Function of intercellular calcite granules in *Achromatium* from Little Sippewissett salt marsh

Abstract

Achromatium is a large sulfide-oxidizing bacteria with unique intercellular calcite granules. The function of the calcite is still unknown. Here I hypothesis that the calcite can be used as buoyancy-regulating mechanism, and I try to prove my hypothesis by "weighting" *Achromatium* cells through optical tweezers and confocal microscopy. Since previous studies mostly focused on freshwater *Achromatium*, as far as I know, it is the first time to study the calcite function on marine *Achromatium*.

Tingting Yang

Marine Sciences

UNC-Chapel Hill

Microbial Diversity Summer Course 2013

Marine Biological Laboratory, Woods Hole, MA

Introduction

Achromatium was first fully described in 1893 by Schewiakoff as a large bacterium living in freshwater sediment (1). It is very unique since it is the only known bacteria that deposits large intracellular calcite and sulfur granules. The mean calcium content of the A. oxaliferum cells was 2.62 + 0.34 ng cell-1 (mean + SE, n = 18) (2). Earlier studies have found that quantity of calcite per cell is somewhat variable; no relationship between the size of a cell and the amount of calcite it contained (2, 3). However, there is no firm answer of the function of calcite inclusions yet. Previous studies had come to some possible functions of the calcite, including dissolution of stored calcite to regulate acidity generated by H_2S oxidation, the use of calcite as a buoyancy-regulating mechanism, the use of calcite as an electron acceptor source in 'carbonate respiration', and the use of calcification to generate CO_2 for carbon fixation, as well as dissolution of sulfide minerals(4), which is not a problem in the ocean. *Achromatium* is large and heavy, it constitutes 90% of the bacterial biovolume in some well-studied fresh lakes, and plays an important role in the rapid recycling of reduced sulfur by oxidizing sulfide to sulfur and further to sulfate in freshwater lakes(5, 6). Therefore, *Achromatium* is a member of giant sulfide-oxidizing bacteria, which belongs to a deep branch of Gammaproteobacteria; free-living. So far no flagella were found in *Achromatium*. This raises an interesting question: how can *Achromatium* manage to stay in the oxygen-sulfide zone which shifts dramatically during day and night? Since most of the more recent studies mainly focus on freshwater and brackish water *Achromatium*, here I would like to study the marine *Achromatium* in the Little Sippewissett salt marsh, since Woods Hole is the only place where marine *Achromatium* was found. Especially I am interested in the looking for the function of the calcite granules. My hypothesis is that *Achromatium* uses calcite inclusions to help it move to more suitable places in the sediment.

Method

Sampling: sediment cores were taken from the Little Sippewissett salt marsh, Berry Pond 1 (see Christa's description) at different time points; the most important two samples were from 4 am and 1 pm. All the sediment cores were collected by plastic core-liners (10 cm diameters) at the shallower edge of the pond within an area of about 4 m^2 to avoid heterogeneous background; we tried not disturbing the water- sediment interface as much as possible. At least two cores were taken back to lab within one hour for cell counting, micro-sensor measurement and sediment DNA extraction. All sediment cores we collected were 5-6 cm deep, then subsamples for cell counts were taken by a cut-off 10 ml pipette, 1 ml sediment were taken from 0-1, 1-2, 2-3 and 3-4 cm by subsampled twice from the cores.

Cell count: as mentioned above, 1 ml sediment sample from each layer was used for cell count. Each 1 ml sample was washed through 20 μm nylon mesh to remove sand and

other sediment particles, by using 15 ml 0.2 μm filter sterilized sea water collected from the berry pond; the "eluate" (sea water with bacteria cells) was collected in plastic petri dish. After settled down for about 10 min, purified *Achromatium* cells were gathered together in the center of the petri dish by slowly rotate-shaking for about 30 s. Under Zeiss dissection microscope, the cells were magnified by 8x times; then *Achromatium* can be distinguished by eyes, and can be transferred into a new petri dish by using specially made pasteur pipette (the glass tip was heated and stretched to make it extremely thin). After washed one time, cell numbers were hand-picked and counted at the second transfer.

