Exploring the effects of salinity and temperature gradients on *Nereocystis luetkeana* sorus incubation and subsequent spore release in the San Juan Islands

Olivia Seyler[1,2], Swetha Sivasubramanian[1,2], Carter Webb[1,3], Ashley Yamamoto[1,2]

FHL Marine Botany 2025
Summer 2025

[1] Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250
[2] Department of Marine Biology, University of Washington, Seattle, WA 98195
[3] Department of Mathematics, Seattle University, Seattle, WA, 98122

Contact Information:
Olivia Seyler
College of the Environment
University of Washington
1410 NE Campus Pkwy, Seattle, WA 98195
olilives7@uw.edu

Keywords: Bull kelp, *Nereocystis luetkeana*, sporulation, salinity, temperature

Abstract

Climate change continues to subject kelps of the Salish Sea to unpredictable and unforeseen conditions. Circumstances such as increased freshwater input from riverine outflow and rising temperatures in the estuarine waters of Washington State and British Columbia have been attributed as leading causes for widespread kelp decline. To assess the potential for compounding deleterious effects induced by large temperature and salinity swings, bull kelp (*Nereocystis luetkeana*) sorus samples were collected from two nearshore habitats along the Friday Harbor coastline. The sori were then incubated in a range of conditions from 8ppt - 32 ppt salinity and 10°C - 22°C in an attempt to mimic recently observed conditions in local waters. The results highlight a small window of viable incubation for the most extreme end (low salinity, high temperature) of the exposure gradient while requiring longer durations for those in control treatments, depending on specific incubation parameters. An exploration into the effects of a range of salinity and temperature exposures during the sorus development of the brown algae, *Nereocystis luetkeana,* returned mixed spore density results, with varying incubation success. The state of the sori also varied during incubation, with boils appearing on all of the 8ppt treatments except for in phase 3 with the 8ppt 10°C treatment sori. For spore density, the highest density observed was in the 32 ppt 16°C treatment, and the most evident relationship visible was shown by lower spore count at lower salinities.

1.0 Introduction

<u>1.1 Biological Context</u>

As climate change intensifies, the ecological consequences of temperature stress on marine algae are becoming increasingly evident. Heat stress, a known contributor to declines in *Nereocystis luetkeana* overall health, growth, reproductive stages, and distribution is becoming a localized driver of kelp sporophyte forest canopy loss (Hollarsmith et al., 2022; Mora-Soto et al., 2024). The loss of production and habitat creation in keystone species such as *N. luetkeana* can be akin to a death sentence for many nearshore ecosystems, causing trophic cascades that can result in the local disappearance of a wide range of species (Hollarsmith et al. 2022). In the Salish Sea, the Kelp Stressor Rating Workgroup (KSRW) identified temperature as one of the most deleterious abiotic stressors affecting kelp, particularly during the sporophyte phase, citing evidence from losses, specifically those seen in southern Puget Sound (Raymond et al. 2024).

However, research into its impact on earlier life stages—namely sori incubation, zoospore settlement, and gametophyte fertilization continues to mature (Weigel et al., 2023; Small & Weigel, 2022). The lifecycle of *N. luetkeana* is complex (Figure 1). In its most simplified form it consists of four phases. It starts with the sporophyte phase where the sorus, a collection of unilocular sporangia, develops on the distal end of the blades until maturity, and falls to the ocean floor when ripe. Zoospores release from these sporangia and settle from the water column onto, typically, hard benthic substrate. Next is the gametophyte stage, where gametophytes develop from settled zoospores into sexually dimorphic gamete-producing thalli . Lastly, the fertilized sporeling begins the cycle anew (Fig 1). The microscopic life stages of this charismatic kelp's life cycle have been deemed a "black box" by many experts as little is known about the recruitment process and overwintering of the gametophytic stage, let alone what factors affect the success of these processes (Berry et al. 2019). Emerging studies suggest that the effects of interacting stressors, such as nitrogen availability, sedimentation, and hypersaline conditions, in conjunction with temperature, can have major deleterious effects on a range of stages within the lifecycle (Lind and Konar 2017, Drakard et al. 2025). While experts initially prioritized nutrient loading and sedimentation at levels similar to temperature, salinity was not considered as significant (Raymond et al. 2024). However, emerging evidence indicates that salinity can play an important role in shaping ecological responses (Drakard et al. 2025). Given this new understanding, it is crucial that we reexamine the impacts of salinity as a stressor.

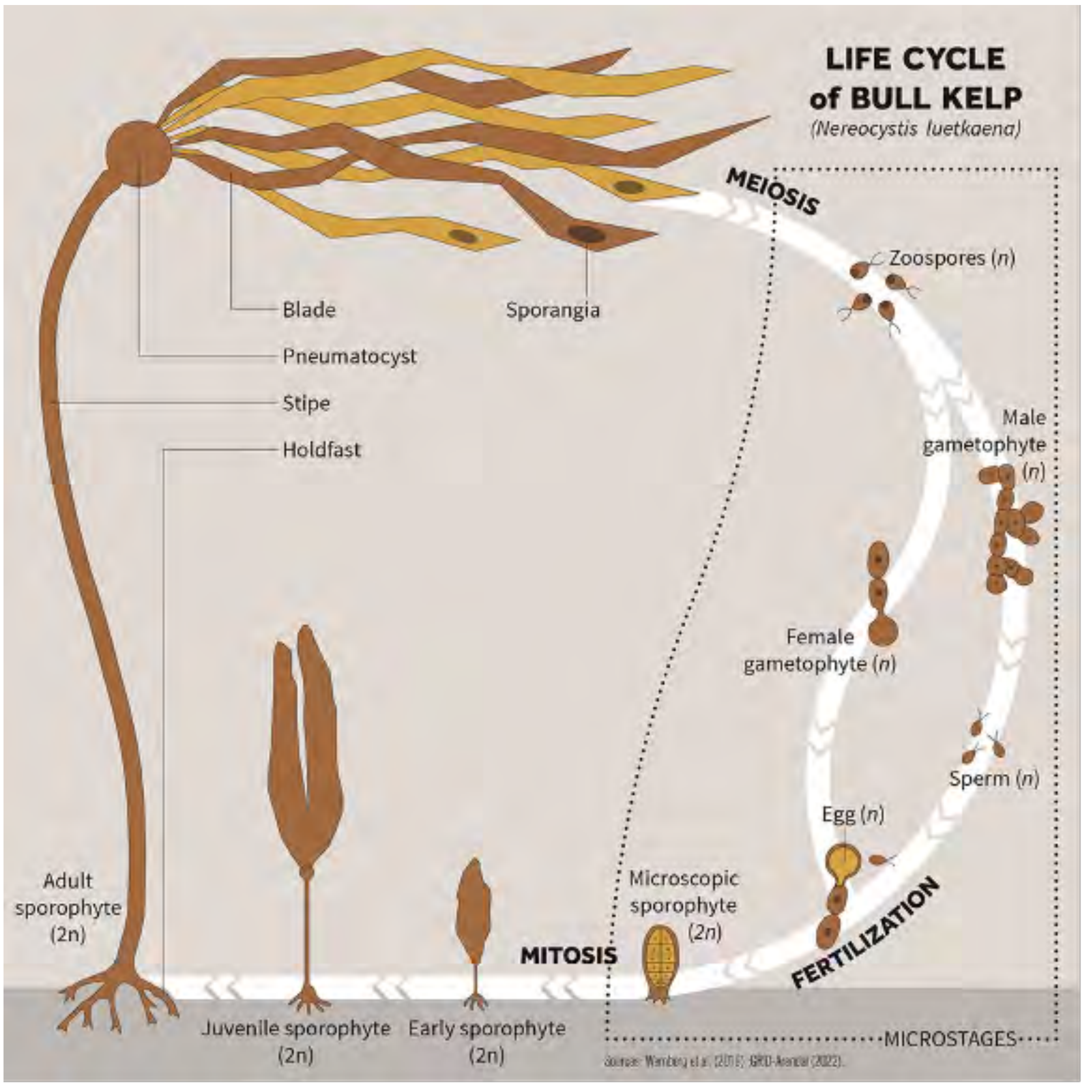

Fig. 1 Bull kelp life cycle.

1.2 Environmental Context

Prior to this research study, data collected from the University of Washington's Friday Harbor Labs Ocean Observatory (FHLOO) showed high variability in salinity and temperature data because of its relatively central position between the Fraser River Delta and the Strait of Juan de Fuca. The two geographic hallmarks are the Salish Sea, and certainly San Juan County's, most influential freshwater and saline water sources respectively ([dataset] Ninokawa, A. T, Broatch and MacCready 2022). Analysis of FHLOO's data depicts salinity exposure ranging from ~23ppt to ~32ppt and temperature patterns ranging from ~7°C to ~16°C (Figs 2 & 3). Over time, these ranges may become more variable and have baseline shifts. In fact, climate-driven shifts in freshwater discharge patterns, particularly near river deltas, may significantly influence local salinity regimes into the future (Broatch and MacCready 2022). Moreover, throughout the western coast of Haro Strait, temperature has been shown to vary between 9.6°C and 25.0°C from 1984-2022, a significantly larger range than seen currently in the San Juans (Mora-Soto et al. 2024). Even anecdotal observations of morphological variation in *N. luetkeana* across salinity and temperature gradients—from the saline Strait of Juan de Fuca to brackish estuarine zones like the Snohomish, Skagit and Fraser River deltas—support concern of salinity effects. Extreme examples of temperature and salinity variations have been experienced at some sites in the area, namely that of Cherry Point, where temperatures can range from 8°C to above 18°C ([presentation] Linhardt, S. 2025). Despite significant losses, this forest has responded with surprising resilience, but more research is needed to understand the causes. Additional experts in *N. luetkeana* restoration and sporulation shared our interest in testing the extreme limitations and possible outliers of both temperature and salinity stressors (pers. comm. Hayford H., Florendo J., Claar D.) For these reasons, testing extreme conditions that the *N. luetkeana* forest may have yet to experience in the Salish Sea became a primary goal of this research.

Furthermore, investigation into these compounding effects will shed light on a persisting knowledge gap in sporulation and recruitment of Laminariales. If an understanding of tipping points and stressor tolerances of *N. luetkeana* sori and their following life stages are illuminated, it could significantly improve restoration and conservation efforts informing what conditions may allow for refuges or persistence through suboptimal conditions.

Missing some literature on effects of salinity on Nereo.

No hypotheses? Careful reading of the literature would help you formulate an hypothesis.

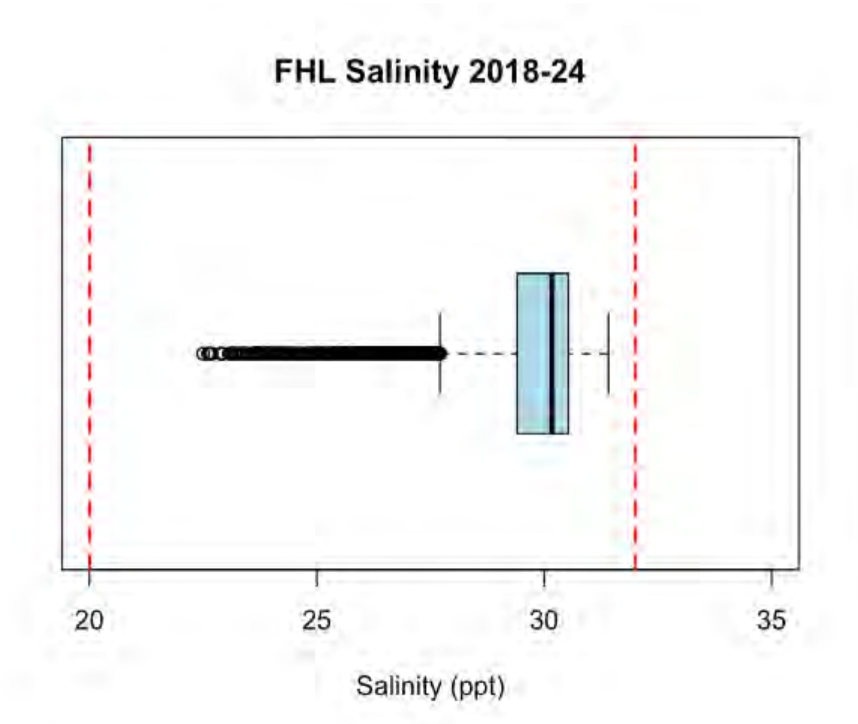

Fig. 2 depicting interquartile range of Salinity Values within Friday Harbor Collected from Friday Harbor Lab's Ocean Observatory from 2018-2024. Red lines depict the chosen values for research at 20 and 32°C. Outlier treatment 8°C omitted on this graph for simplicity.

Data Summary: Min. 22.50ppt. Mean 30.17ppt Max. 31.41ppt

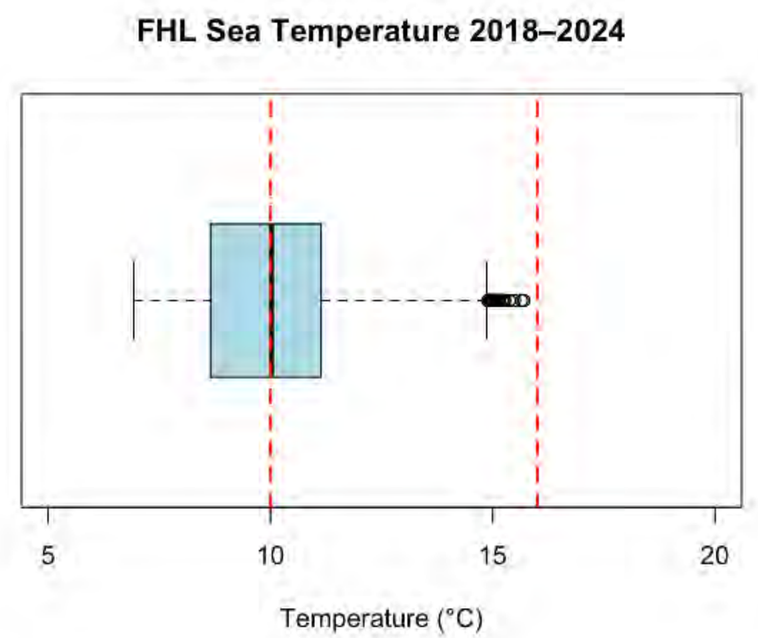

Fig. 3 depicting interquartile range of Temperature Values within Friday Harbor Collected from Friday Harbor Lab's Ocean Observatory from 2018-2024. Red lines depict the chosen values for research at 10 and 16°C. Outlier treatment 22°C omitted on this graph for simplicity.

Data Summary: Min. 6.928°C Mean 9.988°C Max. 15.700°C

2.0 Methods

2.1 General

In seeking an understanding of salinity and temperature stress on developing *N. luetkeana* sori, our research project was divided into three distinct phases. Phase 1 was developed under the simplest construction possible to preserve the entire holobiont that was the incubating sorus. This phase was developed with minimal water sterilization, whole sorus incubation, and long incubation times. Phase 2, on the other hand, was intended to shed light on maximum incubation times for sori subjected to the harshest conditions in our research, with increased water sterilization and the use of sori "cutouts" instead of whole sori. This phase was developed in a response to the loss of many of our most stressed sori in phase 1. Lastly, phase 3 was the culmination of what we had learned in phases 1 & 2, with a stronger emphasis on sterilization and contamination protocols and, again, the use of sori “cutouts” to increase the amount of replicates. For all 3 phases, sori were collected from nearshore environments either in Friday Harbor or just outside the mouth (Fig 4). See section 3.0 for a materials list of all phases.

