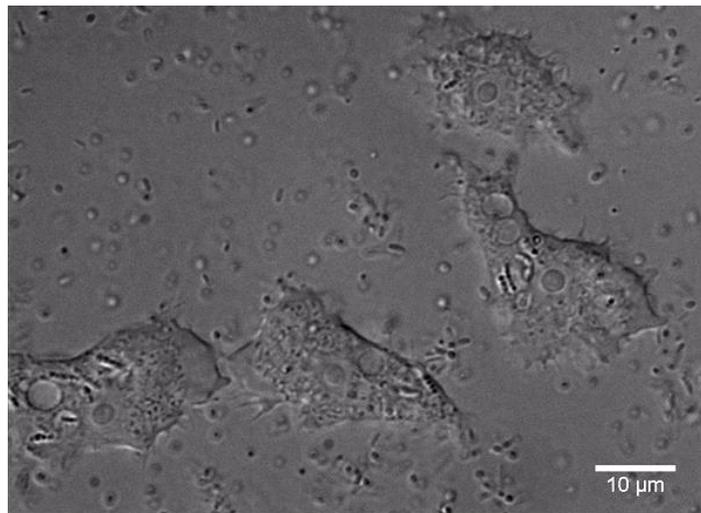


# Characterization of encystation and excystation in a microbial eukaryote, *Nuclearia simplex*



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## Introduction

Amoebae are taxonomically characterized by their ability to alter cell shape and therefore are widely distributed across the phylogenetic tree, occurring in every major eukaryotic lineages. Free living amoebae (FLAs) can form cysts in response to adverse conditions and have been studied in the clinical context as pathogenic bacteria carriers<sup>1</sup>. Mature cysts can persist under stress and starvation conditions. Environmental amoebae are understudied, with their diversity poorly covered. Only a few species have been isolated in the genus *Nuclearia* and their small subunit ribosomal DNA sequences place the genus at the boundary of metazoa and fungi<sup>2</sup>. *Nuclearia* species are diverse in morphology and only a few have been observed to form cysts<sup>2</sup>. Most cysts have been observed to have a double layered wall: ectocyst as the external layer and the endocyst as the inner layer<sup>1</sup>. Both walls are made of mostly proteins and carbohydrates with some species sensitive to degradation by cellulase<sup>3</sup>.

Signalling pathways involved in encystation vary between species. Starvation is a major inducer as well as osmotic stress, extreme temperature, and other signalling molecules<sup>1</sup>. Pathogenic bacteria, such as *Pseudomonas aeruginosa*, can also induce amoebal encystation<sup>4</sup>.

This study aims to characterize encystation, excystation, cyst wall composition, and morphology of *Nuclearia simplex* through imaging. Determination of gene expression changes between free living amoebae and cysts will be attempted through comparative transcriptomic. Conditions under which encystation and excystation are induced will be tested using a flow chamber set up.

## Materials and Methods

### Amoeba cultivation and media composition

An isolate of *Nuclearia simplex* was obtained from Dawson lab at UC Davis and grown in fresh water LTY medium (17.1 mM NaCl, 1.97 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.68 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 6.71 mM KCl, 0.5 mM MOPS at pH 7.2, 0.1 g/L tryptone, 0.1 g/L yeast extract) in the presence of *Maribacter* sp. in cell culture flasks. Seawater LTY medium consisted of 342.2 mM NaCl, 14.8 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 1.0 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 6.71 mM KCl, 0.5 mM MOPS at pH 7.2, 0.1 g/L tryptone, 0.1 g/L yeast extract. TR2, an isolate from Trunk River, was grown in seawater LTY medium in presence of *Maribacter*.

### Bacteria strains

*Maribacter* sp. isolated from Microbial Diversity 2014 was grown in freshwater LTY medium and used as amoeba food source. This was likely not a pure culture as contaminants were detected on plates and under the microscope. A *Pseudomonas aeruginosa* strain that constitutively expresses GFP was obtained from the Host-Microbe Interaction course here at MBL for imaging.

