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Evaluating the Effect of Temperature and CO_2 enrichment on the Red Seaweed *Asparagopsis taxiformis* from Southern CA with Implications for Aquaculture

A thesis submitted in partial satisfaction of the requirements for

the degree Master of Science

in

Marine Biology

by

Hannah M. Resetarits

Committee in charge:

Professor Jennifer Smith, Chair
Professor Eric E. Allen
Professor Brian Palenik

2022

The thesis of Hannah M Resetarits is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

Dedication

I would like to dedicate this thesis to my friends and family for all their support, encouragement, and faith over the past two years.

To my family, Mom, Dad, and Sam, for always being there for me. You have given me such heartfelt and inspiring advice. Your love has kept me going, motivating me to work harder and be the best person I can be.

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This thesis contains unpublished material co-authored with Smith, Jennifer E. and Dishon, Gal. The thesis author was the primary author of this chapter.

ABSTRACT OF THESIS

Evaluating the Effect of Temperature and CO2 enrichment on the Red Seaweed *Asparagopsis taxiformis* from Southern CA with Implications for Aquaculture

by

Hannah M. Resetarits

Master of Science in Marine Biology

University of California San Diego, 2022

Professor Jennifer E. Smith, Chair

The red alga, *Asparagopsis taxiformis*, has recently been recognized for its unique ability to drastically reduce methane emissions from livestock. The main obstacle in using this seaweed is the complete lack of commercially available *A. taxiformis*. Little is known about how to develop large-scale cultivation systems for this red alga and the factors that influence its performance. This study examined the effect of the key factors including temperature and CO_2

concentration, on the growth, photophysiology and the concentration of bromoform, the secondary metabolite responsible for methane reduction in *A. taxiformis*. A series of single and multifactor closed culture experiments, conducted on *A. taxiformis* collected, isolated, and cultured in southern California, identified the temperature and CO_2 concentration required to optimize growing conditions. The findings indicate that through environmental control and addressing limiting factors, significant increases in biomass production can be achieved, making *A. taxiformis* a viable species for large-scale cultivation.

Introduction

As the world comes together in an effort to mitigate climate change, reducing anthropogenic methane has become a priority, as made clear in the recent 2021 UNFCCC conference. A leading source of methane emissions is enteric fermentation in ruminants, otherwise known as cow burps. Currently, there is no commercially available method to reduce methane production in ruminants, but the red alga, *Asparagopsis taxiformis (Delile) Trevisan de Saint-Léon,* provides a unique and potentially viable solution. Recent studies have shown that feeding a minute amount of *A. taxiformis* to ruminants results in a significant reduction in methane production, with no negative effects on the animal (Robert D. Kinley et al., 2016; Machado et al., 2016a). These findings have led to an increased interest in large-scale aquaculture *A. taxiformis;* however, very little is known about the factors that influence the growth, physiology, and secondary metabolite production of this species in captivity or otherwise. The goals of this thesis are to evaluate how key parameters such as temperature and pCO2 concentrations affect the growth and photophysiology of this species. Specifically, I used a series of laboratory experiments to quantify how these factors affect the growth, photophysiology, and concentration of bromoform, the secondary metabolite responsible for methane reduction (Machado et al., 2016b) tetrasporophyte phase of *A. taxiformis.*

Methane (CH_4) is an extremely potent greenhouse gas (GHG) that plays a significant role in global climate change, as it is the most abundant non-CO_2 GHG found in the atmosphere (Montzka et al., 2011). While methane is 28 times more potent than CO_2, it is also a short-lived GHG that breaks down and leaves the atmosphere after about 9 years (Montzka et al., 2011). Because of methane's abundance, potency, and short atmospheric lifespan, reducing it has been

identified as an effective way to mitigate climate change, since small reductions will have rapid positive effects. Scientists have found that nearly two-thirds of methane found in our atmosphere is the direct result of anthropogenic sources, with 17% of global methane production coming from ruminant animals that perform enteric fermentation in their guts (Montzka et al., 2011; Moss et al., 2000; Patra et al., 2017). As a result, researchers and politicians have started encouraging that steps be taken to reduce its production. Most notably, the California state government passed a bill in February 2019 requiring the agricultural industry to reduce its methane emissions to just 60% of the level measured in 2013 by the year 2030 (Senate Bill No. 1383, Chapter 395). With over 5 million cattle in California, the agricultural industry is turning to the dairy and beef sector to reduce methane emissions to reach the levels outlined by this bill. Many strategies are being examined to reduce methane production in cows, such as feeding them lipid supplements and different hydrocarbon compounds as well as a variety of plants, plant extracts, and seaweed which have been shown to have varying anti-methanogenic properties (Patra et al., 2017).

Because of the abundance and variety of secondary metabolites in algae, multiple *in vitro* studies have been conducted to explore the effects of different algae on methane production in ruminant animals (R. D. Kinley & Fredeen, 2015; Li et al., 2016; Machado et al., 2016a; Maia et al., 2016). To date, these studies suggest that *A. taxiformis* has the largest and most consistent impacts on reducing methane production during enteric fermentation. Most recently, a series of experiments coming out of Australia and the University of California, Davis found that the replacement of 1-2% of a cow’s diet with *A. taxiformis* reduces their methane output by up to 90% (Brooke et al., 2018; Robert D. Kinley et al., 2016; Machado et al., 2016a; Roque et al.,

2021). The mode of action has been partially attributed to bromoform and other brominated compounds produced by *A.taxiformis* (Robert D. Kinley et al., 2016; Machado et al., 2016b) where these compounds intervene in two pathways of methane production. It blocks an enzyme necessary for the production of methane in methanogenic microbes, and acts as a competitive terminal electron acceptor in the reduction of H_2, allowing H_2 to be utilized without the byproduct of methane (Patra et al., 2017). Without the production of methane, the carbon that would have otherwise been used by the methanogenic microbes can be used in the animals metabolism. This results in increased efficiency in the digestion of food, thereby theoretically decreasing the total amount of feed needed per animal (Robert D. Kinley et al., 2020). A positive correlation has been identified between the concentration of bromoform within the biomass and the effectiveness of *A. taxiformis* at reducing methane production in ruminants, in both in vitro and in vivo experiments (Brooke et al., 2020; Chagas et al., 2019; Robert D. Kinley et al., 2020; Machado et al., 2016b, 2018; Roque et al., 2021; Vucko et al., 2017).

The ability of *A. taxiformis* to mitigate livestock derived methane production will depend entirely on our ability to commercialize this species. Despite the research efforts that have been focused on the effects of *A. taxiformis* on methane reduction, limited research has been conducted on the seaweed itself. Little is known about how to cultivate this species in captivity, how to close the life cycle or how various environmental parameters affect the growth, physiology or bromoform concentration of the seaweed. A. taxiformis is a red seaweed in the Order Bonamaisoniales with a global tropical to subtropical distribution. While it is fairly cosmopolitan, it is also known to be invasive in some regions such as the Mediterranean Sea where it has been shown to erode habitat and reduce biodiversity (Mancuso et al., 2022). *A.*

taxiformis, is also known as limu kohu or the superior seaweed in Hawaiian culture where it has been consumed as a delicacy for centuries (Andreakis et al., 2004a). It was not until recently that mass cultivation of *A. taxiformis* became a topic of interest (Zhu et al., 2021). The goals of this study were to identify how key environmental parameters affect both the growth and bromoform concentration of the algae to help inform commercial cultivation of this species.

Biogeography and Lineages

Within the genus of *Asparagopsis*, there are two species, *A. taxiformis,* and *A. armata. A. armata* is a temperate species, often found in higher latitudes, while *A. taxiformis* is a tropical/subtropical species, often found between latitudes of 15-45° north and south of the equator (Smith, unpublished data). To date, 6 different lineages of *A. taxiformis* have been identified around the world, via sequencing of the cox 2-3 spacer region (Chualáin et al., 2004; Padilla-Gamiño & Carpenter, 2007; M. Zanolla et al., 2014). While some of these lineages are limited to certain regions, such as L5, which is mostly found around Australia, other lineages have a more global distribution. L2, which is likely the most studied and widely distributed lineage of *A. taxiformis*, is found throughout the Pacific and Atlantic including the Mediterranean where it is considered an invasive species (Andreakis et al., 2004b).

Little is known about whether differences in morphology, physiology, and environmental tolerances exist between and within the 6 lineages. Looking at the global distribution of the species, it seems clear that some lineages, such as lineage 2, are more adaptable to a wider range of environmental variability than others (M. Zanolla et al., 2015). Physiological differences have also been observed in individuals of the same lineage from different locations (Mata et al., 2017). Many ascribed these differences to be the result of specific genetic variation within the lineage,

as well as the effect of environmental cues and long-term acclimation to the surrounding environments. This was shown in the significant differences in both physiological responses and morphologies between the native and invasive samples from lineage 2 (M. Zanolla et al., 2015). This indicates that the surrounding environment likely plays an important role in developing *A. taxiformis'* environmental tolerances. Because of this, it is crucial to consider not only the lineage but also the environmental conditions in which *A. taxiformis* is found when identifying its optimum growing conditions.