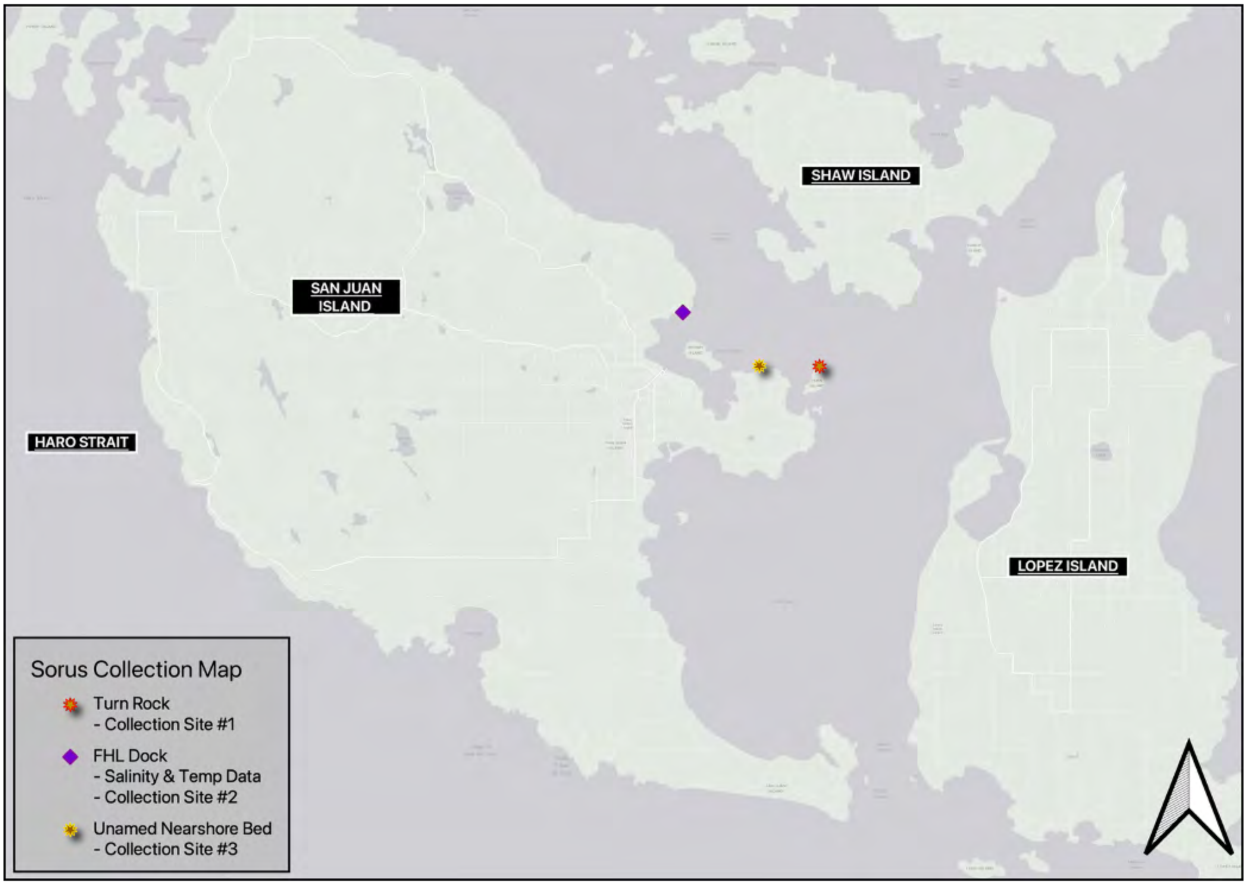

Fig. 4 Depicts collection locations for sorus incubation and data source location for temperature and salinity contextual data seen in figures 2 & 3

2.2 Incubation

Incubation for all phases took place in a 10°C cold room in the basement of the Fernald Building at Friday Harbor Labs. All sori were incubated in one of three aquaria (Appendix 30). These aquaria consisted of a small tank of volume 40,816.5cm^3 , kept at ambient temperature of 10°C, a medium sized tank of volume 52,704cm^3, heated to 16°C and finally, a large tank of volume 80,291.25cm^3 heated to 22°C, acting as our most extreme outlier. Sori were then subjected to different salinity baths within each aquarium 8 ppt (+/- 1), 20 ppt (+/- 1), and 32 ppt (+/- 1). The amount of saline water, time of incubation, and sterilization methods varied by phase. See the methods for each phase in subsequent sections for details. All phases used standard seawater collected from the seawater pump at Friday Harbor Labs campus and was tested using a RHS Series Handheld Refractometer. Tests repeatedly showed that this seawater stayed relatively stable at 32 ppt (+/- 1). Seawater was diluted using the equation $C1V1 = C2V2$ to the desired ppt for each treatment (8, 20, 32) yielding a range of 12 ppt (+/- 1) between each treatment value. Any seawater that was used to clean sori, rinse containers/beakers after sterilization or used in sporulation was first filtered through 0.22μm filters. All tools, containers, beakers, forceps were sterilized before or in between uses with at least 70% isopropyl alcohol, and in the later phases (2 & 3) were sterilized with 91% isopropyl alcohol. If sori were handled at any point during any of the three phases, hands were sterilized with 70% isopropyl alcohol or sterile latex gloves were used. Similarly, all sori were processed before incubation by trimming, though those procedures vary depending on phase, therefore, to read more about those methods see subsequent sections.

2.3 Desiccation

Desiccation procedures remained the same across phases 1 & 3 and were not necessary in phase 2. All desiccation procedures were pursuant to the procedures outlined in the Puget Sound Restoration Fund's Kelp Cultivation Handbook. While some steps were unachievable due to resource and time restrictions, all were adhered to the best of our abilities.

Instead of Betadine used for the Puget Sound Restoration Fund (PSRF) suggested, we used a generic antiseptic solution povidone-iodine 10%.

Appendix 48-87 post incubation before desiccation.

Step 1: Mechanical Cleaning

- Gently wipe each trimmed sorus tissue with a clean dry? wet? paper towel with light pressure.

Step 2: Initial Soak in 0.22µm filtered Seawater

- Place cleaned sori in a 2L wide-mouth Nalgene bottle containing 0.22µm filtered seawater.
- Use multiple containers if necessary.

Step 3: Povidone-iodine Disinfection

- Transfer sori to a new Nalgene bottle containing diluted povidone-iodine solution (5 mL of 10% iodine in 1L 0.22µm filtered seawater).
- Cap and shake vigorously for **30 seconds**.

Step 4: Triple Rinse

- Remove from povidone-iodine solution and transfer to a new Nalgene bottle.
- Fill Nalgene with 0.22µm filtered seawater and shake for **30 seconds**.
- Repeat this rinse **3 times**.
    - Option: remove sorus and rinse in a new Nalgene instead.

Step 5: Blot and Layer Sorus

- Remove sorus one at a time.
- Gently blot dry and alternate layers of wet? Dry? paper towels and sorus (creating a "sorus lasagna").

Step 6: Mild Desiccation Setup

- Place the layered paper towels and sorus in a sterile glass baking dish or large plastic bag.
- Add 1–2 dampened paper towels (with 0.22µm filtered seawater) to maintain slight humidity.

Step 7: Desiccation

- Store the container in a refrigerator or cool room at 4–5°C for **1–24 hours**.
    - **Space in the fridge was limited for our project, so the 10°C cold room was used.**
    - Riper sori needs less time (~4 hours).
    - Less ripe sori may require up to 24–48 hours.

Sporulation (Day 2)

Step 1: Check for Spore Release

- Remove the container from refrigeration.
- Gently peel back the paper towel layer.
- If ready, sori will leave a brown stain, indicating spore release.

These steps were used in phases 1 & 3.

<u>2.4 Sporulation</u>

Sporulation procedures remained the same across phases 1 & 3 and were not necessary in phase 2. All desiccation procedures were pursuant to the procedures outlined in PSRF's Kelp

Cultivation Handbook. While some steps were unachievable due to resource and time restrictions, all were adhered to with the best of our abilities.

Sporulation Procedure (Day 2)

Step 1: Preparation for Sorus Sporulation

- Transfer sorus cutouts to designated containers for continued culture and spore release in 300mL 0.22µm filtered seawater.

Step 2: Incubate for Sporulation

- Cover beakers with clean plastic wrap or lightly tighten lids.
- Place in a temperature-controlled cultivation room set to **10°C**.
- Ensure the room is **well-illuminated**.
- Incubate for **1–3 hours**.

Step 3: Assess Spore Release

- Visually check for spore release — water should appear **cloudy** if release is successful.

These steps were used in phases 1 & 3.

<u>2.5 Spore Counts</u>

Spore Density counts were completed during phase 1 for each of the surviving sori using an Improved Neubauer Lined, Bausch & Lomb Hemocytometer (Fig 5). All counts were done according to the Ocean Approved Kelp Farming Manual (Flavin et al. 2013). This manual outlines two methodologies that are best suited for varying levels of spore density. These methods aim to expedite counting efficiency, especially as spores can lose motility over time when subjected to radiative heat from the microscope slides.

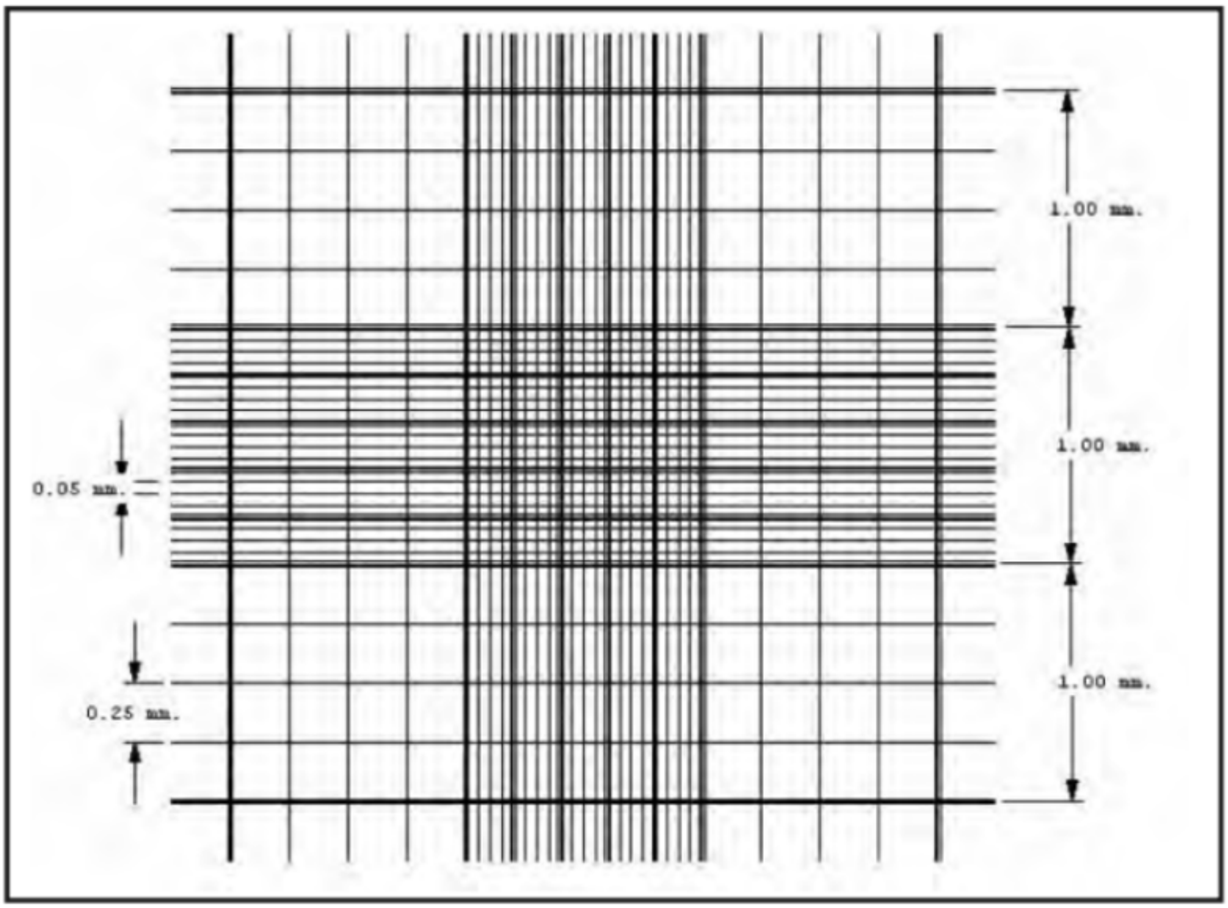

Fig. 5 Depicts the gridded lines of an Improved Neubauer Ruled Hemocytometer. All units are in nanometers (nm) Source: Ocean Approved, Kelp Farming Manual.

Method 1 – Begin by counting the 0.25nm grid boxes, labeled **1, 2, 3, 4** in figure 8. After all four boxes are counted, data is to be processed by the formula seen in figure 6. If spore density was a count of 50 or more spores in any boxes 1- 4 in figure 8, prompting us to abandon method 1 and initiate method 2.

Method 1 counts start in the lower leftmost corner counting each individual in the 0.25nm x 0.25nm box from left to right, then up, then back right to left in a snaking pattern for each box 1-4. Then, it uses the equation from figure 6 below to calculate the final spore density in spores/mL.

$$\text{Zoospore Density (Spores/mL)} = \left(\frac{\text{Sq. 1} + \text{Sq. 2} + \text{Sq. 3} + \text{Sq. 4}}{4} \right) \times 10{,}000$$

Fig. 6 Depicts the recommended equation for spore density in Method 1 of the hemocytometer counts in spores/ml Source: Ocean Approved, Kelp Farming Manual.

Method 2 – Count solely the inner most 0.05nm gridded box, labeled **5** in figures 8 & 9. Count the bottom left box 0.25nm x 0.25nm and continue from left to right then up, then back right to left in a snaking pattern for the entirety of box 5. For our purposes we used this method and the equation from figure 7 below to calculate spores/mL.

$$\text{Zoospore Density (Spores/mL)} = \text{Square 5} \times 10{,}000$$

Fig. 7 Depicts the recommended equation for spore density in Method 2 of the hemocytometer counts in spores/ml Source: Ocean Approved, Kelp Farming Manual.

Alternatively, the PSRF Kelp Cultivation Handbook advises selecting at random a column within box 5 to count for spore density, though this method was not used during our project. Instead, we utilized the Ocean Approved Kelp Cultivation Manual as a guide for hemocytometer counts. As the PSRF's Kelp Cultivation Handbook is derived from said methods, the Ocean Approved manual had a more in depth step by step list for us to follow.

