs tend to emit an excess amount of fluorescence light that oversaturate the light-accumulation capabilities of the CCD-chip. Larger clusters also tend to be more densely packed which renders focusing more difficult; thus, smaller clusters have the advantage as they seem to align in one plane, making it possible to take clearer pictures.

Material: perm. marker
Bunsen burner
20mL sterile glass pipette w/ bulb
100µL Gilson-Pipette w/ sterile tips (yellow)
1 100mL Erlenmeyer flask w/ cotton stopper
2 plastic bullets
Centrifuge (12000rpm)
Vortex mixer
Incubator w/ shaker (37°C)
Microscope (1000x) Nikon used for Fluorescence
Microscope attached to a PC w/ frame grabber
Immersion oil
5 Object slides w/ cover slides
1mL aqua bidestillata
1mL 96% ethanol

Samples:

1µL *Halococcus sp.* H4*
20mL M2-liquid medium*
DAPI-working solution
dilluted with water 1:100
(1mg DAPI/mL H₂O)
TN-buffer (4M NaCl, 50mM Tris-HCl, pH 7.4)*

(*) provided by the tutors; see also appendix - Agar preparation



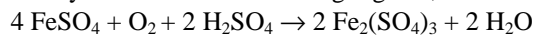
Fig.2.1 Stained *Halococcus sp.* H4 with DAPI solution (1000x)

3. Metal-acids and thermo-acidophile Archaeobacteria

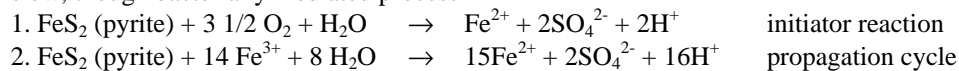
Certain chemo-lithotrophic microorganisms are capable of altering metals from its ionic state into its elemental form.

One of the most common forms of iron and sulfur in nature is Pyrite, which has the overall formula of FeS_2 . Pyrite is formed from the reaction of sulfur with ferrous sulfide (FeS) to form a highly insoluble crystalline structure. The oxidation of pyrite is a combination of chemically and bacterially catalyzed reactions. 2 electron acceptors for this process can function; molecular oxygen (O_2) and ferric ions (Fe^{3+}). However, ferric ions are present only when the solution is acidic, at pH values below 2.5. At pH values above 2.5, ferric ions reacts with water to form the insoluble ferric hydroxide [$\text{Fe}(\text{OH})_3$].

Iron exists in nature primarily in two oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}), and bacterial transformation of these cations is of great geological and ecological importance. Bacterial ferric iron reduction occurs in anoxic environments and results in the mobilization of iron from swamps, gobs, and other iron-rich aquatic habitats. Bacterial oxidation of ferrous iron occurs significantly only at low pH and is very common in coal-mining regions, where it results in a type of pollution called acid mine drainage.



Acid mine drainage occurs because of the attack of *Thiobacillus ferrooxidans* on pyrite, following the steps outlined below; under natural conditions some of the ferrous iron generated by the bacteria leaches away, being carried out into the surrounding environment. However, because oxygen is available in the aerated drainage, bacterial oxidation of the ferrous iron takes place in these outflows and an insoluble ferric precipitate is formed. Reaction 1 occurs spontaneous, catalyzed by bacteria, while reaction 2 is a slow, though bacterially mediated process



3.1 Inoculation & Incubation w/ *Acidianus* strain (Nov. 6th)

Purpose: Sulfur- and sulfur-oxidizing bacteria, which are often chemo-lithotrophs, produce sulfate; sulfate-reducing bacteria use sulfate as electron acceptor in anaerobic respiration and produce hydrogen sulfide (H_2S). $\text{SO}_4^{2-} + 8\text{e}^- + 8\text{H}^+ \rightarrow \text{H}_2\text{S} + 2\text{H}_2\text{O} + 2\text{OH}^-$

