Sulfur-cycle, nitrogenase and carbon utilization in red „Berries“

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Abstract:

Red and green microbial aggregates, which are called “berries”, can be found in great and little Sippewissett Marsh, in Woods Hole. The red microbial aggregates consist of syntrophic bacterial consortia mainly including purple sulfur bacteria (PSB), sulfate reducers (SRB) and cytophaga. In this study I focus on the cryptic sulfur cycle, the nitrogenase activity and the carbon utilization within the berries. In contrast to previous studies the sulfide oxidation assay could not be reproduced because sulfide was degraded even under abiotic condition. But a trend that under dark condition the sulfide oxidation is slower than under light condition could be observed. In addition to that it is shown that under dark condition acetate and hydrogen are used as electron donors for sulfate reduction whereas butyrate and glycolic acid seems not to electron donors for sulfate reduction. Furthermore acetylene reduction assay confirm nitrogen fixation within the Berries, which was already indicated by nanoSIMS experiments in 2010. Under day and night incubation conditions first indications for acetate utilization were observed by HPLC analyzes. These experiments are the first attend to explore the physiology especially on the red berries, but further experiments need to be done.

Introduction:

Red and green microbial aggregates, which are called berries, are found in great and little Sippewissett Marsh, in Woods Hole. In the marsh they exist only in small ponds mainly attached to grass on the bottom of the pond. The red microbial aggregates consist of syntrophic bacterial consortia mainly including purple sulfur bacteria (PSB), sulfate reducers (SRB) and cytophaga. These bacteria geni were identified by 16S bacterial clone library analysis, which were performed by Udi Banin (course participant, 1997). Seitz et al. and Verena S. (course participant, 2011) demonstrated that purple sulfur bacteria are the dominant species in the red berries.

The closest related characterized SRB to the one in the red berries is *Desulfofustis glycolicus*. *Desulfofusitis glycolicus* was characterized by Friedrich M. et al. and gains his name based on its favored carbon substrate glycolic acid. Interestingly sulfate-reducing bacteria obtain their energy by oxidizing organic compounds or molecular hydrogen to reduce sulfates to sulfides (Brock).

The closest related characterized purple sulfur bacterium (PSB) to the one in the red berries is *Halochromatium roseum*. *Halochromatium roseum* is a non-motile, marine, rod-shaped phototrophic gammaproteobacterium with gas vesicles (Kumar et al. 2007). The purple sulfur bacteria belong to the group of proteobacteria, which absorb light in the infrared spectrum for their anoxygenic phototrophic metabolism. These bacteria oxidize hydrogen sulfide and generate granules of elemental sulfur, which in turn are oxidized to form sulfuric acid.

Nothing is known so far about the carbon sources, which are utilized in the microbial consortia of the berries. This study is the first attend to get an idea about the carbon cycle. For the nitrogen and sulfur cycle some work was already done (Report 2010), which could be continued in this study.

METHODS + MATERIALS+ INCUBATION CONDITIONS

For each experiment vial was autoclaved before usage.

Berry sampling

Green and pink berries were collected from a pond at Great Sippewissett Marsh 1day prior to incubation experiments started. The pH of the pond was 7.9 and the temperature was 29 °C. Most of the Berries were associated with grass on the bottom of the pond. The depth of the pond water was about 20 cm. Berries were collected with a sieve and transferred to the lab in a 2L bottle filled with pond water.

Washing and separating of Berries

To remove big particles and sand Berries were roughly washed by filtrated but not sterilized SW. Afterwards the Berries were washed 3 times with filtrated sterilized (poresize= 0.2 μmeter) Marsh water and stored in filtrated water at room temperature. Don't store Berries longer than 2 days prior your final experiment. Washing with sterilizes water is really important to minimize bacterial contamination.

Acetylene reduction assay for N_2 fixation

Acetylene production and GC measurement

Acetylene was formed by a protocol given by Steven Zinder. In a 150 ml serum bottle 0.5 g CaC_2 was added and afterwards the bottle was evacuated. In the anaerobic chamber 1 ml anaerobic water was added with 1 ml syringe. Immediately acetylene gas was generated. Don't put more calcium carbide into the serum bottle because of explosion risk. 5 ml of produced acetylene was transferred to the incubation vials and the amount was measured by gas chromatography (GC) at time point zero. 100μl of the vial headspace was added to the GC for analyzes and the area% of acetylene and produced ethylene was recorded. These values can be converted into mol/L.