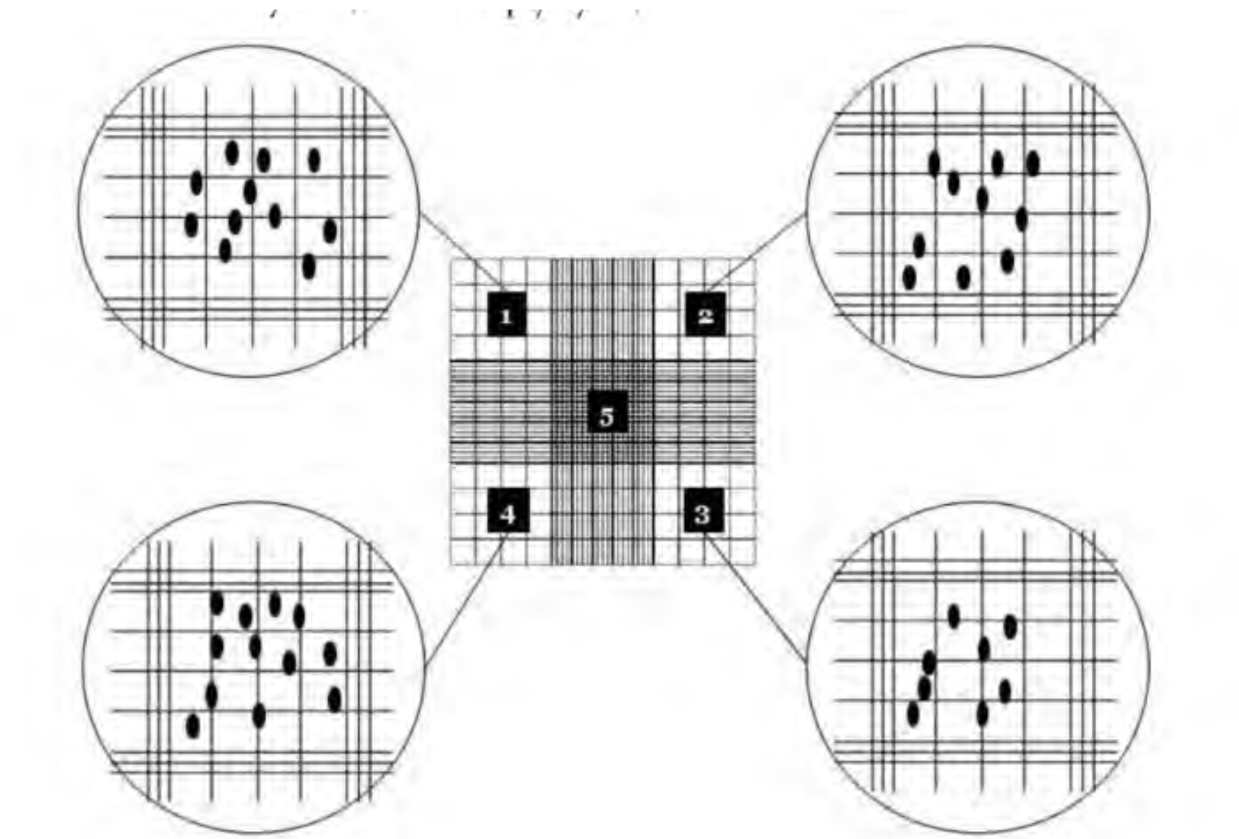

Fig. 8 Depicts the defined counting squares of an Improved Neubauer Ruled Hemocytometer. It also depicts the counting methods for boxes 1-4, mentioned above. All units are in nanometers (nm) Source: Ocean Approved, Kelp Farming Manual.

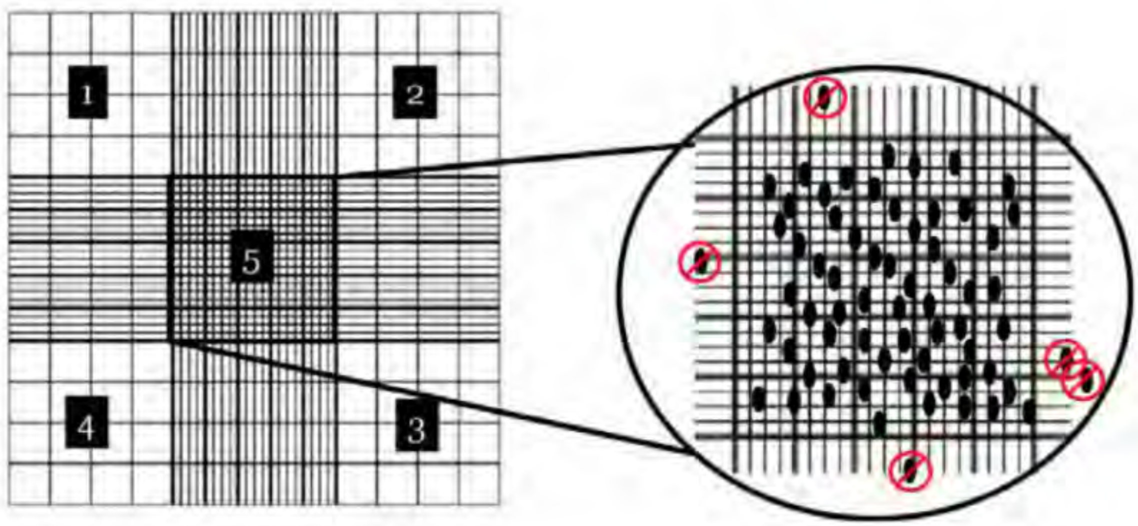

Fig. 9 Depicts the defined counting squares of an Improved Neubauer Ruled Hemocytometer. It also depicts the counting methods for box 5, mentioned above. All units are in nanometers (nm) Source: Ocean Approved, Kelp Farming Manual.

2.6 Phases

Phase 1 began with the collection of 27 sori from the Turn Rock kelp bed (Appendix 18) which were rolled and placed into a cooler immediately after collection. Collected sori were chosen based on maturity and, to the best of our ability, from separate individual plants. The cooler contained a base of ice, four or more layers of paper towels dampened with seawater and lined with a sterile trash bag. Sori were collected at 1:15pm on June 28th 2025 and transported back to Friday Harbor Labs by 2:00pm the same day.

Upon arrival at Friday Harbor Labs, sori were culled based, again, on further maturation screening and processed by cutting the edges of the blades from the sorus up to roughly 1cm away from the sori. Much care was taken to not cut or damage the sori, attempting to maintain the intact holobiont (sorus , surrounding sterile tissue, and mucilage structures as well as the collected microbiome). Initially we intended to incubate three sori in each treatment, however, we quickly discovered our incubation containers, food safe 25oz Glad Sandwich Containers, were not large enough for three large sori . We were then forced to only incubate two sori per treatment (Appendix 21). Sori were then given index names, as shown in Table 1, and placed into incubation. Sorus B4 unfortunately did not make it into incubation and was lost during processing.

Table 1. Outlines incubated sorus index names allowing for the tracking of each sorus through space and time.

Treatment Conditions	32 ppt		20 ppt		8 ppt	
10C	Sorus A1	Sorus B1	Sorus A2	Sorus B2	Sorus A3	Sorus B3
16C	Sorus A4	Sorus B4	Sorus A5	Sorus B5	Sorus A6	Sorus B6
22C	Sorus A7	Sorus B7	Sorus A8	Sorus B8	Sorus A9	Sorus B9

Incubation was planned for 96 hours (4 days) but was shortened to 80 hours (over 3 days) due to the losses of sori A5-B9 before desiccation. Sori were incubated in a bath of once tyndallized water of different salinities in their respective aquaria of different temperature. All incubation baths took place in plastic food safe 25oz Glad Sandwich Containers each with their own air stone (see 2.2 Incubation for further details).

After 80 hours, all five remaining sori then had standard 7.0cm x 2.5cm, microscope slide sized cutouts made which were then put through steps outlined in desiccation (see 2.3 Desiccation for further details). Subsequently, cutouts went through sporulation in plastic embryology containers (Appendix 22-25) where spore release was observed and hemocytometer counts were subsequently taken (see 2.4 Sporulation for further details).

See Appendices 13-26 for photos of the phase 1 steps. See supplementary material phase 1 for detailed accounting of each sorus' life history from collection to hemocytometer counts

Phase 2 began by collection of a single *N. luetkeana* blade from the Friday Harbor Labs Dock which contained two sori. Two, 7.0cm x 2.5cm, cutouts were taken from the more mature sorus which represented the typical maturity of the sori from the other phases 1 & 3. A third sorus cutout was taken from the lesser mature sorus which was significantly less mature than the sori used in other phases 1 & 3. These cutouts were then placed into the most extreme condition from the other phases 1 & 3, at 8ppt and 22°C, to better understand the maximum incubation time for sori at this treatment. Seawater for this treatment was tyndallized twice instead of once to potentially extend incubation time and remove bacterial contamination that may have been a factor in phase 1. Desiccation, sporulation, and hemocytometer counts for this phase were omitted due to time constraints.

See Appendices 27-28 for photos of phase 2. See supplementary material phase 2 for detailed accounting of each sorus' progression from collection to hemocytometer counts (highly recommended).

Phase 3 began with the collection of five sori from different individual plants from a nearshore *N. luetkeana* forest northwest of Turn Rock (Appendix 20) where conditions allowed for more time to choose the best sori. Sori were immediately rolled and placed into a cooler. Collected sori were chosen based on maturity and, again, the cooler contained a base of ice, four or more layers of paper towels dampened with seawater and lined with a sterile trash bag. Sori were collected at 1:15pm on June 7th, 2025 and transported back to Friday Harbor Labs by 2:00pm the same day.

Upon arrival at Friday Harbor Labs, sori were screened further for maturity and culled, finalizing four sori for phase 3 of the experiment. Each sorus was then processed by creating nine 7.0cm x 2.5cm cutouts to be used in each treatment. Cutouts were then assigned index names for tracking throughout the experiment.

Table 2. Phase 3: Index names of sori exposed to different conditions during the incubation period. A1 and B1 are two separate sori cutouts placed in the same container.

Conditions	32 ppt		20 ppt		8 ppt	
10C	A1B1	C1D1	A2B2	C2D2	A3B3	C3D3
16C	A4B4	C4D4	A5B5	C5D5	A6B6	C6D6
22C	A7B7	C7D7	A8B8	C8D8	A9B9	C9D9

Each sorus and its set of cutouts were photographed (Appendix 37-40) then placed, in sets of two, into 91% isopropyl sterilized incubation containers. Each incubation took place in a 15oz plastic or glass wide mouthed round container containing their own air stone and bubbler (Appendix 41-47). Sori were incubated in twice tyndallized seawater (see 2.2 Incubation for further details). Incubation then began and was set to last for 24 hours in their respective salinity and temperature treatments.

After 24 hours, each set of cutouts was removed from their incubation baths and were prepared for desiccation (see 2.3 Desiccation for further details), then taken through the steps of sporulation (see 2.4 Sporulation for further details).

<u>3.0 Materials List</u>

Materials Phase 1

3 aquarium tanks, small, medium, and large, were placed in a 10 ºC cold room
Aquarium tanks:

- One large glass 91.5cm x 32.5 cm x 41cm aquarium tank

- 57,988 cm^3 of tap water in large tank
- One medium glass 61cm x 32cm x 42 cm aquarium tank
- 38064 cm^3 of tap water in medium tank
- One small plastic 50.5cm x 26.5 cm x 30.5cm aquarium tank (resting on two 4.5 cm tall blocks)
- 26,095.875 cm^3 of tap water in large tank

Bubblers:
- SUN YT-304 Magnetic Vibration Air Pump (x2)
- Aquatic Gardens Air Pump 1000 (x2)
- Tetratec AP-150 Air Pump
- 12 bubbler tubes (long enough to reach each container from the air pump)
- 12 AQUANEAT air stones, one for each tube

Heaters:
- Top Fin 50 watt 60 Hz aquarium heater (in the large tank)
- Visi-therm 100 watt aquarium heater (in the large tank)
- Visi-therm 250 watt aquarium heater (in the medium tank)

Container set-up:
- Tupperware container (x9)
- 2 plastic white gratings
- 3 plastic vial trays
- 9 pieces of rope
- 2 120cm Sunco LED 500K shop lights
- 2 19.5cm x 19.5cm x 39.5cm cinder blocks

Tape
Paper towels
2.5 cm by 7 cm microscope slide
Latex gloves
0.22 um filter unit
2500 ml 8 ppt tyndallized seawater
2500 ml 16 ppt tyndallized seawater
2500 ml 32 ppt tyndallized seawater
Refractometer
Sterile syringes
904.5 mL of povidone-iodine 10%
Deionized water
Hemocytometer
Pipettes
Light Meter

Materials Phase 2

1 aquarium tank, set at 22°C
YT-304 Magnetic Vibration Air Pump and its corresponding AQUANEAT Air Stone
25oz Food Safe Storage Container
500ml of twice tyndallized 8 ppt diluted seawater
91% Isopropyl alcohol

Materials Phase 3
13 air pumps
- New Dolphin Aquarium Air Pump Model P-1 (x3)
- Aquatic Gardens Air Pump 1000 (x2)
- Top Fin Air Pump 1000
- Marina 75 Aquarium Air Pump Model:11112 (x2)
- Marina 75 Aquarium Air Pump Model:11110
- Whisper 10 Air Pump (x2)
- SUN YT-304 Magnetic Vibration Air Pump (x2)

18 plastic containers
3 Heaters
- Top Fin 50 watt 60 Hz aquarium heater (in the large tank)
- Visi-therm 100 watt aquarium heater (in the large tank)
- Visi-therm 250 watt aquarium heater (in the medium tank)

21 Bubbler Tubes
21 AQUANEAT air stones
3 aquarium tanks
2 LED lights
2 plastic white gratings
3 plastic vial trays
70% isopropyl alcohol
91% isopropyl alcohol
Tape
Paper towels
2.5 cm by 7 cm microscope slide
Latex gloves
0.22 um filter unit
2500 ml 8 ppt tyndallized seawater
2500 ml 16 ppt tyndallized seawater
2500 ml 32 ppt tyndallized seawater
Refractometer
Sterile syringes
904.5 mL of povidone-iodine 10%
Pipettes

4.0 Results

4.1 Phase 1

In Phase 1 many of the sori in the harshest treatments, specifically sori A8, B8, A9, B9 in the 22°C tank at 8 and 20 ppt, decayed or potentially released spores almost immediately in 2 hours or less after placement in incubation containers on Saturday, June 28th. At this same time all sori in 8ppt treatments were also observed to develop "boils". Sori A7 & B7 were not far behind and all sori in the 20°C were observed to have either decayed due to bacterial contamination or completely released their spores in under 48 hours by 10:00am on June 30th. The water in their containers turned cloudy green and observations under a microscope showed no obvious indications of spores.

As a result, counts were only able to be made on sori that made it through the incubation, dessication, and sporulation process a few days later, including sori A1, B1, A2, A3 and A4. The highest spore density observed was from the 32 ppt 16°C condition (sorus A4) with 760,000 spores per mL, and the lowest density from the 32 ppt 10°C condition (sorus B1) with 25,000 spores per mL (Table 3). However, careful consideration should be taken, as the first spore count collected from A4 was ~8 hours after first sporulation was observed as counts within the recommended one to three hours of sporulation could not be made. . All other first counts were taken within two to three hours and 15 minutes after initial sporulation bath.

Eight hours later, in an effort keep comparable data, a second count was done with the highest density being 1,720,000 spores per mL from the 32ppt 16°C condition (sorus A4, now

~16 hours into sporulation bath) and the least being 20,000 spores per mL from the 8ppt 10°C condition (sorus A3), as shown in Table 4. Between the two counts, the condition that produced the highest spore density was the 32ppt 16°C condition (sorus A4) with 1,720,000 spores per mL in the second count, and the lowest spore density was in the 10°C 8 ppt condition (sorus A3) with 20,000 spores per mL in the second count. Of the hemocytometer counts that were made distinct differences were noticed in the condition of spores released. There were three motile spores identified. Two of them were in the second count of A1, and one of those motile spores was spiraling in motion. The third motile spore was in the second count of B1 and it was also spiraling. Based on hemocytometer calculations, we approximate 20,000 motile spores in the second count of A1 and 2,500 motile spores in the second count of B1. There were no motile spores found in the first count. Spores from A4 (32ppt and 16°C) were found in large masses of deceased spores. A1, B1, A2, and A3, however, were observed to be less dense and spread out. Most interestingly, entire unilocular sporangia were seen to have been shed off in the counts of A3 (8ppt and 10°C).