As a picture is formed of the distribution of the lineages throughout the world, it is clear there is a gap in the known genetics of *A. taxiformis* from the eastern Pacific, specifically along the coast of southern California and Baja. A study by Andreakis et al. (2007) identified a single sample of *A. taxiformis* from Catalina Island, CA, to belong to L2. However, a broad search of the macroalgal herbarium database (link here) clearly indicates that A. taxiformis is present from the southern Channel Islands, CA throughout Baja CA and into mainland Mexico. While it is possible that there are multiple lineages in this region, the latitude, temperature, and distribution of Lineage 2 across the Pacific all suggest that this is the most likely lineage to naturally occur here. Regardless, understanding the variability of this species response to environmental variability will aid in optimizing the cultivation of *A. taxiformis*, as some lineages might be better suited for cultivation than others or require different environmental conditions.

Morphology and Life Cycle

A. taxiformis has a complex triphasic life cycle with an alternation of heteromorphic generations, which as of now has never been completed in captivity (M. Zanolla et al., 2015). *A.*

taxiformis has two morphologies: gametophytes, a haploid form that relies on sexual reproduction, and tetrasporophytes, a diploid form capable of propagation via asexual reproduction or fragmentation. The gametophyte has a complex morphology, while the tetrasporophyte phase, originally named *Falkenbergia,* (Bornet) is characterized by filamentous tufts made up of branches with three pericentral cells surrounding the apical cell (Abbott et al., 1992; Paul et al., 2006). When the branches come into contact with a hard substrate, the alga has been shown to grow holdfasts to anchor itself to the substrate (Bonin & Hawkes, 1987). Because of the nature of its reproduction and ability to be grown in tumble cultures, the tetrasporophyte phase has been targeted for on-land aquaculture (Zhu et al., 2021). While its ability to continually fragment and grow is greatly beneficial for aquaculture, the morphology of the tetrasporophyte, being neither a typical macro nor microalga, creates complications in production, as a filamentous alga of this nature has never been commercially cultivated before.

Within each cell, *A. taxiformis* has a specialized gland that acts as a storage mechanism for the wide variety of secondary metabolites, bromoform, which is the most abundant (Paul et al., 2006). *A. taxiformis* is known to store bromoform at a concentration 10 times higher than other algae (Thapa et al., 2020). While the exact biogenetic pathway from bromoform synthesis in A. taxiformis has not been discovered, it is hypothesized that *A. taxiformis* has a dedicated bromoform biosynthetic enzyme, which converts hydrogen peroxide to secondary metabolites such as bromoform, via a peroxidase reaction (Thapa et al., 2020). Studies have reported large variations in the concentration of bromoform within the cells, ranging from 0.1- to 5%,(Marshall et al., 1999; Mata et al., 2012; Thapa et al., 2020). Similar to environmental tolerances, bromoform concentration has been shown to vary between lineages, as well as between

individuals of the same lineage from different regions (Mata et al., 2017; Paul et al., 2006). The exact cause of bromoform variability is unknown, with some studies suggesting it is the genetic variability between lineages and others turning to environmental factors. Because of the volatile nature of bromoform, it is important to note that the method for quantifying the concentration within alga could greatly contribute to this reported variability (Magnusson et al., 2020; Vucko et al., 2017). The present study explores the effect of physical changes in environmental conditions on bromoform concentration within *A. taxiformis* collected in Southern California.

The cultivation of algae relies on creating a suitable environment in which there is a balance in resource availability and no single limiting factor. In aquaculture, the main limiting factors that influence growth include temperature, nutrient availability, light intensity, and carbon availability (Roleda & Hurd, 2019). The present study focuses on the effects of temperature and CO_2 availability on the growth and bromoform concentration of *A. taxiformis*.

Cultivation Conditions and Limiting Factors

Temperature: Temperature plays an important role in the distribution and growth of algae. Because of its effects on a given alga's physiology, from affecting enzyme activity and cellular metabolism to nutrient uptake and photosynthetic rate, temperature is a crucial factor in the cultivation of alga (Roleda & Hurd, 2012, 2019). When within their optimum temperature ranges, algae have been shown to experience increased growth rates as a result of an increase in the active uptake of NO_3^- and a subsequent increase in photosynthetic rates. When outside this range, there is a decrease in enzyme activity, which can lead to reductions in photosynthesis and

nutrient uptake, all resulting in both reduction in growth and algal health (M. Zanolla et al., 2015).

Sensitivity to temperature has been found to be the main factor in the geographical distribution of algae, including *A. taxiformis* (I. R. Davison, 1991). A study by Zanolla et al. (2018) found that out of a range of environmental variables, temperature was the only factor that explained the geographical distribution of *A. taxiformis* lineages (Marianela Zanolla et al., 2018). As a global species, lineages of *A. taxiformis* have been shown to be better adapted to different temperatures, such as Lineage 3 which prospers in more temperate oceans, while L4 tends to favor more tropical regions. While lineage 2 is found in both tropical and temperate locations, research has shown the lineage's tolerance for temperature variability depends on the current location of the alga. Lineage 2 from its native range of Australia has a smaller temperature range than Lineage 2 from the Mediterranean (M. Zanolla et al., 2015). Other research on a variety of lineages of *A. taxiformis*, from Australia, the Mediterranean, Hawaii, and California, have found that optimal temperature depends on life stage, the season in which the samples were collected, and the location of collection (Mata et al., 2017; Padilla-Gamiño & Carpenter, 2007; M. Zanolla et al., 2015).

Outside of global distribution, temperature has been found to significantly affect the growth rate and bromoform concentration of *A. taxiformis*. A study focused on lineage 6 found a 75% decrease in bromoform when the alga was moved outside its optimum temperature range (Mata et al., 2017). Because of the diversity in temperature ranges between and within *A. taxiformis* lineages, research into the optimum temperature range of other lineages of*A. taxiformis* is needed. In cultivation, having to regulate seawater temperature within a system can

have huge economic costs (A. V. Davison & Piedrahita, 2015; Mata et al., 2017). Because of this, it is important to understand the range of temperatures that a given taxon can tolerate and to identify what the maximum growth rates are at ideal temperatures, as well as the effect of sub-optimum temperatures on growth. This study aims to identify the optimal temperature range for *A. taxiformis*, as well as the lethal temperature limits at which *A. taxiformis* cannot survive.

CO_2 Availability: All algae rely on bioavailable carbon in order to synthesize sugar, their primary energy source, and the building block of biomass. While CO_2 is plentiful in air for use by terrestrial plants, CO_2 availability in the ocean is more variable and can therefore be a limiting resource for some algae (Zeebe & Wolf-Gladrow, 2001). To optimize the growth rate of *A. taxiformis* for the purpose of commercial-scale aquaculture, it is necessary to assess whether CO_2 concentrations in a given source of seawater is limiting. Specifically, CO_2 can be limiting in an algal cultivation system because a) the low concentration at which it is naturally found in seawater is too low, and/or b) because algae's CO_2 uptake rate can surpass the rate at which CO_2 diffuses into seawater (Mata et al., 2007). Dissolved inorganic carbon (DIC) exists in three forms in the ocean: HCO_3^-, CO_3^{2-} and CO_2. CO_2 is the only compound of the three that can diffuse passively through cell walls to be fixed via RuBiSco during photosynthesis. When CO_2 diffuses into the ocean, it reacts with the water to form H_2CO_3 before breaking down into CO_3^{2-} (Equation #1). The ratio of CO_2, HCO_3^-, and CO_3^{2-} in the ocean is dependent on the total DIC, alkalinity, and pH of the surrounding liquid, with HCO_3^- and CO_3^{2-} acting as a buffer. At a pH of 8.1, the current ocean average, CO_2 makes up less than 1% of the available DIC (Fig 1)(Mata et al., 2012). As pH increases, the availability of CO_2 decreases exponentially until reaching nearly zero at a pH of 9.

Because of the limited availability of CO_2 in surface seawater, some algae have evolved Carbon Concentrating Mechanisms (CCMs) to help them use other forms of DIC (Cornwall et al., 2015). CCMs work by either actively transporting other forms of DIC into the cell or transforming the other sources into CO_2 via the enzyme, carbonic anhydrase (Mondal et al., 2016). While CCMs are incredibly useful for algae in limited CO_2 environments, they can require mass amounts of energy (Cornwall et al., 2015). The efficiency of the CCM varies depending on the CCM's affinity for HCO_3^-. Because of these factors, the presence of CCMs does not automatically infer that the culture is not limited by CO_2 (van der Loos et al., 2019). Increased CO2 availability can allow an alga with CCM to downregulate the mechanism, thereby allowing more energy to be directed towards growth, as has been demonstrated with multiple species of algae (Mercado et al., 1999).