### RNA extraction

A seven day old and a ten day old culture were used for total RNA extraction from cysts (> 85% by cell count). A five day old culture was used for RNA extraction from trophozoites (> 90% by cell count).

TRIzol® Reagent from Life Technologies was used to extract total RNA from cultures of free living amoebae and mature cysts. Media was decanted from three large cell culture flasks containing amoebae. 2 ml of TRIzol® Reagent was added to each flask followed by incubation on the shaker for 5 minutes. Each flask was briefly vortexed and the TRIzol solution was transferred into bead-containing tubes on ice. Samples were homogenized for 45 seconds mechanically. RNA isolation and wash steps from the TRIzol Reagent manual were followed. RNA extracts were left in the hood to dry. Quantification, and cDNA library preparations were carried out by Katie (Dawson Lab), Srijak (TA), and Scott Dawson (UC Davis).

### Light Microscopy

Amoebae were imaged on 35 mm and 50 mm uncoated MatTek dishes on inverted microscopes without the lid for DIC and with the lid for phase contrast. The time lapse video of encystment in *Nuclearia simplex* under the 40X objective lens and the cell division image were taken on the Nikon Ti inverted microscope equipped with an Andor Zyla camera. All images and remaining time lapse videos were taken on Zeiss Axio Observer.A1 inverted microscope. Images and videos were processed using Fiji and ZEN.

### Stains used for imaging

Hoechst 33258 was added to a final concentration of 0.1 µg/ml. Stained samples were imaged after a few minutes of incubation and wash with fresh water LTY medium. 40 µl of 1 mg/ml Calcofluor White stock solution was added to 1 ml of amoeba culture in MatTek dishes and incubated for 1 minute. Washing with medium before imaging reduced background signal. Both stains were imaged under the DAPI filter cube.

### Chitin degradation assay

Chitinase from Sigma was suspended to 10 mg/ml in 50 mM phosphate buffer at pH 6 (approximated by pH paper). This stock solution was added to 1 ml amoeba culture in 35 mm MatTek dishes to a working concentration of 1 mg/ml. At each time point, one dish was stained with Calcofluor White, washed with freshwater LTY medium, and imaged.

#### Flow chamber set up and cell counting

A syringe pump was used to continuously flow solution into amoeba cultures and a peristaltic pump was set up to remove solution to maintain a constant volume in cultures. Cell culture flasks were used for RNA extraction and 35 mm MatTek dishes were used for induction of encystation. A flow rate of 4 ml/hr was used for large cell culture dishes and 0.328 ml/hr was used for 35 mm MatTek dishes.



Figure 1. Flow chamber set up for 35 mm MatTek dishes.

Number of cells in the entire field of view was counted manually using a cell counter on the inverted microscope using a 32X objective lens. The number of FLAs and cysts was recorded and the ratio was calculated. Spherical amoebae that were in transition between FLA and cyst were not included in the counting. Encystation study was carried out for five days with eight fields of view counted per day for each condition. The ratio of cyst to FLA and total number of counted cells were calculated and normalized to the corresponding median from day zero. Boxplots were generated from the data and were plotted on RStudio.

### Organic extraction of *Maribacter* sp. culture

50 ml of *Maribacter* sp. culture was extracted with 50 ml of chloroform twice. The organic phase was dried on sodium sulfate and organic solvent was removed in the fumehood under a stream of air. Dried extract remained insoluble in methanol and ethanol. Due to a lack of solvent options, extract was dissolved in a minimal volume of chloroform.

### Solutions used for encystment induction study

Spent media were syringe filtered through 0.22 µm filters from old amoeba cultures that contained mostly cysts. A stationary culture of *Maribacter* in freshwater LTY medium was filtered to obtain the supernatant. Freshwater LTY medium was supplemented with 0.1 mM cAMP or titrated to a pH of 3.3 to test for two additional encystation inducing conditions.

## Results

### *Nuclearia simplex* isolate

The amoeba isolate used here was isolated in Davis, CA and the small subunit ribosomal DNA places this strain as *Nuclearia simplex* (Fig. 2). 18S rRNA sequencing was carried out prior to the start of the course by Dawson lab.

