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Investigations of Pink Berry Cross-Feeding

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Abstract

Pink berries are macroscopic bacterial aggregates that grow on the sediment surface of the small tidal ponds in Great and Little Sippewissett salt marsh. The phylogeny of the Pink Berry organisms has been well described in the resent years with the easy access to high throughput sequencing. The key organisms are purple sulfur bacteria (PSB); sulfate reducing bacteria (SRB) and heterotrophic bacteria of the *Cytophaga*. The cooperation between these organisms has also been studied for decades, but unlike the phylogeny little is still known. In this study the cross-feeding between Pink Berry organisms was investigated. Stable isotope probing was attempted to unveil possible transfer of organic molecules from the PSB to other Pink Berry organisms. Hydrogen has been speculated as a possible electron transfer molecule. In this study it was shown that this is unlikely, though acetate and glycolate should instead be considered as possible transfer molecules. The oxygen penetration of the Pink Berry was investigated using micro sensors. These measurements showed that the majority of the Berry is oxic.

Introduction

Syntrophy between microorganisms is being considered more often these days when describing microbial ecology, though many of these microbe-microbe interactions are difficult to investigate and therefore often unresolved. The "Pink Berry" microbial consortium is no exception. It has been investigated by students of the Microbial Diversity Course for decades and yet most physiological questions still remains unanswered.

The Pink Berries are found in tidal ponds of Great and Little Sippewissett salt marsh. The two salt marshes are characterized by a large tidal exchange of sea water two times a day and by organic and sulfide rich sediment. The Berries lie on the sediment surface of the ponds, but tend to be most abundant in deepest areas of the ponds as well as in small depressions in the sediment. This is due to the density of the berries, which is only few per mill above that of seawater (Seitz, Nielsen et al. 1993). Thereby the Berries remain on the sediment surface where light and oxygen is plenty, but avoid being flushed to sea by the daily tidal fluctuations.

In 2010 a large group of student of the Microbial Diversity course dedicated their project work to reveal secrets of the Pink Berry (Wilbanks 2010). They reported in line with earlier student project that the consortium consists mainly of three key organisms. The most abundant organism in the consortia is purple sulfur bacteria (PSB) of the family Chromatiacaea. Sulfate reducing bacteria (SRB) close related to *Desulfofustis glycolicus* (Friedrich, Springer et al. 1996) and long slim rodshaped *Cytophaga* are also abundant. The students saw that the PSB were located in large compartment while the *Cytophaga* and the SRB were found in the extracellular matrix of the Berries. This observation was confirmed in a student project the following year (Salman 2011). The same group of students showed that the Berries have an internal sulfur cycle where sulfate is reduced by the SRB and directly taken up by the PSB. In a short term experiment, they also proved that CO_2 uptake by the Berries is independent unaffected by sulfide concentrations below 0.5 mM, but inhibited by higher concentrations.

Although comprehensive work has been done on the Pink Berry consortia and some of the fundamental physiological questions have been answered, most still remains a puzzle. One of the major questions is the interaction between the SRB and the PSB. The SRB provide sulfide for the PSB, but it is unclear whether the PSB provide anything in return and thereby feed the SRB to increase the sulfide production. Another key question is the role of the *Cytophaga* in the Pink Berry, which so far remains a mystery. It was proposed that they feed on the extracellular matrix of the berries and that they provide an oxygen barrier for the PSB and SRB.

The aim of this project was to investigate unresolved cross feedings taking place inside the Pink Berries. The role of *Cytophaga* in the consortia was investigated by looking at their uptake of PSB products. Furthermore hydrogen was investigated as possible transfer molecule between the PSB and the SRB within the Consortia.

Material and methods

Sampling

The Pink Berries used in all experiment were freshly sampled from a pond at Little Sippewissett (Supplementary figure S1). The berries were sorted from the sediment by washing the samples through a 1 mm sieve. This way of sampling gives an unintentional selection of berries above 1 mm in size. After sampling the berries were kept at 4 °C until use. Prior to any incubation study the berries were washed twice in 0.2 µm sterile filtered Little Sippewissett pond water to remove all non associated particles.

Enrichment of Pink Berry *Cytophaga*

Berries were sliced in half and placed with the cut surface on either a high (SP2) or a low nutrient (SP6) agar plate based on peptide medium:

SP2:
1 g/L Casitone
0.2 g/L Yeast extract
0.02 g/L Acetate
6.4 g/L $MgSO_4 \cdot 7H_2O$
15 g/L Agar

SP6:
0.3 g/L Casitone
0.1 g/L Yeast extract
6.4 g/L $MgSO_4 \cdot 7H_2O$
15 g/L Agar

PH adjusted to 7.2 using NaOH.
After autoclaving 0.2 g/L of sterile filtered $NaHCO_3$ was added.

Replicates of the enrichment were incubated both oxic and anoxic at room temperature. After two days of incubation the periphery of the colony was sampled and streaked onto new agar plate. This was based on the fact that *Cytophaga* is known to exhibit gliding motility. From each plate the organisms were transferred three times to enrich for a pure culture. The enrichments were followed by microscopy and all end point enrichments were evaluated using PCR with specific 16S rRNA *Cytophaga* primer CF316 (5'- CTGGTACTGAGACACGGA -3') (Kirchman, Yu et al. 2003) in combination with general bacterial 16S rRNA primer 1492R. Amplification of 16S rRNA genes was done with 5 minutes of initial denaturation at 95 °C followed by 30 cycles of 30 sec. denaturation at 95 °C; 1 min. of annealing at 55 °C and 1.5 min. of elongation at 72 °C and a final elongation of 10 min. at 72 °C.

Stable Isotope Probing

To investigate the cross feeding of *Cytophaga* on metabolites or EPS produced by the PSB, heavy labelled $^{13}CO_2$ was used for incubation. One gram of berries was incubated in each of two 150 ml glass vials each containing 30 ml sterile filtered Little Sippewissett pond water. To each of the two vials 292 µmol of $NaH^{13}CO_3$ was added to the normal atmospheric gas phase resulting in a CO_2 concentration of 5 %. H_2S to a final concentration of 20 µM was added each vial to get the CO_2 fixation by the PSB started, relying on the internal berry cycle to provide additional H_2S. As control a similar microcosms was setup using unlabelled $^{12}CO_2$.