CO_2 limitation in cultures of *A. taxiformis* has not been widely studied. However, a study by Mata et al. (2007) investigated the CO_2 uptake strategy of *A. armata*, and while the findings indicate a presence of a CCM, its low affinity for HCO_3^- suggests that *A. armata* would likely be limited by CO_2 in cultivation (Mata et al., 2007; Zhu et al., 2021). The CCM was identified as an external Carbonic Anhydrase mechanism that helped to meet the required amount of CO_2 to reach maximum photosynthetic rates by reducing HCO_3^- to CO_2 between pH of 7.6 and 8.0. However, this mechanism was not efficient enough to supply all the necessary CO_2, as pH rose above 8.0 and CO_2 drastically decreased, as seen by a decrease in the photosynthetic rate (Mata et al., 2007).

Carbon availability has also been shown to have a direct effect on the bromoform concentration within *A. taxiformis*. An increase in bromoform was observed when the ratio of

available carbon to nitrogen was increased, while there was a significant reduction when CO_2 was limited in the presence of excess nutrients (Mata et al., 2012). Therefore, CO_2 limitation could result in significant reductions in bromoform concentration as well as growth.

When considering CO_2 limitation within a culture, it is crucial to consider the interactions it may have with other limiting factors such as nutrient availability, light intensity, and temperature (Roleda & Hurd, 2012). In the present study, two factorial experiments were conducted: one looking at the interaction of temperature and CO_2, and the other on CO_2 availability and light intensity under different densities.

Temperature can also affect the extent to which the growth of a culture is limited by CO_2 while also acting as a secondary stressor (Kübler & Dudgeon, 2015). As already stated, temperature has a significant effect on the metabolic and photosynthetic rate of alga. Algae grown within their optimal temperature range will experience higher CO_2 demands to match the photosynthetic rate than alga outside its optimum range and is, therefore, more likely to be CO_2 limited.

While CO_2 addition can be a powerful tool for increasing growth, acidification has been shown to cause an increase in some algae's sensitivity to potential stress factors. As global warming continues to threaten our ocean, research is needed to understand the effect of rising CO_2 levels and temperature on different species of algae. Increased CO_2 availability and the associated ocean acidification could lead to a shift in the optimum temperature range of algae (Koch et al., 2013). Because of this, it is possible that high CO2 conditions could narrow the optimum temperature range of *A. taxiformis,* as this interaction has not been studied before. By examining this interaction, this study will give insight into potential problems in initiating CO_2

addition in cultivation as well as establish the effect of climate change on *A. taxiformis* in the future.

Similar to temperature, light has a significant impact on the photosynthetic rate of algae and is, therefore, a determining factor in the extent of the CO_2 limitation. Light and CO_2 availability are crucial components to photosynthesis, with light as the driver and CO_2 as the substance (Kübler & Dudgeon, 2015). Because of this interaction, it is important to account for both resources when addressing limiting factors in the growth of *A. taxiformis* cultures. Cultures with high nutrient and high light levels are likely to be CO_2 limited (Cornwall et al., 2015). When determining the amount of light, $CO_{2,}$ and nutrients to give a culture, it is important to know the saturation point of each factor and how that saturation point changes with the availability of other resources. This saturation point depends on many factors, including species, density, and availability of other limiting factors. For light, Dishon et al. (unpublished data) found that at atmospheric levels of CO_2 and with abundant amounts of nutrients, the saturation point for light is around 200 uE PAR at a density of 1g/l. This saturation point is either the result of photoinhibition or other factors becoming limiting. Increasing the CO_2 availability in a culture could increase the saturation point for light. The same goes for CO_2 availability, as increased light levels could allow the alga to take up more CO_2 and thus result in higher growth rates.

The goal of this study is to identify the optimal conditions in which to grow *A. taxiformis* from California, to maximize both the growth and bromoform concentration. Growing *A. taxiformis* for California could lead to a local solution for a local problem that currently has global implications. Since limited research has been conducted on *A. taxiformis* from California, this study will be the first to identify the effect of long-term exposure to different temperatures

and CO_2 concentrations, as well as the interaction between CO_2, temperature, and light. To fully understand the effects, four batch culture experiments were conducted to identify the effect on growth, bromoform concentration, and photosynthetic efficiency. An oxygen evolution and a drift experiment were also conducted to better understand *A. taxiformis'* physiological response to different pH conditions.

Methods

Culturing

Before conducting the experiments, clean cultures of *A. taxiformis* were cultivated at Dr. Jennifer Smith's Lab at Scripps Institution of Oceanography. *A. taxiformis* was collected from 6 different locations around San Diego, CA, and processed via a cut and grow method to isolate the *A. taxiformis*. Establishing a stable culture took between 2 and 4 months, depending on how clean the cultures were after the first round of cutting tips. The first collection site of *A. taxiformis* for this project was an aquarium inside of Hubbs Hall at Scripps. During the length of this work, cultures were cultivated from Mission Bay, San Diego Bay, San Clemente, and Catalina Island. Samples were sent for DNA analysis to identify the lineage found at each location, as well as the variability in genetic composition among the samples. This was in partnership with the Alison Sherwood Lab at the University of Hawaii. The results of the DNA analysis show that the samples collected around San Diego, CA, were all *A. taxiformis* lineage 2.

Closed culture experiments

A total of four closed culture experiments were conducted, including a temperature experiment, a CO_2 addition experiment, and two factorial experiments examining the relationship between 1) CO_2 concentration, density, and light, and 2) CO_2 and temperature. Each experiment lasted between 3-4 weeks, with the response variables measured at the end of each week. The response variables included growth rate (% change in weight per week), bromoform concentration (% bromoform per gram dry weight), and photosynthetic efficiency via dark-adapted fluorescence yield. After the growth rates were recorded at the end of each week, the cultures were returned to their original density. The extra biomass, which was removed from

the cultures, was used to measure bromoform concentration and dark-adapted yield. For samples that experienced less than 10% growth, bromoform concentration and dark-adapted yield were only measured at the end of the experiment due to lack of extra biomass. 500ml or 1000ml laboratory Erlenmeyer flasks were used as the culture vessel. The culture medium consisted of autoclaved (20 minutes at 120 ˚C, at 15 psi), filtered seawater with 100-300µl/l of commercially available F/2 fertilizer solutions, Proline F/2 Algae Food, added to supplement nutrient requirements. Stirring of the cultures, as well as gas exchange, within each flask, was facilitated by bubbling atmospheric air through a 5 ml sterile plastic pipette. Foam plugs were used to hold the pipettes in place and reduce contamination of cultures from the surrounding environment.

The experimental bench was illuminated with a Giesemann light source supplying Photon Flux Density (PFD) of 40-50 µmol quanta m^{-2} sec^{-1} (measured by a hand-held PAR sensor - APOGEE MQ-510 underwater quantum flux meter). The lights were programmed for a 12:12 L:D light cycle. Wet lab temperature was controlled by air conditioning and recorded by a HOBO temperature logger placed inside an Erlenmeyer flask similar to culture vessels. The temperature of the room averaged 21°C.

Response Variables:

Growth rate: The growth rate was measured as the percent increase in wet weight per week. To measure the wet weight, the biomass was strained through a 100 mm mesh bag, gently squeezed, and patted dry with paper towels until no more water was extractable. The biomass was then weighed to +/- 0.01 grams using an analytical scale. The growth rate was calculated using the following equation:

$$Growth\ Rate\ =\ (\frac{(Wf-Wi)}{Wi})\ *\ (\frac{7\ days}{L})\ *\ 100$$

where *Wf* was the final weight, *Wi* was the initial weight, and *L* was the length of the experiment in days.

Bromoform Concentrations: After the required biomass was returned to each flask, 0.10 grams of wet weight *A. taxiformis* was placed in a 1.5ml Eppendorf tube, and put directly in the deep freezer. At the end of the experiment, the samples are packed in a shipping container with dry ice and sent to Dr. Vinayak Agarwal's Lab at the Georgia Institute of Technology where bromoform concentrations were analyzed. The samples were processed and analyzed with a GCMS following standard protocols (Thapa et al., 2020).

Dark Adapted Photosynthetic Yield: A diving-PAM (Pulse Amplitude Modulation Fluorometer) was used to measure photosynthetic efficiency via dark-adapted photosynthetic yield (Fv/Fm). Specifically, 0.2 grams of biomass were collected from each replicate and placed into a well of a clear 24 well culture plate. The wells were then filled with autoclaved seawater and placed in a dark environment for 60 minutes, to allow for dark acclimation before measurements were taken. The Diving-PAM was set to a saturation intensity of 8, a pulse width of 0.8, a measuring intensity of 8, damp of 2, and gain of 2. To calibrate the instrument, the end of the fiber optic cord was placed on the bottom of a well plate filled only with autoclaved seawater. A special attachment on the end of the cord allowed the tip to sit flush with the bottom of the plate. The PAM was then zero-ed. PAM measurements were taken in the dark, with one measurement per well. The Fv/Fm measurement was made on samples with a background fluorescence value between 130 and 400.