Of note, no large spore releases were observed which turned the whole bath cloudy as depicted in many of the kelp cultivation guidelines.

Table 3. First count of spores done with the hemocytometer

Spores/mL	32 ppt		20 ppt		8 ppt	
10C	530,000	25,000	270,000	Sorus B2	310,000	Sorus B3
16C	760,000	Sorus B4	Sorus A5	Sorus B5	Sorus A6	Sorus B6
22C	Sorus A7	Sorus B7	Sorus A8	Sorus B8	Sorus A9	Sorus B9

Table 4. Second count of spores done with the hemocytometer

Spores/mL	32 ppt		20 ppt		8 ppt	
10C	730,000	22,500	450,000	Sorus B2	20,000	Sorus B3
16C	1,720,000	Sorus B4	Sorus A5	Sorus B5	Sorus A6	Sorus B6
22C	Sorus A7	Sorus B7	Sorus A8	Sorus B8	Sorus A9	Sorus B9

4.2 Phase 2

Sori either decayed or potentially released spores after 38 hours of incubation (Appendix 27-28).

4.3 Phase 3

Armed with previous knowledge from phases 1 & 2 we successfully set an incubation time that allowed for all sori to survive until dessication. After which, the sori were left in their sporulation containers for three hours on July 8th, per the recommended protocol from the PSRF Kelp Cultivation Handbook. At that time, none of them had released spores and the decision was made to keep them in the sporulation baths until further observations could be made. They were observed on an opportunistic basis for the next 48 hours, oftentimes within one hour intervals. On the night of July 10th, sporulation began and was not observed until the morning after Unfortunately, due to project time constraints, hemocytometer counts were unable to be produced and this data was lost.

An interesting finding from both incubation and sporulation is that the sori may have produced “mucilage tendrils” from the edges of the cutouts and turned lighter in color during both sporulation and incubation (Appendix 48). While we aren't certain this is the correct terminology, this was something that was not observed in any other phase. See Appendix 29 for phase 3 set up

<u>5.0 Discussion</u>

In this study, the impact of changing conditions caused by global warming and river runoffs were investigated to understand effects on the developmental stages of *Nereocystis luetkeana*. Sporulation proved a challenge during our research. The incubation of *N. luetkeana* sori and the cultivation of their gametophyte stage is a time consuming and tricky process. To optimize cultivation success, we recommend that researchers dedicate their full attention during the incubation, desiccation, and sporulation phases; avoiding other time commitments whenever possible. Continuous, near-hourly monitoring of individual sori is essential during this period.

In phase 1, spores in the hottest temperature condition started releasing spores sooner than expected, leading us to start the desiccation process earlier than originally intended. The expectation was that sori could be subjected to four days of incubatory treatments, however, our results imply that salinity gradients and temperature stressors can potentially induce early spore release when compared to other similarly mature sorus; though significantly more research is needed to have any certainty. The unfortunate loss of many of our crops in the harshest conditions impeded the collection of much anticipated data. Although, the integration of our results from all phases could point to a critical period of time at which all sori can survive and be successfully sporulated. Incubation under that critical point increases necessary sporulation bath time past the recommended amount, while incubation past the point runs the risk of decay of sori in the harshest conditions. This occurred regardless of levels of sterilization and contamination considerations. We would propose this critical point to be somewhere in the 24 hours to 36 hours range.

Focusing back on phase 1, we found, with certainty, blades in the warmest tanks and harshest salinities experienced major morphological changes when compared to their control, or near control, counterparts. These findings were only partially supported by phase 2, but were most consistent in boil development, which was observed in nearly all of the 8 ppt incubation treatments; an utterly unpredicted finding. Importantly, the only sori in the 8 ppt treatment that did not show signs of bubbling is the 8 ppt 10°C. Of note, due to the size of the A and B full sori in phase 1, space within their incubation containers was limited and could have influenced each circulation efficiency of the airstones and contributed to early decay or spore release. It is also worth mentioning, that with the sori being from different plants, they also could have been at different stages of maturity even though the sori were the same shade in appearance.

Similar constraints apply to phase 3 of the experiment, but all A cutouts were from the same sorus, and all B cutouts were from the same sorus and so on. There are a few factors that might have contributed to the lack of sporulation. For example, it could have been that neither sorus was at a mature enough stage in the life cycle, or the incubation period was not long enough. The 24 hour incubation period was informed directly by the findings of phase 2, and it is possible that a longer incubation would have caused them to release spores too soon. Finding the aforementioned critical time period using multiple trial runs and several cutouts should be of importance for any future experiments. Alternatively, if time and availability permit, researchers may monitor sori on an hourly basis and initiate desiccation for each individual sorus at the first sign of decay or sporulation. This approach could serve as a more successful way to test spore release, rather than relying on a standardized incubation duration across all replicates.

A separate confounding variable was that of sterilization procedures. We were intent on creating the simplest, most real world scenario in phase 1, reducing tyndallization to one round, but increasing it to two rounds for phases 2 & 3. A second tyndallization would have killed remaining bacteria and potentially staved off the worst of the decay that was seen in the harshest treatments in phase 1.

The hemocytometer counts from phase 1 that were able to be collected showed little to no pattern in their distribution. The visual observations that were made of these counts did have some important morphological differences. As for spore motility, with only three motile spores viewed across two treatments, there is no clear relationship between salinity, temperature, and spore motility. Please see supplementary phase 1 material for photos of those observations mentioned in the results.

For the cutouts that did release spores, the highest spore densities were in the treatments of A1, B1 and A4. With the first count of spores in phase 1, the A4 cutout had 760,000 spores per mL and the A1 cutout had 530,000 spores per mL. In the second count of spores, the A1 cutout had 730,000 spores per mL and the A4 cutout had 1,720,000 spores per mL. These results aligned with *Nereocystis* preferred living conditions of 32 ppt seawater ranging from 10°C to 16°C (Weigel et al. 2023, Small and Weigel 2022, Drakard et al. 2025). Meanwhile, the cutout in the 20 ppt and 10°C treatment, A2, released 270,000 spores per mL in the first count and 450,000 spores per mL in the second count. This is lower than the number of spores/mL released by cutouts in the 32 ppt salinity water, suggesting that lower salinity may potentially increase spore density. This finding contradicts the hypothesis that lower salinity decreases spore density, however not enough data was collected to generate a strong enough conclusion.

In phase 1, there may have been high spore density in observed treatments, but the spores were arranged in large clumps, indicating possible issues with spore release and separation from one another. This points to a decreased ability of *N. luetkeana* to release effective spores under stressful temperature and salinity conditions, suggesting that riverine outflow and increasing water temperatures could influence the decline of *N. luetkeana* populations in the Salish Sea.

An additional consideration is what happens after spore release; fertilization and germination. Due to time constraints, these processes were not observed in this experiment, so further tests can be performed that follow the life cycle of *N. luetkeana* after initial sporulation. Many questions still remain when it comes to understanding the factors that affect successful *N. luetkeana* sporulation, germination, and survival, but this study provides a basis for further experimentation to draw on.

Acknowledgments

Land acknowledgment

We as researchers would like to take time to acknowledge that we stand and study on traditional lands of the Coast Salish people who have called this area home since time immemorial. To the San Juan Island Friday Harbor and traditional Samish tribe, thank you for stewarding these lands for many years. We would also like to thank the organisms and nearshore communities that call this island home and for their participation in this research.

We would also like to thank Friday Harbor Laboratories for allowing us to occupy the cold room in the Fernald Building and use a few of their aquaria for the experiment, as well as our professors Tom Mumford, D.W. Freshwater and Danielle Claar for their advice and guidance during the process.

Finally, to the Labs stockroom for providing materials in order for the experiment to happen, and to lab 4 for providing microscopes to conduct spore counts with.

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Appendix

APPENDIX

Results of Phase 1

First count of spores extra information - July 2nd, 2025

Time (9:47 pm)
A1: 32 ppt 10C (mid box) - 530,000 spores per mL

Time (10:04 p.m)
B1: 32 ppt 10C (box 4) - 25,000 spores per mL

Time (10:14pm)
A3; 8 ppt 10C (mid box) - 310,000 spores per mL

Time (10:25pm)
A2: 20 ppt 10 C (box 4) - 270,000 spores per mL

Time (7:44pm)
A4; 32ppt 16C (mid box)- 760,0000 spores per mL

Second count of spores extra information - July 3rd 2025

Time (5:45 am)
A1: 32 ppt 10C () - 730,000 spores per mL

Time (6:02 am)
B1: 32 ppt 10C (box 3) - 22,500 spores per mL

Time (6:18am)
A3: 8 ppt 10C (box 2) - 20,000 spores per mL
Whole Sporangia Present (not counted as spores)

Time (6:29am)
A2: 20 ppt 10 C (box 5) - 450,000 spores per mL

Time (6:45am)
A4: 32ppt 16C - 1,720,000 spores per mL

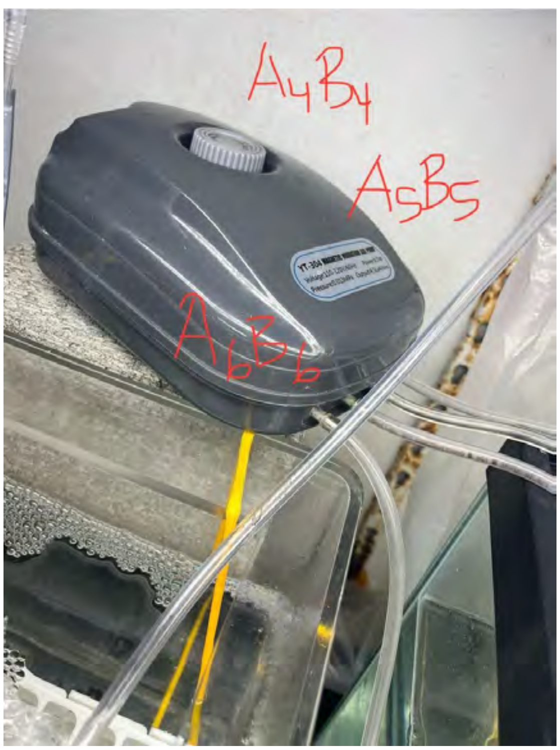
Appendix 1: Air pump used for container holding A2 and B2 during Phase 3

Appendix 2: Air pump used for containers holding A1, B1, C2 and D2 during Phase 3

Appendix 3: Air pump used for container holding A3 and B3 during Phase 3

Appendix 4: Air pump used for container holding C3 and D3 during Phase 3

Appendix 5: Air pump used for container holding C4 and D4 during Phase 3



Appendix 6: Air pump used for containers holding A4, A5, A6, B4, B5 and B6 during Phase 3

Appendix 7: Air pump used for container holding C5 and D5 during Phase 3

Appendix 8: Air pump used for container holding C6 and D6 during Phase 3

Appendix 9: Air pump used for container holding A7 and B7 during Phase 3

Appendix 10: Air pump used for container holding A8 and B8 during Phase 3

Appendix 11: Air pump used for container holding A9 and B9 during Phase 3

Appendix 12: Air pump used for containers holding C7, C8, C9, D7, D8 and D9 during Phase 3

Appendix 13:VISI-THERM 100 W water heater used for 22°C tank

Appendix 14: TOPFIN water heater used for 22°C tank

Appendix 15: VISI-THERM 250 W water heater for 16°C tank

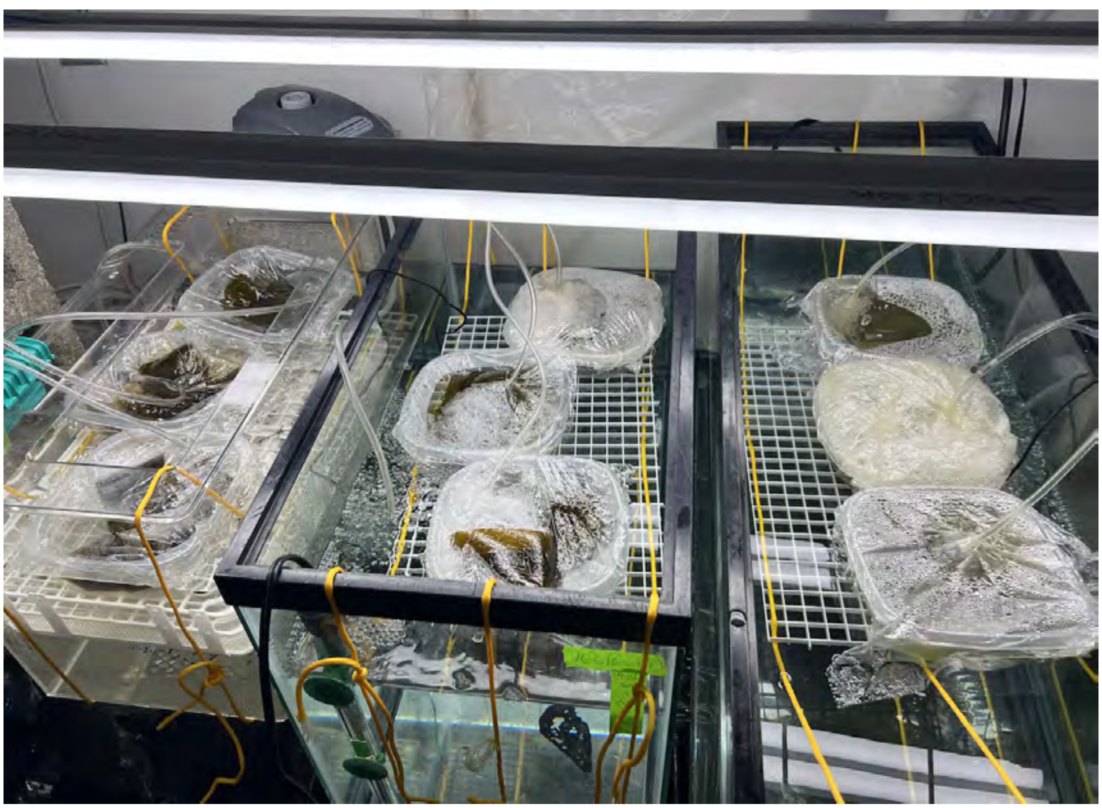
Appendix 16: LI-COR 258 used to measure light intensity

Appendix 17: LI-COR 258 with attached sensor


Appendix 18: Example sorus from Turn Rock, San Juan Island

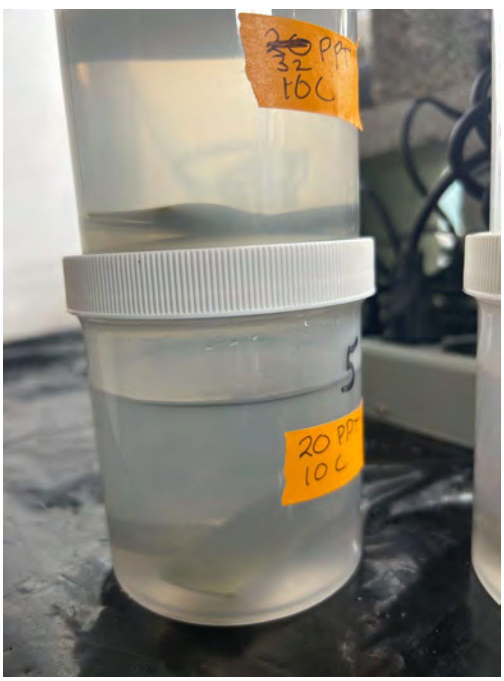
Appendix 19: Turn Rock, San Juan Island, WA *Nereocystis luetkeana* bed collection site for phase 1 on first June 28, 2025

