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Complex Ciliary Flows around *Stentor Polymorphus*
in Solutions of 2% Buttermilk and *Chlamydomonas reinhardtii*

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Biophysics Senior Thesis
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May 4, 2020

Acknowledgements:

First and foremost, I would like to thank Professor Janet Sheung, my primary thesis reader, mentor, and lab director. The COVID-19 pandemic terminated my use of the lab early, and even though my data collection, and thus my quantitative analysis, was cut short, her support and willingness to help me finish my thesis never wavered. Also in the lab, I'd like to thank Keir Keenan and Isaac Reyes for doing a lot of the initial work on figuring out which type of milk would be the best for this project. Also within Keck, big thank you to Dr. Sadie Ottie from the organic chemistry lab for providing many of the chemicals I needed and Professor Wiley for being my second thesis reader. `

Outside Keck, thank you to Dr. Mark Slabodnick, of UNC Chapel Hill, who sent us some *S. polymorphus* cells from his own culture and was so incredibly helpful by sharing all the information he knew about the species. Thank you to Evan Burns, Professor Sheung's previous student at Vassar College who laid down the groundwork for both culturing *Stentor* and filming the vortex. And thank you Athena Lin from UCSF who guided me in the culturing of *Chlamydomonas reinhardtii*.

Finally, huge thank you to my family and friends who supported me during this year-long process. You were understanding when everything in the lab took longer than I guessed and kept my spirits up when I was struggling.

Abstract

Stentor are large, unicellular ciliates of the Heterotricha order. They live in both freshwater and marine habitats and are mostly found in ponds. I studied *Stentor polymorphus*, which is a species of *Stentor* only recently discovered to be lab culturable. They range from 0.5-1.5mm in length and are unusual because they live with endosymbiotic algae and are much more likely than other, more widely studied, species of *Stentor* to form aggregates while they are eating. There are three main components to this thesis: First, I established protocols for keeping a viable *S. polymorphus* culture, since no protocols had been published on the care and upkeep of a culture of this species. Second, I established protocols for and collected preliminary data on a single *S. polymorphus* cell feeding. Third, I developed imaging and data analysis protocols to investigate the ciliary flows of *S. polymorphus*.

In the wild, *Stentor* do not differentiate between active or passive food sources. Thus, I tested the usefulness of *Chlamydomonas reinhardtii* and 2% buttermilk as particle tracers to study the ciliary flows of *S. polymorphus*. Even with a limited data set due to the COVID-19 pandemic, there was a significant difference between the relative vortex sizes. *S. polymorphus* was able to create a larger vortex relative to its cell size in a buttermilk solution than it could make in a *Chlamydomonas reinhardtii* solution. To further my research, videos of *S. polymorphus* eating in colonies could be compared to them eating individually to analyse whether the aggregates are evolutionarily advantageous in any way.

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Introduction

Stentor are large, trumpet-shaped ciliates of the Heterotrichida order. They live in both freshwater and marine habitats and have been found all over the world. Even though they are only single-celled organisms, they have many complex systems, including regenerative abilities (outside the scope of this thesis), and complex use of their cilia located around their oral cavity and along the length of the cell. They use their cilia to swim, bind to surfaces in their environment, and eat, creating a vortex-shaped microflow that forces vegetation into their oral cavity. I studied *Stentor polymorphus*, a species of *Stentor* only recently discovered to be lab culturable. This species measures at 0.5-1.5mm in length, and displays a striking desire to form clusters to eat, seemingly “choosing” to feed in clusters even though their food is evenly distributed around the culture.

The biology of *S. polymorphus* is rather complex. They have two main morphologies: a round teardrop shape when contracted (mainly due to a “startle response”) and a trumpet shape when swimming or feeding (Fig 1A). They have endosymbiotic green algae living within its cells that has been identified as the *Mychonastes* species (Fig 1B) [5]. Each *Stentor* consists of an oral cavity, a long, trumpet-shaped body, and a holdfast that the animal uses to bind to materials in its environment. The anterior end of *S. polymorphus* consists of the peristomal cap surrounded by membranelles that spiral downward to form the oral cavity, or cytopharynx, and the internal buccal cavity (Fig 1C) [11]. Each cell has approximately 250 membranelles encircling the membranellar band and each membranelle has 3 rows of 20 to 25 cilia [11]. Additionally, larger and longer cilia are attached to the outer membranelles of the *Stentor* [11]. Coordinated cilia movement allow the cells to swim and feed.