***In situ* pore water sampling**: sediment cores for pore water were collected by special core liners with holes every 1 cm. These sediment cores were taken at the same place together with sediment used for cell counts. We inserted 20 μl pipette tips into each hole from 4-5, 3-4, 2-3, 1-2, and 0-1 cm; about 1 ml pore water then was collected in 15 ml falcon tubes and was diluted 10 times by Milli-Q water, filtered through 0.2 μm syringe filters into sterilized glass tubes for IC measurement. Overlying sea water of each core was collected as well and was treated the same as pore water samples.

Micro-sensor measurement: we measured O_2 and HS^- concentration as well as pH by using microelectrodes. Sensor calibration was performed every time before each measurement; the motometer controlled the sensors to move 200 μm every 3 seconds, starting from the very beginning of the overlying seawater. Total [H_2S] was measured together with pH at each core, and was calculated and calibrated by the correlated pH value.

Sediment DNA extraction, PCR and 454 pyro-sequencing: two sediment cores from the berry pond 1 were taken home for molecular work. DNA samples from 0-1 and 1-2 cm were extracted using the MoBio PowerSoil DNA isolation kit and following the manual. PCR amplification was carried out in a 30 μl reaction mixture containing ca. 10-50 ng DNA (quantification of DNA was performed via Thermo Scientific Nanodrop UV/Vis Spectrophotometer), 15 μl of a Phusion 2x HF MasterMix, as well as 2.4 ul 100% DMSO (dimethyl sulfoxide), 25 μM of a universal reverse primer oligo 907R, 6.25 μM of a barcoded universal forward primer oligo 515F and filled up with nuclease free water. The PCR product was subsequently checked on a gel along with a DNA ladder before the sample was sent out for sequencing. Analysis of the 454 pyrosequencing data was performed via QIIME, for quality control the minimum and maximum length of sequences were set to 400 and 450 bp, respectively. Taxonomic classification of the 454 sequences was performed via the RDP (ribosomal database project) website, RDP classifier.

Achromatium cell DNA extraction, PCR and 16S clone library: cells collected for DNA extraction were washed exhaustively: four times wash in sterilized sea water, then 1

time wash in Milli-Q water. About 30 well-washed cells stored in 8 µl Milli-Q water were pre-heated at 98°C for 5 min, and then used as template for PCR reaction. For the full length 16S rRNA gene, PCR amplification was carried out in a 25 µl reaction mixture with 13 µl Promega Go-Taq Green 2X Mix, 2 µl 16S_8F, 2 µl 16S_1492R with our prepared templates up to the total volume. 16S clone library was constructed by using TOPO vector kit. 48 colonies were picked up for forward and reverse Sanger sequencing.

Microscopy: Zeiss PALM Microdissection system (optical tweezers) and LSM 780 Confocal Scanning Microscope were used for force measurement and component imaging. Zen blue 2012 software was used for confocal microscopy image analysis. Lasers of 405, 561 and 638 nm wavelengths were used for separating different cell components and 7 images of *Achromatium* cells were taken from z stack so we can have a 3D image. Scaling X and Y were every 0.208µm, scaling Z was every 3.241 µm.

Ion Chromatography: 10 times diluted and 100 times diluted pore water samples were used for IC to determining ion concentrations. The pump setting: 1 ml/min; dectetor setting-supp. Current: 112 mA; EG setting-Target conc.: 10 mM. Each sample takes 32 min to run.

Raman spectroscopy: at WHOI, we were able to scanning fresh cells by using Raman spectroscopy, which could distinguish different chemical bonds. Sample cells were washed 4 times in sterilized sea water.

Results

Although we were very confident that we collected *Achromatium* cells, we still performed Raman spectroscopy. The result (data not shown) confirmed our cells contain calcite granules and sulfur granules. Therefore, we can be sure that our hand-picked cells for sure belong to *Achromatium*.

Cell morphology:

Purified cells were observed under phase contrast microscope. Most of the cells are about 10-20 µm long, 8-10 µm width, rod or spherical shaped, intercellular calcite granules can be clearly seen around the periphery of the cells, with sulfur inclusions mostly “trapped” at the edge where two calcite granules reach each other (Fig 1). It is obvious that some cells have smaller but more abundant calcite granules, while others have larger but low abundant calcite granules. *Achromatium* cells can lose their calcite in few second if treated by acid (30 min to few hours if treated by Milli Q water), however they can maintain most sulfur granules and cell shape for a longer time; cells that lost calcite were extremely bright under microscope, and became extremely fragile. *Achromatium* cells can slowly roll at different directions, no jerking was observed.

