RUNNING TIDE

Macroalgae at Alda
Q1 2024 Report

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Macroalgae Cultivation Report

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Summary

- Cotton danks and *Paulownia* have been seeded with sugar kelp gametophytes for OOGE-24-1
- First iteration of substrate seeding stress test revealed 48-72 hour cure time tolerance of Ulva spore/binder mixture
- CO_2 administration to the Ulva cultivation process improves growth rates by 39.4%
- Biomass and nitrate dynamics of Ulva cultivation can be accurately described using hybrid models
- Yield of induced Ulva sporulation can be described by moon, age of Ulva and nitrate consumption
- Storage of Ulva biomass in a cold environment for 2 weeks prior to sporulation and seeding is possible

Introduction

Following the Macroalgae Summit in Portland in January 2024, the main macroalgae-related goals for 2024 were clear. A plan for the year in Alda was outlined, where the first tasks were investigating the potential of storing Ulva biomass prior to seeding, and stress testing of seeded substrate. Along with those priorities the deployment season for the open ocean growth experiments (OOGE) is coming up, with the first substrates being inoculated in Q1. For those deployments, we'll seed cotton danks and *Paulownia* wood with Ulva spores and/or sugar kelp gametophytes. This will be performed four times (OOGE-24-1 to 4) from April to July 2024.

Much of the work presented in this Q1 report was conducted under the assumption that the company would make significantly sized deployments this summer of seeded substrate. As known to everyone at RT priorities have been fluid in Q1 and changing rapidly, which has impacted short to medium term experimental planning. Around Easter the team started slowing down efforts on continuous Ulva cultivation in our large PBRs to free up time to shift more focus onto substrate seeding and attachment.

Ulva lactuca/fenestrata

The first quarter of 2024 was spent on gathering more detailed data than previously obtainable for the Ulva cultivation process. A method to quantify nitrate levels using a UV-vis spectrophotometer was developed so the nutrient status of the PBRs could be monitored. Since beginning the CO_2 administration to the larger PBRs, we have also spent more effort on monitoring the pH levels to prevent them from becoming too low. The improved data gathering effort has allowed us to perform more detailed data analysis and modeling of the Ulva cultivation and sporulation processes, which will be presented in this chapter. First stress testing of seeding/attachment were carried out as well with positive results.

Effect of CO_2 on Ulva growth

One of the goals of early 2024 was to quantify the improvement in growth rates of Ulva when CO_2 is added to the PBRs. When growth of Ulva in all batches (i.e., individual runs in PBRs) is compared for Q1 2024, it is clear that the CO_2 addition improves the growth rate significantly (**Figure 1**). The average daily specific growth rate (SGR) for Ulva without CO_2 addition was 13.26 ± 3.38% whereas the average SGR for CO_2-supplemented Ulva was 18.48 ± 4%, which is an increase of 39.4%.

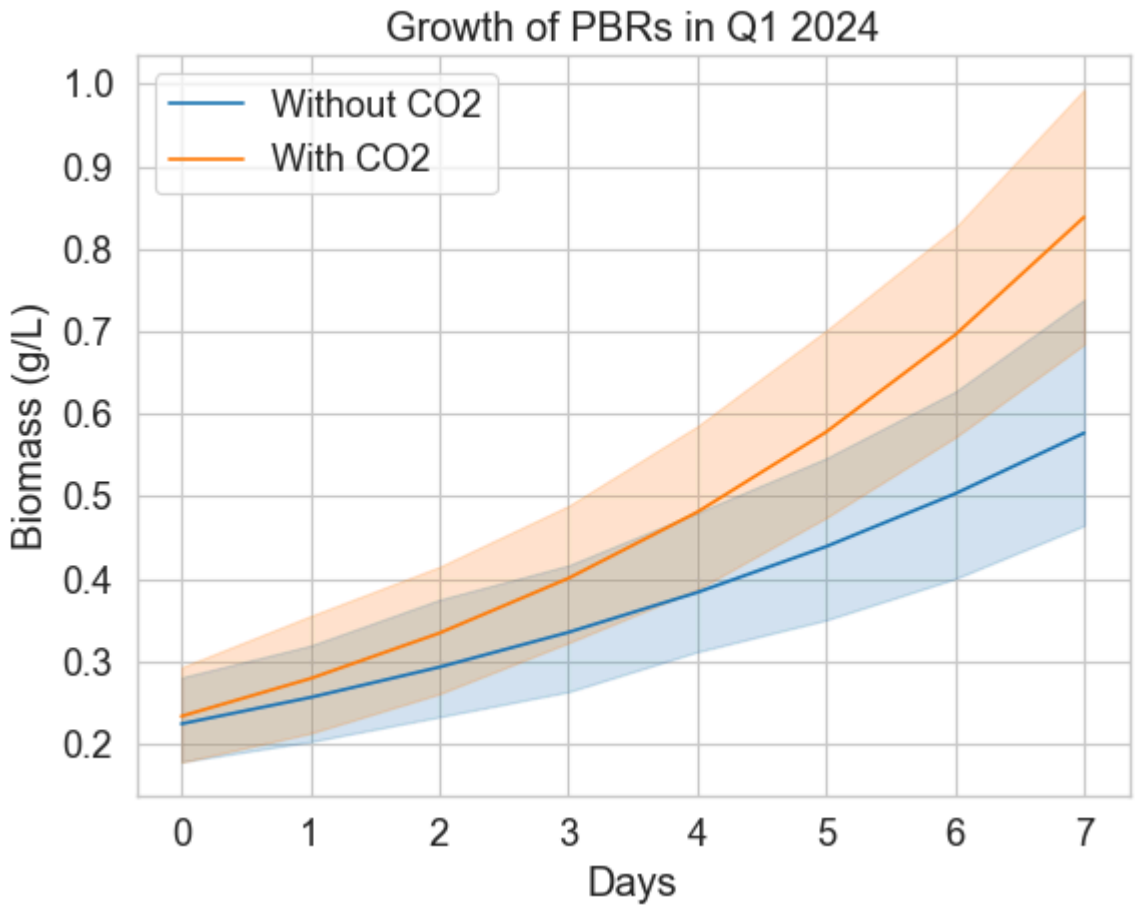

Figure 1. Growth of Ulva in 100L, 500L and 1000L PBRs in Alda in Q1 2024*. The orange line represents the Ulva batches with CO_2 supplementation. The blue line represents the Ulva batches without CO_2 supplementation. The shaded area represents the 95% confidence interval.*