Independent Variables:

1. Temperature Experiment: A three-week temperature experiment was conducted, covering a range of temperatures from 12˚C to 31˚C. This temperature range includes both the average minimum and maximum seawater temperatures along the coast of southern California, as well as anomalies recorded in the recent past. While the maximum recorded water temperature was around 26˚C, the maximum temperature for the experiment was raised to 30˚C to account for possible future temperature spikes and to identify the maximum temperature at which the *A. taxiformis* survives long-term exposure. Water baths were used to control the temperature in the flasks. Three temperatures of seawater flow into the Smith Lab, ambient (19°C, or temperature of the ocean at the time of the experiment), chilled (12.0°C), and warmed (26.0°C). By controlling the ratio of seawater from the three sources, five water baths were created: 12.0 °C, 15.0°C, 19.0°C, 22.5°C, and 26.0°C. The warmest water bath, at 30°C was created using an aquarium heater to ensure equal heating throughout the water bath. HOBO temperature loggers (Onset HOBO UA-002-08 Pendant Light and Temperature Data Logger), set to measure the temperature every 15 minutes, were deployed in flasks without biomass within each water bath to record the temperature. The experiment consisted of 24 flasks, with four replicates per treatment. The biomass used was originally collected from the aquarium in Hubbs Hall. Each flask contained 1 gram of biomass, for a density of 1g/l.

2. CO_2 Addition Experiment: The initial CO_2 experiment explored the effect of increased CO_2 availability on the growth rate of *A. taxiformis*. To increase the amount of available CO_2 within the seawater, CO_2 enriched air was bubbled into half the flasks while the other half was bubbled with atmospheric air. pH was used as a real-time proxy for CO_2 availability as pH

decreases with increased CO_2 availability. Large bubbles were required to ensure the continued circulation of biomass through the flask. As such, air with a higher concentration of CO_2 was needed to reach the desired reduction in pH. The flasks bubbled with atmospheric air (300ppm CO_2) held at a pH of 8.1, while the CO_2 enriched air (1500ppm CO_2) was held at a pH of 7.65. For the first CO_2 experiment, pre-mixed cylinders of CO_2 and atmospheric air were used. The pH in the control flasks was measured using Durafett sensors (Honeywell 51453503-501 Durafet III pH Electrode) attached to a Honeywell UDA2182 Dual Input Analyser, which recorded pH and temperature every 5 minutes. The experiment consisted of 12 flasks, with 6 flasks in each treatment. Half the flasks in each treatment were stocked with biomass that originated from Catalina Island (CI) while the other had biomass from Mission Bay (MB). All biomass had been cultivated and grown in the Smith Lab for over six months prior to the experiment. The experiment lasted for four weeks with each culture being returned to the original density (1 g/l) at the end of each week.

3. CO_2 availability and Temperature Experiment: To understand the effect of temperature on *A. taxiformis*' response to increased CO_2 availability, a factorial experiment was conducted which included three pH levels and three temperatures. The experiment was designed to emulate different climate change scenarios predicted in the IPCC Climate Report and to provide insight into how temperature affects *A. taxiformis* growth in large-scale cultivations under different pH conditions. The experiment followed a 3x3 factorial design. There were three pH conditions, 8.1 (current), 7.9 (RCP 6.0), and 7.7 (RCP 8.0) which were created by bubbling CO_2 enriched air at 300ppm (atmospheric), 800ppm, and 1200ppm, respectively. The CO_2 enriched air was created by mixing pure CO_2 with ambient atmospheric air using two Mass Flow Controllers, one

per pH treatment. An LI-COR CO_2 Gas Analyzer was used to measure the ppm of CO_2 within the mixed air in real-time. The concentrations of CO_2 were determined by adjusting the Mass Flow Controllers until the pH of the control flasks became constant at the predetermined pH values. pH was monitored for three days prior to the experiment to ensure limited variability of pH within the control flasks. pH was monitored throughout the experiment using Durafetts (Honeywell 51453503-501 Durafet III pH Electrode) attached to a Honeywell UDA2182 Dual Input Analyser, which recorded pH and temperature every 5 minutes. Flasks were kept at three temperatures, 19℃, 22.5˚ C, 26˚ C to represent the current average summer temperature in southern California, a 3.5°C increase predicted in the RCP 6.0 model, and a 7.0°C increase, as predicted in the RCP 8.5 model. The temperature was controlled using water baths as previously described in the first temperature experiment. HOBO temperature loggers (Onset HOBO UA-002-08 Pendant Light and Temperature Data Logger), set to measure the temperature every 15 minutes, were deployed in flasks in each water bath to record the temperatures. These pH and temperature conditions were combined to create nine different environmental treatments for the experiment. The experiment consisted of three replicates per environmental treatment for a total of 27 x 1000ml flasks. The biomass used was originally collected from Catalina Island. Each flask contained 1 gram of biomass, for a density of 1g/l. The experiment lasted three weeks.

4. CO_2 x Light x Density Experiment: The final experiment explored the interaction between increased CO_2 concentration and light intensity across multiple densities. The variables included two CO_2 concentrations, two light intensities, and two densities. The pH reduction, as a proxy for increased CO_2 concentration, was set at 8.1 and 7.7, which required atmospheric air with 300ppm CO_2 and CO_2 enriched air with 1500ppm CO_2 respectively. Unlike the previous

two CO_2 addition experiments, the CO_2 was only delivered to the system during the "day" and turned off at "night", to better simulate an aquaculture setting. The light cycle was 12L:12D. The light intensity was set to a PAR of 50 uE for the low light and 300 uE for the high light. To create a high light environment, two additional Kigung LSS 3ft 8P4H LED lights were used in addition to the Giesemann light source. A solid plastic divider was used to separate the low and high light environments. The density was set at 1g/l for the low density and 2g/l for the high density. For each variable, the "low" setting was equivalent to the control settings in past experiments. The combination of these three variables resulted in 8 different environmental conditions. There were four replicates per treatment, for a total of 32 x 500ml flasks. For the first week of the experiment, the pH was continuously monitored in the control flasks. The pH of each flask was measured and recorded once a day.

Oxygen Evolution Experiment

The photosynthetic and respiratory rates of the A. taxiformis were measured under a variety of pH conditions. The photosynthetic rate was calculated by measuring the rate of O_2 production within a closed container, using a Hach Dissolved Oxygen Probe. Four oxygen electrodes were placed in 265 ml clear water-tight containers along with magnetic stir bars and 265 mg of *A. taxiformis* for a final density of 1g/l. The containers were placed in a temperature-controlled environment with magnetic stirrers in the bottom. The temperature was held at 21.0 °C. The pH of the seawater in each container was manipulated through the titration of NaOH 0.1M. For each treatment, 2L of pH-controlled seawater was prepared. 100 ul/ l of F/2 Proline Algae Food solution was added. The final pH of the seawater was 8.2, 8.4, 8.6, and 8.94. The respiration rate was measured during an initial phase of 20 minutes in darkness. The

photosynthetic rate was measured in 10-minute intervals under 100 µE PAR of illumination. The seawater within each container was replaced between each run. Oxygen was measured every 30 seconds for both the respiration and photosynthetic trials. The photosynthetic rate was calculated as the change in O_2 per gram of *A. taxiformis* per minute.

Drift Experiment

The drift experiment, which was conducted directly following the CO_2 x Temperature experiment, used 0.5g of biomass from each of the nine environmental conditions. The biomass was placed in a 500ml flask for a density of 1g/l. The flasks were placed in a temperature-controlled environment with an array of magnetic stir rods in the bottom. Additional lights were added to increase the illumination to 150 µE Par. A magnetic stir bar was placed in the bottom of each flask to create movement and reduce the stagnation of the surrounding seawater in the flask. A stopper was placed in the top of each flask to prevent CO_2 from being absorbed from the surrounding air. The initial pH of each flask was 8.1. The biomass was left for 24 hours in the high light condition before the pH was measured again. The pH was measured with a Hach pH Probe. The probe was left in each flask for 1.5 min to provide time to fully equilibrate. A maximum of nine samples could be run at one time. The experiment was repeated 3 times, with a replicate from each environmental combination present for each run.

Data analysis

Repeated measures, multifactor ANOVA were used to analyze the interactions between variables including time, location, temperature, CO2 availability, light intensity, and Density on growth rate, dark-adapted yield, and bromoform concentration. Analysis was conducted in R, using the rstatix package. The repeated measures, multifactorial ANOVA was run with the Anova_test function. To follow up, either a t-test for paired samples was run with the *pairwise_t_test* function, or a one-way ANOVA with the *aov* function followed by a Tukey-Kramer test with the *HSD.test* function.