Incubation condition

Fifteen red Berries were incubated anaerobically in 5 ml anoxic, filtered marsh water in a 55 ml vial. The pH was 7.9. Additionally to the filtrated marsh water 0.5 mM NO_3^-, 0.5 mM NH_3 or N_2 (in headspace) were added as N-sources. Each vial was sealed

with blue thick rubbers and incubated at the window board at room temperature. In all cases, except the abiotic conditions, fifteen berries were added to the sterile pond water. An overview of the experimental setup is also provided below.

Incubation condition
SW+0.5 mM NO_3^-+acetylene
SW+0.5 mM NO_3^-+acetylene+berrries
SW+N_2+acetylene
SW+N_2+acetylene+ berries
SW+0.5 mM NH_3+acetylene
SW+0.5 mM NH_3+acetylene+ berries

Cline assay

For sulfide concentration determination the cline assay is a rapid method to do so. In this assay sulfide reacts with N,N,-dimethylphenylenediamine to form the leuco form of methylene blue, which is oxidized by Fe (III) to blue form. This change can be determined by its absorbance at 670 nm. The protocol was kindly provided by Steven Zinder.

Sulfide consumption assay

The incubation conditions for this experiment are shown in table 1. All incubations were carried out anaerobically, at pH 7.9 , 30 °C and in replicate. In all cases, except the abiotic conditions, fifteen berries were added to the sterile pond water.

Table1: Incubation condition for sulfide oxidation

Incubation condition	
Abiotic+500µM H_2S	light
Abiotic+500µM H_2S	dark
Red Berries 500µM H_2S	light
Red Berries 500µM H_2S	dark

Sulfide production by SRB

The incubation conditions for this experiment are shown in table 2. All incubations were carried out anaerobically, at pH 7.9, 30 °C and in replicate. In all cases, except the abiotic conditions, fifteen berries were added to the sterile pond water. Importantly these experiments were carried out in dark condition to reduce the purple sulfur bacteria activity to explore which compounds might be utilized by sulfate reducer.

Table2: Incubation condition for sulfide production

	Incubation conditions
1	SW+0.5mM sulfate+Berries
2	SW+0.5mM sulfate+Berries+**1 mM acetate**
3	SW+0.5mM sulfate+Berries+**1 mM butyrate**
4	SW+0.5mM sulfate+Berries+**1 mM glycolic acid**
5	SW+0.5mM sulfate+Berries+**H_2**

HPLC analysis – Carbon source determination

Sample treatment

450 μL of the incubation medium was taken from each sample and 50 μL 5 N H_2SO_4 was added to the sample. The acidified sample was spin down for 5 min highest speed. Supernatant was applied on a HPLC vial and 20 μL were analyzed.

HPLC analyzes to determine the carbon utilization within the berries

I selected six different carbon sources for the berries to cover a huge range of the most promising and diverse carbon sources. As shown in the table 3 I planed to use CO_2 because nanoSIMS data indicated ^{13}C sodium bicarbonate accumulation within the berries. Acetate, ethanol, butyrate and butanol were chosen because of different potentials to donor or accept electrons and because they differentiate in their carbon skeleton size. Smaller and uncharged molecules enter cells much more easier, if no transporter are available. Glycolic acid was chosen because *Desulfofustis glycolicus*

is the closest related to the SRB within the berries and the main carbon source is glycolic acid.

Table 3: Putative carbon sources for berries

CO_2	**acetate**	**ethanol**	**butyrate**	**butanol**	**glycolic acid**
^{13}C sodium carbonate accumulation detected by nanoSIMS	C-2 acid, electron donor	C-2 alcohol, electron acceptor	C-4 acid, electron donor	C-4 alcohol, electron acceptor	utilized by *Desulfofustis glycolicus*

As shown in the picture 1 I planed to incubate the different carbon sources without berries, with only red berries, with green and red berries and only with green berries. These samples were unfortunately not analyzed by HPLC because the machine did not work most of the time.

Picture 1: Selection of incubation vials for HPLC analysis.