Appendix 20: *Nereocystis luetkeana* forest collection site, northwest of Turn Rock, San Juan Island, WA, for phase 3 on July 7, 2025

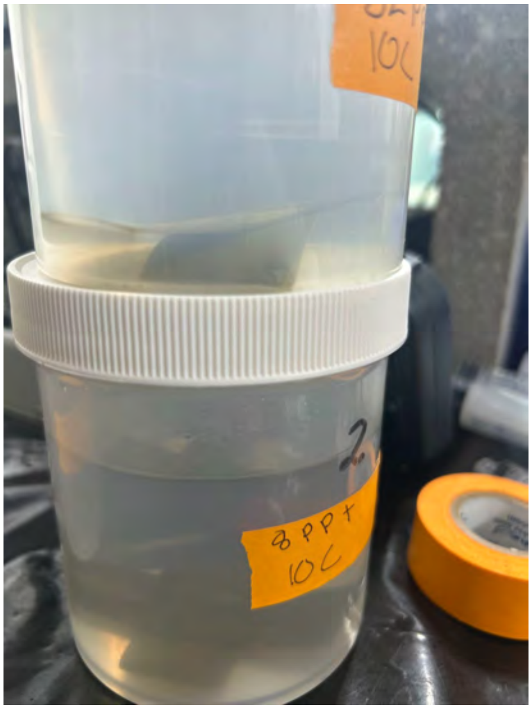


Appendix 21: Phase 1 set up (front to back) 32ppt, 20ppt, 8ppt and (left to right) 10°C, 16°C, 22°C

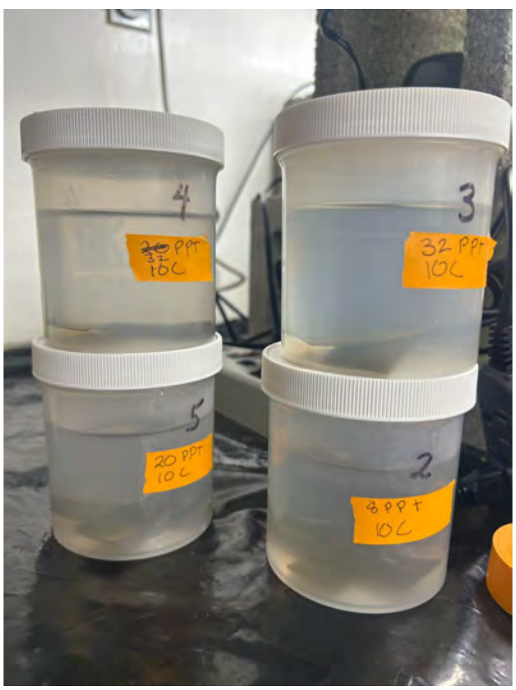
Appendix 22: First image of the sporulating sori for the first spore count, taken on July 2, 2025 at 8:47 p.m.



Appendix 23: Second image of the sporulating sori of one of the 32 ppt and 10°C containers and the 20 ppt and 10°C container for the first spore count, taken on July 2, 2025 at 9:05 p.m.



Appendix 24: Second image of the sporulating sori of the second 32 ppt and 10°C container and the 8 ppt and 10°C container for the first spore count, taken on July 2, 2025 at 9:05 p.m.



Appendix 25: Third image of the sporulating sori for the first spore count, taken on July 2, 2025 at 9:25 p.m.

$$\text{Zoospore Density (Spores/mL)} = \left(\frac{\text{Sq. 1} + \text{Sq. 2} + \text{Sq. 3} + \text{Sq. 4}}{4} \right) \times 10{,}000$$

$$\text{Zoospore Density (Spores/mL)} = \left(\frac{\_\_\_\_\_\_\_\_}{4} \right) \times 10{,}000$$

$$\text{Zoospore Density (Spores/mL)} = \_\_\_\_\_\_\_\_$$

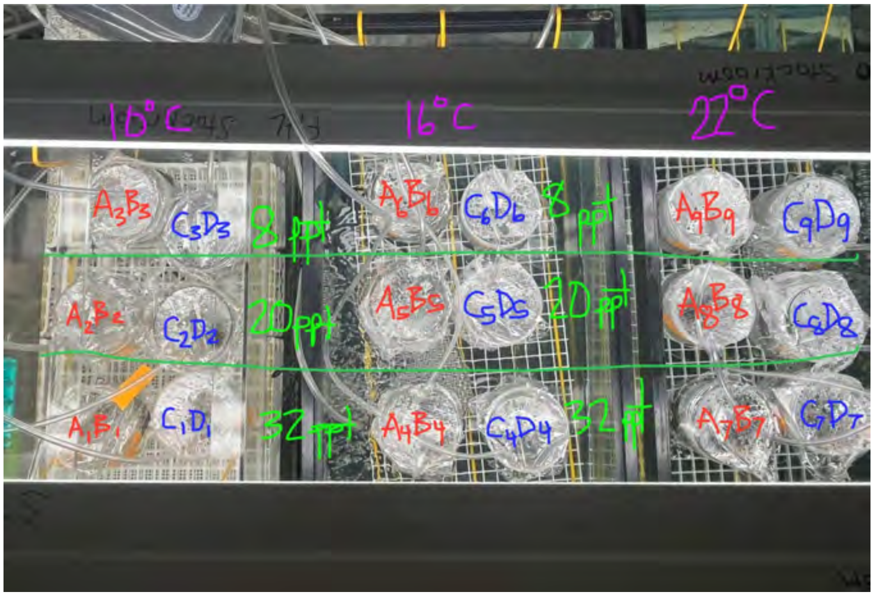
$$\text{Zoospore Density (Spores/mL)} = \text{Square 5} \times 10{,}000$$

$$\text{Zoospore Density (Spores/mL)} = \_\_\_\_\_\_\_\_$$

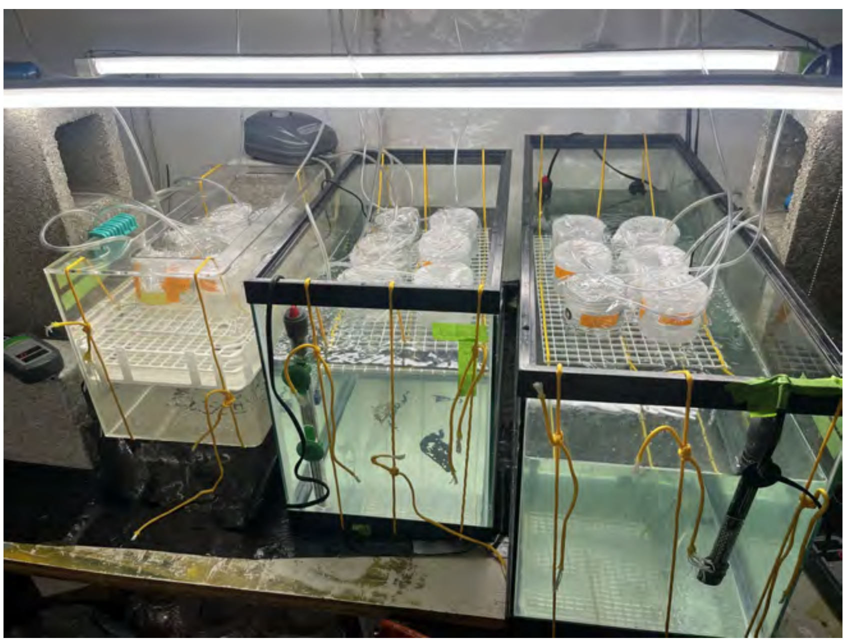
Appendix 26: Calculation for spore density from Ocean Approved, Kelp Farming Manual

Appendix 27: Sorus cutout after the 38 hour incubation in 8ppt 22°C condition for phase 2.

Appendix 28: Sorus cutout after the 38 hour incubation in 8ppt 22°C condition for phase 2.



Appendix 29: Set up for Phase 3. The top row of containers consists of sori exposed to 8 ppt, the middle row is 20 ppt seawater, and the bottom row is 32 ppt seawater. Meanwhile, the left tank is 10°C, the middle tank is 16°C, and the right tank is 22°C.


Appendix 30: Front view of experiment set up for Phase 3

Appendix 31: Desiccation set up during Phase 3

Appendix 32: Filtered seawater used to rinse the sori

Appendix 33: Phase 3, sorus A. One cutout before desiccation

Appendix 34: Phase 3, sorus B

Appendix 35: Phase 3, sorus C

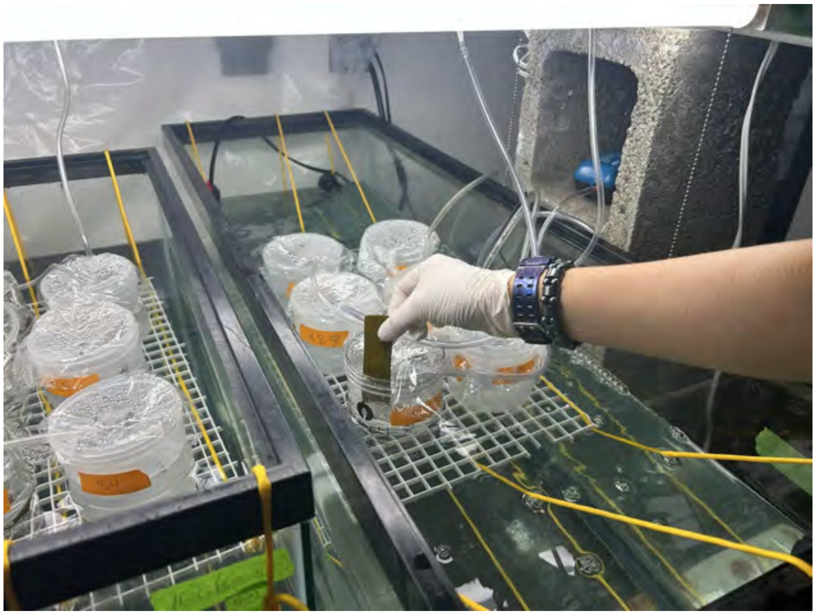
Appendix 36: Phase 3, sorus D

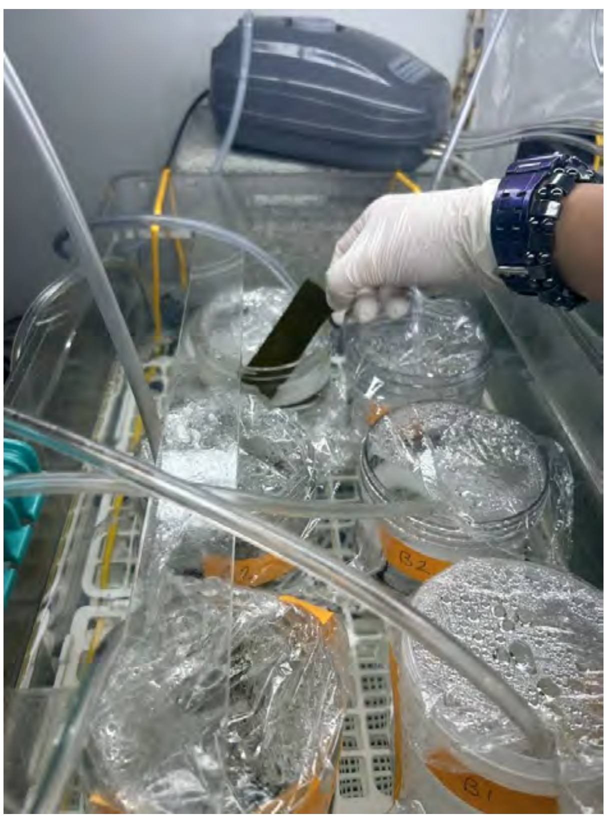
Appendix 37: Image of all nine cutouts from phase 3 sorus A.

Appendix 38: Image of all nine cutouts from phase 3 sorus B.

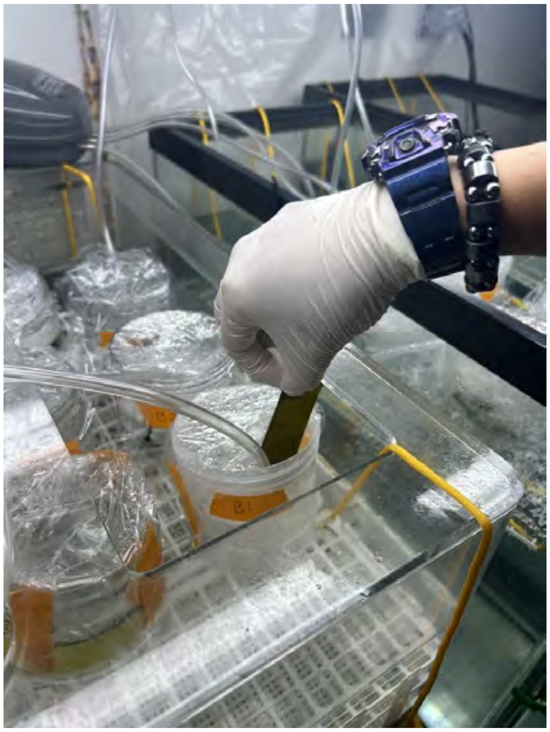
Appendix 39: Image of all nine cutouts from phase 3 sorus C.