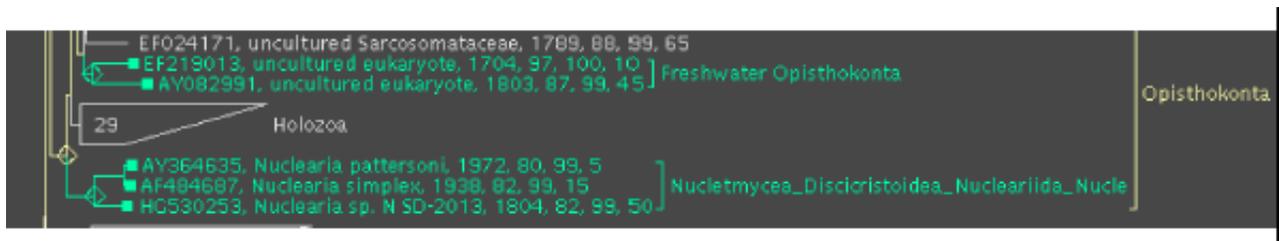


Figure 2. Phylogenetic tree constructed using 18S rRNA sequence places the amoeba isolate as *Nuclearia simplex*.

### Comparative transcriptomic

Total RNA was extracted using TRIzol Reagent for comparative transcriptomic on free living amoebae and cysts (Table 1). RNA extracts were passed on for cDNA library preparations by Katie (Dawson lab), Srijak (TA), and Scott Dawson. Due to time constraints, sequencing data will be obtained after the course period.

Table 1. Percentage of cyst in samples used for RNA extraction. Calculated based on cell counts on the inverted microscope.

<b>Sample</b>	<b>% cyst</b>
<b>Cyst 1</b>	0.87 ± 0.06
<b>Cyst 2</b>	0.96 ± 0.04
<b>FLA</b>	0.088 ± 0.007

### Characterization through light microscopy

From preliminary observations under the microscope, free living *N. simplex* can be over 30 µm in length (Fig. 3a). Precysts are approximately 10-12 µm (Fig. 3b) in diameter and mature cysts tend to be slightly smaller at 8-10 µm (Fig. 3c). After transferring an aliquot of *N. simplex* into a MatTek dish, amoeba population density increased and cell replication by binary fission was frequently observed in the first time lapse video taken overnight (Fig. 3d). Encystation was induced at a higher population density when the MatTek dish was left open without the lid and the medium partially dried (Video 1). Encystation was also imaged at a higher magnification where the process took about an hour to form precysts (Video 2). Excystation from mature cysts was not observed within the time frame of time lapse videos, but was observed from precysts. Single amoebae exited from each precyst through a pore-like structure, leaving an empty cyst wall behind (Video 3).