DAPI staining

Before starting the experiment the filter tower was assembled with a moistened cellulose nitrate support filter and then the white polycarbonate membrane filter both with a diameter of 17.5 mm. Importantly the shiny side of the polycarbonate membrane filter has to face up. Subsequently 1 ml of sample was applied to a filter tower and the entire sample has passed through at a gentle pressure (200 mBar) to filter the sample. After washing the filter with 1 ml of sterile 1x PBS buffer, the membrane was marked with a pencil and cut into peaces (circa 1/8 of the entire filter). The filter were sucked in the DAPI embedding solution (provided by Sara / TA)

and covered by a cover slide. Prior counting the cells the slide was incubated in -20°C for 10 minutes.

Results:

The cryptic sulfur cycle

In this section I was working on the cryptic sulfur cycle, which is shown in figure 8. For this purpose I have performed two experiments. In the first experiment I focused on sulfide consumption most likely driven by purple sulfur bacteria (PSB) and in the second experiment I investigated the production of sulfide by sulfate reducing bacteria (SRB) to close the S-cycle.

<u>Sulfide consumption by red berries</u>

Sulfide consumption was investigated under different incubation conditions, which are mentioned in method and material section. To determine the sulfide consumption the cline assay was used. In this experiment it is crucial to work under strictly anaerobic condition to avoid sulfide oxidation by oxygen. Unexpectedly the sulfide concentration decreased even under abiotic (without berries) condition. I repeated the assay three times, but all results were similar to the one, which is shown in figure 1. Sulfide was degraded faster under light condition than under dark condition.

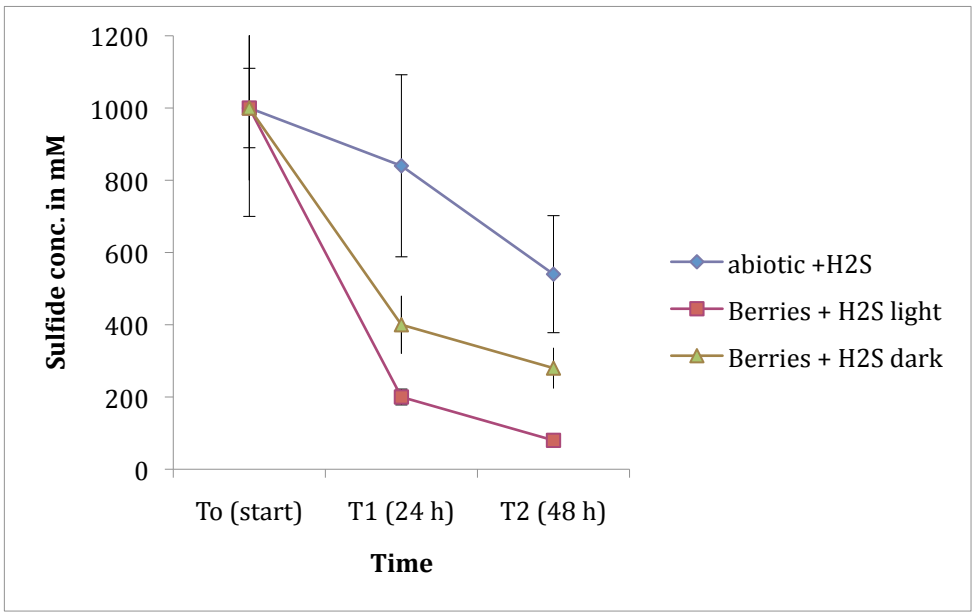

Fig.1: Sulfide consumption by red berries. Abiotic control represents incubation condition without red berries. Medium including red berries are incubated under light or dark condition.

Sulfide production by SRB within berries

Sulfide production was investigated in the presence of various possible electron donors. The incubation conditions are mentioned in method and material section. To determine the sulfide production again the cline assay was used.

Also for this experiment it is crucial to work under strictly anaerobic condition to avoid sulfide oxidation by oxygen. The obtained data indicate sulfide production in the presences of hydrogen and also but in much smaller amounts for acetate condition. In the abiotic, butyrate and glycolic acid condition no production of sulfide was detected (fig.2).