Appendix 40: Image of all nine cutouts from phase 3 sorus D


Appendix 41: Placing phase 3 sorus A7 into its container for incubation, taken July 7, 2025 at 2:01 pm

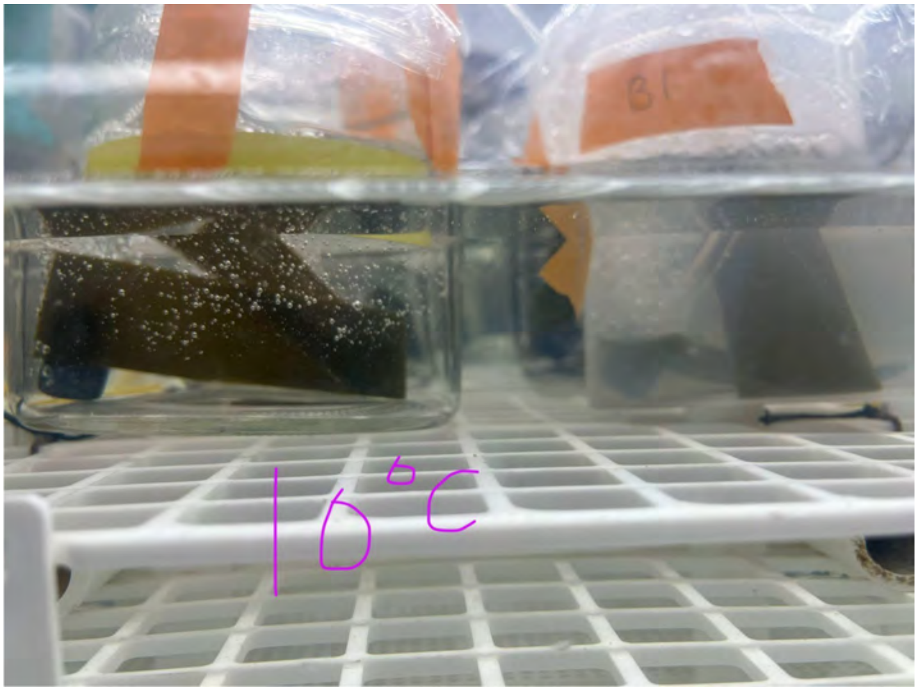


Appendix 42: Placing phase 3 sorus B3 into its container, taken July 7, 2025 at 2:09 pm

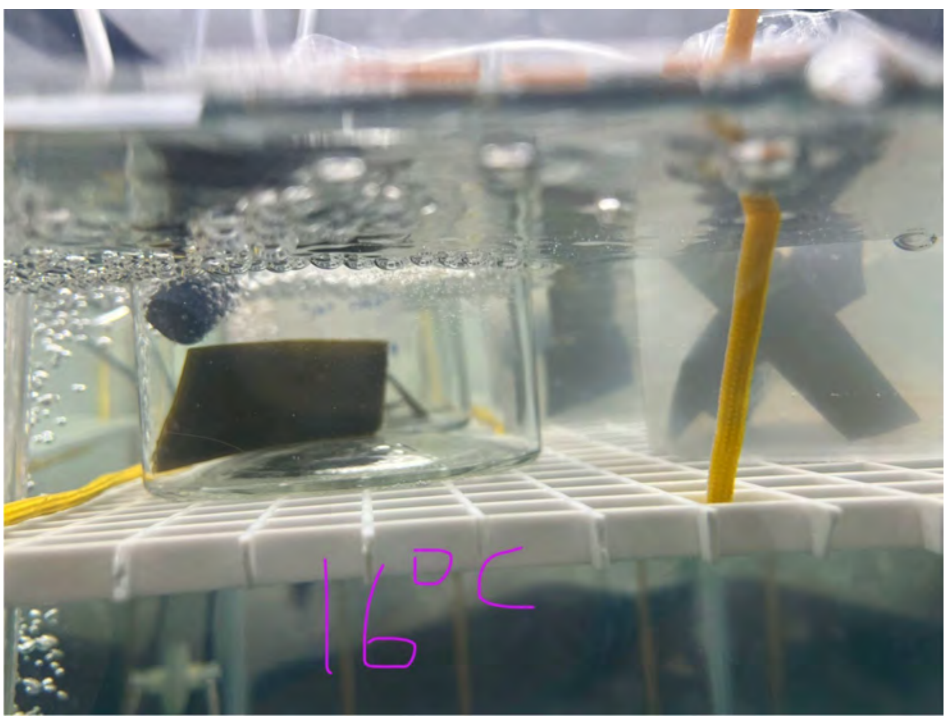


Appendix 43: Placing phase 3 sorus C1 into its container, taken July 7, 2025 at 2:17 pm

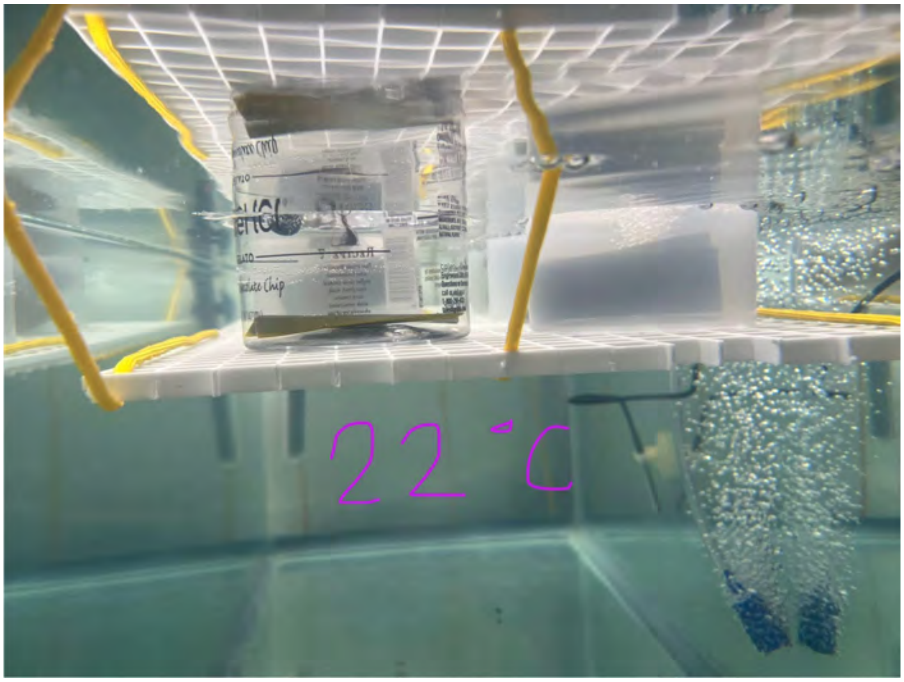
Appendix 44: Placing phase 3 sorus D3 into its container, taken July 7, 2025 at 2:27 pm



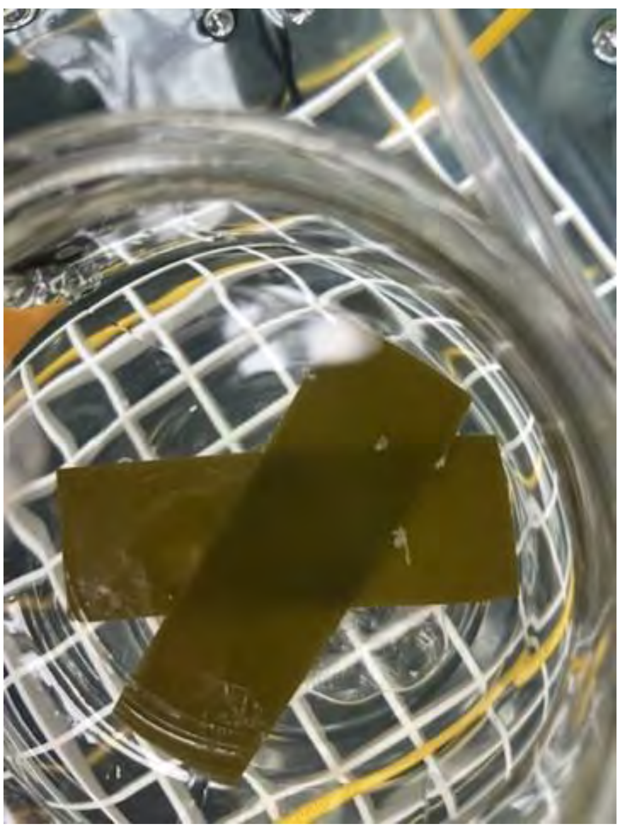
Appendix 45: Sori incubating in the 10°C tank at 11:45 am on July 8, 2025



Appendix 46: Sori incubating in the 16°C tank at 11:45 am on July 8, 2025



Appendix 47: Sori incubating in the 22°C tank at 11:45 am on July 8, 2025



Appendix 48: Mucilage tendrils noticed for A6B6

Appendix 49: Phase 3 A1 B1 sori after incubation and before desiccation

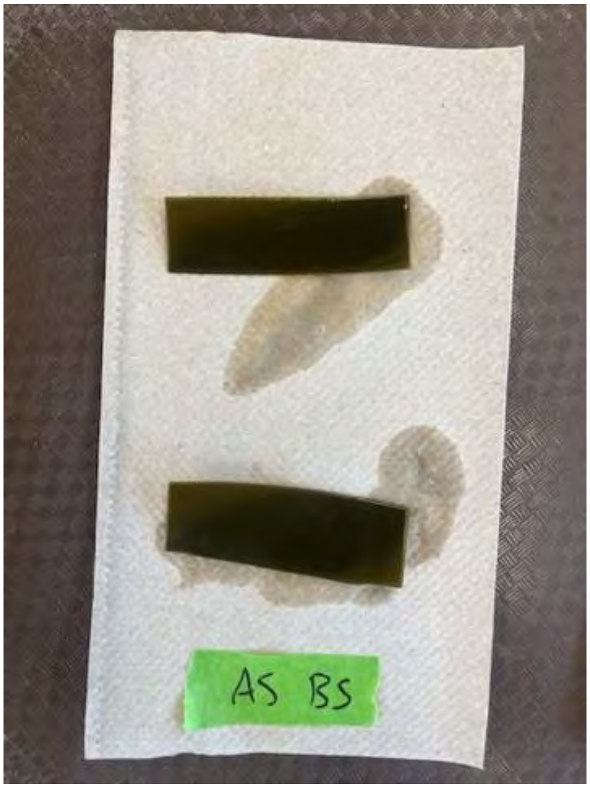
Appendix 50: Phase 3 C1 D1 after incubation and before desiccation

Appendix 51: Phase 3 A2 B2 sori after incubation and before desiccation

Appendix 52: Phase 3 C2 D2 sori after incubation and before desiccation

Appendix 53: Phase 3 A4 B4 after incubation and before desiccation

Appendix 54: Phase 3 C4 D4 after incubation and before desiccation



Appendix 55: Phase 3 A5 B5 after incubation and before desiccation

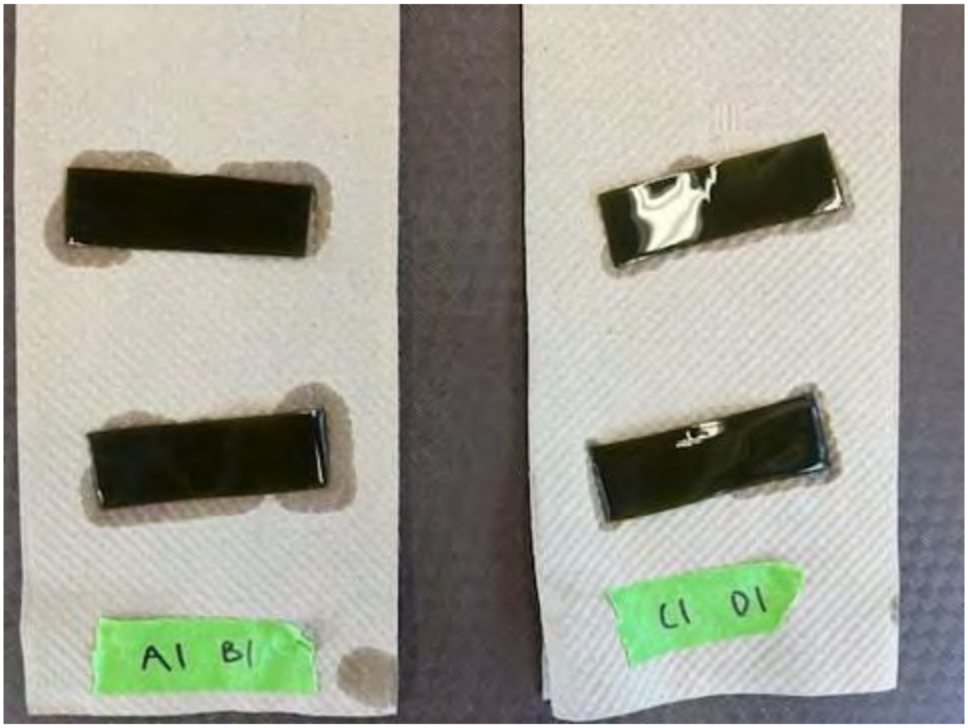
Appendix 56: Phase 3 C5 D5 after incubation and before desiccation

Appendix 57: Phase 3 A6 B6 after incubation and before desiccation

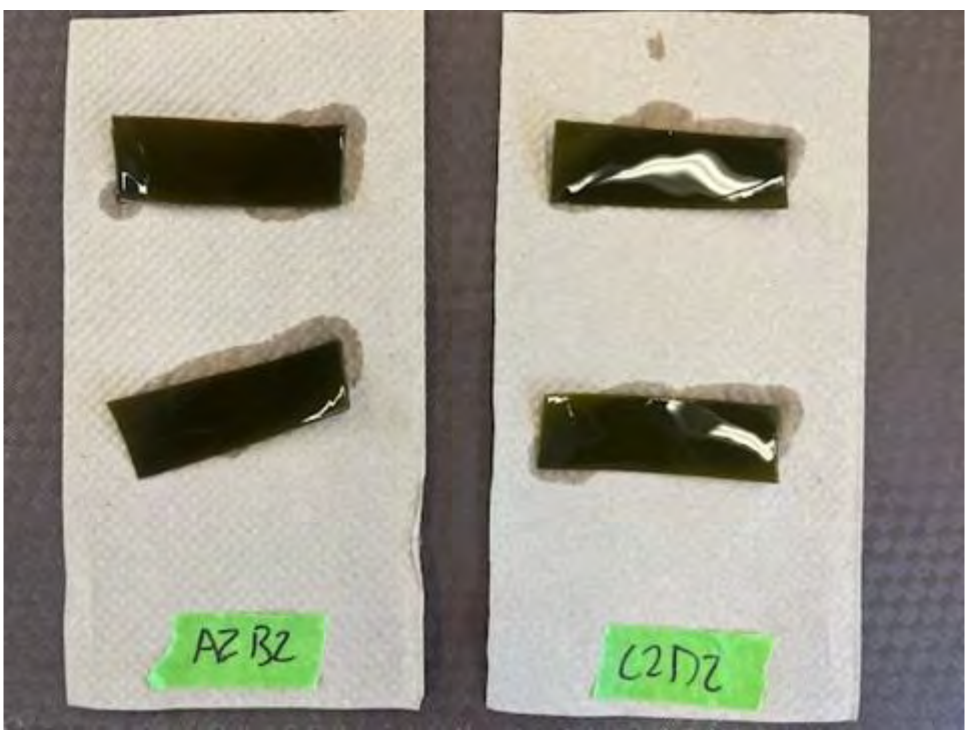
Appendix 58: Phase 3 C6 D6 after incubation and before desiccation

Appendix 59: Phase 3 A9 B9 after incubation and before desiccation

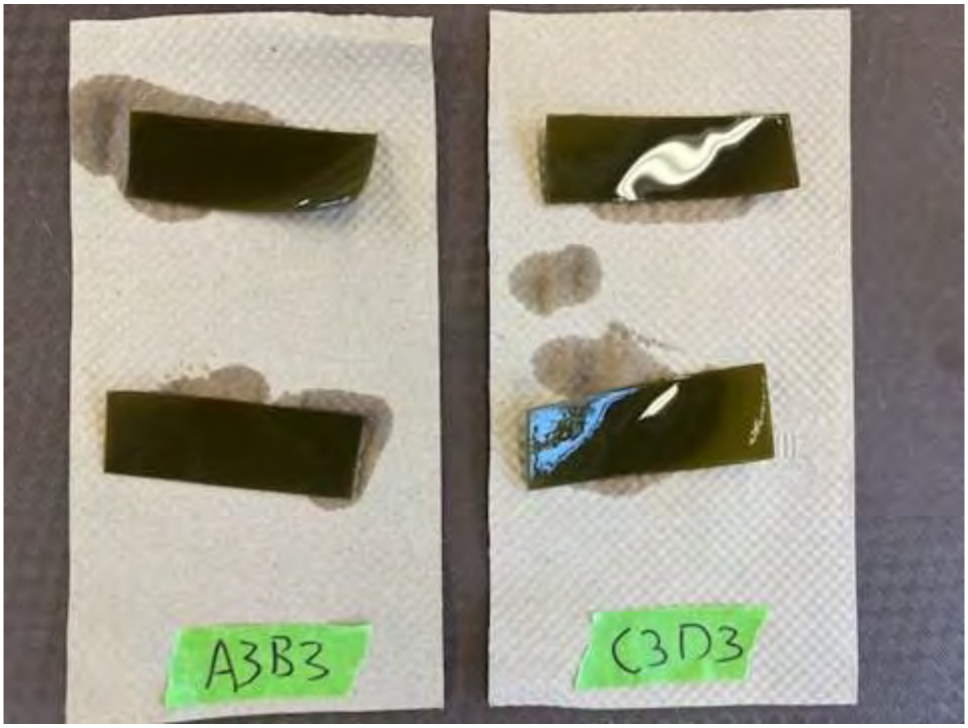
Appendix 60: Phase 3 C9 D9 after incubation and before desiccation