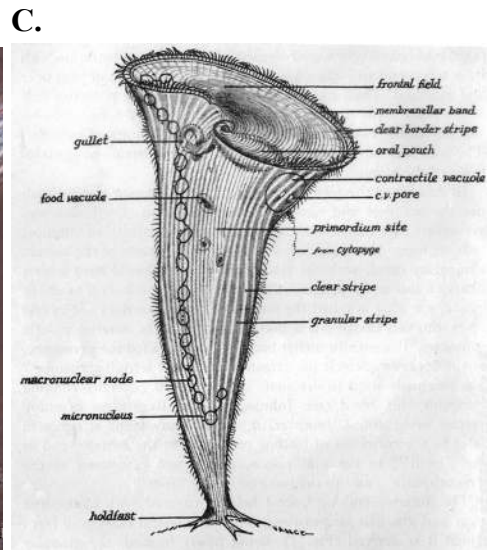
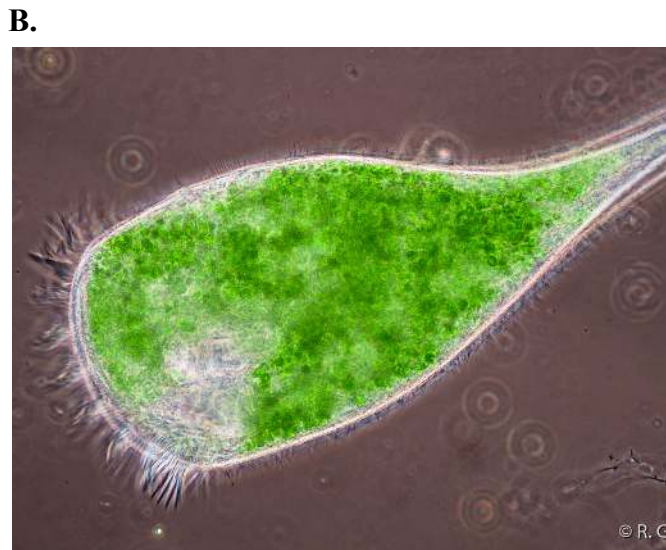
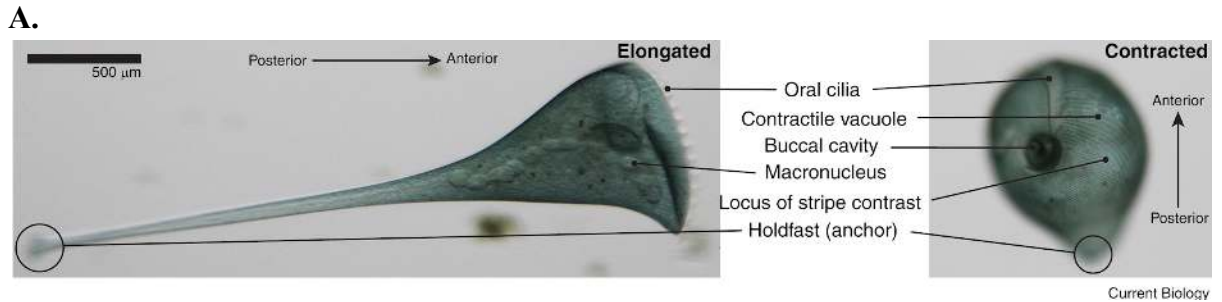


Figure 1. **A.** Different morphologies of *Stentor* (this image is of *Stentor coeruleus*, a more widely studied species of stentor) [13]. **B.** Endosymbiotic *Mychonastes* algae living in a *S. polymorphus* cell [4]. **C.** Basic structure of *S. polymorphus* [9].

Stentor has been shown to be aware of and react to elements in their surrounding environment. Evidence has been collected that demonstrates that they are food selective, which means they will only accept viable food particles and will reject indigestible particles like glass and sulphur. They will even demonstrate selectivity between different species of *Phacus*, an organism that they are able to digest [12]. When swimming, *Stentor* generally swim in a straight line, but are also capable of reversing direction when they encounter a noxious environment or scooting along a surface exploring it with their oral cavity [14]. Besides reacting to elements in their environment while swimming, *Stentor* also react when they encounter noxious stimuli while eating. Depending on how recently they've been fed, how intrusive the stimuli is, and how

persistent it is, *Stentor* will turn away and continue to eat, briefly reverse their direction of flow to send the substance away, quickly contract (Fig 1), or detach and swim away [14]. They are also capable of learned responses, where after being repeatedly exposed to a minorly negative stimulus for extended periods of time they cease their contraction response and continue to feed while “ignoring” the stimulus [14].

Stentor feeding has been studied extensively on an individual level, mostly with *S. coeruleus*. They use ciliary differentiation between cilia located on their short body for swimming and cilia located along the membranellar band for eating [15]. When they eat, they will attach their holdfast to a certain material and form an elongated trumpet shape, allowing their oral cilia to beat. These cilia beat metachronously, creating an “alimentary vortex” [15] that pumps food from their environment into their oral cavity (Fig 2). The mechanism of the holdfast involves an amoeboid disk of naked cytoplasm that is extruded and sticky combined with ectoplasmic projections, termed “radicules”, and cilia that have been converted into attaching pseudopods [1]. When *Stentor* are forcibly detached, the holdfast remains sticky for some time before being withdrawn.



Figure 2. Vortex created by feeding *Stentor*

As mentioned above, *S. polymorphus*, unlike other species of *Stentor*, are much more likely to form aggregates while they are eating, often forming them in groups of 100 or more *Stentor*. The clusters form many different shapes but almost never involve individual *Stentor* spreading themselves evenly throughout an environment, or even evenly across a popular binding surface. In all the clusters, they point radially outward (Fig 3). *Stentor* are capable of binding to a wide range of materials (we have witnessed glass, coconut fibers, and other kinds of vegetation) [14], so it seems as if they are capable of “choosing”, or have a preference for, certain materials and environments over others. Their clustering within the same material demonstrates that not only do they prefer one material, they also prefer to be clustered together on this “chosen” material. This seeming coordination between species to form clusters has not been studied previously in *Stentor*, but has been seen in a few other microorganisms. The amoeba *Dictyostelium discoideum*, for example, is single-celled only in conditions of nutritional abundance, combining to form essentially one multicellular organism upon starvation [16]. Similarly, quorum sensing, or cell-cell communication, has been found and explored in multiple different species of bacteria [6]. The binding patterns of *S. polymorphus* suggests a non-random element to where they choose to eat, similar to the two previously mentioned organisms.



Figure 3. Examples of *S. polymorphus* clustered on pieces of coconut fiber

It is unknown if there is an evolutionary advantage to *S. polymorphus* congregating on certain surfaces. In order to study this behavior, first *S. polymorphus* must be culturable in the lab. At the time of writing this thesis no protocols had been established on the culturing of *S. polymorphus*, but detailed protocols had been published on the culturing of *S. coeruleus* [8]. These protocols were followed closely when establishing the first *S. polymorphus* culture in the lab.