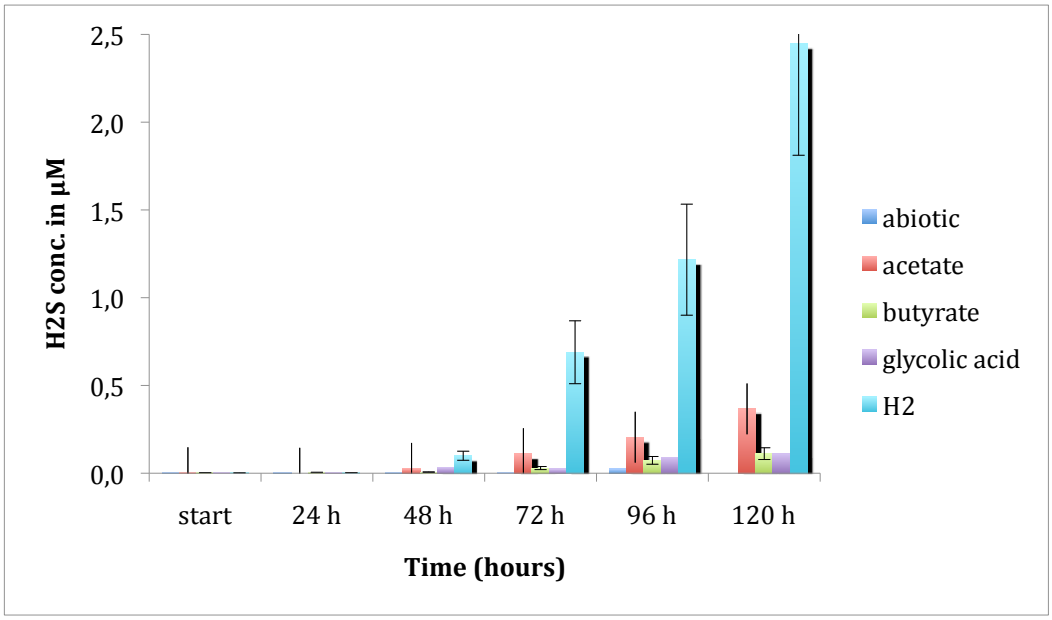

Fig. 2: Sulfide production in red berries under various possible electron donors in dark condition. Sulfide is mainly produced in hydrogen and in minor quantities in acetate incubation.

Sulfide production started after 2 days. Expected was a more rapidly production of sulfide driven by red berries. To verify that the production of sulfide was not conducted by sulfate reducer except the berries associated I counted the „free living“ cells via DAPI staining in the media. For the abiotic, acetate and hydrogen condition I counted $7.5x10^5$, $3x10^6$ and $8x10^5$ cells/ml, respectively. These results show that the cell numbers in hydrogen supplied condition is comparable to the cell numbers to the abiotic condition, where no sulfide production could be observed.

Assaying Nitrogenase: Acetylene Reduction

To assay nitrogenase within the red berry the acetylene reduction assay was performed. For this purpose red berries were incubated with different N-sources (N_2, NO_3^- and NH_3) and the ethylene production was measured by gas chromatography. As shown in figure 3 no ethylene was produced when ammonium was added. The slightly different ethylene concentrations between the abiotic control and the incubation with red berries are due to standard error for the ethylene detection by GC. In contrast to that ethylene was significantly produced in the presence of nitrate and nitrogen, latter 3 times up to 320μM ethylene after 5 days.