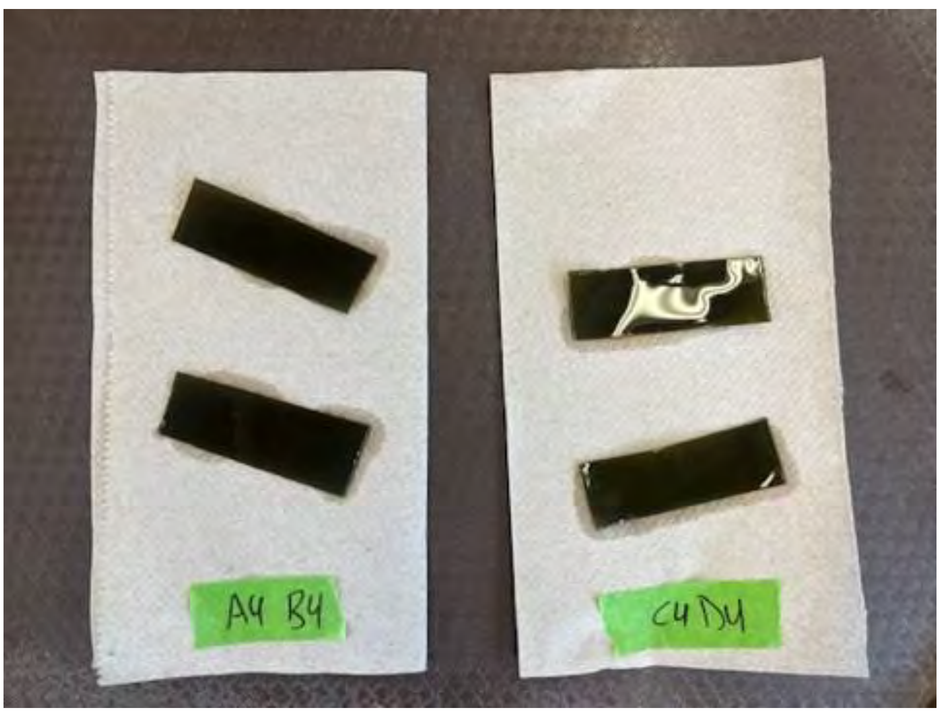
Appendix 61: Phase 3 A1B1 and C1 D1 after incubation and before desiccation



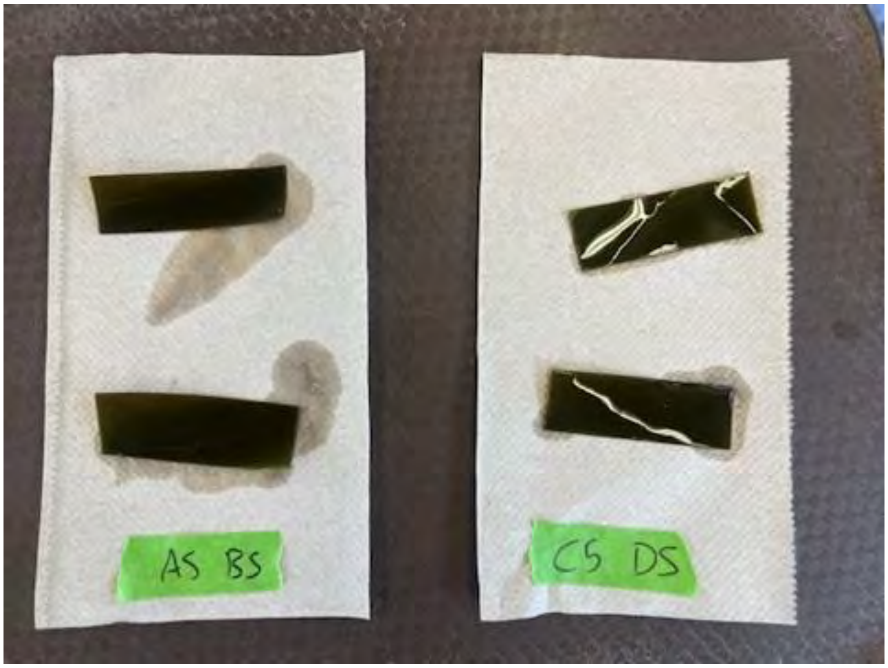
Appendix 62: Phase 3 A2B2 and C2D2 after incubation and before desiccation



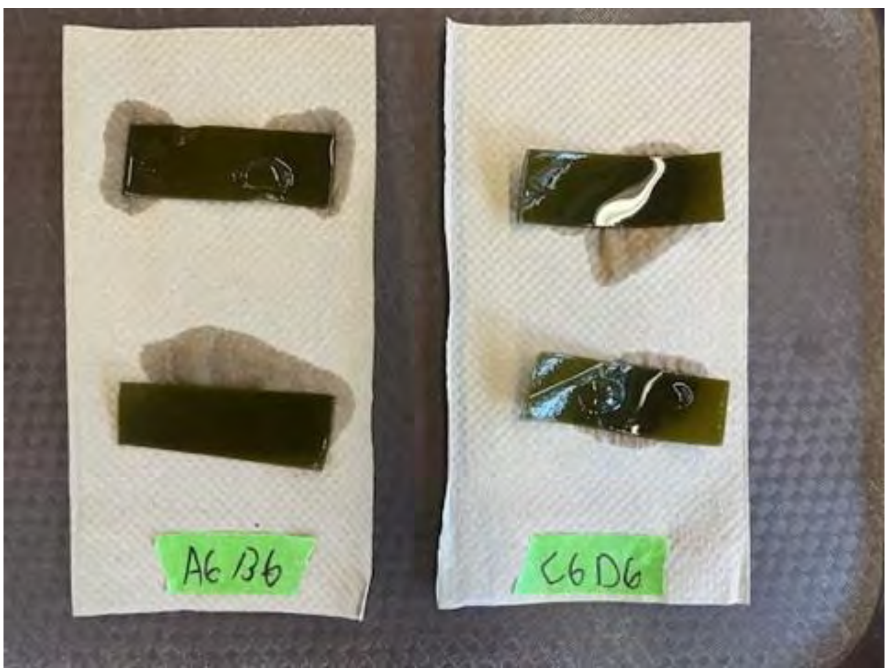
Appendix 63: Phase 3 A3B3 and C3D3 after incubation and before desiccation



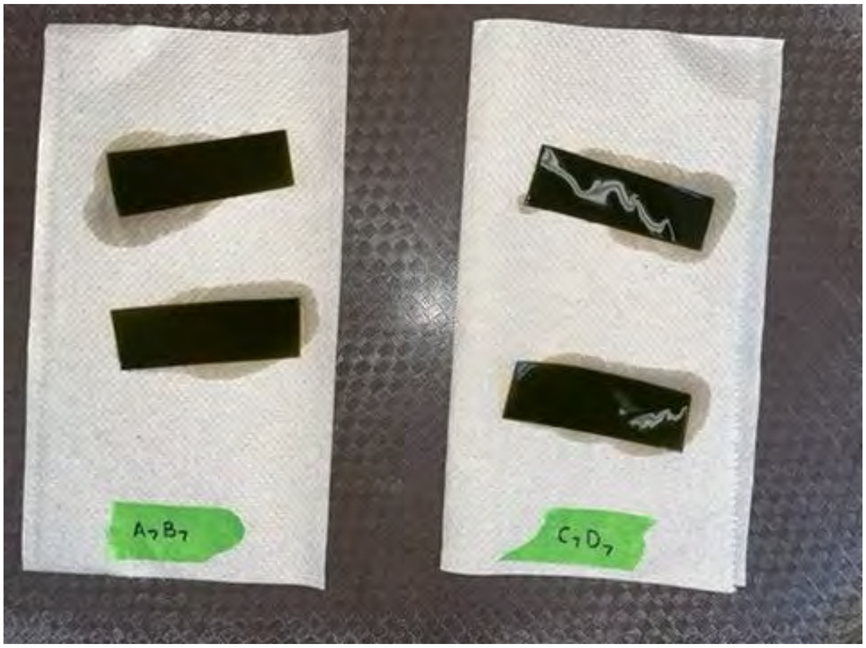
Appendix 64: Phase 3 A4B4 and C4D4 after incubation and before desiccation



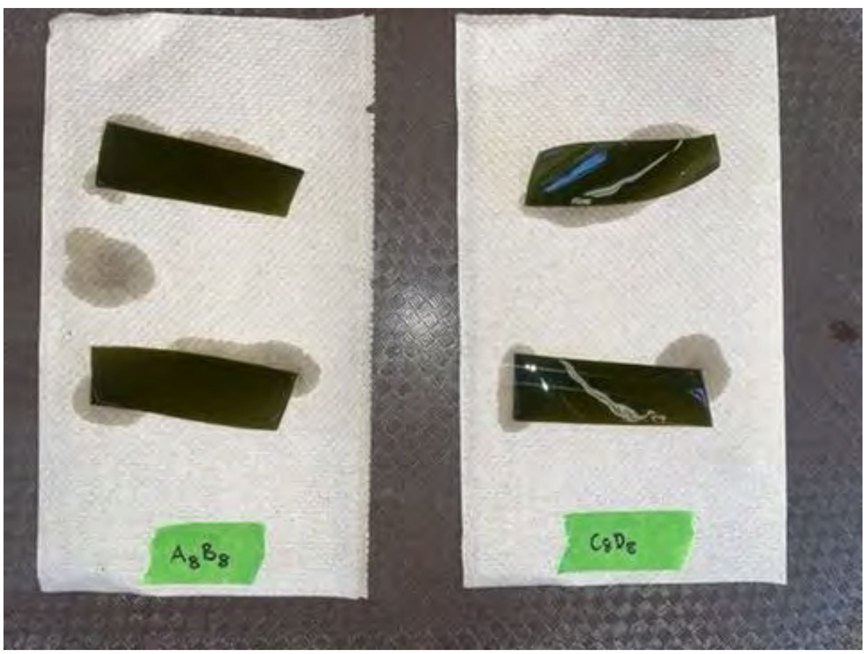
Appendix 65: Phase 3 A5B5 and C5D5 after incubation and before desiccation



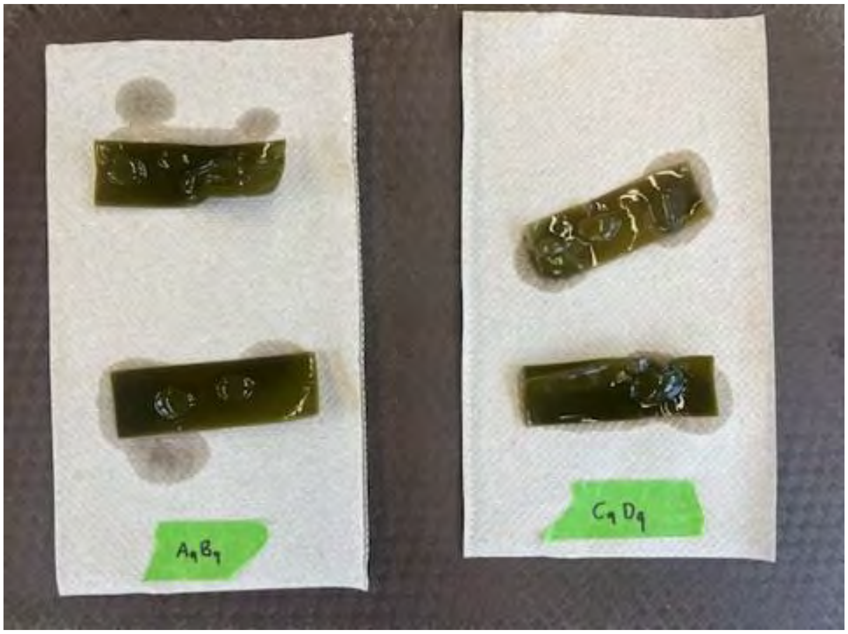
Appendix 66: Phase 3 A6B6 and C6D6 after incubation and before desiccation



Appendix 67: Phase 3 A7B7 and C7D7 after incubation and before desiccation



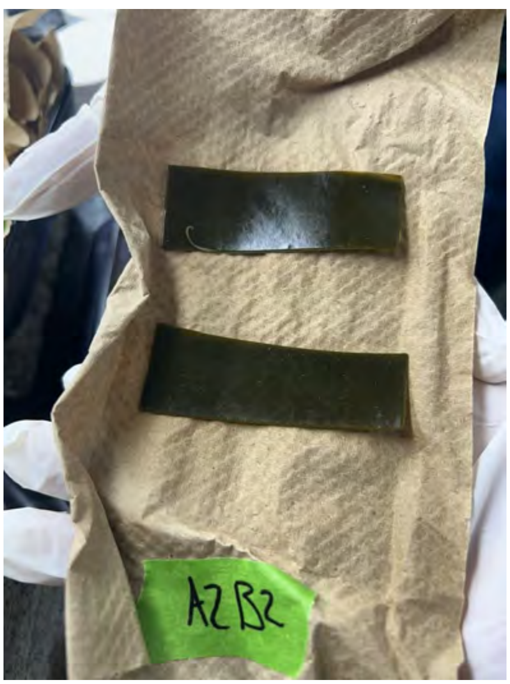
Appendix 68: Phase 3 A8B8 and C8D8 after incubation and before desiccation



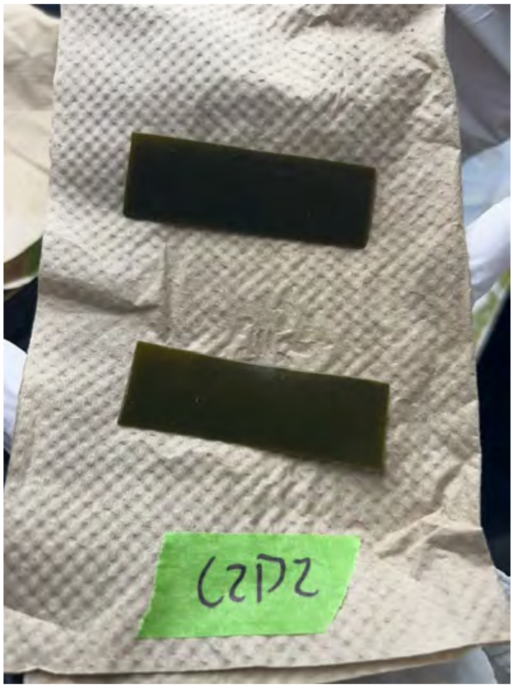
Appendix 69: Phase 3 A9B9 and C9D9 after incubation and before desiccation