Second, an appropriate tracer particle must be identified that reveals the movement of the surrounding fluid while imitating the natural feeding style of *S. polymorphus*. Thus, I explored the benefit of using milk and *Chlamydomonas reinhardtii* to study the eating habits of *S. polymorphus* so the effects of the aggregates could be more closely examined. Previous research on cooperation within colonies of ciliates has been studied with *Opercularia asymmetrica* [17]. Similar to *Stentor*, these ciliates use cilia beats to create fluid flow in order to obtain nutrients from their environment. They also tend to live in colonies [17]. They have two stages in their life cycle in which they create micro-flows of nutrients. First, colonies of ciliates settle on other organisms and create a continuous nutrient flux to improve the colonization process. Second, once they have grown and a granule and core zone has developed, the ciliates inhabiting the outer layer contribute to further growth of the granula by creating a micro-flow of nutrients toward the surface of the granule [17]. Because of the cooperative nature of *Opercularia asymmetrica*, the colony's flow fields were studied instead of individual flow fields, and Zima-Kulisiewicz and Delgado did find improved efficiency in the nutrient seeking process [17], demonstrating with similar microorganisms to *Stentor* that feeding in colonies is more efficient than feeding alone.

Many things must be taken into account when studying the fluid flow dynamics in *S. polymorphus*. I aimed to study the *natural* behavior of *S. polymorphus*, so the environment under which we did all of our testing had to guarantee biocompatibility, or not stray far from environments they encounter in the wild [17]. Thus, the lighting used must be adjusted accordingly, as *Stentor* are light sensitive [14]. Additionally, the particles we use to track the fluid flow must fulfill the biocompatibility requirement, as *Stentor* are capable of food selection and are likely to either reject or perish from indigestible particles. Zima-Kulisiewicz and Delgado were able to use milk in their analysis of fluid flow [17], and this technique will be compared to *Stentor* eating *Chlamydomonas reinhardtii*, an algae they eat in their natural habitats. Even though milk is not their natural habitat, studies performed by Kowalczyk et al. also found milk to be the best tracers for fluid flow visualisation [7]. Milk allowed more detailed presentation of the flow field, created a “smoothing effect”, and can be used at a higher magnification [7]. However, the organism used in these studies, *Opercularia asymmetrica*, is 50 to 100 um in length [7], and *S. polymorphus* is much larger, with an average length of 0.5 to 0.7 mm [11], so larger tracing particles were used.

The MATLAB code developed by Gilpin, et.al [2], allowed me to create images that reflect the movement of the tracer particles surrounding a feeding stentor. The schematic below demonstrates how the MATLAB program completes the flow visualization (Fig 4). The blue dot represents the tracer particle, whose movement is represented by summing the 4 yellow frames together and adjusting for contrast. This motion is condensed into a final streak in the processed image, represented by the gray box that contains all 4 stages of movement. This same effect

happens to every particle surrounding the feeding stentor, and together represents the flow generated by the cell. The length of the streak corresponds to how fast the particle is moving.

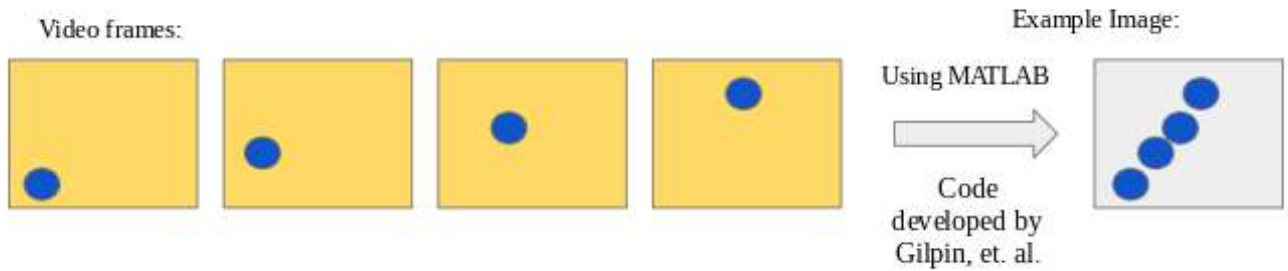


Figure 4. Schematic demonstrating how MATLAB code creates flowtrace images.

Materials and Methods

Maintaining a healthy *S. polymorphus* culture

First, Tris-Acetate-Phosphate, or TAP, media, was made to be used for our *Chlamydomonas reinhardtii* culture [3]. To make TAP media, 2.42 g Tris base, 1 mL Hunters Trace elements, 10 mL Solution A (Table 1), 1 mL Phosphate buffer (Table 1), and 1 mL Glacial Acetic Acid were dissolved in 850 mL DI water. I made sure to swirl the mixture until all solids were completely dissolved. Then, DI H₂O was added to reach the desired 1000 mL volume, and NaOH was used to adjust the pH until a desired pH of 7.0 was reached. The entire solution was then autoclaved to sterilize and prevent unwanted growth. It was stored in a refrigerator when not in use.

Table 1. Specific solutions needed to make TAP media

	Substance	Amount (g)	Concentration (mol/L)
Solution A (ingredients dissolved in 0.5 L of DI H ₂ O)	<i>NH₄Cl</i>	20.05 g	0.75 mol/L
	<i>MgSO₄</i>	5.06 g	0.83 mol/L
	<i>CaCl₂</i>	2.49 g	0.045 mol/L
Phosphate Buffer (ingredients dissolved in 0.1 L of DI H ₂ O)	<i>KH₂PO₄</i>	5.59 g	0.41 mol/L
	<i>K₂HPO₄</i>	10.84 g	0.62 mol/L

To start the *Chlamydomonas reinhardtii* culture, using sterile technique, small patches of *Chlamydomonas reinhardtii* were transferred from a commercial supplier using a sterilized inoculation loop into 50 ml screw-cap centrifuge tubes. They were then placed on the window sill in an orbital shaker to grow (Fig 5). If the culture became oversaturated, some of the original culture was poured into a new centrifuge tube filled with TAP media, while the original culture

was diluted with pure TAP. If any culture became contaminated, the culture was restarted with the original *Chlamydomonas reinhardtii* culture that came from the manufacturer.



Figure 5. *Chlamydomonas reinhardtii* culture on orbital shaker in window sill.