The microcosms were incubated for seven days on a window self facing east to provide as natural light conditions as possible. The ambient temperature ranged from 25 to 35 °C.
DNA was extracted at the end of incubation using PowerBiofilm™ DNA Isolation Kit (MoBio) including one minute of bead beating. The extract was purified on a 1.5 % agarose gel followed by cutting out DNA fragments above 3000 bp and gel extracting. The dissolved DNA was concentrated by vacuum centrifugation. The amount of purified DNA was quantified using Nanodrop2000.
An ultracentrifugation tube was setup up by adding 7.5766 g CsCl to 4.3 ml of gradient buffer (15mM Tris-HCl, pH=8.0; 15mM EDTA; 15mM KCl), which results in a final gradient density of 1.69 g/ml. The exact density of the gradient media was achieved using a refractometer and adjusted using additional gradient buffer.

$$Density\ \left(\frac{g}{ml}\right) = 10.9276 \times Refractive\ index - 13.593$$

To each ultracentrifuge tube 5.5 µg of DNA and 200 µl of TE were added and all tubes were balanced with additional TE. The ultracentrifuge tubes were mixed by inverting and centrifuged at 55000 rpm for 65 hours, at 20°C in vacuum.

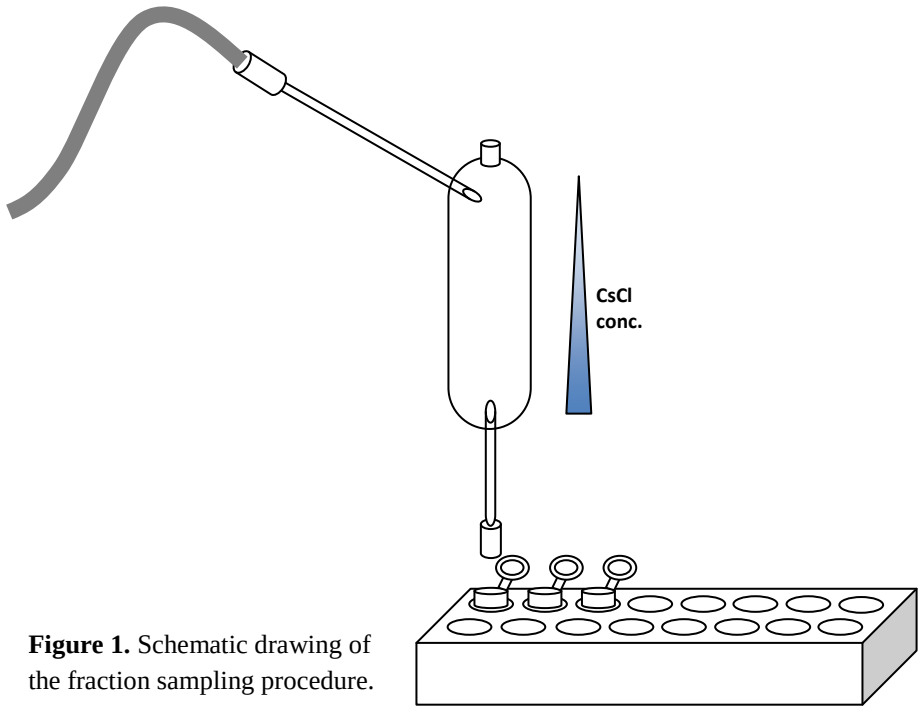

Figure 1. Schematic drawing of the fraction sampling procedure.

After the gradient was formed it was separated in fractions of approximately 0.1 ml.. This was done by introducing 0.1 ml of water via a syringe needle at the top of the column and collecting the same amount trough a syringe needle at the bottom (figure 1). Immediately after sampling of each fraction the refractive index was measured and converted to density. Before further processing was possible each fraction was washed to remove the CsCl salt. This was done by adding 3 volumes of

DNA free water and 2.4 volumes of isopropanol followed by mixing and 30 min. of centrifugation at 15,000 rpm. After centrifugation the supernatant was poured of and the pellet was washed two times by adding 1 ml ethanol, vortexing and centrifugating at 15,000 rpm for 15 min. The ethanol supernatant was poured off and the pellet was air dried upside down on paper towel until no liquid was visible. The dried pellet was resuspended in 50 µl DNA free water.

Quantitative PCR

The fractionation of the Stable isotope probing DNA was evaluated using qPCR. One ml of each cleaned fraction was transfer to an 96 well optical plate containing QuantiFast SYBR Green and general bacterial primers 515F and 907R. The amplification was done by a two-step cycle with an initial denaturation at 95 °C for 5 min. follow by 35 cycles of denaturation at 95 °C for 5 sec. and annealing and elongation at 60 °C for 30 sec.. A plasmid solution diluted in salmon sperm DNA was used as standard.

Hydrogen production and consumption

Internal hydrogen production by the Berries were investigated by adding 0.5 g of Berries to a 38 ml air tight glass vial containing 5 ml sterile filtered Pink Berry pond water. Sodium molybdate was added to the glass vial to inhibit SRB and the vial was flushed with nitrogen gas for 1 minute to remove all oxygen. Two replicates were made with H_2S added to a concentration of 500 µM and 50 µM, respectively. The vials were placed in front of a day light lamb at 37 °C and the hydrogen concentration was followed the next five days.

The Berry hydrogen consumption was investigated by a setup similar to the hydrogen production experiment. However, instead of adding molybdate and H_2S 1 % hydrogen of the total gas phase was added to the glass vials. Six of these microcosms were fabricated. Two were incubated in front of a day light lamp at 37 °C, of which one contained intact berries and one completely grinded Berries. Two were incubated in the dark at 30 °C and two in front of a 850 nm LED lamp all at 30 °C. All vials were followed for three days.

Hydrogen measurements were done on a Gas Chromatograph.

Oxygen profiling of berries

Pink berries washed twice in sterile filtered pond water were embedded in 1 % low melting point agarose (Promega) using sterile filtered pond water. The pond water agarose was made either with no addition of organic compound or with addition of acetate or glycolate. The pH of the media was adjusted to 7.2 and autoclaved. When the media had cooled to 37 °C it was poured into a Petri dish and the cleaned berries were distributed in the agarose. When solidified each single berry was cut out in a cube of agarose and placed upside-down onto a clean agarose surface and incubated at room temperature for 2 hours.