Results

Experiment 1: Temperature

The temperature experiment, which sought to identify the optimum temperature range for *A. taxiformis* cultivation*,* found a significant effect of temperature on the alga's growth rate, photosynthetic efficiency, and bromoform concentration. The highest growth rates were observed in flasks between 22°C-26 °C. While the growth rates were statistically similar between the 17.7°C, 21.4°C, and 26.3°C treatments, the 21.4°C had the highest growth rate through out the experiment, reaching 64.25% increase in weight per week during the final week of the experiment (Table 2). In comparison, there was a slight decrease in growth in the 17.7°C treatment, and a significant reduction in growth for the cultures grown at 12.1°C, 15.0°C, and 31.6°C (Fig 2a). For growth rate, there was a significant interaction between time and temperature (ANOVA, Temperature x Time, F= 3.80, p =1.00E-3, Table 1). Between weeks 1 and 3 there was an increase in the growth rates of cultures at temperatures between 17.7°C and 26.3°C. The 21.4°C treatment experienced a statistically significant increase over time (ANOVA, Time, F = 11.2, p = 9.0E-3) while the 17.7°C and 26.3°C treatments showed a slight non-significant increase. Over time, there was a decrease in the growth rate of the cultures at 12.1°C, 15.0°C, and 31.6°C. The 15.0°C culture experienced a reduction in the growth rate from 11.25% to -7.2%5 between weeks 1 and 2 (ANOVA, Time, F=6.37, p = 3.3E-2). The growth rate increased slightly during week 3, with a final growth rate of 1.55%. The 12.1°C treatment experienced a significant reduction in growth from 2.75% during week 1 to -9.69% during week 3 (ANOVA, Time, F=5.77, p = 40.E-2). There was a non-significant reduction in growth, from

6.27 to -6.85% between weeks 1 and 3 in the 31.6°C treatment (ANOVA, Time, F=1.46, p = 0.304).

There was a significant effect of temperature on the dark-adapted photosynthetic yield of *A. taxiformis,* measured at the end of week 3 (ANOVA, Temperature, F = 38.38, p = 5.49E-9, Table 2). The highest yield was recorded in the 26.3°C treatment, followed by a slightly lower Fv/Fm value in the 21.4°C treatment (Fig 2b, Table 2). The dark-adapted yield continued to decrease at temperatures below 21.4°C, resulting in the lowest measured value in the 12.1°C treatment. There was a significant reduction between the 26.3°C and 31.6°C treatments, resulting in the second-lowest Fv/Fm measurement.

Bromoform was measured at the end of week 1 and week 3. The bromoform concentrations within the experiment varied from 0.176% dw to 3.736% dw, with the highest concentration measured in the 21.4°C treatment. Similar to growth, bromoform concentration decreased at temperatures above and below 21.4°C. The results show a significant interaction between time and temperature (ANOVA, Temperature x Time, F= 7.90, p = 5.27E-4). The 21.4°C treatment increased by 112.5% between week 1 and week 3. There was no change in bromoform concentration at the 12.1°C, 17.7°C, and 26.3°C treatments, however, in the 15.0°C treatment, there was a significant reduction in bromoform concentration, from 1.81% dw to 0.28% dw between weeks 1 and 3 (Fig 2c).

Experiment 2: CO_2 Addition + Oxygen Evolution

Prior to the CO_2 cultivation experiment, a photosynthetic oxygen evolution experiment was conducted to measure the rate of photosynthesis under different pH conditions. The results from two trials show that pH had a significant effect on the rate of photosynthesis, with a

significant reduction in oxygen production with an increase in pH (ANOVA, pH, F =6.76, p = 9.2E-6). The maximum recorded O_2 production, at 0.085 mgO2/gFW/min occurred at a pH value of 8.25. The rate slowly decreased, until there was a significant reduction in O2 production once the pH was above 8.6. At the max pH of 8.95, there was a 70% reduction in O2 production from the max rate at 8.25 (Fig 3).

The daily fluctuation in pH was measured in the ambient and CO_2 enriched cultures during the second experiment. As a result of CO_2 uptake by the *A. taxiformis*, the pH of the culture rapidly increased during the photo-light period, reaching pH values between 8.65and 8.90 for the ambient conditions(Fig 4). During the dark period, pH decreased due to respiration, resulting in minimum pH levels between 8.04 and 8.28. A slight increase in the daily max and min pH values was observed over the course of each week. For the ambient culture, this increase maxed out after 48-72 hours for a final max pH value of 8.9. The daily max of the CO_2 enriched cultures rose for the duration of the week, with the initial daily max at 7.9 and the final at a pH value of 8.27. After the weekly full water replacement and the subsequent reduction in density, the daily pH maxes returned to their initial values. The daily change in pH for the ambient flask averaged at 0.63, while the daily change in pH of the CO_2 flask averaged at 0.45. The daily change in pH did not vary significantly throughout the week.

Overall, cultures with CO_2-enriched air experienced higher growth rates than those with atmospheric air. The extent of this increase depended on both time and location (ANOVA, CO_2 x Location x Time, F= 5.59, p = 5.0E-3, Table 3). Cultures from Catalina Island experienced an average increase of 63 % between cultures grown with and without additional CO_2 for an average growth rate of 72.8% and 44.6% respectively (Figure 4a). The highest weekly growth

rate, at 93.5 %, occurred in the CO_2 enriched flask during week 2. The Mission Bay cultures showed no significant difference between flasks with and without additional CO_2 addition during the first two weeks of the experiments for an average growth rate of 43.5%. This shifted during the third and fourth weeks when the cultures with CO_2 enriched air experienced growth rates 61.1% and 34.6% higher than the cultures with atmospheric air, respectively (Fig 4a, Table 4). The growth rate in the MB cultures reached 66.9% at the end over week 4, but overall the average growth rates in cultures from Mission Bay with CO_2 addition were lower than those from Catalina Island. The growth rates in the ambient conditions did not vary between locations.

Unlike growth rates, the photosynthetic efficiency of *A. taxiformis* did not change significantly in the presence of increased CO_2 availability (ANOVA, CO_2 availability, F = 1.82, p = 2.15E-1, Table 3). There was a significant effect of time and location on Fv/Fm (Table 3). The cultures that originated in Catalina Island experienced higher Fv/Fm value on average than the cultures from Mission Bay. As for time, on average there was an increase in Fv/Fm during the first three weeks, followed by a slight decrease at the end of the final week (Fig 4a).

The bromoform concentration was measured at the end of the first and fourth weeks of the experiment. The bromoform concentration varied from 1.5to 4.85% dw. Overall there was no significant effect of increased CO_2 availability (ANOVA, CO_2 availability, F =0.747, p=4.12E-1, Table 3) or time (ANOVA, Time, F =0.018, p=8.96E-1, Table 3) on the bromoform concentration, however, there was a significant effect of location(ANOVA, Location, F =19.61, p=2.00E-3, Table 3). Cultures from Mission Bay, on average, had a significantly lower bromoform concentration than the Catalina Island cultures. The bromoform concentrations from Mission Bay were variable, with a slight reduction in bromoform for cultures with CO_2 addition.

At the end of week 4, the Bromoform concentration within the Catalina Island sample with CO_2 addition, was 32.88% higher than the sample cultured without CO_2 enriched air.

Experiment 3: CO_2 addition x Temperature

The results show a significant effect of both temperature (ANOVA, Temperature, F = 19.92, p=2.7E-5, Table 5) and CO_2 availability (ANOVA, CO_2 Availability, F = 104.51, p=1.24E-10) on the growth rates of *A. taxiformis*, but no significant interaction between the two factors (Temp x CO_2 availability, F = 2.62, p=6.90E-2, Table 5). On average, there was a significant increase in growth rates between cultures with and without CO_2 addition across all three temperatures with the highest average growth rate of 49.40% in the 19°C treatment with a pH of 7.7. There was a 42.88% increase in growth when the pH decreased from 8.1 to 7.9, independent of temperature. This increased an additional 8% with a reduction in pH to 7.7. Within each temperature treatment, there was a statistically significant effect of CO_2 availability on growth, with a 74.86% increase in growth between cultures at a pH of 8.1 and 7.7 at 19 °C (ANOVA, F=38.3, p = 3.37E-8), a 54.95% increase at 22.5°C (F= 11.6, p = 2.92E-4) and a 39.09% increase at 26 °C (F= 10.01, p = 6.92E-4).

For the CO_2 availability, the results found a statistically significant increase in growth between cultures at a pH of 8.1 and both 7.9 and 7.7. However, there was no significant increase in the growth rate between a pH of 7.7 and 7.9 for any of the three temperatures (Table 6). There was also a significant effect of temperature on the growth rate of the *A. taxiformis*. Cultures at 22.5°C experienced the lowest average growth rates for each CO_2 treatment compared to the same treatment in the other temperatures.There was no significant difference between the 19 and

26°C treatments, but there were significant reductions in the growth rates when comparing from 19°C to 22.5°C or 26°C to 22.5 (Table 6).

While there is a statistically significant effect of time on the growth rate of the cultures when grouped by treatment conditions, the significance was limited to the 19 °C flasks at a pH of 7.7 (ANOVA, Time, F=34.1, p = 3.0E-2). The culture's growth rate decreased from 52.3% to 41.3 % between weeks 1 and 3. There was no significant effect of time on any other conditions.

For the experiment, Fv/Fm was measured at the end of each week. Across all treatments, there was no significant effect of CO_2 or Temperature on the Fv/Fm measurements (ANOVA, CO_2 availability, F= 2.57, p=01.04E-1, Table 8),(ANOVA, Temperature, F=0.061, p = 9.41E-1). There was a significant effect of time, with an average increase in Fv/Fm measured between the end of weeks 1 and 3 (Table 6).