To prepare *Chlamydomonas reinhardtii* to feed the *Stentor* cultures, using sterile technique, the desired amount of *Chlamydomonas reinhardtii* culture (mixture of *Chlamydomonas reinhardtii* and TAP) was poured into a 17x100mm culture tube. For small starter cultures, approximately 4 mL of *Chlamydomonas reinhardtii* and TAP medium was used per 2 oz jar of *Stentor*. For larger cultures, approximately 10 mL of *Chlamydomonas reinhardtii* and TAP medium were used per 2 cup container of *Stentor*. The 17x100mm culture tube was then centrifuged at 3000 rpm (1308 rcf) for 1 min. Centrifugation speed does not need to be exact, the goal is to form a stable pellet. Supernatant was poured out, and the *Chlamydomonas reinhardtii* pellet was resuspended with pasteurized spring water: 1 mL pasteurized spring water

(PSW) per 2 cup container of *Stentor* or 0.25 mL PSW per 2 oz jar of *Stentor*. This resulting mixture of *Chlamydomonas reinhardi* and PSW was then pipetted into the *Stentor* culture.

To maintain a healthy culture, *Stentor* were fed either every 2-3 days to grow the colony, or every 3-4 days to maintain colony size. Once a week, the culture was examined under a microscope and, if it contained many contaminating microorganisms or an overgrowth of *Chlamydomonas reinhardtii*, any large clusters of these contaminants were pipetted out of the culture, and the culture was diluted with PSW. The *Stentor* culture was stored on the lab bench in an area of low light below 25 °C. Once *Stentor* concentration approximately exceeded 20 cells/mL, they were carefully poured into a larger container.

Collecting flow patterns of *S. polymorphus*

The vortices created by *S. polymorphus* while eating in a solution of *Chlamydomonas reinhardtii* were compared to *S. polymorphus* in a buttermilk solution. Both solutions have to be made same-day, because they do not keep well. To make the milk solution, 100ul of 2% buttermilk was combined with 8 mL of PSW (or equivalent concentration) and mixed well. To make the *Chlamydomonas reinhardtii* solution, one-half a saturated 17x100mm culture tube of *Chlamydomonas reinhardtii* was centrifuged at 1308 rcf for 1 min, supernatant was poured out, and a 17x100mm culture tube was filled with PSW to resuspend the *Chlamydomonas reinhardtii* pellet and dilute it to improve visibility of flow. It is difficult to standardize the concentration of *Chlamydomonas reinhardtii*, but doing the procedure above with a fully concentrated culture of *Chlamydomonas reinhardtii* produces the best results. When looked at under 55x magnification,

the solution should be concentrated enough to track the fluid flow well without blocking the view of the *Stentor*.

Once the solutions were made, a clean glass slide was placed on the microscope at least partially on a kimwipe (for easy sliding of glass slide). This could be achieved with either half the slide on a whole kimwipe or with cut slivers of kimwipe on each side of the slide (Fig 6).



Figure 6. Glass slides on microscope: either half (left picture) or partially (right picture) on kimwipe

Next, approximately 100ul to 200ul of the flowtrace solution was pipetted onto the glass slide. For best filming results, the bubble was spread out so it was thick enough for the *Stentor* to swim around but thin enough to not have a thick layer blocking the view of the *Stentor*. Using as small of a pipette as possible, approximately 10-15 *Stentor* were transferred from their cultures to the milk bubble. The gentler you are with the *Stentor* the faster they will adjust to their new environment and start eating. *The order with which the previous two steps is done depends on how much experience the researcher has with pipetting *Stentor*. It may be easier to choose *Stentor* from large culture, put them into a separate dish for easy access, and then put the solution onto the glass slide and transfer the *Stentor* onto the glass slide. If you wait too long after

pipetting the solution onto the glass slide the particles in the solution will sink and will not work as a tracer particle. Stirring the solution on the slide was not effective.

The *Stentor* were then allowed to sit for approximately 30-60 minutes, or for however long it took them to bind to the glass. Turning off the microscope light quickened the process of binding, but turning the light on after it had been off often causes an eating *Stentor* to flinch. The process here might depend on what works for your individual colony. Optimal lighting was then set up for filming fluid flow. The overhead lab lights were turned off to reduce glare and the lighting from the microscope was reflected off frosted glass to illuminate the *Stentor* at an angle from below (Fig 7).



Figure 7. Illumination of glass slide from below

Finally, find a *Stentor* that is bound and eating, and film for approximately 10 seconds.

Using Matlab code to analyse flow fields

The videos taken were processed using the code and methodology developed by Gilpin, et.al. [2].

MATLAB code, taken from www.flowtrace.org, was used to analyze the videos taken of *S.*

polymorphus eating, creating a simple visualization of the flow fields generated by the organism.

The videos were processed using a moving window of 60 frames merged in each image, with no extra parameters.

Results

By feeding *Chlamydomonas reinhardtii* to the culture regularly and keeping the culture clean and out of direct light, a healthy culture of *S. polymorphus* was established and maintained throughout the year. However, this process was not without some setbacks. The first method of cleaning involved transferring individual cells to a new, clean, bowl approximately once every two weeks. This process produced a dramatic enough change in the water to cause some mass deaths. It is critical to the survival of the colony to maintain a relatively clean culture (invasive species can take over the colony and overtake the *Stentor*) but cleaning it too fast can also cause death. Even with a non-contaminated *Chlamydomonas reinhardtii* culture, there was always overgrowth of various contaminating organisms in the culture. At its peak there were two contaminating rotifers in our colony along with the usual algae-like overgrowth (Fig 8). By feeding the colony enough and taking out large clusters of the contaminating organism they were kept at bay and the *S. polymorphus* were still able to thrive.