The oxygen profile of the berries was measured using oxygen micro sensors (unisense.com) with a tip diameter of 25 µm. The oxygen profile of the berries was measured all the way through the centre of the consortia. Oxygen flux to the berries was calculated using the linear regression of the diffusive layer of each profile. The oxygen diffusion flux was calculated (J) by Fick's 1. Law:

$$J = -D \times \frac{dC}{dx}$$

Where D is the diffusion coefficient in the given medium and dC/dx is the slope of the change in concentration with depth in the diffusive layer.

Results & Discussion

Enrichment of Pink Berry *Cytophaga*

After two days of incubation berries on both high and low nutrient agar plates and plates from both oxic and anoxic showed colony formation (figure 2A-C). No single colonies were observed on any of the restreaks. Instead transparent branching colonies were observed on all plates (figure 2D-F).

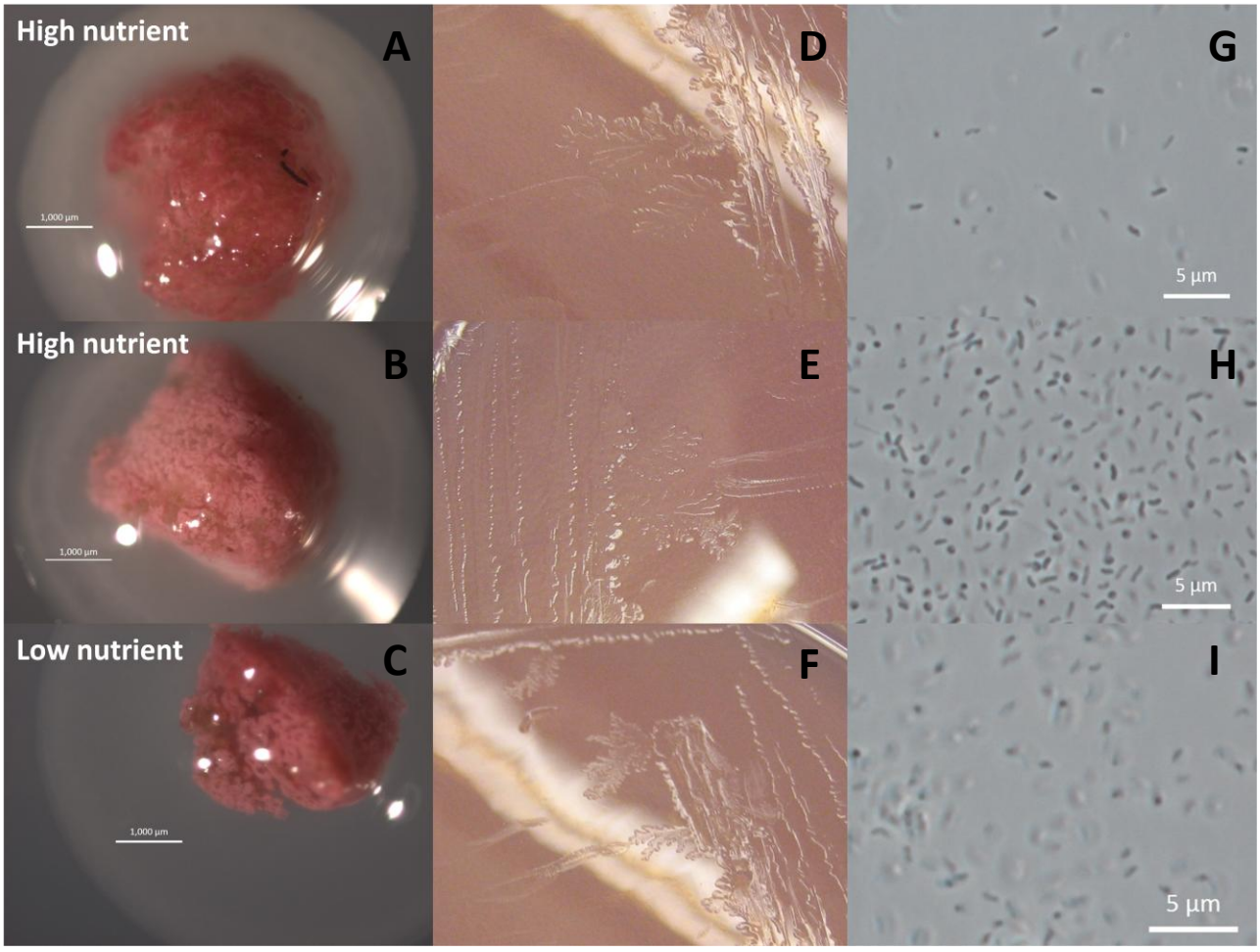

Figure 2. Microscopy observations of the *Cytophaga* enrichment during (A-C) sliced Berries colony formation; (D-F) restreaking to look for single colonies; and (G-I) single cell morphology of third streak enrichment.

As shown in figure 2, colonies forming from a Berry cut in half tended to be thinner and with larger diameter when grown on low nutrient agar compared to colonies on high nutrient agar. Furthermore the colonies showed branch-like spreading after restreaking, both behaviors in good correlation with *Cytophaga* gliding motility. The observation that no single colonies were forming when streaking

for isolation was an expected possibility, based on the fact that these organisms live as a consortium and therefore might be dependent on mutual growth.
All third generation plates were investigated for presence of *Cytophaga* using specific *Cytophaga* primers and using the whole Berry extract as positive control. None of the enrichments amplified and were therefore not containing any *Cytophaga*. Observations of the third generation enrichment showed presence of 1 µm long rod-shaped cells (figure 2G-I). *Cytophaga* were expected to be long slim rod shaped (Madigan 2012).
All enrichments were checked for SRBs using sodium molybdate as SRB-inhibitor. No inhibition of the enrichments was observed compared to the sodium sulfate control (Figure 3).