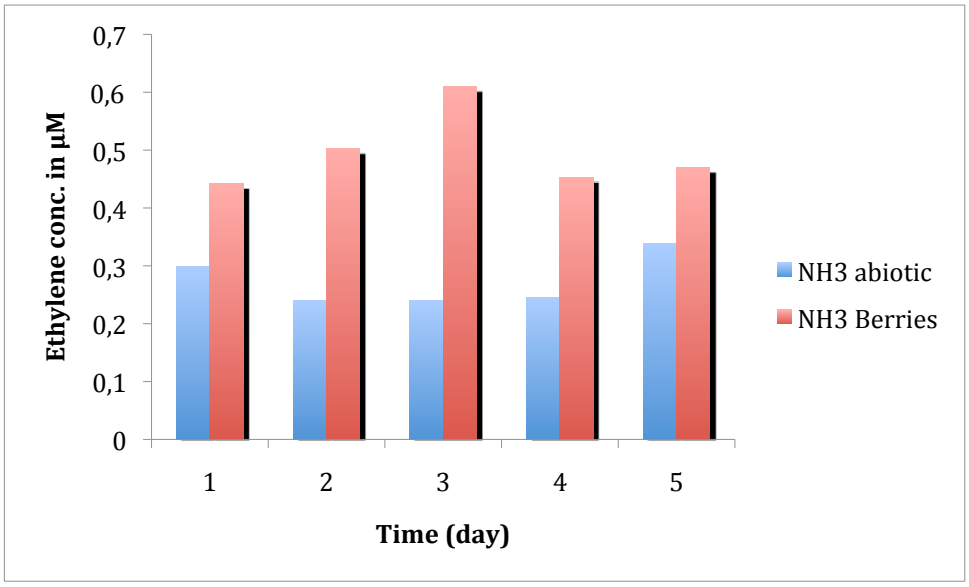

Fig. 3: Ethylene production in red berries in the presence of 500μM ammonia

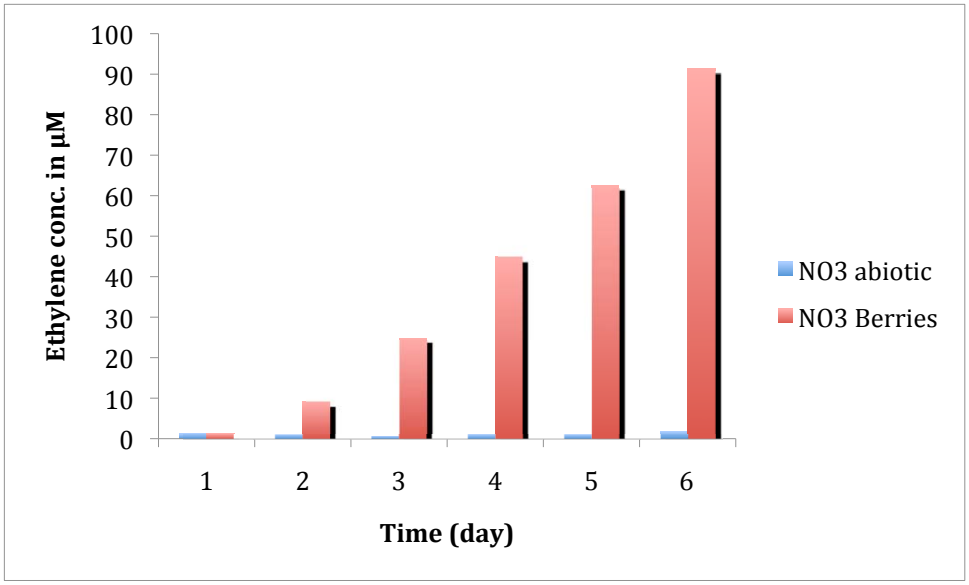

Fig. 4: Ethylene production in red berries in the presence of 500*µ*M nitrate.

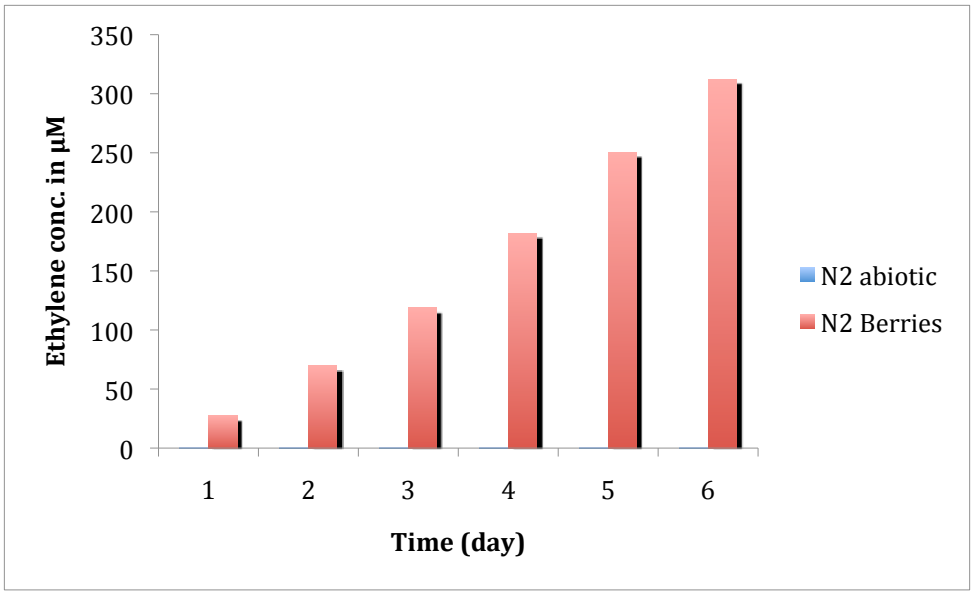

Fig. 5: Ethylene production in red berries in the presence of nitrogen.