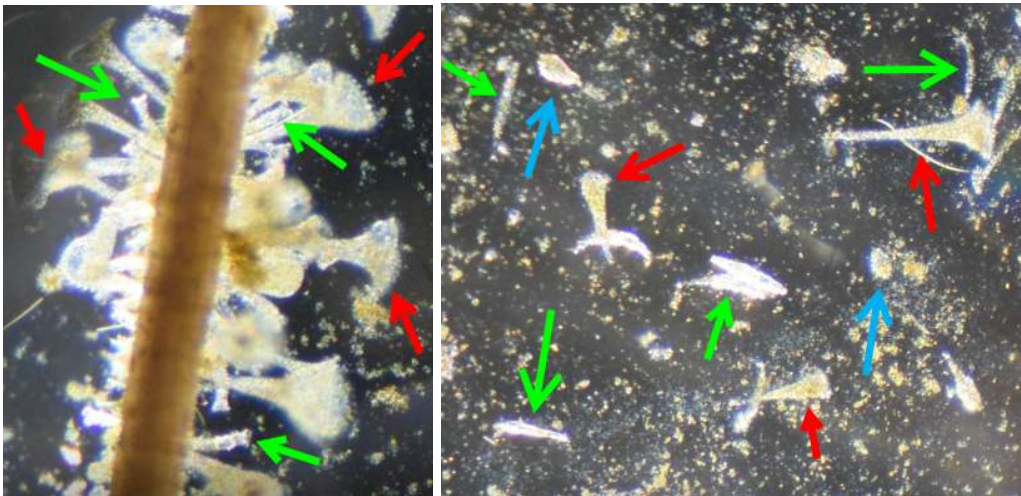


Figure 8. Contaminating rotifers and algae in *S. polymorphus* culture. *S. polymorphus* is the trumpet-shaped cell (red arrows), rotifers are the long worm-like organisms (green arrows), small round organisms (blue arrows), and green debris.

A total of 15 videos were analysed of *S. polymorphus* in *Chlamydomonas reinhardtii* and a total of 8 videos were analysed of *S. polymorphus* in 2% buttermilk. All the videos of *S. polymorphus* in *Chlamydomonas reinhardtii* solution were taken using 55x magnification using 1/60 shutter speed on the same camera and microscope. The videos of *S. polymorphus* in the buttermilk solution were taken at an unrecorded magnification, but were still at 1/60 shutter speed with the same camera. Using MATLAB, the videos were processed using a moving window of 60 frames merged in each image, with no extra parameters (Fig 9). To control for the orientation of the *Stentor*, only videos with both vortexes visible were used in this analysis.

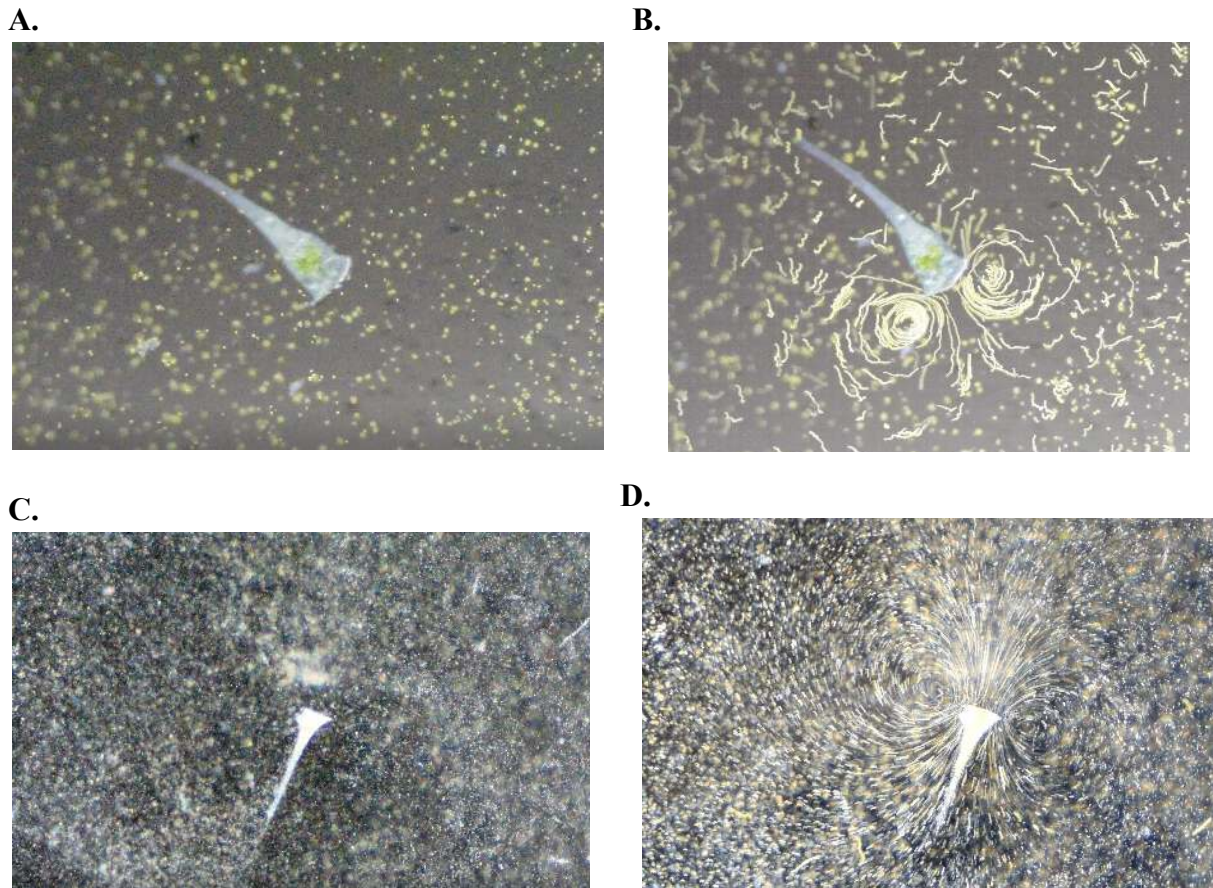


Figure 9. **A.** Unprocessed snapshot of video of *S. polymorphus* in *Chlamydomonas reinhardtii* **B.** MATLAB processed video of *S. polymorphus* in *Chlamydomonas reinhardtii* **C.** Unprocessed snapshot of video of *S. polymorphus* in buttermilk **D.** MATLAB processed video of *S. polymorphus* in buttermilk