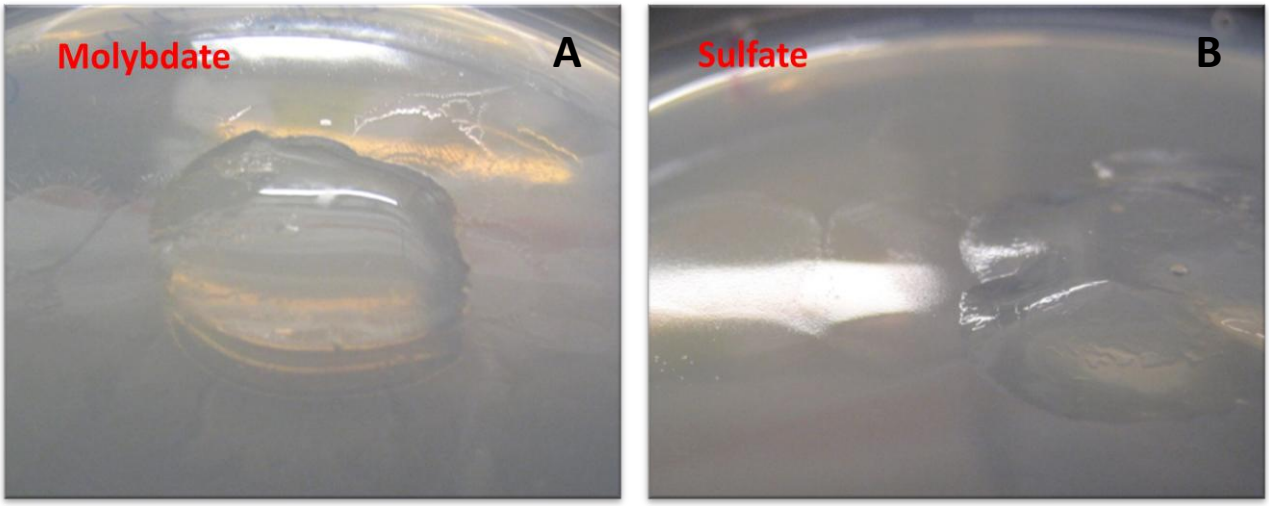

Figure 3. Test for presence of sulfate reducing bacteria with molybdate (A) added to inhibit the sulfate reducers and sulfate (B) is added as control.

Stable Isotope Probing

qPCR of each fraction from both labelled and unlabeled samples were plotted with copy number as a function of fraction density (figure 4; S4). The graph shows that the unlabelled sample contains DNA in fractions that are both lighter and heavier than those of the labelled sample. This means that no or very little DNA was labelled in the sample where $^{13}CO_2$ was added. If there had been a high incorporation of $^{13}CO_2$ there would have been a shift towards higher density in the qPCR curve of the heavy labelled sample compared to the unlabelled. The lack of heavy label incorporation is likely due to insufficient growth of the Pink Berry. When the incubated cells do not divide DNA is not amplified and therefore the label in not incorporated.

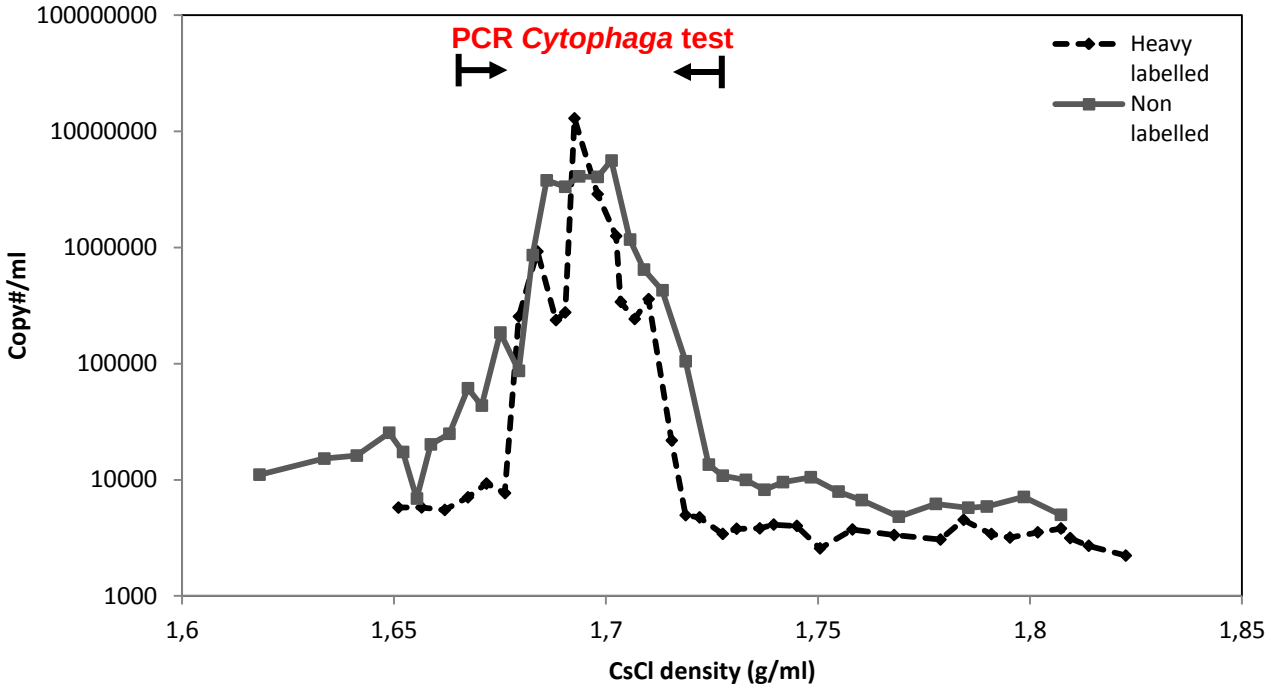

Figure 4. Quantitative PCR of density fractionations from $^{13}CO_2$ labelled berries and of control berries. Arrows indicate fractions tested for presence of *Cytophaga* using specific primers.