Therefore, to maximize the growth in our cultivation batches, we have connected a CO_2 system to all our larger PBRs (100L-1000L). The main issue with the current CO_2 administration is that it is manual. We do expect to improve that setup in the future with an automatic CO_2 dosing system that is regulated by the pH. This way we might get even higher growth rates. But to further optimize the cultivation process, we have to consider more variables than CO_2 and pH. We need to look at how the process variables interact with one another. One approach to do this is through process modeling.

Modeling of cultivation process

The Ulva cultivation process exhibits a high degree of complexity, owing to its intricate interplay of various factors such as biomass growth, nutrient dynamics, light intensity, pH levels, moon phase, moon distance, batch size, and CO_2 status. Each of these factors contributes uniquely to the overall dynamics, making it challenging to model using

conventional approaches alone. Recognizing this complexity, a hybrid modeling approach integrating ordinary differential equations (ODEs) with kernel ridge regression emerges as a robust solution. In recent years, the hybrid modeling approach has been getting widespread attention, particularly within the biopharmaceutical industry to model the production process of biologics (Narayanan et al., 2023, Pinto et al., 2022, Schweidtmann et al., 2024)

By leveraging the flexibility and non-linearity of machine learning models (e.g., kernel ridge regression or neural networks), the model captures the nuanced relationships between the process variables and the specific rates governing biomass and nutrient dynamics. This hybrid structure allows for a more comprehensive representation of the cultivation process, accommodating the diverse array of influencing factors and yielding insights that may elude simpler modeling strategies. In essence, by combining the strengths of mechanistic ODEs with the adaptability of machine learning, the hybrid model offers a powerful framework to navigate the intricate dynamics of the cultivation process effectively.

In our case, the 100L, 500L and 1000L PBR runs/batches were divided into distinct train and test sets to facilitate hybrid model training and evaluation. Specifically, the train set (n = 23 batches) was resampled 25 times with replacement (75% of the train set batch number for each resample). For each bootstrap resample, a kernel ridge regression model was trained to capture the intricate relationships between process variables and the specific rates governing biomass and nutrient dynamics. Subsequently, the ensemble of bootstrap resampled trained models' performance was assessed by examining their fit to the training set batches (**Figure 2A**). The ensemble model demonstrated a strong fit, achieving an average error of 6.2% for biomass and 4.9% for NO_3. Subsequently, the ensemble model's performance was evaluated on an independent test set (n = 5 batches), representing unseen data (**Figure 2B**). Despite the inherent variability across batches, the ensemble model maintained a reasonable fit with the test set, albeit with a slightly higher average error of 12.3% for biomass and 13% for NO_3.