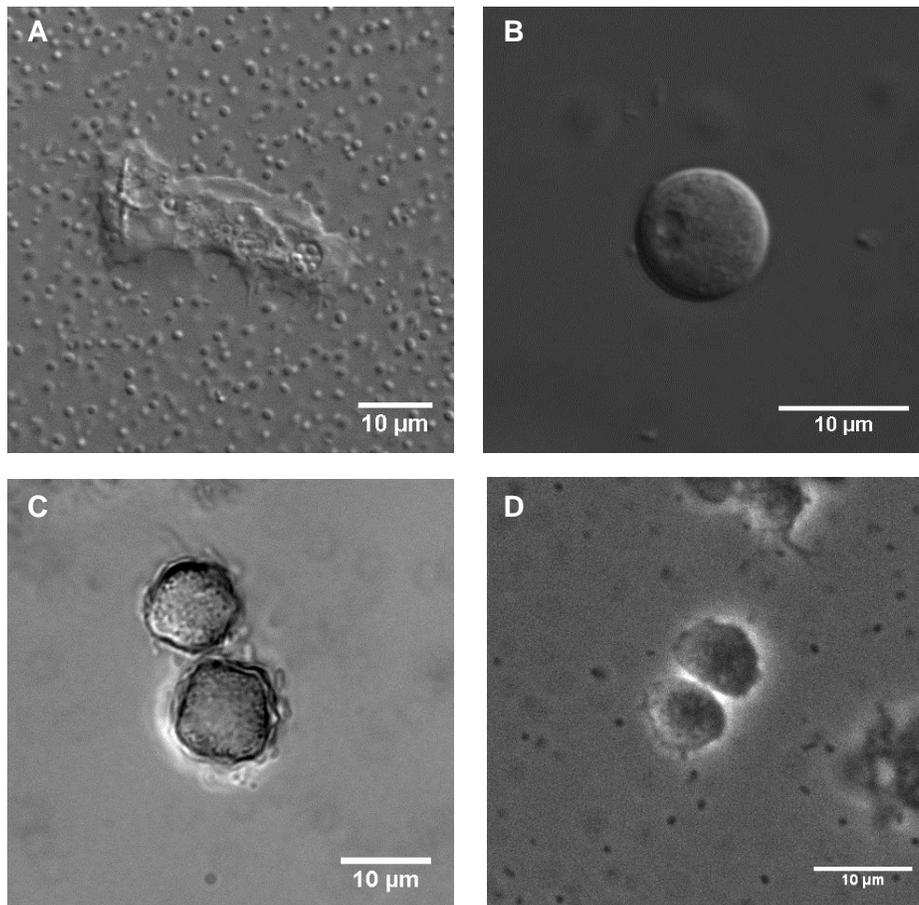


Figure 3. DIC images of a) a FLA, b) a precyst, c) mature cysts, and d) a cell undergoing binary fission.

Composition of the cyst wall was investigated through Calcofluor White staining and a chitin degradation assay. Calcofluor White stained only precysts, mature cysts, and empty cyst walls (Fig. 4). Ectocyst layer seemed to be more preferentially stained, which is indicative of a high cellulose and/or chitin composition. Cyst cultures in MatTek dishes were incubated with chitinase and imaged over a time period of 19 hours. Samples were stained at various time points with Calcofluor White to determine any obvious change in cyst wall composition (images not shown). No difference in the intensity of staining nor cyst wall structure were observed under identical microscope settings.