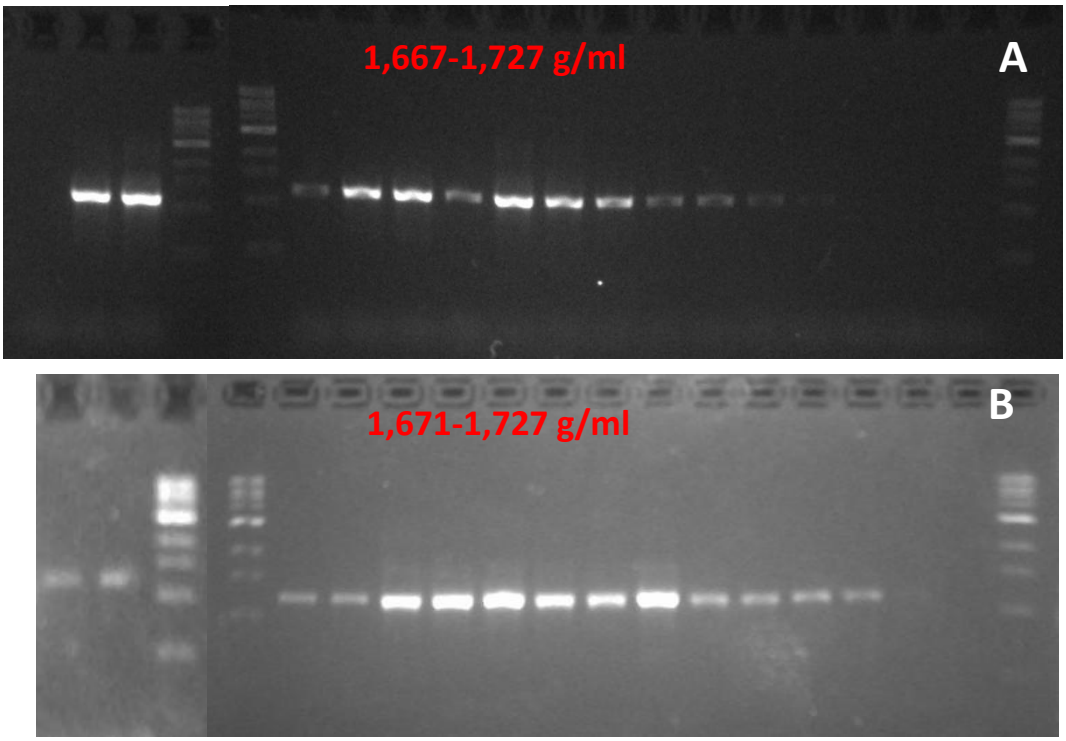

Figure 5. PCR to test for presence of *Cytophaga* in (A) heavy labelled SIP fractions with densities of 1,667-1,727 g/ml and in (B) control fractions with densities of 1,671-1,727 g/ml.

To test if there were an incorporation of $^{13}CO_2$ in the *Cytophaga* that was not detected by qPCR a simple PCR test was done. Fractions within the expected density span were tested using specific *Cytophaga* primer (figure 5). In correlation with the result of qPCR the *Cytophaga* PCR test showed that the unlabelled control had *Cytophaga* 16S rRNA genes in higher density fractions than those of the heavy labelled sample.
From the qPCR graph (figure 4) it is also clear that the total amount of DNA quantified is greater in the unlabelled sampled compared to the labelled sample, even though equal amounts of DNA were added to the ultracentrifuge tube prior to centrifugation. This could be due to a high loss of DNA during the isopropanol washing and explain the misleading result of the qPCR and the PCR test.

Hydrogen experiments

The hydrogen production experiment was followed for 5 days and no production of hydrogen was observed during that time. This can either mean that hydrogen is not used as transfer molecule between PSB and SRB or that that the given conditions were not sufficient for hydrogen production. At the end of the experiment it was observed that the Berries turned brown and that liquid turned blue. This indicates that the growth conditions were unfavorable likely due to the addition of molybdate or the absence of oxygen.
The hydrogen consumption experiment was monitored for 76 hours and during that period only half of the added hydrogen was consumed in all of the different treatments. If organisms within the Berries were capable of taking up hydrogen it would have been consumed much faster, therefore the slow uptake must be caused by a contaminating organism. This confirms the rejection of hydrogen as possible transfer molecule.

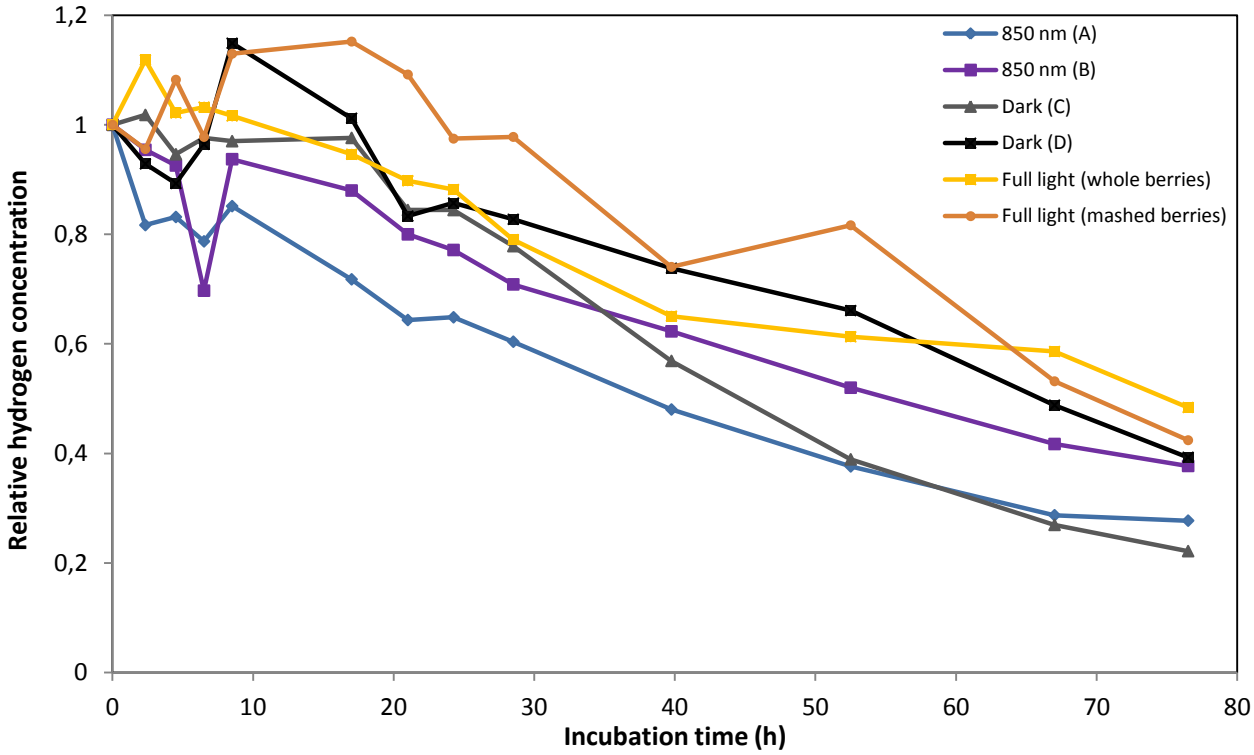

Figure 6. Relative hydrogen consumption as function of time in all six microcosms. All values have been corrected for volume removed at each sampling point.