Even though there is natural variation in size amidst a *S. polymorphus* culture (Fig 10A), the videos of *S. polymorphus* in buttermilk appeared to have been filmed at a different magnification than *S. polymorphus* in *Chlamydomonas reinhardtii*. To test this, the average length of each *S. polymorphus* cell and width of each oral cavity was compared between data sets. These measurements were taken using ImageJ software, which measures the lengths in pixels. There is a significant difference between the average size of oral cavities ($t(7,14) = -5.20434$, $p = 0.000037$) and average length of each *S. polymorphus* cell ($t(7,14) = -8.02444$, $p < 0.00001$) in the buttermilk solution when compared to the *Chlamydomonas reinhardtii* solution. The cells in the *Chlamydomonas reinhardtii* solution were longer and had a bigger oral cavity (Fig 10B) due to a magnification difference, not due to natural variation in the colony.

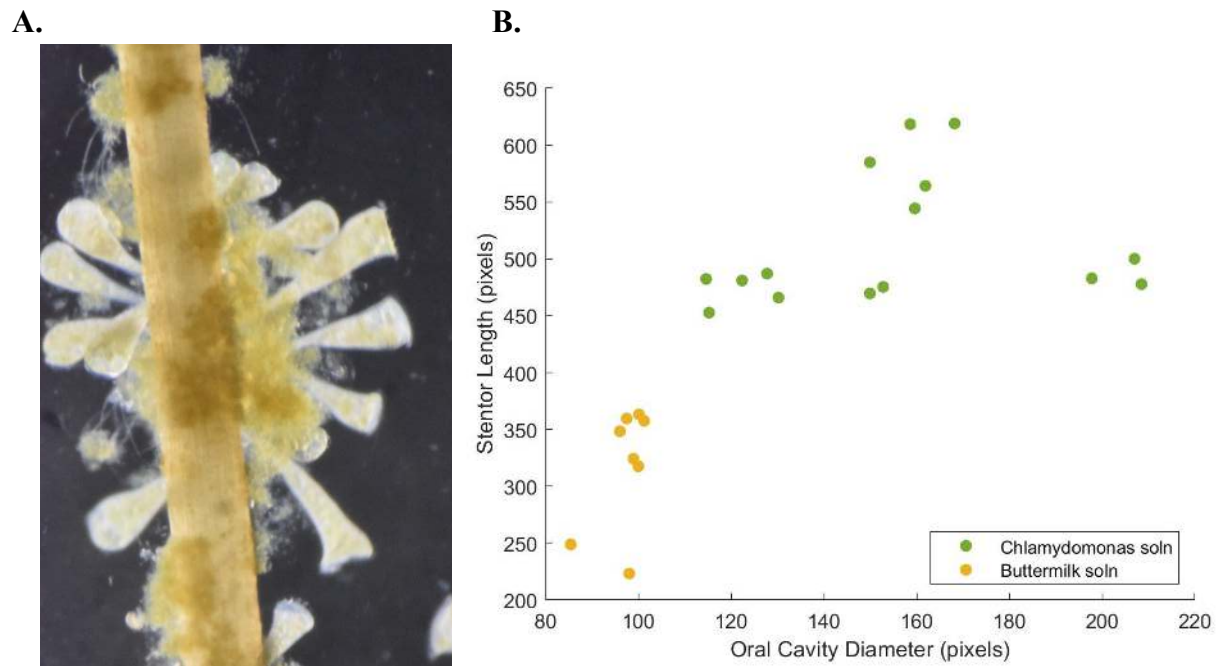


Figure 10. **A.** *S. polymorphus* cluster at 55x magnification using the same camera and microscope that was used to collect flowtrace data. **B.** Oral cavity diameter vs total length for *S. polymorphus* in *Chlamydomonas reinhardtii* solution vs Buttermilk solution.

To compare vortex size between the two tracer particles, a ratio of vortex size:oral cavity size was compared using all the images processed by MATLAB. I chose the end of the vortex from the last complete curve (Fig 11A). There is a significant difference between the average ratio of vortex size to oral cavity size when the *S. polymorphus* in the buttermilk solution is compared to the ratio in *Chlamydomonas reinhardtii* solution ($t(14,7)=-3.03339$, $p=0.00632$). *S. polymorphus* was able to create a larger vortex relative to its cell size in a buttermilk solution than it could make in a *Chlamydomonas reinhardtii* solution (Fig 11B).