- Use the hammer to chip apart the pyrite sample;
- **Note:** use protective eyewear!
- with piston and mortar grind fragments of the pyrite sample to powder;
- weigh 150mg of ground powder and place it in the sterile test tube;
- fill the test-tube with about 5mL of *Acidianus* medium; besides marking the tube properly with name and date do not forget to outline the upper surface level with waterproof marker as well;
- place sealed test-tube in the incubator (70°C) and activate shaker mode at moderate oscillation over the next few weeks;
- upon determination of metal ion concentration, use the Pasteur pipette to fill up the evaporated volume of *Acidianus* medium till to the outlined mark; shake thoroughly on vortex;
- extract 2x 200µL from it and pipette 200µL in each bullet;

pH determination with Merck's pH indicator paper:

- use the remaining droplets of the tips to verify the solutions pH;

Fe²⁺/ Fe³⁺ determination with Merck's quickset Fe²⁺ test-strips:

- extract a few µL of the 1st bullet and pipette it onto the Fe²⁺-test stripe; after 1secs, shake off excess liquid and compare color change due to the ionic metal concentration with the color scale on tube;
- add a spatula tip full of ascorbic acid to the 1st bullet and shake well;
- extract a few µL of the 1st bullet and pipette it onto the Fe²⁺-test stripe; after 1secs, shake off excess liquid and compare color change due to the ionic Fe³⁺ concentration with the color scale on tube;

Cu²⁺ determination with Merck's quickset Cu²⁺ test-strips:

- add 4drops of Na-acetate the 2nd bullet and shake well;
- extract a few µL of the 2nd bullet and pipette it onto the Cu²⁺-test stripe; after 1secs, shake off excess liquid and compare color change due to the ionic Fe³⁺ concentration with the color scale on tube;

<p>Material: perm. marker Small spatula Bunsen burner Protection eyewear Hammer Piston and mortar Digital flat-pan balance 1 sterile test tube w/ cap Test-tube rack Sterile Pasteur pipett w/ bulb 200µL Gilson-Pipette w/ sterile tips (yellow) Incubator w/ shaker (70°C) Vortex mixer 6 plastic bullets pH-indicator paper (Merck) Merckoquant kit for Fe²⁺ (-"-) Merckoquant kit for Cu²⁺ (-"-) Microscope (1000x) Leitz used for Phase Microscopy</p> <p>Samples: 50g sample of pyrite 25mL <i>Acidianus</i> Medium set at pH 2.0 few grams of ascorbic acid few mL of Na-acetate</p>

3.2 Results and Discussion (Nov. 6th, 13th, 20th, 27th):

Microscopic analysis of the *Acidianus* broth revealed a prospering culture; it seems obvious that the bacteria under the preset conditions are well able to utilize the sulfur present in the crushed pyrite (fig. 3.1).

As can be seen in the table below the metabolic utilization is further proved by the increased ionic concentration of both Cu^{2+} and $\text{Fe}^{2+}/\text{Fe}^{3+}$ metallic ions (fig. 3.2).

It is essential to keep the pH of the broth at a constant level (in this case it was always found to be in-between 1 and 2), which is nothing more than the optimum pH-value for this particular strain.

The rather more elevated level of Fe^{3+} could be the result of extended exposure times of the test-strips with the culture medium; therefore, it should be considered as unrealistically elevated.