Oxygen profiling of berries

Oxygen profiles were measured in Berries embedded in agarose either without addition of organic compound or with addition of acetate or glycolate (figure 7). The profiles showed that oxygen penetration of all Berries was below 1 mm. Oxygen penetration of the berries without additional carbon source is approximately 0.6 mm and the Berries diameter is approximately 2.5 mm (measure by microscopy). From this the oxic volume of the Berry can be calculated:

$$V_{total} = \frac{4}{3} \times \pi \times r^3 = \frac{4}{3} \times \pi \times (1.25\text{mm})^3 = 5.24\ \text{mm}^3$$

$$V_{oxic} = \frac{4}{3} \times \pi \times (1.25\text{mm})^3 - \frac{4}{3} \times \pi \times (0.95\text{mm})^3 = 3.60\ \text{mm}^3$$

This means that more than 2/3 of the Berry is oxic. The Pink Berries in their natural environment live on top of the sediment in the small ponds of Sippewissett where oxygen is present. Therefore it seems unlikely that the PSB only grow under anoxic conditions. Furthermore this also rejects the hypothesis of the *Cytophaga* providing an oxygen shield for the PSB.

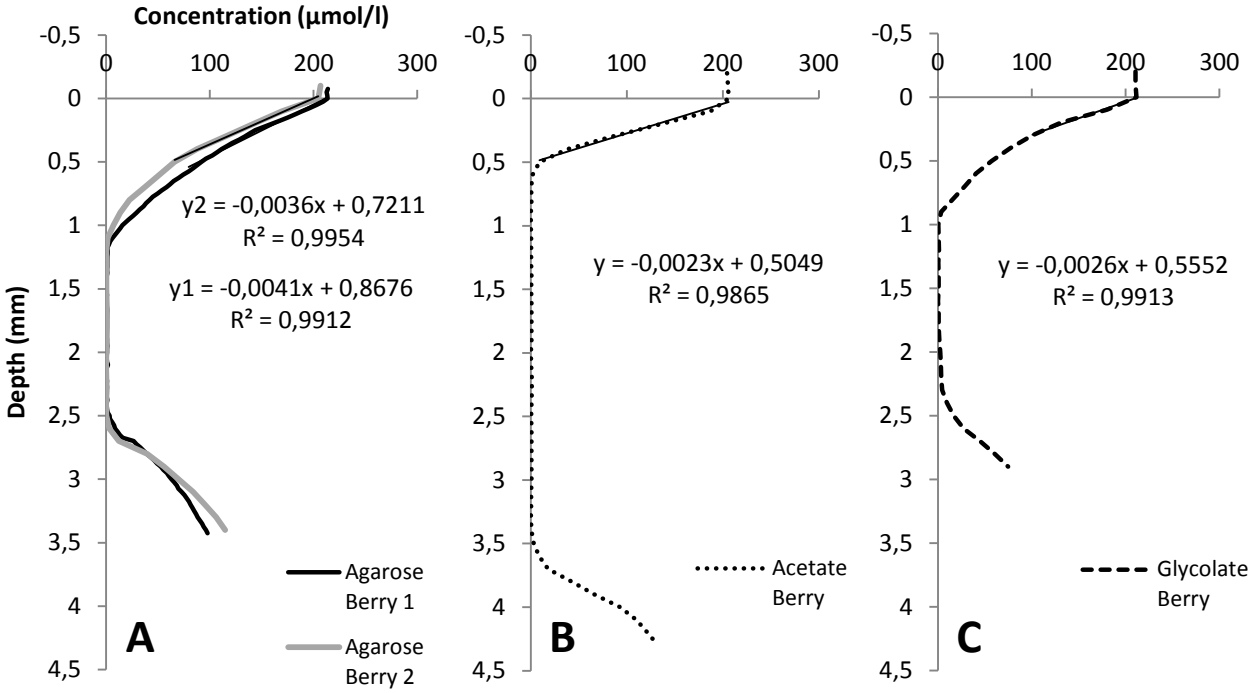

Figure 7. Oxygen profiles of berries embedded in agarose with (A) no organic compound; with (B) acetate or with (C) glycolate added. The equation in each profile indicate the slope and R^2 value of the difusive layer above the berries.

Calculation of oxygen flux to the berries

In order to calculate the flux of oxygen to the berry (i.e. the berry oxygen consumbtion) the oxygen diffusion coefficient was assume to be 2.2×10^{-5} $cm^2\ s^{-1}$ (FERRELL 1967).

Conversion of slope:

$$Slope(Agarose\ Berry\ 1) = -0.0036\frac{mm}{\mu mol/l} = -0.0036\frac{mm}{nmol/cm^3} = -0.00036\frac{cm^4}{nmol}$$

$Inverted\ slope(Agarose\ Berry\ 1) = -2777.78\frac{nmol}{cm^4}$; $Inverted\ slope(Agarose\ Berry\ 2) = -2439.02\frac{nmol}{cm^4}$;

$Inverted\ slope(Acetate\ Berry) = 4347.83\frac{nmol}{cm^4}$; $Inverted\ slope(Glycolate\ Berry) = 3846.15\frac{nmol}{cm^4}$

Diffusionflux without addition of organic compound:

$$J(Agarose\ Berry\ 1) = -2.2 \times 10^{-5}\frac{cm^2}{s} \times -2777.78\frac{nmol}{cm^4} = 6.1 \times 10^{-2}\frac{nmol}{cm^2 \times s}$$

$$J(Agarose\ Berry\ 2) = -2.2 \times 10^{-5}\frac{cm^2}{s} \times -2439.02\frac{nmol}{cm^4} = 5.4 \times 10^{-2}\frac{nmol}{cm^2 \times s}$$