Bromoform concentration was measured at the end of week three. The results show no significant effect of CO_2 availability (ANOVA, CO_2 addition, F = 0.218, p = 8.06E-1, Table 5) or Temperature (ANOVA, Temperature, F = 0.636, p = 5.41E-01, Table 5) on the bromoform concentration. The bromoform concentrations within the experiment ranged from 0.633% to 1.437% with an average concentration of 1.067% (Fig 6b).

At the completion of the drift experiment, the final pH of the flasks varied between 8.8 and 8.9 with no cultures recorded to increase the pH above 9.0. There was no significant effect of prolonged CO_2 addition or temperature on the final pH of the closed cultures (ANOVA, CO_2 addition, F = 0.087, p = 0.917, Table 5) (ANOVA, Temperature, F=0.676, p = 0.546, Table 5) (Fig 6c).

Experiment 4: CO_2 addition x Density x Light

The results of a mixed model repeated measures ANOVA found a significant interaction between CO_2 availability, light, and time on the growth rate of *A. taxiformis* (ANOVA, CO_2 x Light x Density x Time, F= 3.94 p= 1.2E-2, Table 7). The data shows that on average, cultures with CO2-enriched air experience a 42.38% higher growth rate than cultures with atmospheric air (Fig 7a). To understand the effect of CO_2 addition, the growth rate of cultures with and without CO_2 was compared across the four environmental conditions. On average, there was a significant difference in growth between cultures with and without CO_2 addition in three of the four environments, with no significant difference in the high light, low-density cultures (Table 8). The largest average increase, as a result of supplemental CO_2, was in the low light high-density condition, with an increase in growth of 58.29%. This was followed by the high light, high-density conditions, with an increase of 55.28%, then the low light, low density with an increase of 41.46%. The high-light, low-density condition resulted in the smallest difference, with an increase of 14.5%. When comparing the growth rates, the high light, high-density cultures were very similar to the low light low density, with average growth rates of 30.14% and 33.60% for the ambient flasks, and 46.80% and 47.53% for the flasks with CO_2 enriched air.

There was a significant effect of time on 5 of the 8 conditions. Both the ambient and CO_2 enriched cultures in the low light, low-density cultures, as well as the low light, high-density environment with supplemental CO_2 experienced an increase in growth over time from 24.22%, 27.27%, and 18.20% to 38.20%, 57.57%, and 29.59%. Lastly, there was a significant reduction in the growth rate within the high light, low-density environment for the cultures with CO_2 addition. These cultures exhibited the highest growth rates of the experiments, at 88.29%, during

the first week. This was followed by a significant reduction between weeks 1 and 2 as well as between weeks 2 and 3 to 65.7% and 33.21% respectively. Two of the four replicates experienced mass bleaching events and were terminated after week 3.

The dark-adapted yield (Fv/Fm) was measured at the end of weeks two, three, and four. No data was collected at the end of week 1 due to technical errors with the PAM. For the dark-adapted yield, there was a significant interaction between CO_2 availability and light intensity (ANOVA, CO_2 availability x Light, F= 5.75, p= 2.5E02, Table 7). When examining the four different environments, there was a significant effect of CO_2 on three of the four environments, with no significant effect in the low light, low-density conditions (Table 8).

The two high light environments showed a significant reduction in the Fv/Fm measurements between the flasks with and without CO_2 addition. The high light, high-density flasks experienced a 33.93% decrease, while the high light, low-density flasks had a 73.15% decrease. There was also a small, non-significant reduction in Fv/Fm between the flasks with and without CO_2 addition in the low light, high-density culture. There was little to no significant difference between cultures with and without CO_2 addition when broken down by week (Table 8). This is due to the high variability of the Fv/FM data. The highest Fv/Fm measurements were recorded in the low light, high density for both the cultures with and without CO_2 addition. The lowest was in the high light, low density with CO_2. This condition also had the lowest Fv/Fm value for an ambient flask, out of the four environmental conditions.

The bromoform concentration was measured at the end of week 2 and week 4. The concentrations varied from 0.337 to 1.34% DW. Due to the multiple interaction terms, a series of paired T-tests were conducted. The results showed a significant effect of time on the bromoform

concentration, with a decrease in bromoform across all four environments with ambient air between weeks 2 and 4. There was no significant effect of time on three of four environments with CO_2 addition (Table 9). Examination of the effect of CO_2 on the bromoform concentration found that there was a significant reduction in bromoform with the addition of CO_2 in the high light low-density environments for both weeks 2 and 4 (Table 8).

Discussion

To the best of our knowledge, this study is the first to report on the effects of and interactions between temperature, light, and CO_2 concentration on the growth rate, photosynthetic efficiency, and bromoform concentration of *A. taxiformis*, specifically lineage 2 from southern California. Manipulation of environmental conditions within the experiments resulted in significant changes in growth (% change in weight per week), from 10-88%, and bromoform concentration (% dw), from 0.18% to 4.85% across the four experiments.

Growth Rate

The growth response of the *A. taxiformis* identified four temperature ranges for this cultivar: optimal, suboptimal, long-term stress, and short-term lethal. The optimum temperature range of *A. taxiformis* from southern California was found to be between 22°C and 26°C, with significant decreases in growth above and below this range, following a standard temperature response curve (Eggert, 2012). An acclimation period was observed within this identified optimal temperature range, resulting in a significant increase in the growth rates between weeks one and two, followed by a smaller increase between weeks two and three. The decrease in the acceleration of the growth rates between weeks two and three indicates that the acclimation period was ending, therefore the growth rate from week three may be indicative of future growth rates in these conditions.

Temperatures between 17.7°C and 22.0°C were identified as sub-optimal, as there were significantly lower growth rates during weeks two and three in comparison to the optimal temperature range identified above. Growth rates in the suboptimal temperature range did not vary over time. Below 17.7°C and above 26.0°C, *A. taxiformis* started exhibiting signs of stress.

This temperature stress response can be split into two ranges; short term, in which the alga showed significant signs of stress within the first week, and long term, in which the effects of temperature stress did not become apparent within the first week. The mechanism that results in a stress response to lethal temperatures depends on whether the alga is exposed to temperatures above or below its viable temperature range. Stress response to low temperatures can include a decrease in membrane fluidity, reduction in the enzyme activity within the Calvin cycle and damage to the pigment-protein complex needed for photosynthesis (Eggert, 2012). Long-term exposure to heat stress can lead to excessive damage of the plastid, resulting in cell damage, reductions in growth, and eventual death of the alga (Kumar et al., 2020; L. Wang et al., 2013).

The relative short-term lethal temperature range was identified as below 15°C and above somewhere between 26.3°C and 31.3°C, as the extreme temperature conditions of 12°C and 31°C resulted in the death or near-death of *A. taxiformis*, with growth rates below 5% during the first week of cultivation. These cultures experienced negative growth rates during weeks 2 and 3 due to biomass loss as their pigments and tissue began to break down. Similar observations have been noted in past studies where Padilla-Gamino and Carpenter (2007), who found that *A. taxiformis* from California experienced high-temperature inhibition above 30°C and lower lethal temperatures between 10°C and 15°C (Padilla-Gamiño & Carpenter, 2007).

While the effects of extreme temperatures were apparent within the first week of the experiment, the full effect of the 15°C treatment was not observed until the second week when the growth rate dropped significantly. Because this response was time-dependent, it is likely that the alga would have experienced limited negative effects from short-term exposures. However,

there was no growth in the 15°C culture by the end of week 3, indicating that this temperature became lethal after long-term exposure.

When considering the implications of these temperature ranges on the distribution of *A. taxiformis* in southern California, it is important to note that there were gaps in the temperature conditions tested in this experiment, particularly in the higher temperature range. While the cold temperature treatments identified the optimum, suboptimal, long-term stress, and lethal temperatures ranges, the higher temperature skipped straight from the optimum temperature range to the lethal range. As such, the exact response of A. taxiformis between 26.3°C and 31.6°C is unknown, and more research is needed to identify the potential presence of a suboptimal or prolonged stress temperature range.

The CO_2 experiments found that *A. taxiformis* growth is limited by CO_2 availability, as indicated by a significant increase in growth in cultures with CO_2 enriched air as opposed to those ambient air. This result was supported by the oxygen evolution and drift experiment, which all indicate *A. taxiformis* relies on CO_2 as its main source of DIC.

The oxygen evolution experiment, which was conducted prior to the initial CO_2 experiment, examined the effect of changing pH on the photosynthetic rate of *A. taxiformis*, with the understanding that photosynthetic rate is tightly linked to algae health and growth rate. To measure this, the O_2 production rate of *A. taxiformis* was measured in seawater under a variety of pH conditions. The data indicated that there was a significant decrease in the photosynthetic rate of *A. taxiformis* in pH conditions above 8.6. This is likely due to the depletion of available CO_2 within the seawater as the pH rises, reaching a concentration of nearly zero at a pH of 9. Since there is a 90% reduction in CO_2 concentration between 8.0 and 9.0, and only a 30% reduction in

HCO_3^-, the reduction in photosynthesis indicates that *A. taxiformis* relies on CO_2 as its primary form of DIC (Mata et al., 2007). These results were supported by the finding of Mata et al. (2007), whose study of CO_2 limitation in *Asparagopsis armat*a found that *Asparagopsis* mainly relies on CO_2.