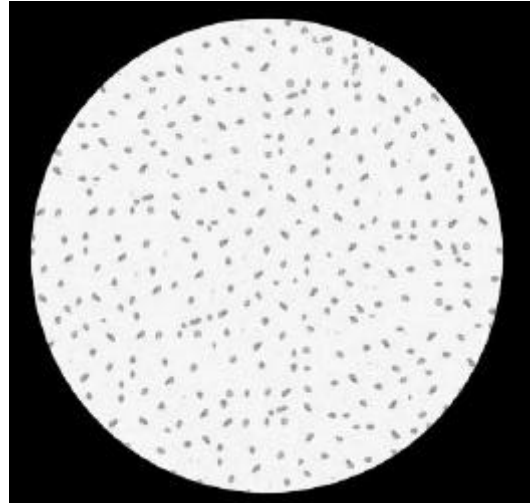
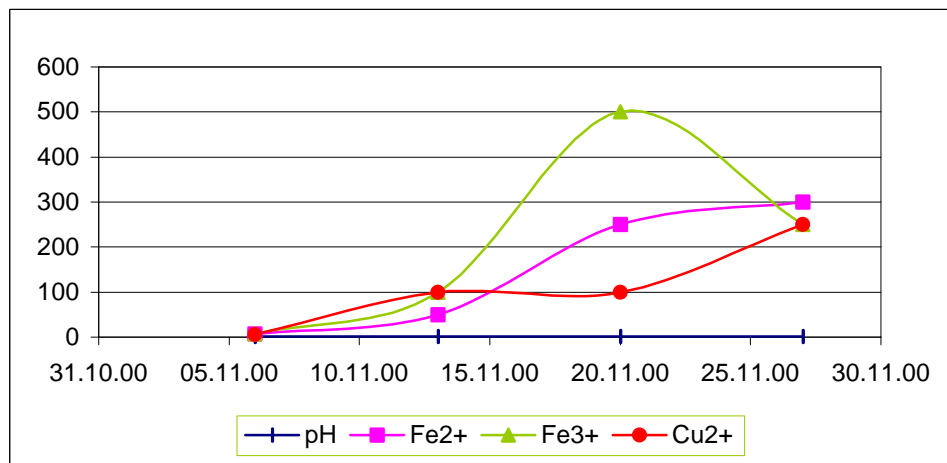


Fig.3.1 visual census of *Acidianus* medium (1000x)

Table 3.1: Ionic metal concentrations in [mg/L]

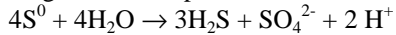
6.11.2000			13.11.2000			20.11.2000			27.11.2000		
Fe^{2+}	Fe^{3+}	Cu^{2+}	Fe^{2+}	Fe^{3+}	Cu^{2+}	Fe^{2+}	Fe^{3+}	Cu^{2+}	Fe^{2+}	Fe^{3+}	Cu^{2+}
3-10	3-10	0-10	50	100	100	250	500	100	300	250	250

Fig.3.2 graphical representation of the various metal concentrations liberated by the *Acidianus* culture;



4. Thermophilic Sulfur-utilizing strains from volcanic substrates

Bacteria play a major role in both the oxidative and reductive sides of the sulfur cycle. Sulfur- and sulfide-oxidizing bacteria such as *Acidianus* sp., which are often chemo-lithotrophs, produce sulfate. Sulfate reducing bacteria use sulfate as the final electron acceptor in anaerobic respiration and produce hydrogen sulfide. Because sulfide is toxic and also reacts with various metals, sulfate reduction is an important biogeochemical process.



Acidianus strains being thermophilic grow best at temperature between 65° and 95°C at pH-values of 1-5.

4.1. Filtration and incubation (Nov. 13th)

Purpose: It should be shown that sulfate-reducing bacteria are able to reduce elemental sulfur to sulfate; because the reduction of sulfate requires first its activation by a reaction with ATP, this reduction process is not very efficient energetically as it requires the disproportionation of the end products; i.e. one product is more oxidized (H₂S), while the other is more reduced (SO₄²⁻).

Procedure:

- Pipette about 10mL of *Acidianus* medium to the large test tube;
- Inoculate the test tube with about 0.5mL of the Santorini sample;
- before grabbing the small test tube, sweep tweezers through the flame of the Bunsen burner, and fill the tube with the *Acidianus* medium (roughly 1.5mL);
- carefully place the small tube up-side-down into the large tube, cover with cap, and incubate for a week or more at 65°C.

Note: try to avoid loss of liquid that might end up in a trapped gas bubble of the drowned small test tube.

Material: Waterproof marker

Adhesive tape

Bunsen burner w/ igniter

1 sterile small test tube

1 sterile large test tube w/ cap

Test tube rack

Tweezers

1000µL Gilson-Pipette w/
sterile tips (blue)

5mL Gilson Pipette w/ sterile
tips (white)

Incubator (65°C)

Samples:

15mL sterile *Acidianus*

medium w/ elemental sulfur
adjusted to pH 6*

1mL Santorini-sample*

(*) provided by the tutors; see
also appendix

4.2 Results and Discussion (Nov. 13th, 20th, 27th):

Initially, the reaction seem not to take off properly, only the pH-depression suggested a moderate activity (table 4.1). During the third week, though, the reaction became not only measurable, but was also evident by the concomitant production of H₂S-gas trapped in the small test tube. This became even more evident in the final round of observation and pH-measurement (fig. 4.1).