Diffusionflux with addition of acetate:

$$J(Acetate\ Berry) = -2.2 \times 10^{-5}\frac{cm^2}{s} \times -4347.83\frac{nmol}{cm^4} = 9.6 \times 10^{-2}\frac{nmol}{cm^2 \times s}$$

Diffusionflux with addition of glycolate:

$$J(Glycolate\ Berry) = -2.2 \times 10^{-5}\frac{cm^2}{s} \times -3846.15\frac{nmol}{cm^4} = 8{,}5 \times 10^{-2}\frac{nmol}{cm^2 \times s}$$

These flux calculations show that the oxygen consumption of Pink Berry incubated with glycolate is 48 % higher than the average consumption by berries without addition of organic compound, while berry incubated with acetate is 67 % higher. Profiling of a secondary set of berries with acetate and glycolate show the same tendency as described above (data not shown). This strongly indicates that organisms in the Berry are capable of using these two compounds for aerobic respiration. The result could also indicate that SRBs use the organic compounds for reduction of sulfate and release sulfide which is then oxidized by the PSB using O_2 terminal electron acceptor, as described in an earlier study (Seitz, Nielsen et al. 1993). If the latter is true acetate and glycolate should be considered as possible transfer molecules between the PSB and the SRB.

Based on this study it is unlikely that hydrogen function as an electron transfer molecule in the Pink Berry consortia. Therefore future investigations of the transfer mechanism should target different organic molecule as possible carrier. Oxygen profiles with berries incubated with acetate and glycolate point towards these two organic compounds as target for a new investigation. The oxygen profiles also indicate that the Pink Berries should be considered as a mainly aerobic consortia.
The enrichment for *Cytophaga* in this study was not successful. Nonetheless based on observations during the enrichment it should be possible with the right techniques and trouble shooting.

Similar unsuccessful was the stable isotope probing. The experiment is an obvious approach to investigate the cross-feeding within the Berry and therefore highly recommended for future experiments along with MAR-FISH and nanoSIMS, though optimization of Pink Berry growth will be necessary before such an experiment should be conducted.

Acknowledgement

The summer at MBL Microbial Diversity has been both really hard and really exiting and it would not have been the same without all the fantastic people. I would like to like to thank the course directors Dan Buckley and Steve Zinder for being so friendly and helpful. I would also like to thank all the TAs and Susanna for being so enthusiastic and hard working for our sake. And thanks to all my fellow students for making hard work and long hours in the lab so cheerful.
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Supplementary information

Figure S1. Sampling location of Little Sippewissett salt marsh.

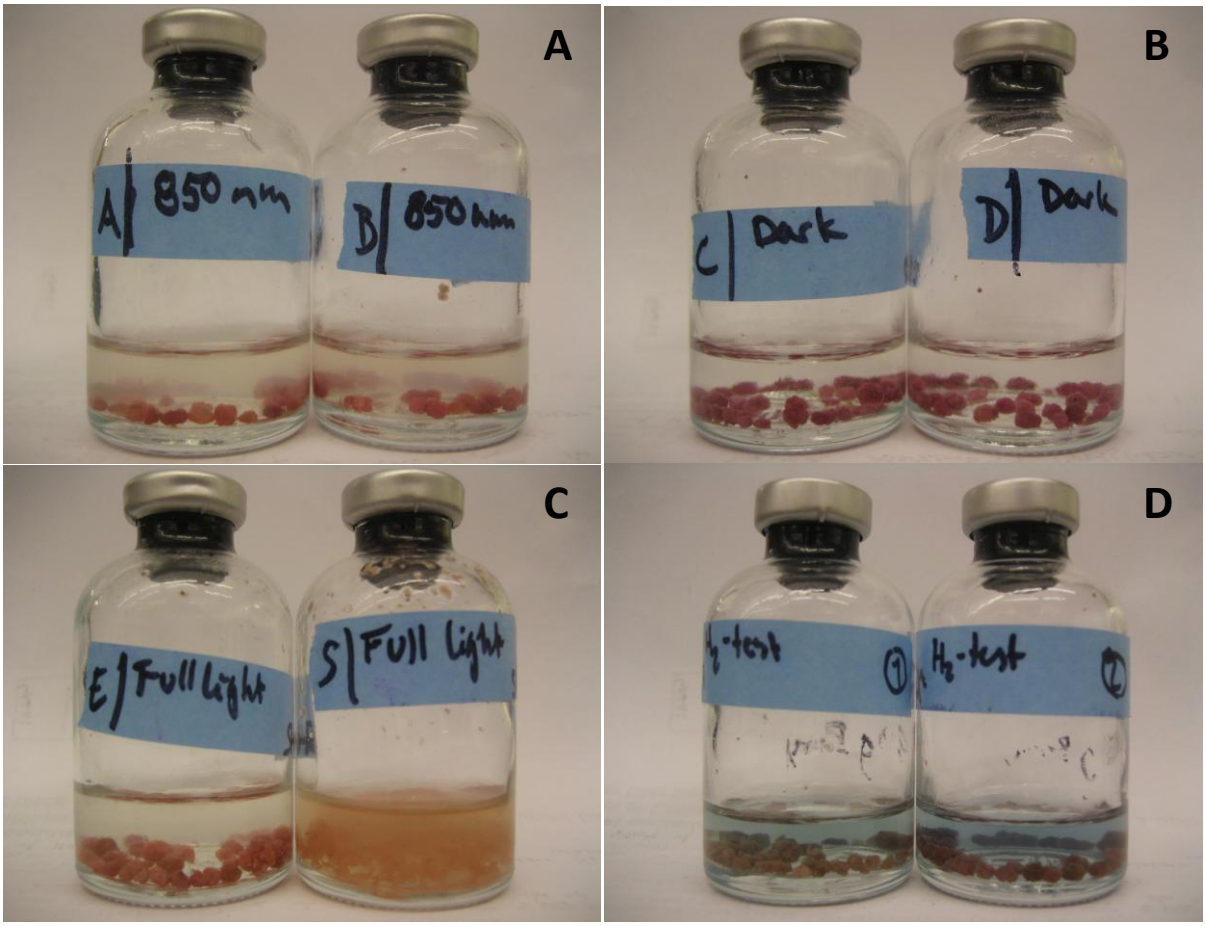

Figure S2. Hydrogen experimental microcosms at the end of incubation.