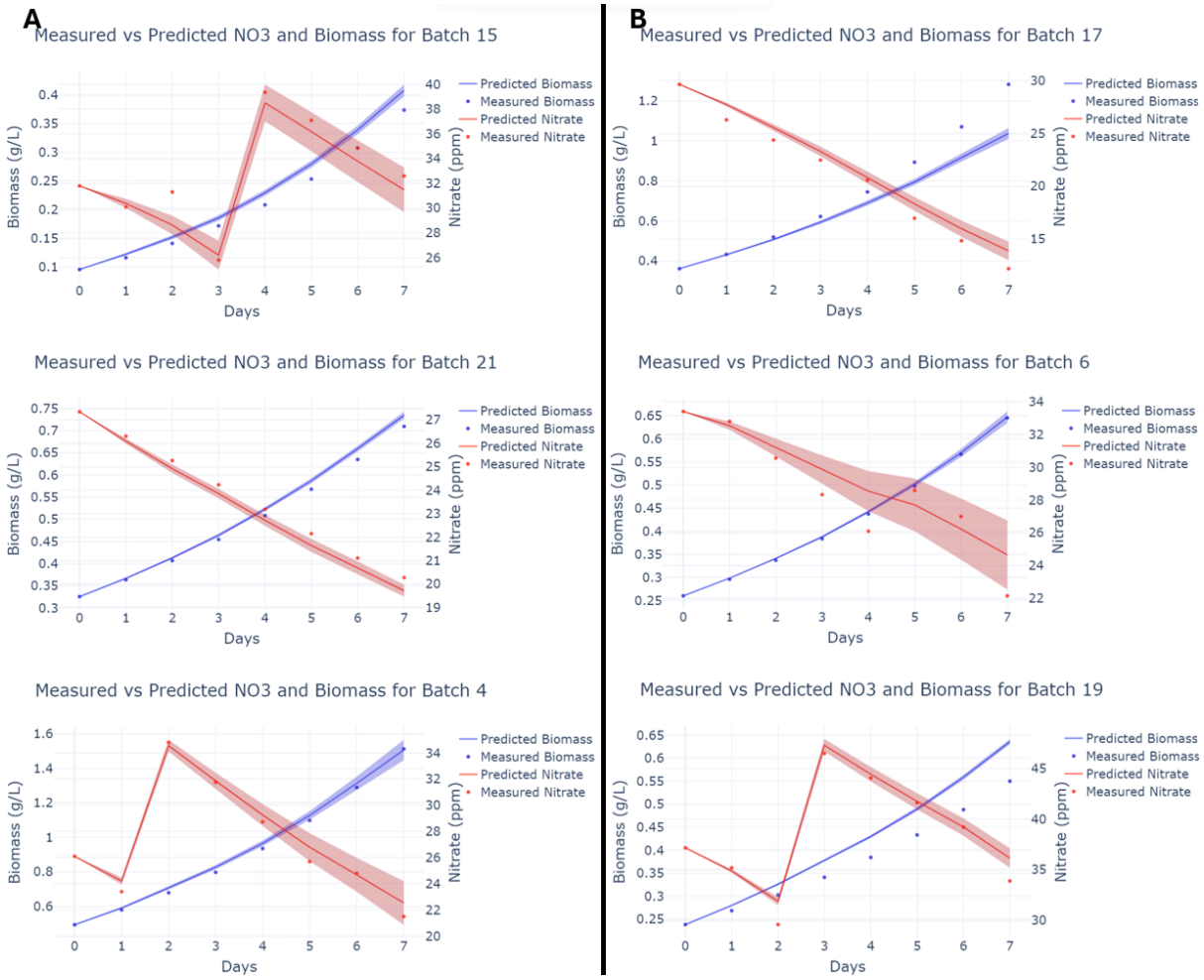

Figure 2. Predicted and Actual Dynamics of Cultivation Process for Training and Test Batches. *A) Predicted (lines) and actual (dots) dynamics of the cultivation process for three randomly selected batches from the training set. B) Predicted (lines) and actual (dots) dynamics of the cultivation process for three randomly selected batches from the test set. Each subplot illustrates the temporal changes in biomass and nutrient levels. The test set batches were not used to train the kernel ridge regression models and are therefore representative of unseen data. The shaded area represents 95% confidence intervals of predictions based on bootstrap resampling.*

This evaluation underscores the model's capacity to generalize beyond the training data and its potential utility in predicting cultivation dynamics across various scenarios. After successful modeling of the cultivation process, we next turned our attention to the sporulation process.

Modeling of spore yield

During deployment season, we will need to reliably produce spores on a large scale. Therefore, we need to understand how we can optimize our spore production. To do that, we need to understand the sporulation process and what factors influence the spore yield

(i.e., the amount of spores produced per microgram of fresh weight Ulva). Since the beginning of 2024, we have been carefully monitoring our cultivation processes, including daily measurements of pH and NO_3 levels, thorough assessment of the light intensity in different PBRs, and careful, manual administration of CO_2 in selected batches. This has allowed us to gain a deeper understanding of the cultivation process, and helped us build the cultivation process model presented above. In addition, we have been conducting large scale sporulations (in a 1000L PBR tank) on a regular basis (**Table 1**).

Table 1. Large-scale Sporulation Inductions performed in Alda in Q1 2024. *Shown are experimental variables and results that were collected for each of the batches. Only the maximal spore yield values were considered.*

Batch number	Sporulation date	Amount biomass (gFW)	Sporulation Volume (L)	Max spore density (sp/mL)	Max spore density day	Spore yield (spores/ug)
UL_LAB2_290124_1000L_A	2024-02-05	1025.5	500.00	405000	4	197.464651
UL_LAB2_290124_1000L_B	2024-02-05	1025.5	500.00	405000	4	197.464651
UL_LAB2_070224_1000L_A	2024-02-14	2046.0	500.00	92500	4	22.605083
UL_LAB2_070224_1000L_B	2024-02-14	2046.0	500.00	92500	4	22.605083
UL_LAB2_220224_1000L_A	2024-02-29	847.0	500.00	55000	5	32.467532
UL_LAB2_010324_1000L_A	2024-03-08	2233.0	550.00	85000	6	20.935961
UL_LAB3_120324_1000L_A	2024-03-19	784.5	500.00	220000	5	140.216699
UL_LAB3_200324_1000L_A	2024-03-26	791.5	500.00	107500	3	67.909033

In an attempt to further understand the relationship between the environment, the cultivation process and the subsequent sporulation induction efficiency, we merged the sporulation data from the batches in **Table 1** with their cultivation process data (i.e., biomass, pH, NO_3, light intensity, CO_2 supplementation). Moreover, we used the *astropy* Python module to retrieve data on moon phase angle and distance from earth for the dates when the sporulation inductions were performed (The Astropy Collaboration, 2022). Using this merged dataset, we sought to investigate whether the spore yield could somehow be described by the behavior of the cultivation process and/or the lunar effect. More specifically, we used the merged dataset variables to train a regression model to predict the spore yield.