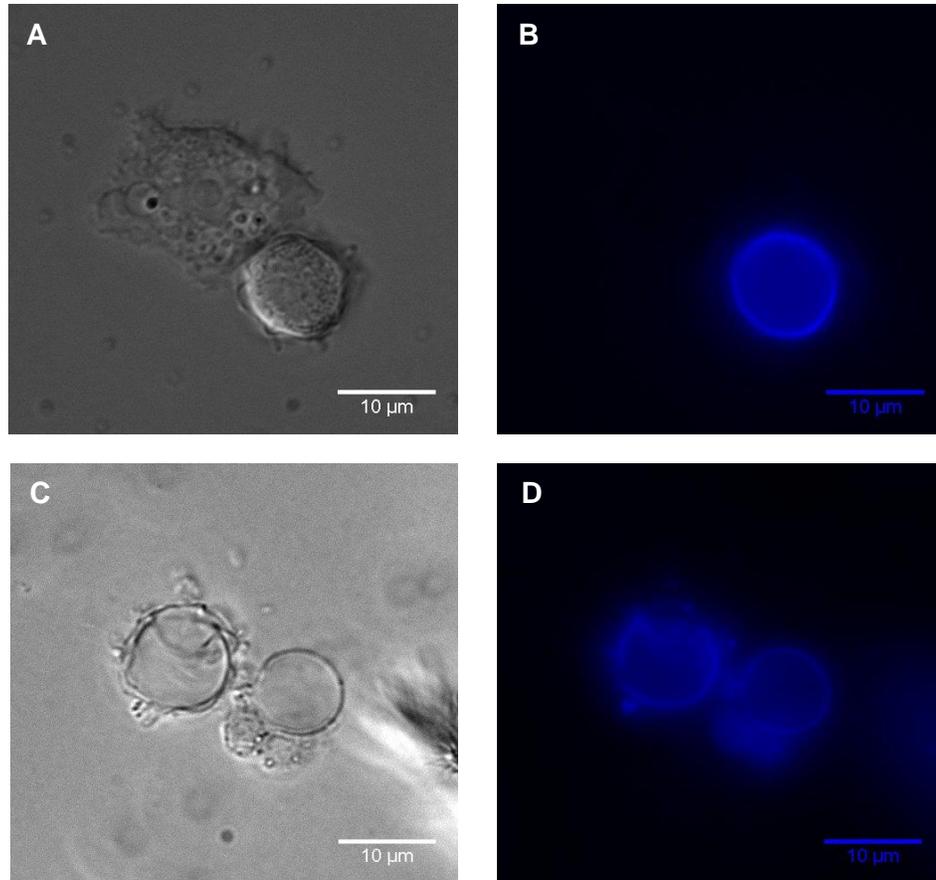


Figure 4. Calcofluor White staining of a-b) FLA and mature cyst, and c-d) empty cyst shells.

Multiple vacuoles per cell were detected when FLAs were stained with Hoechst 33258 (Fig. 5), which are difficult to distinguish from the nucleus and the number of nuclei present could not be determined. During epifluorescent imaging, FLAs lysed under UV irradiation and staining of a single nucleus and the nucleolus within was observed (Video 4) after lysis. Cysts did not stain, likely due to impermeable cyst walls.

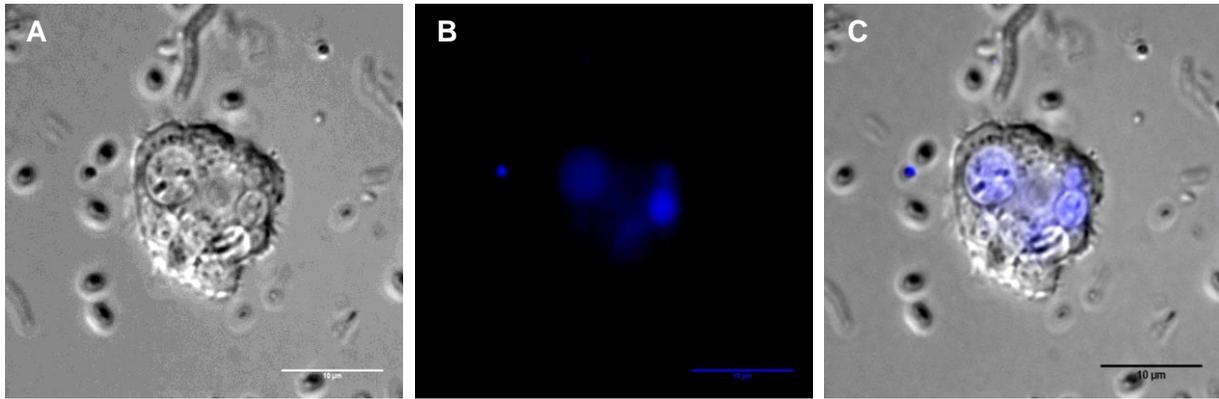


Figure 5. Hoechst staining of a FLA in a) DIC, b) DAPI fluorescence, and c) overlay.

### Induction of encystation

A *Pseudomonas aeruginosa* strain that constitutively expresses GFP was added to amoeba cultures for imaging time lapse videos. No cyst formation was observed and *P. aeruginosa* density decreased over time (Video 5). *P. aeruginosa* was observed in food vacuoles and seems to preferentially attach to posterior ends of moving amoebae.

Seven 35 mm MatTek dishes were inoculated and set up as flow chambers with six different solutions pumping through at 0.328 ml/hr for five days. Although large variations were observed within each MatTek dish between different fields of view, two conditions yielded significant changes (Fig. 6). Filtered spent medium from TR2 amoeba isolate (isolated in 2014 from Trunk River) induced an increase in the cyst/FLA ratio. *Maribacter* in freshwater medium increased population size of *N. simplex* significantly. Spent medium from the TR2 cyst culture was at a pH of approximately 5 and spent medium from *N. simplex* cyst culture was at a pH of approximately 6. Two additional MatTek dishes were incubated in freshwater medium at pH 3.3 and freshwater medium containing 0.1 mM cAMP for 24 hours. No changes in cyst/FLA ratios nor total cell counts were observed. An organic extraction of a stationary phase *Maribacter* culture was carried out and the final extract was dissolved in chloroform due to lack of non-polar solvent options. A minimal volume of the extract was added to a cyst culture and the total cell count decreased over time.