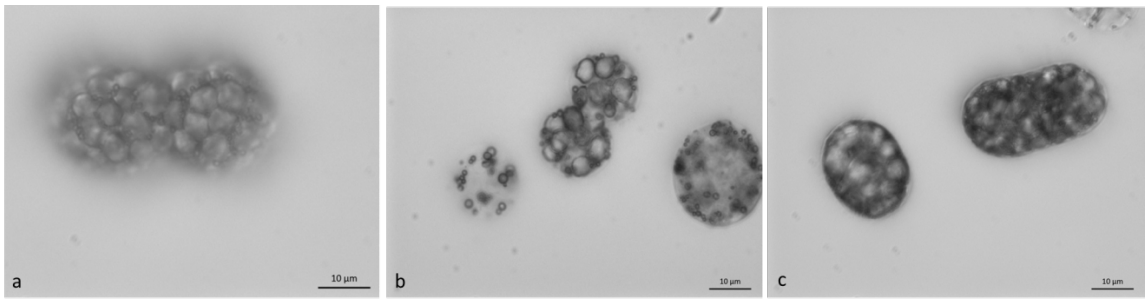


Fig 1. Cell morphology: a) *Achromatium* cell with calcite and sulfur granules; b) the cell at the left was bleached out, the one in the middle is still healthy, the one on the right has lost a lot of the calcite granules. However, all of them have sulfur granules; c) the cell on the left has less but bigger size calcite granules.

Cell count:

Sediment samples from different time points (1 pm, 7 pm, 4 am, 9 am and 10 am) were used for cell count (Fig 2 a, b). It is obvious that *Achromatium* cells are most abundant in 0-1 cm in all of the sediment cores, especially from the 4 am core, surface sediment cell number was as high as 2148 cells per ml; while the sediment core from 1 pm has the lowest surface sediment cell number (377 cells/ ml). Compare to the absolute numbers, relative cell abundant in 0-1 cm and 1-2 cm sediment layers would be more convincing: in the 1 pm core, 0-1 cm sediment had only 53% of the total cells (total cell number counted from 0-4 cm), 1-2 cm layer had 40% of the total cell; other cores (from 7 pm, 4 am, 9 am and 10 am) had 93%, 70%, 79% and 87% of total cells in their 0-1 cm layers, and 5%, 25%, 14% and 10% of total cells in the 1-2 cm layers, respectively.

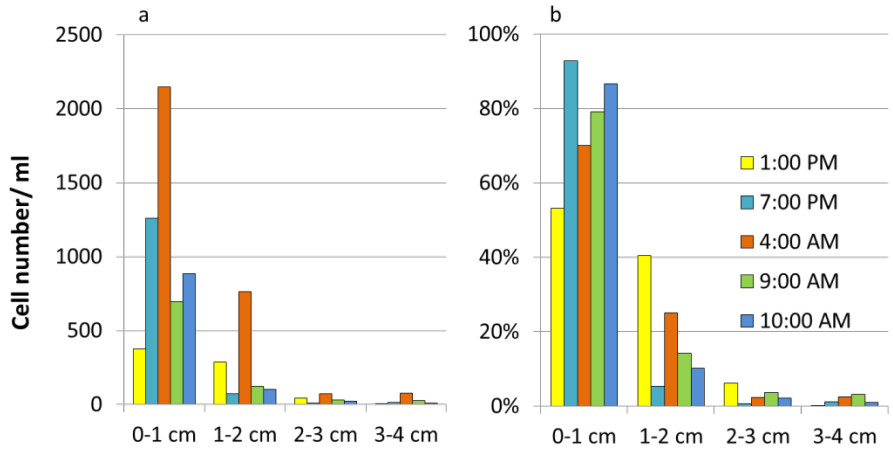
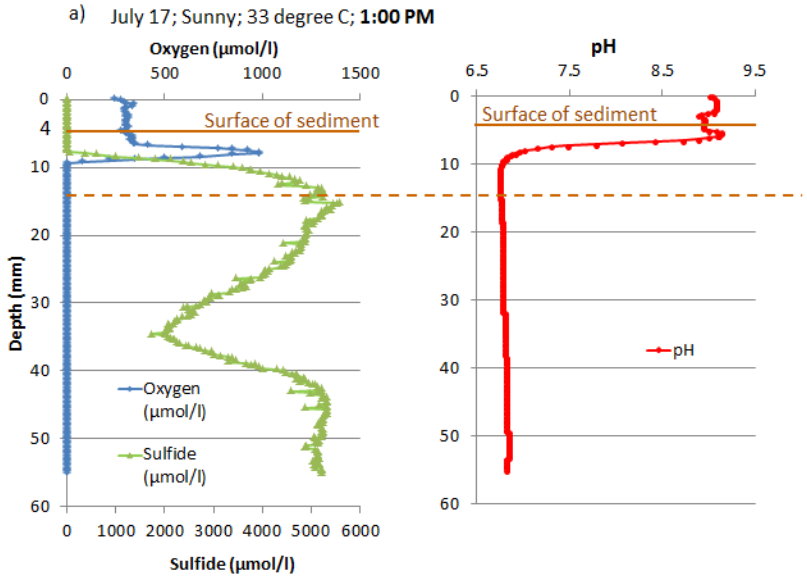