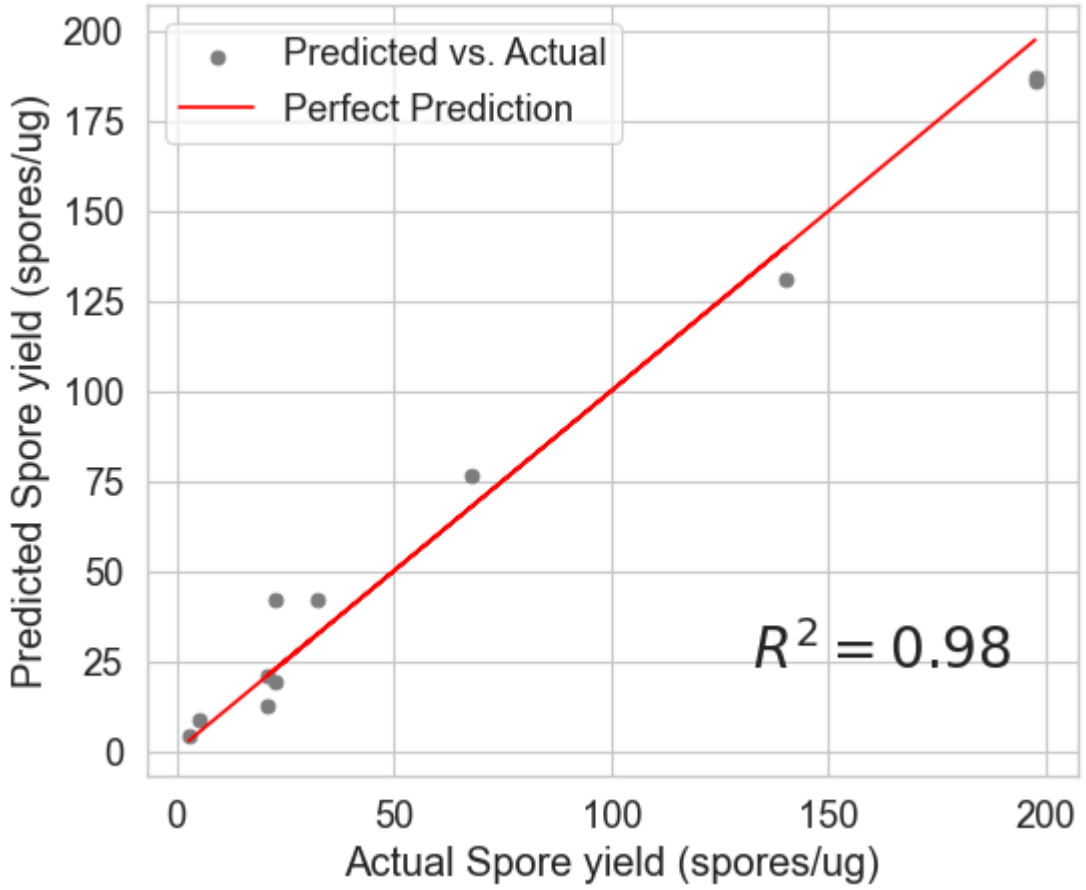

Figure 3. Predicted vs. Actual Spore Yield using Penalized Regression*. The scatter plot illustrates the comparison between predicted and actual spore yield values obtained using Lasso regression. The gray scatter points represent the predicted spore yield values plotted against the corresponding actual spore yield values. The red line depicts the line of perfect prediction. The coefficient of determination (R2) is shown on the plot.*

Using penalized linear regression (Lasso) with a bootstrap-resampling approach, we found that there was high consistency (R^2 = 0.98) between predicted spore yield and actual spore yield (**Figure 3**). Lasso regression was chosen for its ability to perform variable selection by imposing a penalty on the absolute size of the regression coefficients.

To ensure robustness and reliability of the model, a bootstrap-resampling approach was utilized. This approach involved randomly sampling the dataset with replacement to create multiple bootstrap samples. For each bootstrap sample, a Lasso regression model was trained, and the coefficients of the predictor variables were recorded. The mean coefficient of all variables across the bootstrap samples was then calculated, providing a stable estimate of the variable importance. The coefficients and the 95% confidence intervals are displayed in **Figure 4**.

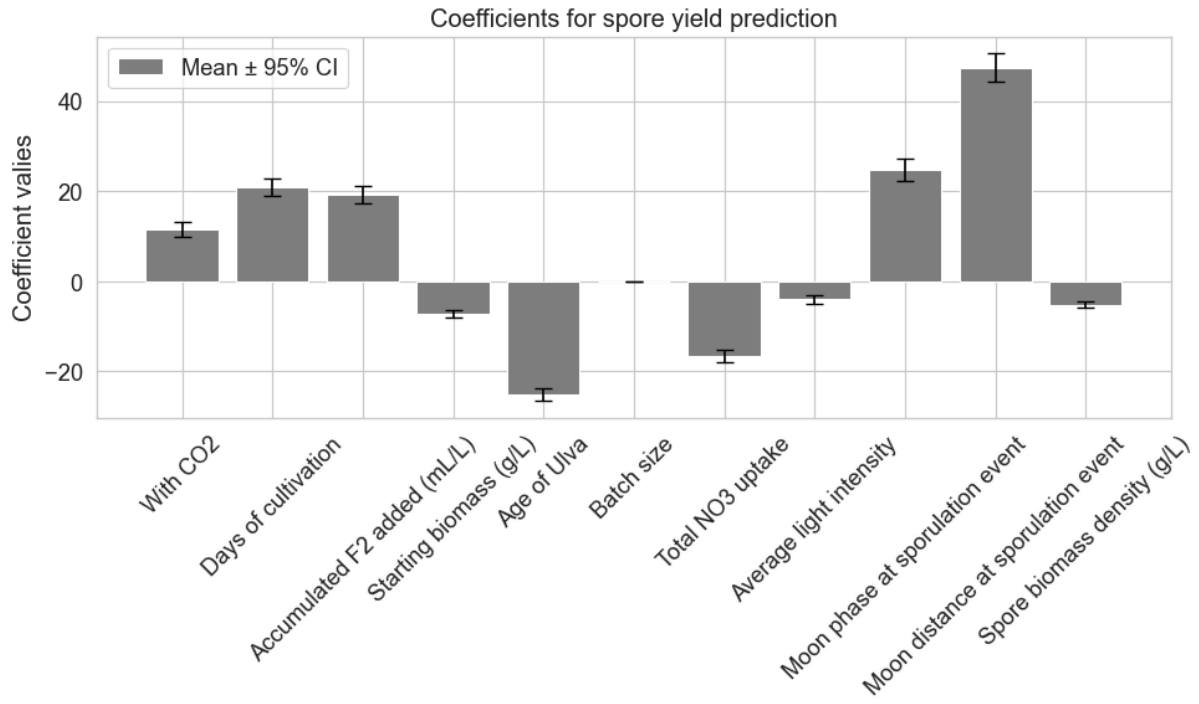

Figure 4. Coefficients of the Spore Yield Model. *The plot depicts coefficients of predictor variables derived from a bootstrap resampling procedure, alongside their 95% confidence intervals (CI). Coefficients were estimated by repeatedly fitting regression models to bootstrap samples, and 95% CI were computed using percentile-based bootstrap intervals.*

By observing the coefficients, the effect of each of the variables on the spore yield can be readily interpreted. For example, it appears that the older the Ulva is (higher “Age of Ulva”), the lower the spore yield. Also, the closer the moon is to being full (moon phase angle of 180, which is the highest possible angle), the higher the spore yield. To further investigate how important each of the variables is to the prediction of spore yield, we performed permutation-based importance scoring (**Figure 5**). Overall, the moon phase angle and the moon distance were the most important variables for determining the spore yield. The most important biological variables were Ulva age and the total uptake amount of NO_3 throughout the cultivation process period.