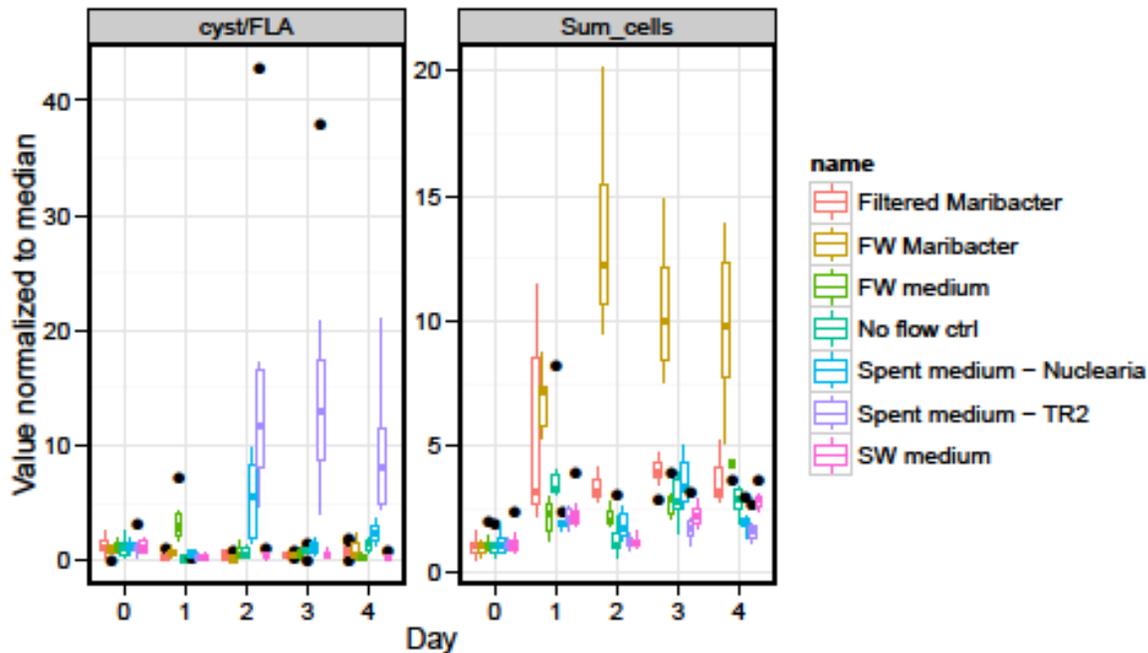


Figure 6. Boxplots of cyst/FLA ratio and total number of cells from cell counts for amoeba cultures incubated under different media in flow chambers.

## Discussion

Different life stages of *N. simplex* were observed and imaged in DIC. Locomotive cells displayed pseudopodia and bacterial particulates were observed within vacuoles. Non-locomotive cells were spherical and stained under Calcofluor White, which is indicative of cyst wall formation and suggests a precyst stage. Mature cysts exhibited at least two wall layers with the ectocyst being irregularly shaped and wrinkled. Excystation seemed to occur through a pore and the empty cyst wall seemed to be resistant to decomposition over time. Addition of chitinase resulted in no detectable change in Calcofluor staining over 19 hours, which might suggest that the ectocyst had little to no chitin and consisted of mostly a different polysaccharide polymer like cellulose. Unfortunately, no positive control was included and therefore the possibility of inactive chitinases remains open. No conclusion can be made from this preliminary data without running a positive control containing chitin and chitinase. *N. simplex* potentially has a single nucleus in its free living form based on cell lysis in presence of Hoechst 33258. Cysts in other amoeba species have been observed to form multiple nuclei. This could not be studied as Hoechst did not stain cysts under conditions used here.