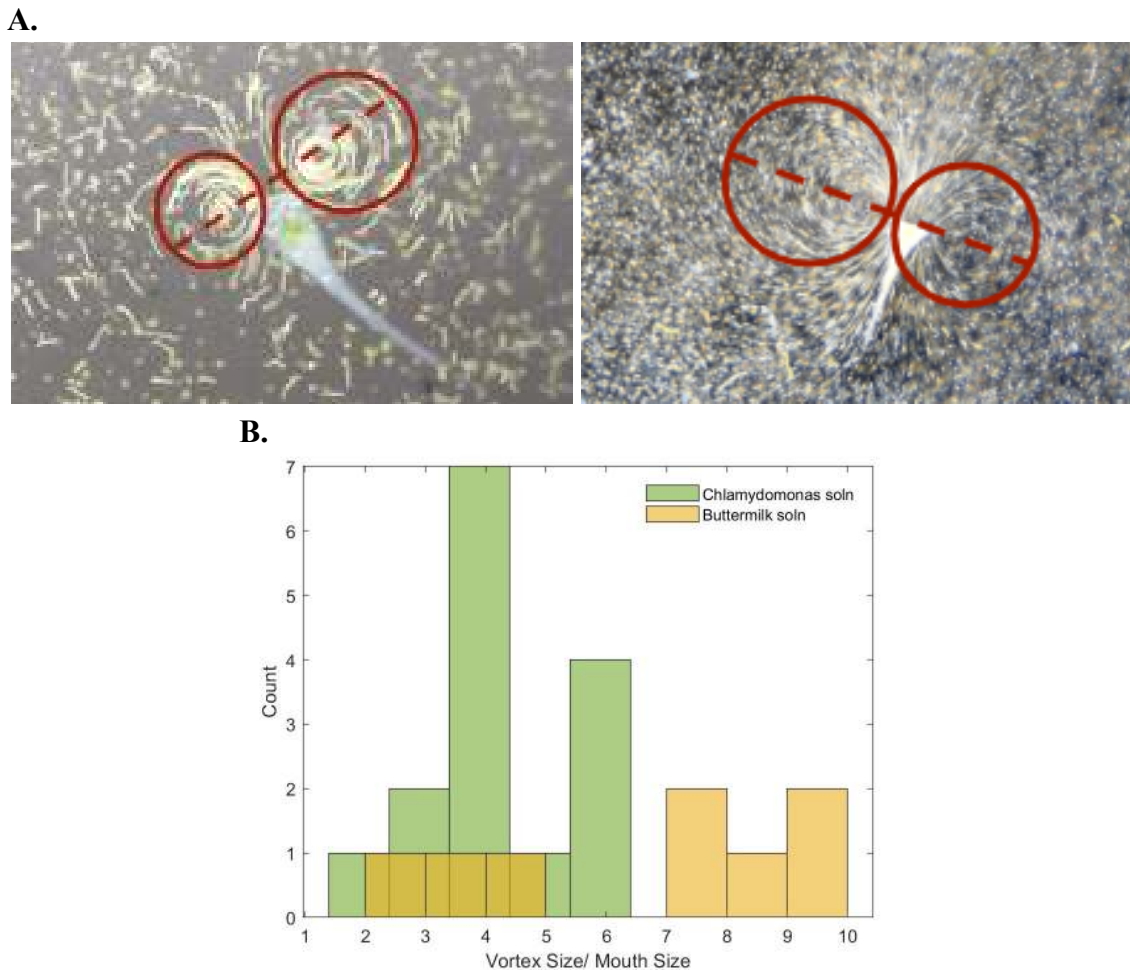


Figure 11. **A.** How vortex size was measured for histogram. The two red circles represent the two visible vortices and the dotted line represents total vortex size. **B.** Vortex size relative to oral cavity size of *S. polymorphus* in Buttermilk (mean = 6.642, SD = 1.126) and *Chlamydomonas reinhardtii* solutions (mean = 4.217, SD = 2.734).

Discussion

The videos in the buttermilk solution and the videos in the *Chlamydomonas reinhardtii* solution produced similar-shaped vortices (Fig 9). All eating *S. polymorphus* attached their holdfast to the glass and formed an elongated trumpet shape, allowing their oral cilia to create the “alimentary vortex” [10] that was consistent with previous research done on *Stentor*. However, *S. polymorphus* was able to produce a larger vortex relative to its cell size (Fig 11) with more consistent fluid movement around the cell (Fig 9) in the buttermilk solution. This is likely due to a difference in movement of the *Chlamydomonas reinhardtii* and the concentration of the tracer particle in the pasteurized spring water. A greater concentration of buttermilk was achieved because the *Chlamydomonas reinhardtii* sank to the bottom much more quickly than the buttermilk. When the *Chlamydomonas reinhardtii* was especially active, it produced an extremely different image, because the *Chlamydomonas reinhardtii* moved on its own in addition to being pulled by the *S. polymorphus* cell (Fig 12).



Figure 12. MATLAB processed image of *S. polymorphus* in active *Chlamydomonas reinhardtii* solution

It is normal for there to be wide variation in size among individuals in a colony of *S. polymorphus* cells (Fig 10a), but there is clearly a variation in magnification between the videos taken in buttermilk and the videos taken in *Chlamydomonas reinhardtii* solutions (Fig 10b). This is because the videos taken in buttermilk are preliminary data and were thus not at the same magnification as the *S. polymorphus* in the *Chlamydomonas reinhardtii*, which was data taken later. Preliminary data was used because of the evacuation of Scripps campus due to the COVID-19 pandemic, so there wasn't enough time to collect more data. The outliers on the scatterplot for the *S. polymorphus* in *Chlamydomonas reinhardtii* is likely because as *S. polymorphus* eats they sometimes will swing around their holdfast while creating the vortex (video of this behavior can be found at <https://youtu.be/Wa12S5NPC3M>), and the side-to-side movement of the cell would make it appear in the MATLAB processed image like it was wider than it is. However, it is not different enough to warrant throwing it out as a data point.

To compare the size of the vortex between the buttermilk and *Chlamydomonas reinhardtii* solutions, a ratio of vortex size to oral cavity size was compared. This ratio controls for the varying magnification levels that the videos were taken at. There is a significant difference in vortex size between the two solutions (Fig 11), which was expected of the two tracer particles, because *Chlamydomonas reinhardtii* is capable of swimming away from the pull created by the feeding *S. polymorphus* cell, but obviously buttermilk cannot swim. In the wild, Stentor do not differentiate between active or passive food sources, so either could replicate situations seen in their natural habitats. Most importantly, *S. polymorphus* was able to use their oral cilia to create the “alimentary vortex” in both solutions, which is consistent with previous research done on Stentor.

This data does not tell the full picture, however. Even though an attempt to control for the orientation of the cell was made, there wasn't enough data and there isn't currently enough known about how to identify the orientation of *S. polymorphus* to fully control for this variation. Thus, even though each image had a vortex on each side of the cell, the orientation was not always exactly the same (Fig 13).