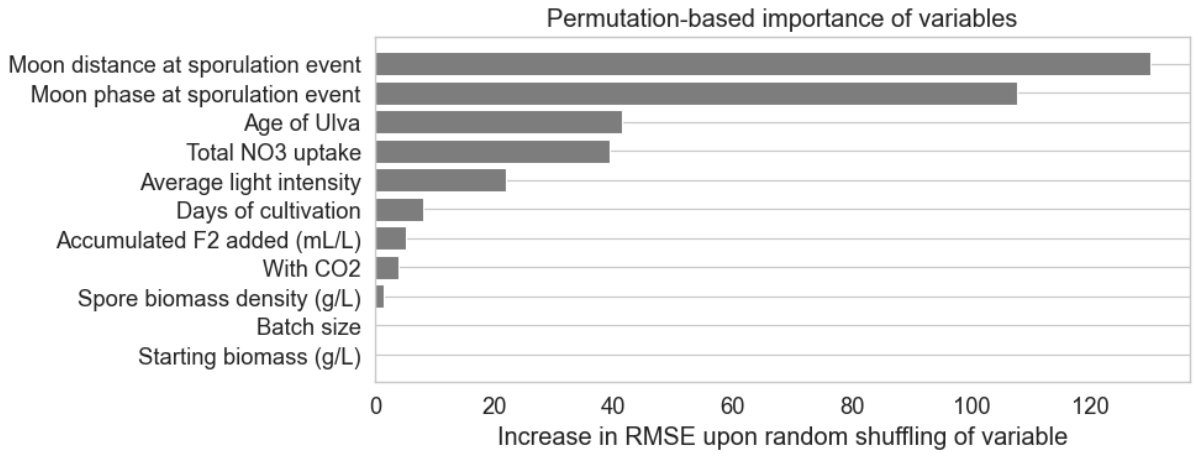

***Figure 5. Permutation-based Variable Importance**. The bar plot illustrates the permutation-based variable importance scores for predicting spore yield. Each bar represents the importance score of a predictor variable, with higher scores indicating greater importance in predicting spore yield.*

The finding of the moon as the most important contributing factor in determining the spore yield is major. Previous research has indicated that there is a connection between the moon and sporulation of Ulva (Luning et al., 2007), but to our knowledge, no one has accurately modeled Ulva sporulation events from moon data. Sure, Ulva age has been used for modeling of sporulation dynamics (Obolski et al., 2022), but our modeling efforts used both biological and environmental variables to accurately describe spore yield. Furthermore, the model was a simple linear regression, meaning that the contribution of each of the variables to spore yield can be readily interpreted. As more data is gathered and more time is spent on data modeling, we believe we will be able to use both the cultivation process model and the sporulation model in tandem to identify: 1) The optimal time to start a cultivation, 2) the optimal strain (based on age) to use for cultivation, 3) the optimal cultivation process parameters and settings, all to maximize spore production during deployment season. We may even use the models to identify the upper limits of our spore production capacity, which will help with further scaling of our production.

In an attempt to improve the Alda capacity even further, we have been investigating the possibility of storing Ulva biomass for an extended period of time prior to sporulation and seeding. The results from these efforts will be discussed in the next section.

Cold storage of biomass - Effect on spore yield and seeding

As mentioned above, we have been conducting biomass storage experiments in Q1 2024. The maximum Ulva production capacity of Alda is currently 6kgFW/week. Upon sporulation of that biomass (and assuming a high spore yield of 200 spores/ug), we can produce 1.2 billion spores per week. Depending on the size of the substrate buoy we will use for

deployments, we are able to seed between 444 and 1326 tonnes of substrate per week. This is all assuming continuous production in Alda throughout the deployment period.

However, if we could stock up on biomass prior to the deployment season, and that biomass remains viable (i.e., we can produce healthy, germinating spores from it), it is clear that we could increase our spore production capacity significantly. Therefore, we need to investigate the possibility of storing biomass for extended periods of time (1-12 weeks).