*N. simplex* effectively used *P. aeruginosa* as a food source without any other significant changes detected. Spent medium from a seawater amoeba isolate, TR2, induced encystation while seawater medium itself did not. pH of this spent medium was slightly lower than the spent medium from *N. simplex* cyst culture. It is not known if encystation was induced by the lower pH or a different signal. It would have been interesting to follow the culture at pH 3.3 for a longer period of time to give sufficient time for encystation to occur. Unsurprisingly, when bacterial food was provided, the total cell number increased over time. Extraction and fractionation of the spent medium can be carried out to look for bioactive fractions.

## Reference

1. E. Fouque *et al.*, *Eukaryotic Cell*, 382-387 (2012).
2. M. Yoshida *et al.*, *European Journal of Protistology*, **45**, 147-155 (2009).
3. R.A. Barrett and M. Alexander, *AEM*, **33**,670-674 (1977).
4. X. Lee *et al.*, *Microbes and Infection*, **14**, 268-272 (2012).

## Appendix - Videos

Video 1 - encystation overnight

<http://youtu.be/VEZ0tkp0D6g>

Video 2 – encystation, close up

<http://youtu.be/FscOM20gEhc>

Video 3 – excystation

<http://youtu.be/6h7IGSZBwvM>

Video 4 – cell lysis stained with Hoechst

[https://youtu.be/dPHPDGxOG\\_o](https://youtu.be/dPHPDGxOG_o)

Video 5 – feeding on *Pseudomonas aeruginosa* expressing GFP

<https://youtu.be/OCRat7GJ0Jk>

## Extras:

Free living *N. simplex* - <http://youtu.be/Ez9k7uuH8ww>

Grazing on bacteria - <https://youtu.be/ZekCF2ZIkZs>

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## **Side project: Enrichment for anaerobic L-hydroxyproline reducers**

Given the abundance of 4-hydroxyproline in collagen, it is surprising that hydroxyproline degradation is not well studied in bacteria. Currently, there is one pathway characterized that involves an initial step of L-hydroxyproline epimerization to D-hydroxyproline followed by oxidation to pyrroline-4-hydroxy-2-carboxylate. The enzymes involved are found in aerobic bacteria, including *Pseudomonas* species. This project aims to enrich for anaerobic L-hydroxyproline reducers from sampling sites around Woods Hole.

Briefly, a medium composition was designed to enrich for L-hydroxyproline reducers under anaerobic conditions. Hydroxyproline concentration was monitored on the HPLC.

### Enrichment medium

20 mM L-hydroxyproline, 40 mM sodium formate, 1 mM acetate, 10 mM MOPS pH 7.2, 10 mM  $\text{NH}_4\text{Cl}$ , 1 mM phosphate buffer, 250  $\mu\text{M}$  thiosulfate, 250  $\mu\text{M}$  sulfate, 150  $\mu\text{M}$  sodium sulfide, 1X trace element, 1X vitamin, 1X freshwater base, 0.0001% resazurin

### Derivatization of amino acids for HPLC

Hydroxyproline, proline, and samples were derivatized through the van Slyke reaction to obtain the corresponding  $\alpha$ -hydroxy acids. 70  $\mu\text{l}$  of 1 M potassium nitrite was added to 350  $\mu\text{l}$  of standard or sample in 1.5 ml Eppendorf tubes. Reaction was started by adding 14  $\mu\text{l}$  of 12 N HCl and was stopped by adding 28  $\mu\text{l}$  of 5 N NaOH. Derivatization was carried out at 45°C for 90 min. Derivatized solutions were mixed with 5 N  $\text{H}_2\text{SO}_4$  at a 9:1 ratio for HPLC runs. HPLC method and retention times

Quantitation of  $\alpha$ -hydroxy acids was carried out on Aminex HPX-87P column with isocratic elution of 5 mM  $\text{H}_2\text{SO}_4$  at 0.4 ml/min and 60°C for 40 minutes. Detection by the RI detector was used to make standard curves. Hydroxyproline eluted at 18 min under these conditions.

### Sampling sites

Little Sippewissett Marsh, Trunk River, Cedar Swamp, School St. Marsh