Fig 2 Cell counts: a) absolute cell counts from each time point and layer; b) relative abundant of each layer to the total cell number.

Micro-sensor profiles:

Micro-sensor profiles indicated that the oxygen concentration, sulfide concentration as well as pH change dramatically at different time points. Here I only listed two profiles, one was from a 1 pm core (with about 4 mm overlying water), another one was from a 4 am core (with about 7 mm overlying water) (Fig 3 a, b). The 1 pm profile showed that oxygen concentration was extremely high (super saturated) at 0-0.6 cm sediment layer, with highest [O2] as about 1 mM/l. [Sulfide], in contrast to O2, was the lowest at the surface sediment, but increased to maximum at about 1 cm sediment depth. pH value of the overlying sea water was about 9; as soon as the microsensor hit the sediment, pH dropped dramatically, reached 6.8 within 0.6 cm.



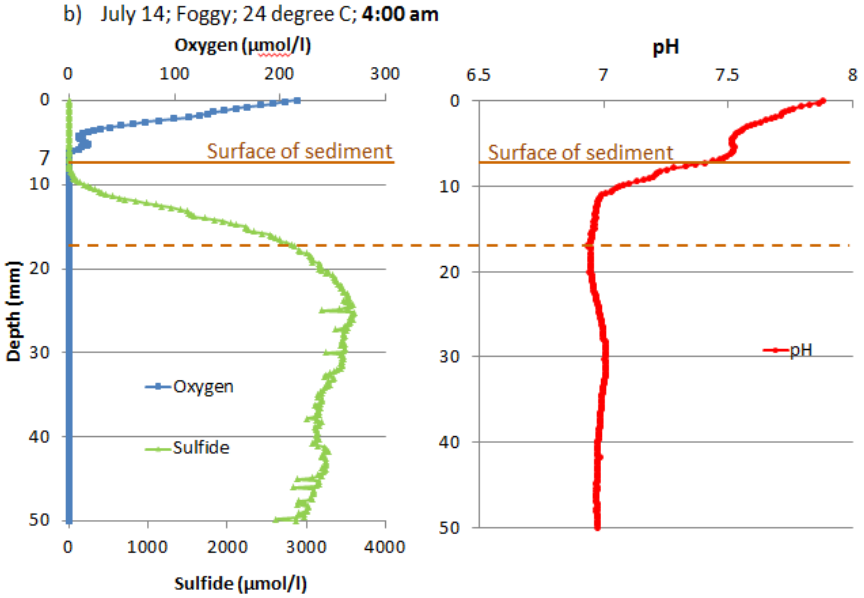


Fig 3 Microsensor profile: a) profile of 1 pm core; b) profile of 4 am core.

The core collected from 4 am had almost no oxygen at all in the surface sediment; the whole core was anoxic. Although the oxygen concentration was extremely low in 0-1 cm of sediment, but sulfide concentration curve showed that the maximum value was reached at about 1.7 cm of sediment. pH of the overlying water was lower than day time (7.8), and it dropped to 6.9-7 at 1 cm sediment depth.