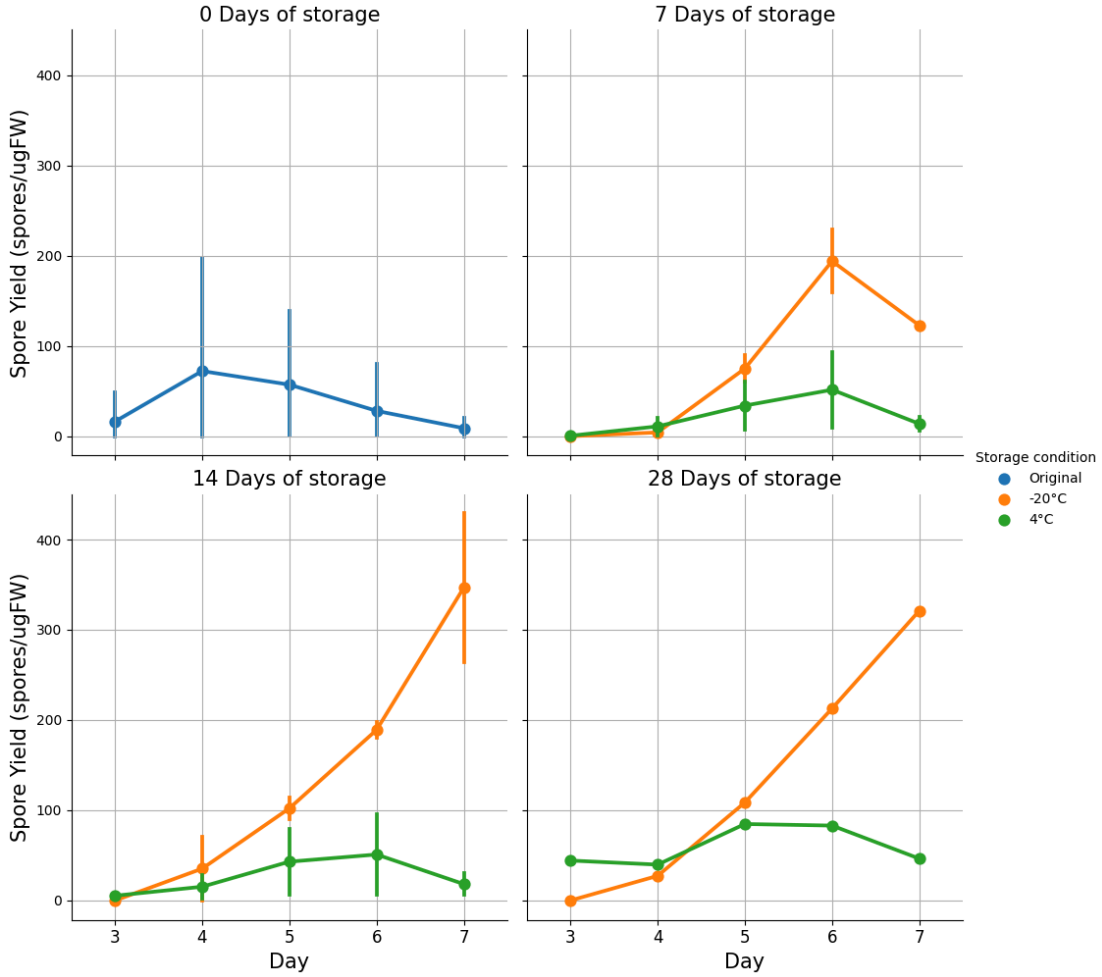

Figure 6. Spore Yield of Stored Biomass at 4°C and -20°C. *Ulva biomass was stored for 0-28 days at the two different temperatures prior to sporulation induction. The graph depicts the spore yield values 3-7 days after sporulation induction for all treatments. Results from two independent experiments are shown. The bars represent the standard deviation from 2 independent experiments.*

We have already performed two identical storage experiments. Briefly, Ulva biomass was harvested from 1000L PBRs. A portion of that biomass was induced to sporulate immediately (Control group), another portion was stored at 4°C and a third portion was stored at -20°C. After 7, 14 and 28 days, the biomass was taken from storage and induced to sporulate. The spore yield numbers for all different storage methods and timepoints are shown in **Figure 6**. In summary, storing the biomass at 4°C for up to 28 days did not

decrease the spore yield when compared to the control group. Surprisingly, the spore yield increased when biomass was stored at -20°C. However, the spores from the -20°C-stored biomass were not viable (i.e., they did not germinate when used to seed substrates). In contrast, the spores from the 4°C-stored biomass were viable for up to 14 days in storage (28 day stored biomass was not tested for seeding).

Moving forward, we will try to combine the storage methods. We will store the biomass at 4°C for 1-4 weeks. Then, we will try freezing the biomass for 1-24 hours prior to sporulating it. The hypothesis here is that freezing temperatures (for a short time) might boost the spore yield for the 4°C-stored biomass, while still maintaining spore viability. We will then seed substrates with the resulting released spores and monitor germination and growth of Ulva.

In addition to investigating the effect of storage conditions on Ulva sporulation and germination, we were also interested in the tolerance of the spores themselves to extended periods of drying. This is particularly important for practical reasons during the deployments themselves, as will be discussed in the next section.

Stress testing for seeding/attachment

In Q1 our first round of stress testing was conducted. The idea behind these tests is to try to simulate the challenging conditions that Ulva spores will encounter if Running Tide were to seed substrate on land, possibly multiple days prior to deployment. Being able to seed substrate on land would be technically easier and RT would have more control over the seeding conditions compared to seeding taking place at sea. The main question being: do the spores survive for multiple days without seawater after being attached to substrate?

Specifically for this test multiple cure times for the spore/binder mixture were tested, the cure times being: 1h, 24h, 48h and 72h. Since this test we have changed out the 72h test for a 5 day test to simulate deployment time in Canada. So far, these tests are only a YES/NO test, meaning that there is no detail yet in the result other than did macroalgae grow or not. Detail in these experiments will increase as the sophistication of the tests increases.

The first substrate that we tested were BM Valla bricks. The bricks were leached and dried prior to the test. The test with the BM Valla bricks will serve as a worst case scenario as the bricks are dry and absorb moisture extremely fast, meaning that when they are sprayed with spore/binder solution the bricks dry fast, exposing the spores to quite an extreme environment. The team did not expect any Ulva growth on BM Valla bricks that received the extended cure time in this test.

The team was however positively surprised to see that Ulva growth was **confirmed on all 24 bricks seeded** (each cure time being a triplicate of two bricks), even on the bricks that

received the extended cure time. This should be considered as a great indicator that Ulva spores can survive harsher conditions than previously thought.

The main reason for using BM Valla bricks for this test was mainly that it was the only substrate available in enough quantity at the time, and similar tests on wooden substrate are already underway or being planned by the team using both Ulva spores and kelp gametophytes.