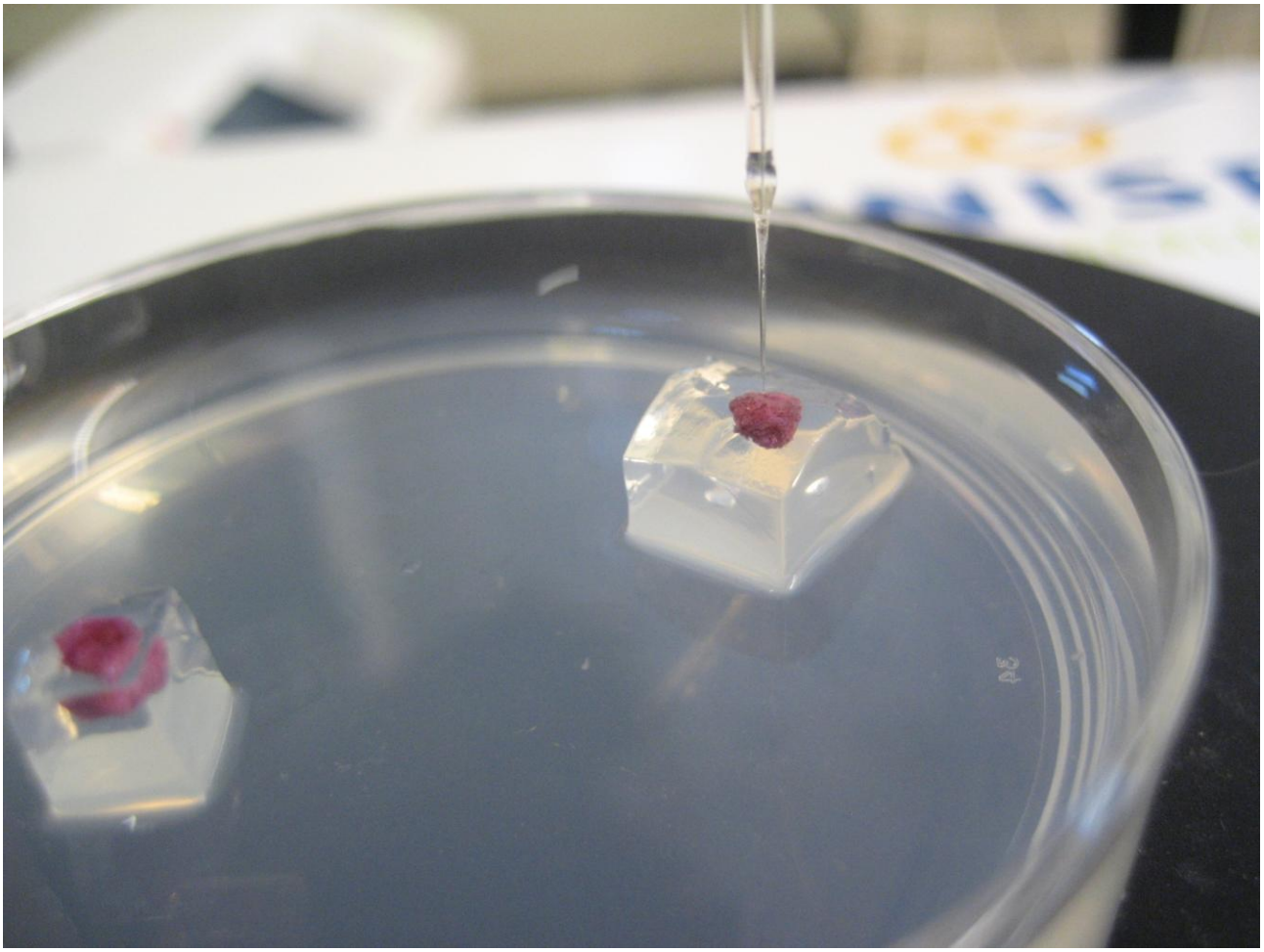

Figure S3. Microsensor entering an agarose embedded Pink Berry.

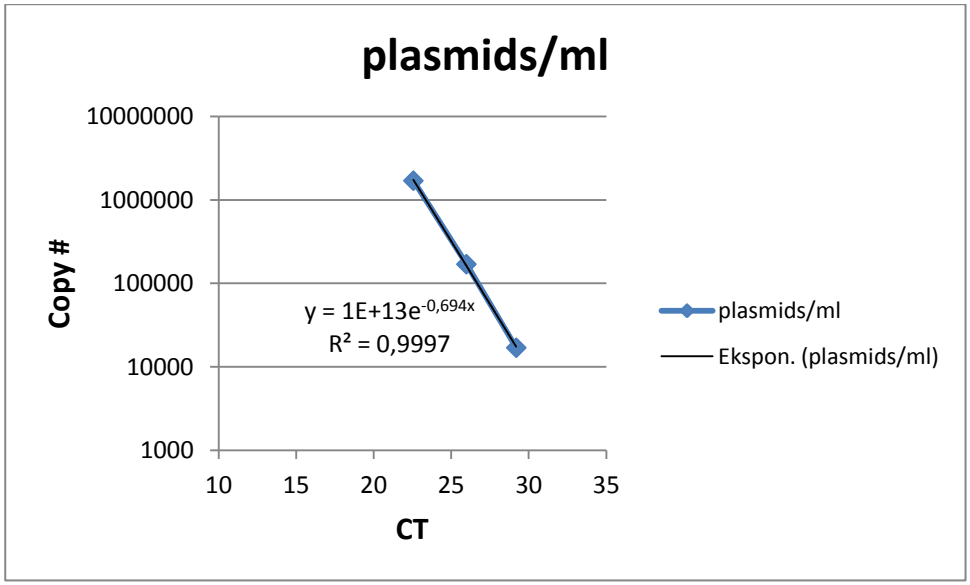

Figure S4. Standard curve for quantitative PCR.