Appendix 70: Phase 3 A1B1 post desiccation before placing in sporulation bath

Appendix 71: Phase 3 C1D1 post desiccation before placing in sporulation bath



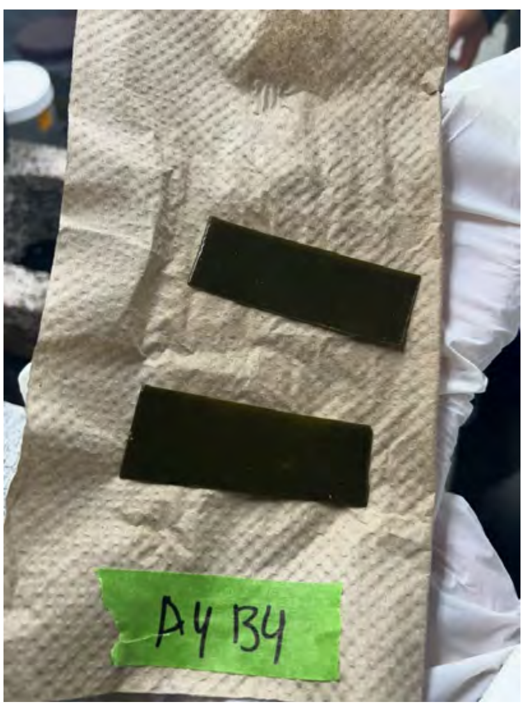
Appendix 72: Phase 3 A2B2 post desiccation before placing in sporulation bath



Appendix 73: Phase 3 C2D2 post desiccation before placing into sporulation bath

Appendix 74: Phase 3 A3B3 post desiccation before placing into sporulation bath

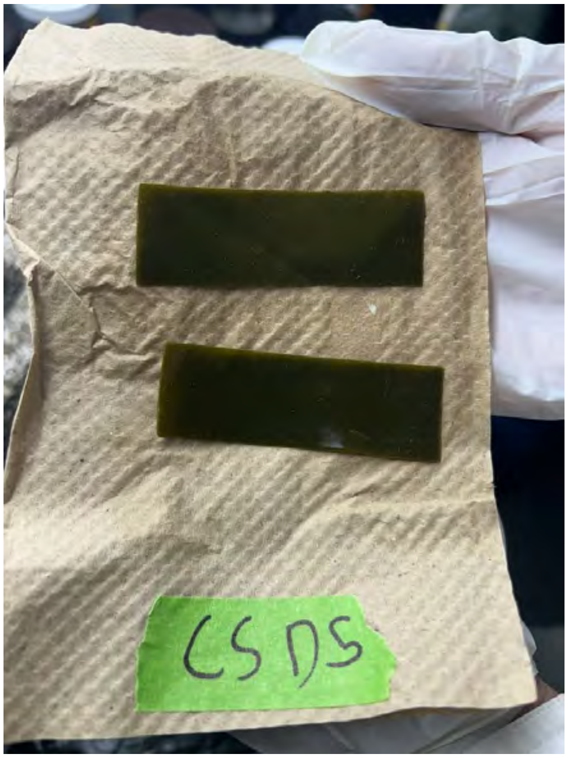
Appendix 75: Phase 3 C3D3 post desiccation before placing into sporulation bath



Appendix 76: Phase 3 A4B4 post desiccation before placing into sporulation bath

Appendix 77: Phase 3 C4D4 post desiccation before placing into sporulation bath

Appendix 78: Phase 3 A5B5 post desiccation before placing into sporulation bath



Appendix 79: Phase 3 C5D5 post desiccation before placing into sporulation bath

Appendix 80: Phase 3 A6B6 post desiccation before placing into sporulation bath

Appendix 81: Phase 3 C6D6 post desiccation before placing into sporulation bath

Appendix 82: Phase 3 A7B7 post desiccation before placing into sporulation bath

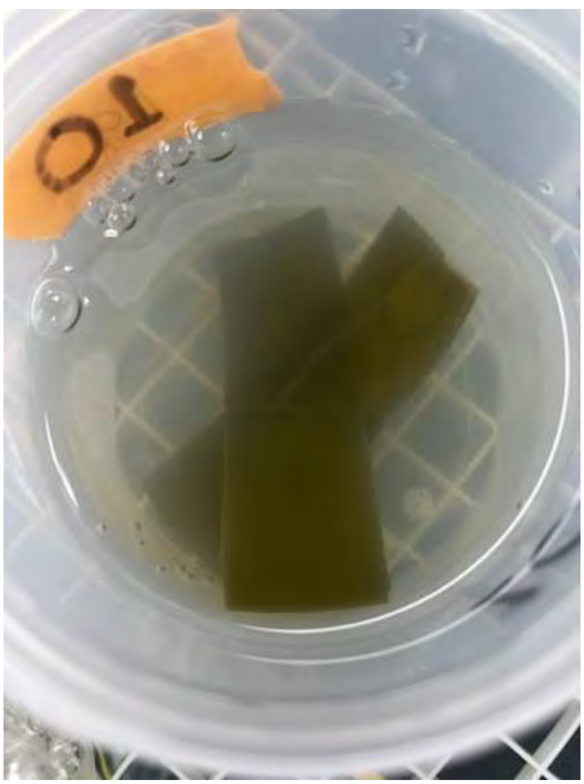
Appendix 83: Phase 3 C7D7 post desiccation before placing into sporulation bath

Appendix 84: Phase 3 A8B8 post desiccation before placing into sporulation bath

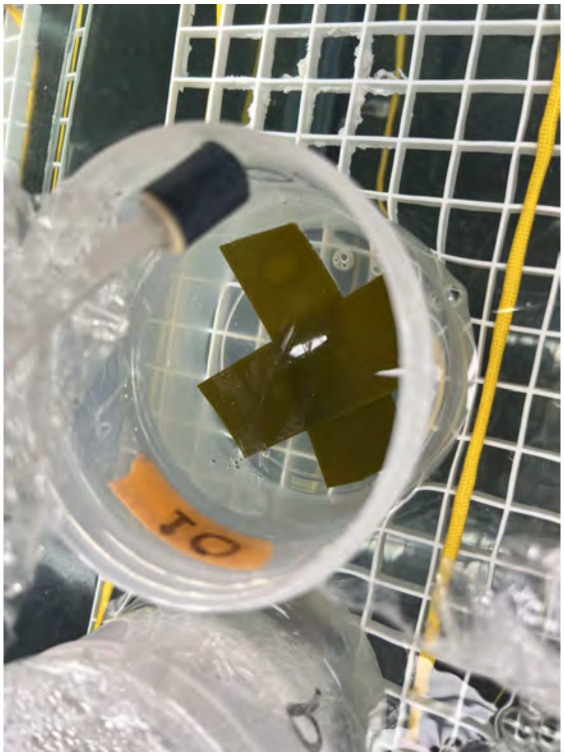
Appendix 85: Phase C8D8 post desiccation before placing into sporulation bath

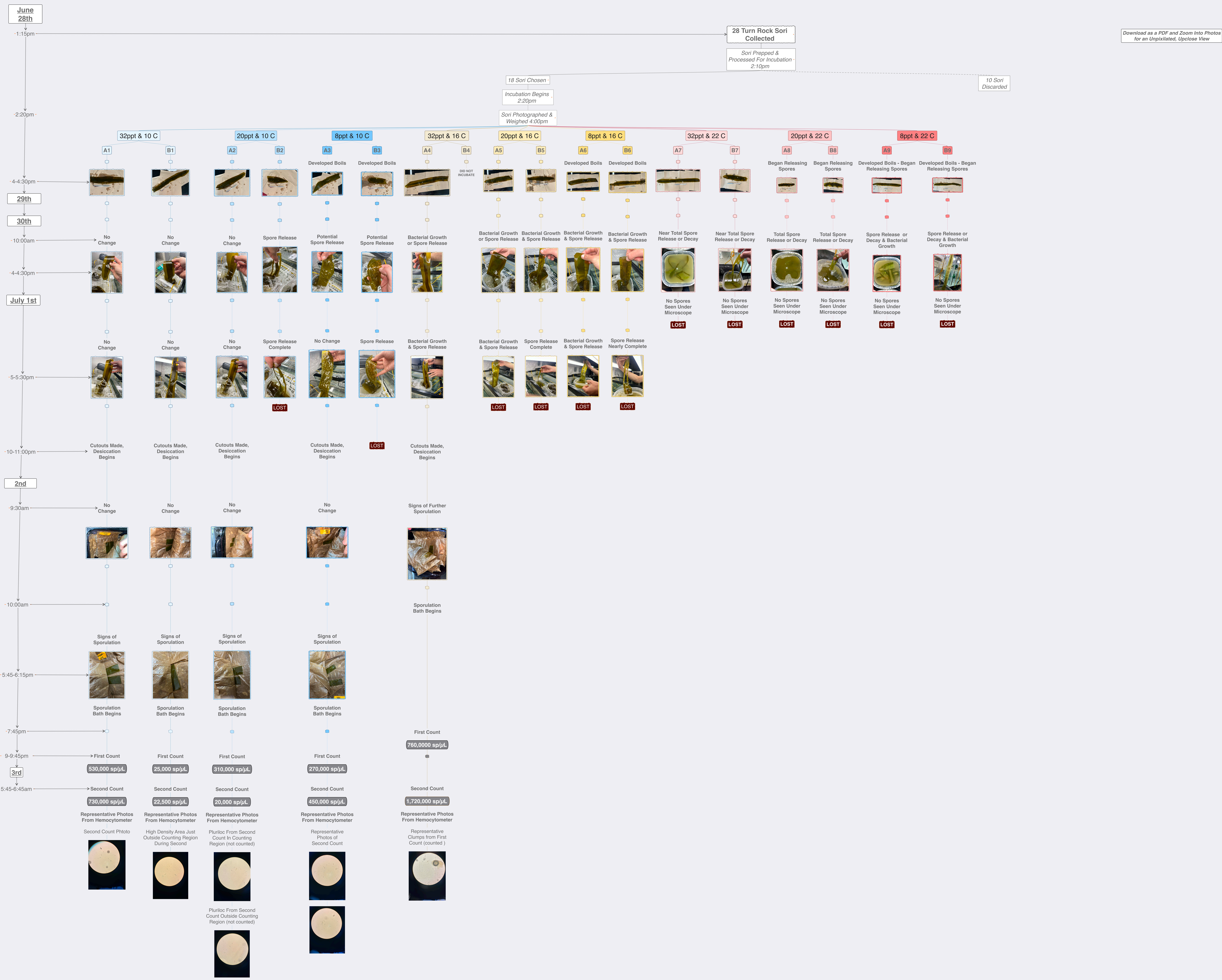
Appendix 86: phase 3 A9B9 post desiccation before placing into sporulation bath

Appendix 87: phase 3 C9D9 post desiccation before placing into sporulation bath



Appendix 88: A9B9 Phase 3 during incubation


Appendix 89: phase 3 incubation



June 28th
1:15pm
28 Turn Rock Sori Collected
Sori Prepped & Processed For Incubation 2:10pm
18 Sori Chosen
10 Sori Discarded
Incubation Begins 2:20pm
Sori Photographed & Weighed 4:00pm
2:20pm
32ppt & 10 C
20ppt & 10 C
8ppt & 10 C
32ppt & 16 C
20ppt & 16 C
8ppt & 16 C
32ppt & 22 C
20ppt & 22 C
8ppt & 22 C
A1
B1
A2
B2
A3
B3
A4
B4
A5
B5
A6
B6
A7
B7
A8
B8
A9
B9
Developed Boils
Developed Boils
DID NOT INCUBATE
Developed Boils
Developed Boils
Began Releasing Spores
Began Releasing Spores
Developed Boils - Began Releasing Spores
Developed Boils - Began Releasing Spores
4-4:30pm
29th
30th
10:00am
No Change
No Change
No Change
Spore Release
Potential Spore Release
Potential Spore Release
Bacterial Growth or Spore Release
Bacterial Growth or Spore Release
Bacterial Growth & Spore Release
Bacterial Growth & Spore Release
Bacterial Growth & Spore Release
Near Total Spore Release or Decay
Near Total Spore Release or Decay
Total Spore Release or Decay
Total Spore Release or Decay
Spore Release or Decay & Bacterial Growth
Spore Release or Decay & Bacterial Growth
4-4:30pm
No Spores Seen Under Microscope
LOST
July 1st
No Change
No Change
No Change
Spore Release Complete
No Change
Spore Release
Bacterial Growth & Spore Release
Bacterial Growth & Spore Release
Spore Release Complete
Bacterial Growth & Spore Release
Spore Release Nearly Complete
5-5:30pm
LOST
10-11:00pm
Cutouts Made, Desiccation Begins
2nd
9:30am
No Change
Signs of Further Sporulation
10:00am
Sporulation Bath Begins
Signs of Sporulation
5:45-6:15pm
Sporulation Bath Begins
7:45pm
9-9:45pm
First Count
530,000 sp/µL
25,000 sp/µL
310,000 sp/µL
270,000 sp/µL
760,0000 sp/µL
3rd
5:45-6:45am
Second Count
730,000 sp/µL
22,500 sp/µL
20,000 sp/µL
450,000 sp/µL
1,720,000 sp/µL
Representative Photos From Hemocytometer
Second Count Phtoto
High Density Area Just Outside Counting Region During Second
Pluriloc From Second Count In Counting Region (not counted)
Pluriloc From Second Count Outside Counting Region (not counted)
Representative Photos of Second Count
Representative Clumps from First Count (counted)
Download as a PDF and Zoom Into Photos for an Unpixilated, Upclose View

1 Sorus Collected from FHL Dock

Dowlaond As a PDF and Zoom Into Photos for an Unpixelated, Upclose View

July 2nd

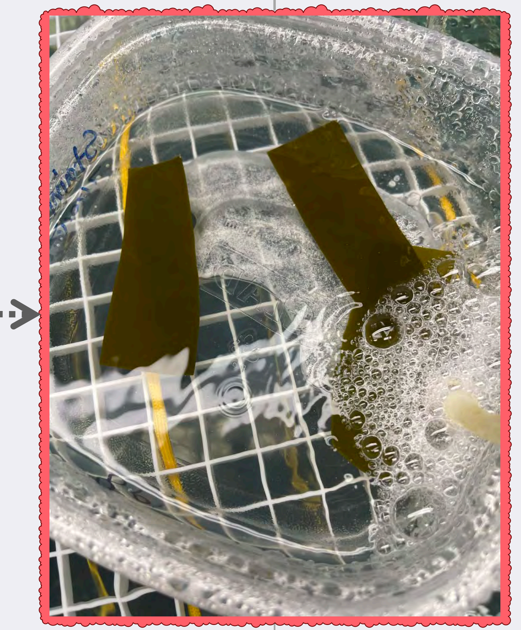
3 - 7cm x 3cm - Microscope Slide Sized Cutouts Proccessed

- 10:00pm

Incubation Begins Using Twice Tyndallized Water

8ppt & 10 C

- 11:53pm



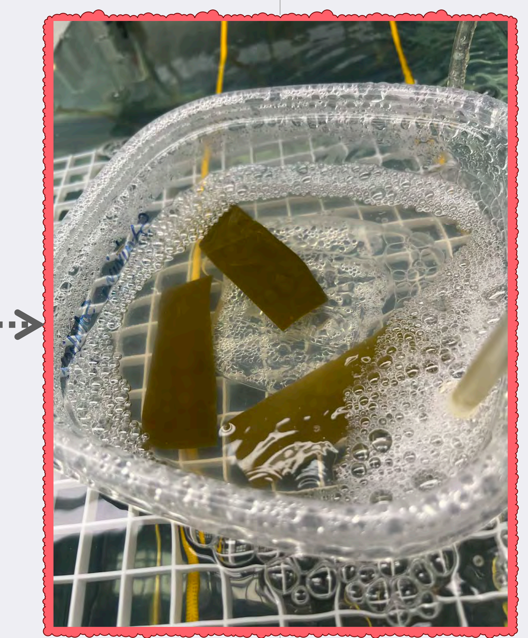
3rd

Boils Developed

-6:41am

Boils Continue to Develop - Slight Water Discoloration

-2:42pm



4th

Spore Release or DecayProgresses Past Desired Conditions

- 11:42am