Saccharina latissima

The first quarter of 2024 was spent increasing the densities of the gametophyte cultures, creating and refining a gametophyte density standard curve utilizing UV-vis absorbance measurements, and preparing for the OOGE-24-1 deployment at the beginning of the second quarter.

Alda gametophyte bank

Two 20L PBRs were started this quarter and are a first for Running Tide's gametophyte production capabilities. We are currently refining their maintenance procedures and developing SOPs. As of publishing this report, the Alda gametophyte bank consists of 28 PBRs of production cultures, ranging in volume from 500mL to 20L, and nine 1L PBRs that are being used for experiments, but are still available for use in production (**Table 2**). We estimate that these cultures could be used to produce over 300L of seeding solution at a density of 10,000 fragments/mL. Future seeding and deployment experiments will allow us to convert the solution volumes to more meaningful units (i.e. the amount of substrate that can be seeded per liter).

Table 2. Alda Gametophyte Bank Cultures. ** - indicates cultures undergoing experimentation.*

Alda Gametophyte Bank Cultures			
20L PBRs			
Ind1_4a_20L	Grótta1.2_2_20L		
4L PBRs			
Ind1_4a_4L	Grótta1.2_2_4L	Ind1_3a_4L	Ind1_4b_4L
1L PBRs			
Ind1_1_1L	Ind1_2_1L	Ind1_3_1L	Grótta1.2_2_1L
Grótta1.2_4_1L	Grótta16_1L	Ind1_4b_1L	Ind1_4c_1L
Ind1_4d_1L	Ind1_4_Exp01*	Ind1_4_Exp02*	Ind1_4_Exp03*
Ind1_4_Exp04*	Ind1_4_Exp05*	Ind1_4_Exp06*	Ind1_4_Exp07*
Ind1_4_Exp08*	Ind1_4_Exp09*		
500mL PBRs			
Male22	Male05	Ind1_4a	April23_1
April23_2	April23_3	Female09b	Female04b
Male01	Sept23Female1	Sept23Female2	Sept23Male
Grótta3			

Also of note, the majority of the 500mL PBRs are composed of previously isolated gametophytes that are now being fragmented to start our sex segregated monoclonal cultures. These cultures are expected to take at least a year to reach densities that allow us to experiment with seeding based on controlled sex ratios. The continued maintenance and regular fragmentation of these isolations and cultures is the beginning of the next phase of the Alda gametophyte bank.

Gametophyte standard curve

Of particular importance this quarter, we have established a well defined and validated standard curve for the calculation of gametophyte fragment densities based on UV-vis absorbance measurements (**Figure 7**). The curve is under perpetual refinement but currently consists of 28 points acquired through serial dilutions of cultures, UV-vis absorbance measurements, and visual fragment counts utilizing microscopy and a hemocytometer. Due to the difficulty in differentiating fragments on the hemocytometer at densities greater than 800,000 fragments per mL, we have yet to validate a continued linear relationship beyond an absorbance of 1.25 AU; however, the gametophyte densities we are currently utilizing for substrate seeding fall comfortably along our validated curve (see **OOGE-24-1** below).

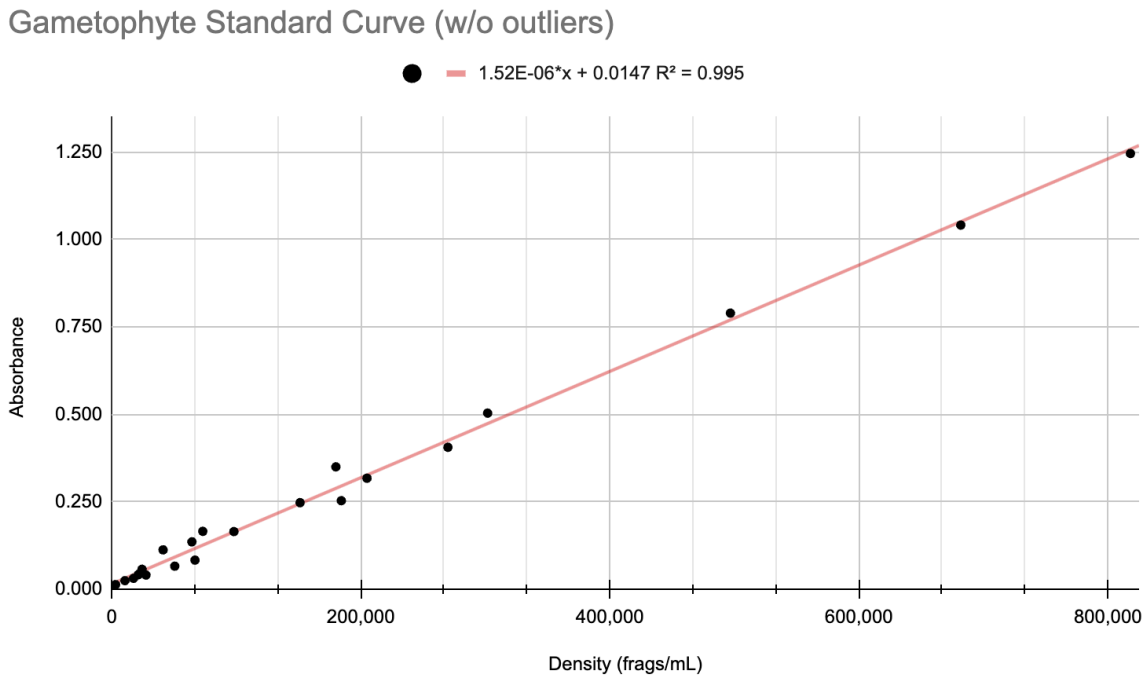

Figure 7. Gametophyte Standard Curve. *The absorbances and fragment counts of 28 samples have been plotted, which shows a clear linear relationship between the two measurements. Using this information we are able to extrapolate fragment densities of samples based on their absorbances, providing increased control of future experiments and seeding efforts.*

The gametophyte standard curve has opened up numerous avenues for experimentation this quarter and beyond. First, we are able to more accurately prepare seeding solutions

based on predetermined gametophyte densities. Second, we can quantitatively monitor gametophyte cultures and determine their growth rates (density), instead of previous qualitative visual designations. Because we can now measure growth rates, we can also experiment with cultures to determine effects on growth rates. In fact, the nine experimental cultures in Table Kelp1 are currently undergoing nutrient experiments in an effort to improve gametophyte growth rates and/or reduce production expenses.