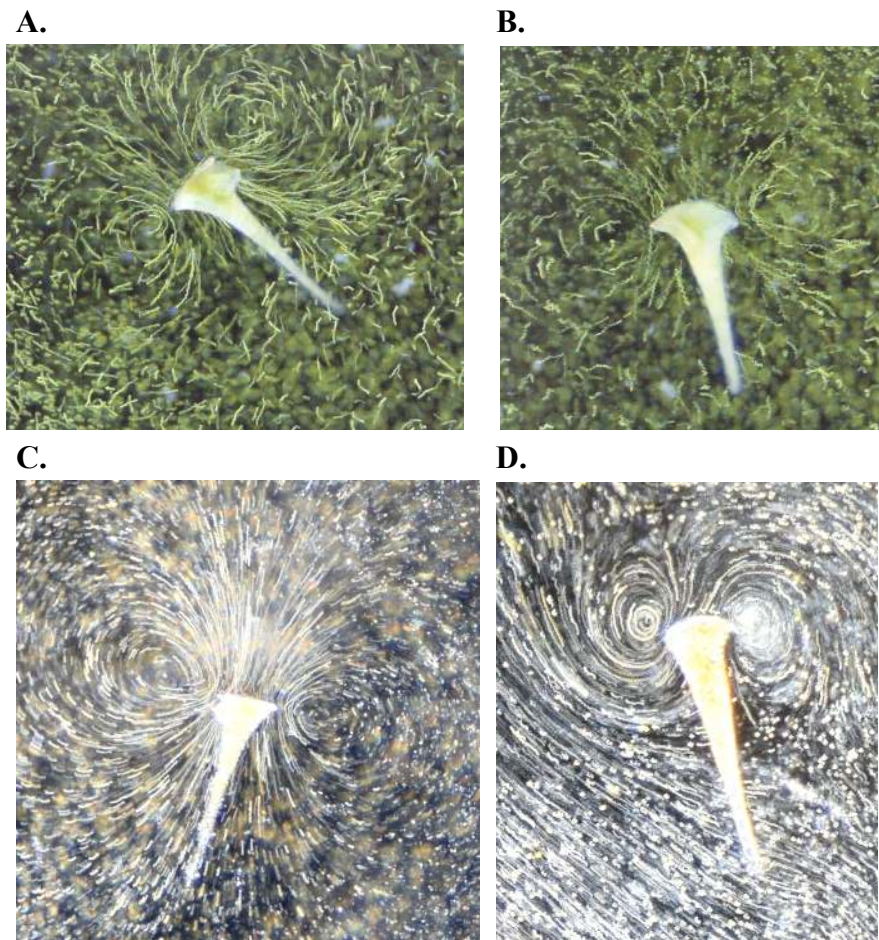


Figure 13. Despite there being a clear vortex on both sides of each *S. polymorphus*, **A** & **C** both have a gullet clearly visible on the side of the oral cavity, whereas **B** & **D** have a smooth oral cavity with the gullet not visible.

Conclusions

With consistent feeding and cleaning a healthy *S. polymorphus* culture was able to be maintained. In some ways, the *S. polymorphus* culture turned out to be more resilient than the *S. coeruleus* culture. They multiplied in numbers more quickly and experienced fewer deaths when fed less, which is likely due to their endosymbiotic algae. In maintaining the culture, it is important to only feed with clean, uncontaminated *Chlamydomonas reinhardtii*, without feeding too much or too little each time (too much food encourages contaminating organisms to grow and makes the culture “dirty” much faster). Also, it is important to never change more than 50% of the pasteurized spring water at one time in the culture to prevent shocking and killing the entire culture. If possible, it is helpful to have two cultures growing that you clean at slightly different times so there is a backup culture available if you experience a mass death.

The flow patterns created by a feeding *S. polymorphus* cell also turned out to be quite similar to the flow patterns previously studied with *S. coeruleus*- *S. polymorphus* was able to create an alimentary vortex in both solutions of 2% buttermilk and in *Chlamydomonas reinhardtii*. However, there were some differences between the active and passive tracer particles that should be explored further. Even though they both replicate conditions seen in the ponds where *S. polymorphus* live, they created significantly differently-sized vortices. Active tracer particles, like *Chlamydomonas reinhardtii*, are capable of swimming away from the flow created by a feeding *Stentor*, but by swimming around, they will also repopulate any areas of lower concentration created by the cell pulling in particles from the surrounding area. This likely results in a smaller vortex because force created by the cell on the surrounding fluid would be

counteracted by the swimming *Chlamydomonas reinhardtii*, and they would need to reach less far because the area around them would just be repopulated.

The differences between vortices should be analysed using particle image velocimetry (PIV). To further my research studying the flow patterns created by *S. polymorphus*, more data should be collected on individual *S. polymorphus* cells feeding in the two different particle tracers. This more quantitative analysis could then be performed to study the eating habits of *S. polymorphus* and truly parse out the difference between the two tracer particles.

This same analysis could be used to study the eating patterns of aggregates of *S. polymorphus*. *S. coeruleus* does not display the same binding patterns as *S. coeruleus*, even though the vortices created by both species appear to be comparable. To further my research, videos of *S. polymorphus* eating in colonies could be compared to them eating individually to analyse whether the aggregates are evolutionarily advantageous in any way. This could then be compared to the eating patterns to *S. coeruleus*. From our observations so far it appears that multiple *S. polymorphus* cells from the same colony feed at the same time, but we have also witnessed individuals in an aggregate switching off, or potentially stealing, each other's flow. Using the quantitative analysis mentioned above, both the width of the vortices and how fast each particle is moving could be compared between one feeding *S. polymorphus* cell and many to determine if there actually is a difference.

This is all to ultimately figure out if *S. polymorphus* cooperates. Specifically, can we discover this by quantifying the flows of individuals in a colony? This could illuminate single-celled intelligence or communication, both of which would greatly add to our understanding of *Stentor* as a species.

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