Table 4.1. Summary of the gathered results:

Date	13.11.2000			20.11.2000			27.11.2000		
Parameter	pH	growth	gas	pH	growth	gas	pH	growth	gas
Results	6	-	-	3	little	2mm	2	little	4mm

As the build-up of H₂S gas proceeded, the stock of elemental sulfur dwindled rapidly; this became most evident during the last round of observation as the flocks of sulfur usually aggregated at the bottom of the large tube seemed to be trapped in a more or less homogenous network of sulfur fibers; this network is most likely made entirely of Sulfur-reducing bacteria that aggregated firmly first at the sulfur crystal and as it gradually becomes depleted, at the bottom of the tube. Altogether, the reaction taking place under this condition leads to an acidification of the previously almost neutral medium.

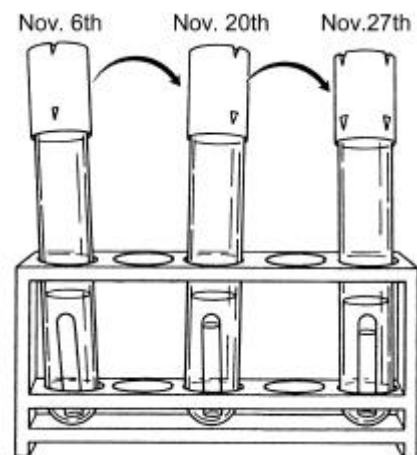


Fig 4.1. H₂S-gas formation as reaction speeds up

Appendix

A. Complex medium for neutrophile *Halobacteria* (experiment 1,2):

amounts given to obtain 1L of culture medium;

Distilled water	800mL
Yeast extract	5g
Casein-hydrolysate (HyCase, Sheffield)	5g
NaCl	200g
Tris	12.1g
Salty solution*	50mL

*) Salty solution

MgCl ₂ ·6H ₂ O	40g
KCl	4g
CaCl ₂ ·2H ₂ O	0.4g
Distilled water	100mL

It is required to adjust the pH of the solution to 7.4 by adding few drops of HCl; fill up the container with distilled water to obtain 1L of culture medium; finally, autoclave medium for 15min at 121°C.

B. M2-Agar (20% NaCl medium for experiment 1):

As under point A; to obtain a solid culture medium, add 20g of agar per L of solution before autoclaving the culture medium;

C. M2.5 (5% NaCl medium experiment 1):

As under point A; instead of adding 200g of NaCl only 50g are added to the solution; likewise, to obtain a solid culture medium, add 20g of agar per L of solution before autoclaving the culture medium;

D. *Acidianus* Medium (experiment 3 and 4):

Enriched minimum medium for *Acidianus brierleyi*; amounts given to obtain 1L of culture medium;

(NH ₄) ₂ SO ₄	3g
K ₂ HPO ₄ ·3H ₂ O	0.5g
MgSO ₄ ·7H ₂ O	0.5g
KCl	0.1g
Ca(NO ₃) ₂ ·4H ₂ O	0.01g
Distilled water	980mL
Yeast extract	0.4g
Elemental Sulfur	10g

Adjust pH with diluted H₂SO₄ to the desired values (i.e. pH 2 for experiment 3 and pH 6 for experiment 4); autoclave solution and yeast extract /w/o solution) separately at 121°C for 15mins and mix them after the sterilization procedure.

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Web related sites:

- Introduction to Archaea: <http://www.ucmp.berkeley.edu/archaea/archaea.html>
- Domain Archaea - phylogenetic tree: <http://trishul.sci.gu.edu.au/~bharat/courses/ss13bmm/archaea.html>
http://www.sidwell.edu/us/science/vlb5/Labs/Classification_Lab/Archaea/
- Interesting links to Archea: <http://geta.life.uiuc.edu/~nikos/archaealinks.html>
- International Microbial Journal: <http://archaea.ws/>
- Archaea: <http://www.microbe.org/microbes/archaea.asp>