OOGE-24-1 seeding

The mission for OOGE-24-1 is the deployment of *S. latissima* seeded substrates on CamLite buoys in the North Atlantic Ocean with the goal of verifying kelp growth in the open ocean. Cotton danks and blocks of *Paulownia tomentosa* were spray seeded with gametophytes (and binder) at a concentration of 10,000 fragments per milliliter. These substrates were seeded on two occasions to test two different cultures and provide potential backups for the deployment. Therefore, a total of 12 cotton danks and 24 *Paulownia* blocks were seeded. After two weeks of growth only the first seeding of danks showed significant kelp attachment/growth (**Figure 8**).

Figure 8. Seeded danks. *Kelp danks 17 days after spray seeding at a density of 10,000 fragments/mL.*

Franken buoy

Due to the difficulty and expense of growing *S. latissima* sporophytes in a laboratory environment, we are initiating a coastal growth experiment utilizing our kelp gametophytes and outmoded camera buoys that were rescued from the shores of Iceland. Our plan is to seed cotton hanks with the same gametophyte cultures used for OOGE-24 deployments, install them in a refurbished second generation camera buoy (CB2), and anchor the buoy in the coastal waters a few hundred meters from the Alda facility. This experiment should provide us with valuable and relatively inexpensive information that can aid in future kelp experiments.

The planning and execution of the experiment will primarily fall to Matthew (kelp seeding, maintenance, and measuring), Ívar (buoy construction and maintenance), and Íris (logistics). Additionally, we will need to communicate and coordinate with the engineers in Portland (Brook, Tim, and Philipp) to make sure the buoy software is working properly. Once the buoy is refurbished, reconnected, seeded, and moored we can monitor the growth of the kelp via the camera then occasionally collect, dry, and weigh blades to begin the process of quantifying its carbon content. At the time of submitting this report, we have begun seeding the hanks and creating the franken buoy (**Figure 9**).

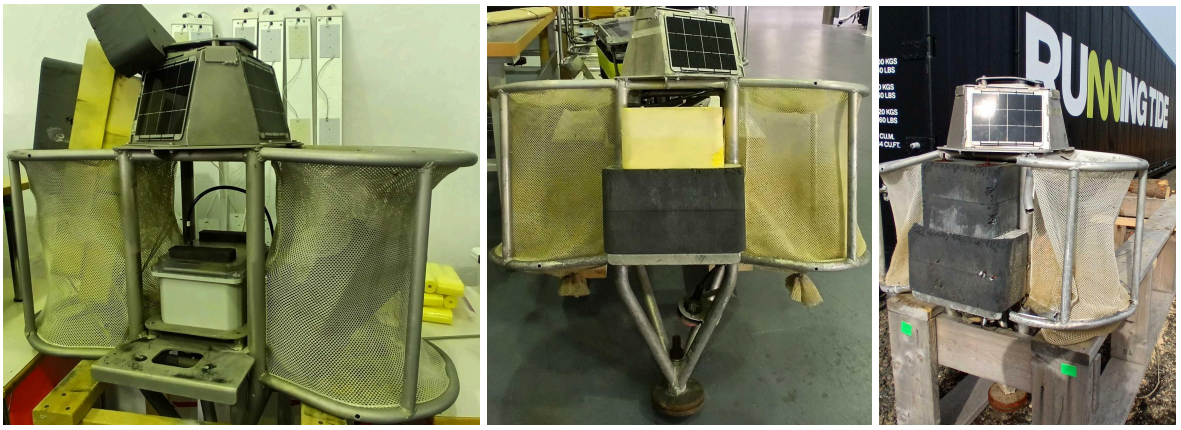

Figure 9. Franken buoy donors. *Three camera buoys (CB2.25, CB2.19, CB2.36) that ran ashore in Iceland and were rescued are having parts salvaged so that a single functional buoy can be created and used for a kelp growth experiment in the coastal waters close to the Alda facility.*

Ongoing Issues

To date we have a single, ongoing issue with the kelp gametophyte cultures that we are attempting to solve. We are occasionally burdened with unannounced “greenings” of our brown algae cultures, which results in us terminating the culture. Essentially, upon viewing our regularly maintained cultures, a seemingly fine brown culture the day before will be incredibly green and no longer safe to maintain with the other cultures. We are currently unsure of the cause of these greenings but believe they are resulting from either a bloom of naturally occuring diatoms that have been carried along since the collection of the kelp sorus or a potential contamination from an undesired green microalgae. The greenings have occurred across all culture volumes, from 500mL to 20L, and in cultures that range across the age of our gametophyte bank. Anywhere from 0 to 5 cultures may go green in a given month but no pattern has been discernable to date as to what is causing the issue or if it is spreading or spontaneously occurring. In the coming year, it will be worth the time and financial investment to understand what is causing the greening, so that we can efficiently and effectively remove the ongoing complication.

Planned Experiments

Several experiments are being planned for the coming months, particularly after the deployment season ends. First, as previously mentioned, nutrient experiments are currently underway and will most likely be added upon by introducing a nitrogen measurement component to fine tune the gametophyte renewal schedule. Second, we are planning various seeding experiments to hone in on ideal fragment densities and seeding procedures. Finally, experiments are being planned to determine the sex of isolated gametophytes that have lost their dimorphic features, which will aid us in improving our sex based gametophyte bank.

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