Carbon utilization within the berries – HPLC analysis

-HPLC machine was almost the whole time out of order-

Nothing is known so far about the carbon sources, which are utilized in the microbial consortia of green and red berries. A first attend was started with six different carbon sources covering the most promising and diverse carbon sources as substrates for berries. They are listed and described in the method section.

Because the machine was only running a few days and many participants of the course used the HPLC I could only run a small fraction of my prepared samples. This small fraction was in part contaminated by other participant samples!

Two time points for acetate (fig.6) and glycolic acid (fig.7) incubation were finally analyzed. The acetate concentration did not change in abiotic and red and green berry mix condition. For the red and green berry only incubation the acetate concentration drop down indicating consumption of acetate.

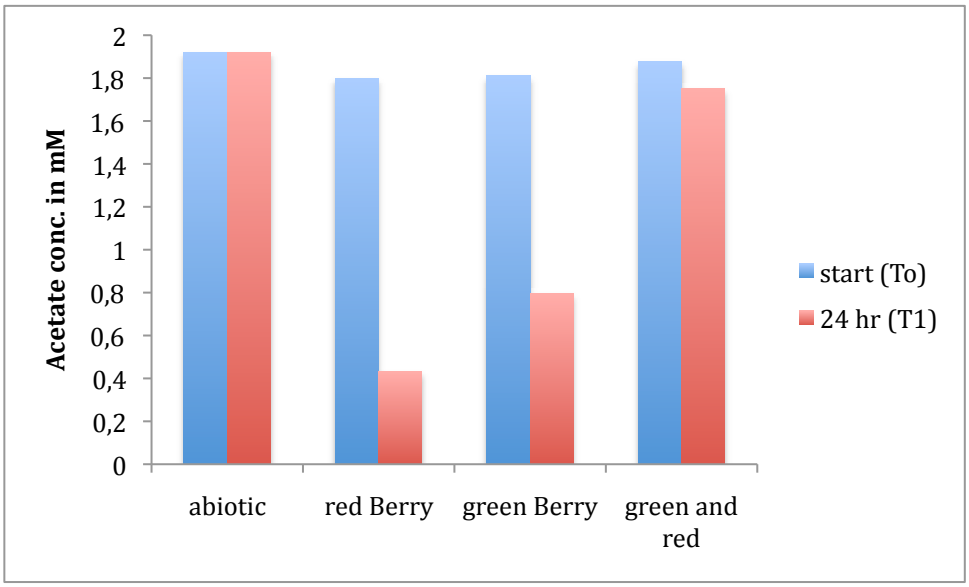

Fig. 6: HPLC analysis of acetate consumption in berries.

Glycolic acid concentration did not change in any incubation condition indicating that glycolic acid is not a carbon source for berries.