IC data:

IC data provide background information of the salt marsh. As expected, Chloride and sulfate have the highest concentrations since the salt marsh is basically sea environment. Comparing IC data of 1 pm and 4 am samples after removed high peaks of other ions, seems the 0-1 sediment at 4 am had a much higher phosphate concentration than 1 pm. The data from two time points were listed as table 1

	1:00 PM	unit: mg/L					**4:00 AM**	unit: mg/L				
	Overlying	0-1 cm	1-2 cm	2-3 cm	3-4 cm	4-5 cm	Overlying	0-1 cm	1-2 cm	2-3 cm	3-4 cm	4-5 cm
Fluoride	1.303862	1.59437055	1.407487	1.865176	1.239291	0.992028	1.401083	1.514714	1.419665	0.86467	0.788034	1.318771
Chloride	20372.87	20858.47	20824.83	21119.19	23024.25	20160.3	17788.39	18601.01	18492.19	18909.96	18694.49	18392.54
Bromide	77.03704	69.0295517	66.69249	67.0853	66.61011	63.80763	56.47894	60.58847	60.13761	61.24852	59.56615	59.70632
Nitrate	1.207556	0.5633831	0	0.700892	0.904053	0.6112	0.640244	0.706532	0.743441	0.718182	1.344038	0
Carbonate	13.88774	1.94093482	12.21939	8.531065	8.543301	2.833677	10.70532	12.15876	10.53548	8.27507	2.249817	2.155402
Sulfate	2687.32	2624.86306	2543.622	2567.136	2551.578	2418.998	2325.072	2479.7	2418.443	2467.389	2414.194	2386.677
Phosphate	8.163109	5.01793653	7.904856	7.753977	6.799782	7.769406	5.090494	337.8011	3.662415	0	0	1.645179

Table 1: IC data of pore water from each layer

Optical tweezers

The easiest way to test my hypothesis is to “weighting” cells from different time points. Zeiss PALM Microdissection system allows force measurement at the range of 0.2 to 100 nN; by using 2 laser traps, microscopic particles can be confined with optical tweezers. The force applied on the object depends linearly on its displacement from the trap center. In other words the trap acts just like a linear spring where the restoring force F is proportional to the trap stiffness k_{trap} and displacement x. Once the trap stiffness is calibrated monitoring the displacement of the object directly results in a force measurement (from force measurement manual). Therefore, it would be possible to use laser traps to measure the force of moving each *Achromatium* cell from day time and night time, from 0-1 and 1-2 cm sediment, then perform a relative compare. However, since the *Achromatium* cells are generally 10 – 20 μm long and 8 – 10 μm width and thick, while the optical traps were only 2 μm in diameter, it was very hard to drag one cell to move, even if it moves, it is hard to say if it moves by itself or by the trap. Therefore, the force measurement failed.

Confocal microscopy and image analyzing

Thanks to whoever put the Zeiss LSM 780 confocal microscope right next to the PALM optical tweezers! As suggested by one embryologist, Maggie Wu, I tried to take images of my *Achromatium* cells under different wave lengths since everything is autofluorescent! In the spectrum of the image it showed clearly there were two main components inside the *Achromatium* cells, at exactly where the calcite and sulfur granules are. I could clearly separate these two components at different wavelengths! Further, with the help of Zeiss specialists we took a 3D image which includes fresh cells, unhealthy cells and one cell without any calcite granules. The spectrum showed that the blenched cell gave off fluorescence at a different wave length than calcite and sulfur in healthy *Achromatium* cell. By separating these three components, I could measure the intensity of pixels at each wave length at each of the 7 images, and then perform the relative compare (Fig 4). Therefore, if I have more time, I could image cells from different conditions to compare the intensity of pixels and determine if cells from special time points or specific layers would heavier than others.