The results of the initial CO_2 experiment found a significant increase in growth rate in the presence of additional CO_2. As noted, the specific temporal response to the addition of CO_2 varied between locations, with Catalina Island cultures experiencing increased growth within the first week, while cultures from Mission Bay did not show a significant increase until week 3. The ambient conditions from both locations exhibited equivalent growth rates throughout the experiment. This indicates that the variability in growth rate was the result of the CO_2 addition. Therefore, the acclimation period to the increased CO_2 availability can vary between individuals of the same lineage from different collection locations.

The pH, which was measured continually within the flasks with and without CO_2 enriched air, showed a daily variation in pH, with increasing pH during the light period as the CO_2 was utilized by photosynthesis, and a decrease during the dark period as the stores of CO_2 were replenished due to respiration. The daily variation in pH within the ambient conditions, which stabilized at 8.1 without biomass, resulted in the culture pH remaining above 8.6 for 29.4% of the photolight period. As seen in the O_2 evolution experiment, when the pH rose above 8.6 there was a significant reduction in photosynthesis, which corresponded to a reduction in growth. As such, under ambient conditions, the growth of the alga was greatly limited by CO_2 availability for up to 3.6 hours per day. The addition of CO_2 enriched air kept the pH below 8.6, with a maximum daily pH of 8.25. As such, the conditions with CO2 enriched air allowed for at

least 30% more growth than in the ambient condition. Since a more than 50% increase was observed, it is likely that there was a reduction in photosynthesis leading up to the significant reduction seen at a pH of 8.6.

The first factorial experiment, which examined the interaction between temperature and CO_2 availability, identified a significant effect of both factors on the growth rate of *A. taxiformis*. However, there was no significant interaction between the two variables, indicating that the differences in the response to changing environmental conditions were independent of one another. While this signifies that there was no positive synergistic effect on the growth rate, there was also no significant negative effect, such as a stress response, caused by the different environmental combinations. Unlike the univariable experiment, the factorial experiment compared the response of *A. taxiformis* to three different pH conditions, which resulted in the general identification of the saturation point for CO_2 addition in terms of increasing growth rate. The experiment showed a significant difference in growth between seawater with a pH of 8.1 and 7.9, but no significant difference between 7.9 and 7.7. This indicates that at a pH of 7.9, *A. taxiformis* was no longer significantly limited by CO_2 concentration and therefore did not utilize the additional CO_2 made available by lowering the pH to 7.7. This saturation point was the same across the three temperatures examined but would likely differ under different light or nutrient conditions.

As for temperature, the 19°C treatment resulted in the highest growth rates of cultures with CO_2 enriched air, while the highest growth rate of samples in atmospheric air was in the 26.0°C treatment. The increase in growth at the lower temperature was likely due to higher diffusion rates of CO_2 into the seawater, since decreasing temperature increased the solubility of

gas within the water. While it was hypothesized that the highest growth would be in the 22.5°C treatment, as that was within the optimum temperature range identified in experiment one, the results found that this temperature resulted in the lowest average growth rate when compared within the CO_2 treatments. Examination of the temperature data found that the 22.5°C treatment had the largest variability of temperature, due to the nature of mixing the ambient and warmed seawater sources in the lab. While the temperature never left the optimal range (19°C -26°C), there were several large fluctuations during the first week of the experiment. It is possible that this variation in temperature resulted in a reduction in growth rates. Further experimentation would be needed to determine the effect of temperature variability on algal stress and growth rates.

Overall, the changes in pH resulted in a larger variation in growth rate than changes in temperature (28.99% to 45.07% and 34.24% to 40.68%, respectively). While this could be the result of the relatively narrow temperature range that was used, a study conducted by Kübler & Dudgeon (2015) on the effects of climate change found that CO_2 has a stronger effect on photosynthesis at low light, while temperature tends to have a larger effect on the alga in high light. The light intensity of the second experiment, as it relates to the saturation point identified by Dishon et al. (unpublished data), indicates that the experiment was run under low light conditions, thereby supporting the model put forth by Kübler & Dudgeon (2015).

The second factorial experiment identified an additive interaction between light intensity and CO_2 addition. CO_2 addition, which reduced the pH from 8.1 to 7.65, resulted in a 50% increase in growth independent of light intensity and density. Similarly, increased light intensity, whether by doubling the illumination intensity or reducing the density by half, resulted in around

a 50% increase in the growth rate. However, when a low-density culture was exposed to high light and additional CO_2, there was a negative interaction resulting in biomass bleaching with substantial mortality in half of the replicates. While this additive stress response has rarely been reported, an experiment focused on pelagic marine microorganisms found a significant reduction in productivity when the organisms were exposed to both high light and increased CO_2 (Gao et al., 2012). The mechanism that causes an extreme stress response in *A. taxiformis* when these two factors, which by themselves only increase growth, are combined is unknown. This negative interaction could have large implications for the use of CO_2 addition in *A. taxiformis* cultivation. As such, caution must be used when adding CO_2 to outdoor cultures of *A. taxiformis*, where the light intensity is likely to be significantly higher.

Considering the evidence that CO_2 availability can limit growth, it is likely that *A. taxiformis* does not have an efficient CCM which was also supported by the drift experiment, which found no indication of an efficient CCM. Across the experiment, pH maxed out at 8.9, indicating that *A. taxiformis* is unable to utilize other forms of DIC. It is important to remember that while the findings suggest no CCM mechanism, Mata's study identified the presence of a CCM with low affinity for HCO_3 in *A. armata* which helps supplement CO_2 at lower pH values (Mata et al., 2007). While understanding the CCM in *A. armata* provides insight into the carbon uptake strategy of *A. taxiformis*, CCMs can vary between species and even within morphologies of the same species (Y. Wang et al., 2019). Since a low-affinity CCM would not be identified in the drift test, more experimentation is needed to confirm its presence and identify the potential for downregulation under increased CO_2 conditions.

Dark Adapted Photosynthetic Yield:

The dark-adapted photosynthetic yield (Fv/Fm) of *A. taxiformis* was measured throughout the experiments to identify the effects of changing environmental variables on the physiology of *A. taxiformis*. The Fv/Fm measures the efficiency of *A. taxiformis'* photosystem II, an important mechanism in photosynthesis and a measure of algal health (Padilla-Gamiño & Carpenter, 2007). The results of the four experiments indicate that the photosynthetic yield was significantly affected by changes in temperature as well as CO_2 availability when combined with changing light intensity.

The response of Fv/Fm to different temperatures mirrored the growth response, with the highest Fv/Fm values measured between 22.6°C and 26.3°C, followed by a significant reduction between 26.3°C and 31.3°C, as well as at temperatures below 22.6°C. The significant reduction in the Fv/Fm between 26.3°C and 31.3°C is likely the result of *A. taxiformis* reaching its maximum lethal temperature. Prolonged exposure to high temperatures can cause inhibition in the ability of algae to repair photosystem II. This can be accompanied by a build-up of ROS which causes more damage to the algae (Allakhverdiev et al., 2008).

The results of the temperature experiment were supported by studies conducted by Padilla-Gamino and Carpenter (2007), which found a significant reduction in Fv/Fm at 30ºC for *A. taxiformis* from California (Padilla-Gamiño & Carpenter, 2007). However, the study also identified that the photosynthetic yield varied by season, with a higher tolerance for warmer temperatures during the summer and for colder temperatures during the winter. Because the material used in Padilla-Gamino's study was freshly harvested, the *A. taxiformis* was likely less affected by culture conditions. The biomass used in this study's first temperature experiment had

been isolated in non-variable culture conditions for over 6 months prior to the experiment. Since an alga's tolerance for varying temperatures can depend on local conditions, it is possible the mere act of cultivation in closed systems could limit the optimum temperature range of *A. taxiformis* cultures (Nejrup et al., 2013). This could result in the algae becoming stressed by temperatures found within its natural temperature range. This was seen with the unexpected long-term stress response of cultures in the 15°C treatment as this temperature falls within the annual temperature range of San Diego. The extent to which this isolation could affect *A. taxiformis'* ability to survive at different temperatures is unknown but is a crucial question to address in order to understand how temperature tolerance will change over time in an aquaculture setting.