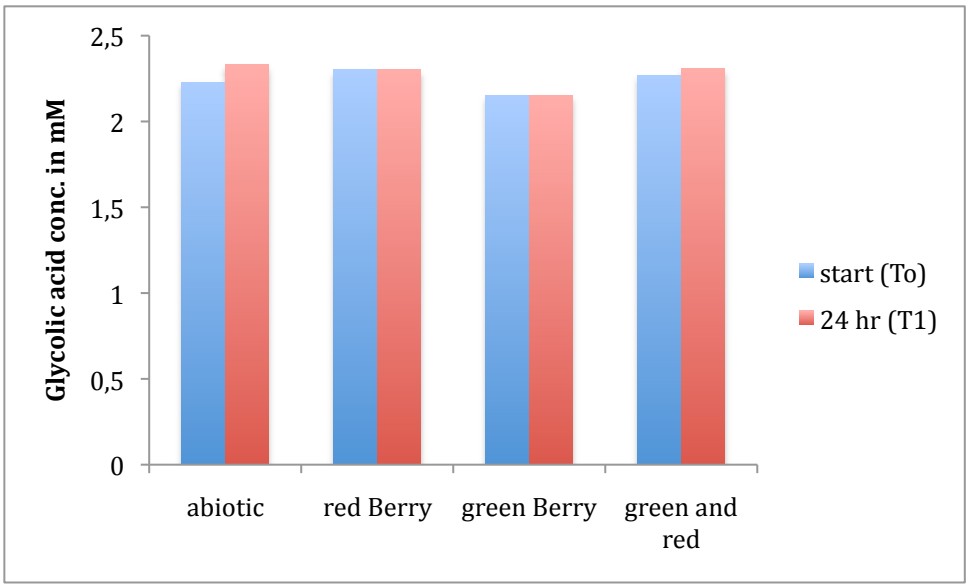

Fig. 7: HPLC analysis of glycolic acid comsumption in berries.

Discussion

In the first part of my study I investigated the sulfide consumption and production in two different experimental approaches. The sulfide consumption experiment was performed under dark and light condition. As shown in figure 8 sulfide consumption is executed by the PSB, which absorb light in the infrared spectrum for their anoxygenic phototrophic metabolism. Under dark condition the PSB cannot use the anoxygenic photosynthesis, which should inhibit their metabolism. And indeed the sulfide consumption in dark condition was slower compared to light condition. The obtained results fit nicely to the S-cycle hypothesis but the experiment has to be repeated because in the abiotic condition (without berries) sulfide was also slowly oxidized.

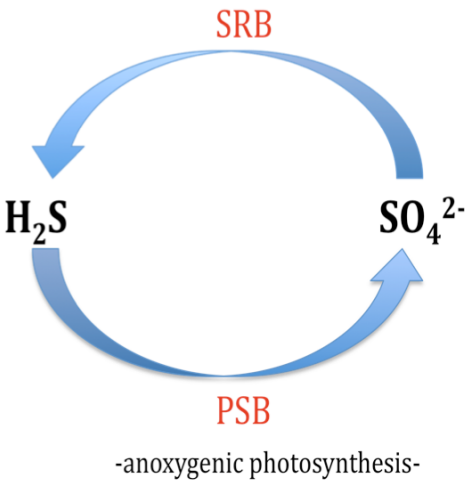

Fig.8: Simplified proposed S-cycle in red berries

For the sulfide production experiment I incubated the berries in dark condition with sulfate and different putative electron donors. Dark conditions were required to reduce the PSB activity because of the following reasons: 1) I wanted to determine the electron/carbon sources especially of the SRB 2) active PSB could still feed SRB with electron rich compounds which would impair the uptake of the external compounds (no difference would be visible between the different incubations) 3) active PSB could immediately oxidize the produced sulfide which would make it impossible to measure sulfide production. **Hydrogen** seems to play the major role for the tested compounds.

This is an agreement with the experiment from Verena Salmann (also this year participant).

What is about the nitrogen fixation in the red berries? NanoSIMS experiment revealed $^{15}N_2$ fixation in red berries. To confirm these results I performed the acetylene reduction assay, which is a rapid and sensitive method to measure nitrogenase activity. The results clearly show an ethylene production when no ammonia is present in the medium. Interestingly the ethylene production rate is faster in the nitrogen-supplied condition in contrast to the nitrate condition even so that both conditions are equally depleted for ammonia. The only reasons I can imagine are that nitrogen gas has a stimulating effect or/and nitrate has an inhibiting effect on the nitrogenase activity.
At the end I want to mention the acetylene reduction assay does not show a direct nitrogen fixation but at least the assay demonstrates that the cells want to fix nitrogen, because acetylene is a competitive substrate/inhibitor to nitrogen.

Nitrogenases catalyzing the following reaction: $\mathbf{N_2 + 8\ H^+ + 8\ e^- \rightarrow 2\ NH_3 + H_2}$**.** Interestingly per two molecules ammonium one mole hydrogen is formed as a side product. We know from the sulfate reducing experiment mentioned above that hydrogen is most probably an electron donor for SRB. This finding offers another cycle between PSB and SRB, when nitrogen is fixed in the PSB.

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