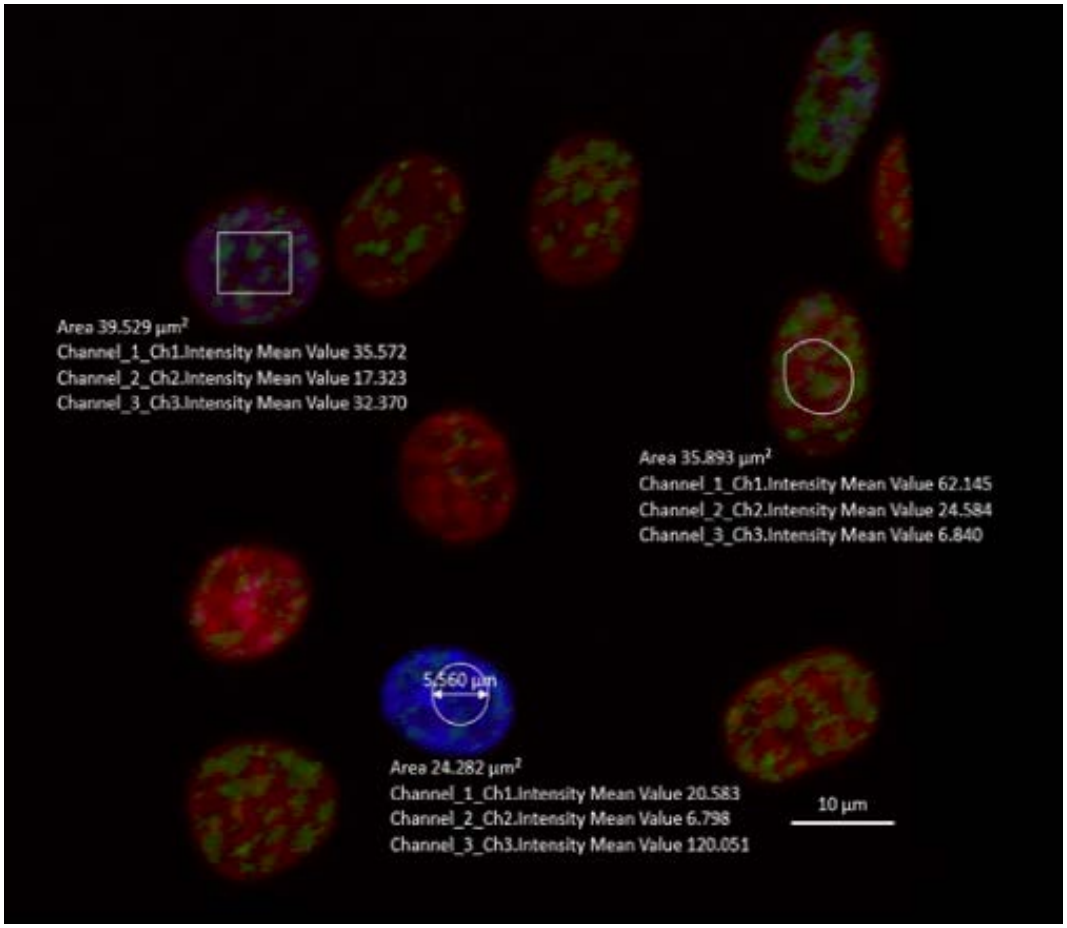


Fig 4. Confocal microscopy image with information of pixel intensity from each channel. Red color indicates calcite, green color indicate sulfur, blue is for cells with dissolving calcite.

16S rRNA gene sequencing and 454 sequencing

The 16S rRNA clone library doesn't contain any *Achromatium* sequences; that remind me that a lot of bacteria are much smaller than *Achromatium* and cannot be very well seen by dissection microscope. Anyway, the 16S clone library has a lot of marine sourced bacteria, which is clear evidence that *Achromatium* in Woods Hole are living in marine environment.

Our 454 data showed that except to the 1-2 cm at site A, all other samples have 1488 to 14323 fragment reads. The background bacteria community at phylum/sub-phylum level doesn't change a lot in the adjacent area in the berry pond 1; neither the first two depths tell us any difference. The main groups belong to Gamma, Delta, Alpha-proteobacteria, Bacteroidetes, Planctomycetes. 194 fragments are more than 97% similar to *Achromatium* species; these fragments showed once or twice in the environmental samples, but more abundant in two hand-picked-cell samples.

Discussion

Since Little Sippewissett salt marsh at Woods Hole is so far the only marine environment where *Achromatium* was found, it is very interesting to compare marine *Achromatium* to fresh water lake *Achromatium*. For all the *Achromatium* cells I have observed, they all can roll slowly, but jerking was never be seen. No difference was found in cell morphology; cells with different calcite granule sizes were observed and the inclusion size has nothing to do with cell size, which is the same as studied in the fresh water *Achromatium* (2, 3). My cell count result showed that *Achromatium* has the potential to move inside the sediment; the microsensor profile indicated that oxygen seems like is not the most important factor to restrain where *Achromatium* can go. However, all my time points are single point, to further prove my opinion, more sampling and statistical analysis are required. Also, 1 cm interval of sampling was too broad to get useful information, maybe 2 mm each layer would be better. Although people had used confocal microscopy to calculate the abundance of calcite, they didn't use fresh samples (2). By using 3D scanning and calculating pixel intensities of autofluoresense from different wave lengths, the relative abundance of cells can be compared.

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