Unlike temperature, which has a predictable response, the effect of CO_2 on Fv/Fm varies greatly from study to study, with one study reporting a 5% increase under elevated CO_2 conditions and another reporting a reduction under pH of 7.7 under low light conditions (Connell & Russell, 2010; Yıldız, 2018). Overall, there was no significant effect of increased CO_2 availability on the Fv/Fm in the univariable CO_2 experiment or the factorial CO_2 and temperature experiment. This changed during the 2nd factorial experiment which explored the relationship between CO_2 addition and light intensity. The low light, low-density treatment, which mirrored the light intensity and density conditions of the previous CO_2 experiments, indicated no significant difference in Fv/Fm for cultures with and without CO_2. However, when the density was increased, resulting in a reduction in light availability, the Fv/Fm in the ambient flask increased. This contrasts with the high light treatments, in which there was a significant reduction in the Fv/Fm for the flasks with CO_2 addition. This reduction in Fv/Fm with increased

light was similarly identified in another study on the red alga *Porphyra leucosticta* (Figueroa et al., 1997). The lowest Fv/Fm, which was measured in the high light, low-density flasks with CO_2 addition, corresponded with the significant decrease in growth caused by the bleaching event. The decreased Fv/Fm reading was recorded in week 2, while the growth rates did not significantly decrease until the end of week 3, indicating that the photosystem was experiencing stress prior to the decrease in growth rate.

Bromoform:

There was significant overall variation in bromoform concentration measured throughout the experiments, varying from 0.18 to 4.85% dw. While temperature appeared to have a direct effect on bromoform concentration, CO_2 addition did not directly affect bromoform concentration. However, when coupled with secondary stressors, such as increased light intensity, a reduction in the bromoform concentration within the alga was observed.

Bromoform concentration paralleled the growth rates and photosynthetic yields in the temperature experiment. Similar to growth, bromoform concentration increased when exposed to optimum temperatures for a prolonged period of time. The difference in bromoform concentration across the different temperatures became more significant across time, with an increase in the concentrations in the optimal temperature range and a significant reduction in the lethal temperatures. Since the bromoform concentration was only measured twice, at the end of week one and week three, the rate of change over time can not be calculated. This means it is unknown at which point in time bromoform reached its maximum concentration within the optimal temperature range, or if it was still increasing past the end of the three-week experiment. Past research has also documented a relationship between temperature and bromoform, with the

highest bromoform concentration measured at 20.2°C and the lowest at 28.1°C (Mata et al., 2017). While Mata's study reported a linear relationship between bromoform and temperature, the extreme low temperatures from our study, which resulted in a decrease in bromoform, creating a response curve, were not tested in Mata's study (2017).

The effect of temperature on bromoform concentration was not significant in the CO_2 x temperature factorial experiment. While this appears to differ from the results of the first temperature experiment, the temperature range that was used in the factorial experiment fell within the suboptimal temperature range identified in the first experiment. This is significant because it indicates that the cultures in the factorial experiment exhibited little to no stress, thus reducing the variability in bromoform and resulting in no significant effect of temperature on bromoform concentration.

Similar to Fv/Fm, there was a significant interaction between CO_2 addition and high-light on bromoform concentration in the final experiment, resulting in an overall reduction in the concentration. Since this reduction was only present in the flasks showing significant signs of stress and mortality, it was likely the result of biomass degradation. This phenomenon was seen throughout the experiments, in which low bromoform concentrations were only observed in the replicates inhabiting unfavorable conditions. This connection puts into question the correlation between growth rates and bromoform concentrations observed in the temperature experiment. Since decreased growth rates and bromoform concentrations are both the result of increased stress, a false correlation between growth and bromoform concentrations could be inferred, one which does not continue as growth rate and bromoform concentrations increase.

While past studies have shown a positive relationship between environmental stressors and increased production of secondary metabolites in both plants and algae, this relationship between bromoform and stress has not been studied in *A. taxiformis* (Ramakrishna & Ravishankar, 2011). While secondary metabolites can aid in a variety of functions, bromoform has been identified as an anti-predation, antibacterial compound (Mata et al., 2012). As such, it has been hypothesized that bromoform will increase in the presence of such threats, but there is no clear connection between environmental stress and increased bromoform concentration. Short-term increases in secondary metabolites have been observed in A. taxiformis after the addition of H_2O_2, which resulted in a significant increase in the bromoform concentration followed by a subsequent decrease in a matter of hours (Mata et al., 2011). However, the current experiment examined the long-term effect of stress, with our first time-point at one week after exposure. It is possible that if the stress response resulted in an increase in secondary metabolites, it was a short-term increase and was therefore unlikely to be sustainable for one week or more. An increase in bromoform as a result of increased CO_2 availability or sub-optimum temperatures might have been missed due to the lack of samples collected early in the experiment.

Implication for Population Distribution

Taken as a whole, the data from the experiments provides insight into the current and possible future distribution of *A. taxiformis* in southern California. In comparison to its global distribution as a tropical to subtropical species, the population of *A.taxiformis* in San Diego is inhabiting temperatures on the colder end of its range (Chualáin et al., 2004). As such, the alga's distribution and population density is likely limited by cold temperature stress. This was

supported by the findings of the first unifactorial experiment, which identified cold temperature stress starting at 15°C, a temperature often recorded in the ocean in southern California between January and March.

While the seasonal distribution of sporophytes of *A. taxiformis* has not been studied in San Diego, it is likely that the population is drastically reduced during the winter due to temperature stress. It likely rebounds in the spring before reaching its maximum population size during the summer, when temperatures are more consistently within the identified optimum temperature range of *A. taxiformis*.

The first factorial experiment provided insight into the effects of climate change on the future distribution of *A. taxiformis* in southern California. The conditions used in the first factorial experiment simulate future climate conditions in San Diego under different climate change models, including current conditions, RCP 6.0, and the RCP 8.5 "business as usual" model. It was hypothesized that an increase in acidification would result in a more narrow temperature range, resulting in increased stress at the edges of the optimal zone. This was not apparent in the bromoform, Fv/Fm, or growth rate data from this experiment. The findings indicate that the *A. taxiformis* will likely be unharmed by changing climate conditions, at least those studied here. In fact, the population of *A. taxiformis* in southern California will likely become more abundant as an increase in growth was observed in the future climate scenarios, but there should be no change in the concentration of secondary metabolites.

While these experiments indicate that population expansion will be limited by temperature, other research has shown *A. taxiformis*, especially lineage 2, to have high-temperature plasticity and adaptability (Dijoux et al., 2014; M. Zanolla et al., 2015). As

such, it is likely that between increased temperature due to climate change and prolonged acclimation, *A. taxiformis* will migrate northward. It is these characteristics that have resulted in it becoming a successful invasive species in the Mediterranean. Here in CA, *A. taxiformis* will likely 1) continue to migrate north, especially as ocean warming increases temperatures up the coast of California, and 2) be a durable species for cultivation.

Conclusion:

The results of this study reveal that both temperature and CO_2 availability play a crucial role in the optimization of growth conditions of *A. taxiformis* in an aquaculture setting. For *A. taxiformis* collected in southern California, maintaining a steady temperature between 22°C-26°C is necessary to optimize growth rate and bromoform concentration. While this temperature range will likely change depending on collection location and time in captivity, the relationship between growth rate and bromoform will likely remain correlated. This makes identifying the ideal temperature ranges of different geographical groups of *A. taxiformis* crucial in the cultivation of *A. taxiformis* worldwide.

Overall, the results of the CO_2 experiments indicate that *A. taxiformis* is limited by CO_2 availability in batch culture systems, independent of light and temperature conditions. To maximize the growth rate of *A. taxiformis* in an aquaculture setting, carbon will need to be supplemented. The addition of CO_2 can account for a major increase in the operational costs of growing algae, and if not done correctly could result in a decrease in both the sustainability and effectiveness of *A. taxiformis* as a climate change fighting tool (Mata et al., 2007). Other strategies to increase CO_2 concentration should be explored, such as using CO_2 collected from power plants or effluent from a fish farm (Mata et al., 2007).

These findings have larger implications for the global distribution of *A. taxiformis* under future climate change conditions (Kübler & Dudgeon, 2015). As temperature and ocean acidification increase, it is likely that *A. taxiformis* will migrate farther away from the equator, as well as become more prolific in its current habitat as growth rates increase.

While this study provided insight into the temperature and carbon requirements of *A. taxiformis*, further research into the interaction of other limiting factors is needed. This includes, but is not limited to, the relationship between carbon availability and nutrient addition and its effect on bromoform concentration, as well as the underlying cause of the negative stress response initiated by high-light and CO_2 addition as it pertains to outdoor aquaculture.

Despite these remaining questions, this study has shown that through controlling the culture environment and addressing limiting factors, a significant increase in the growth rate of *A. taxiformis* in cultivation can be achieved in unison with high bromoform concentrations. This will be crucial for producing large amounts of *A. taxiformis* with high bromoform concentration to be utilized as a cow food supplement. Yet the question remains as to whether the environmental benefits associated with using *A. taxiformis* as a methane mitigator in cows will outweigh the resources required to achieve its maximum growth and bromoform concentration. Both an in-depth life cycle analysis and a focus on sustainable aquaculture design will be needed to ensure a net positive outcome, in terms of GHG. With this in mind, *A. taxiformis* is on track to be the first commercially available method to reduce methane production in ruminants, making it a crucial component in the fight against climate change.

This thesis contains unpublished material co-authored with Smith, Jennifer E. and Dishon, Gal. The thesis author was the primary author of this chapter.

Figures:

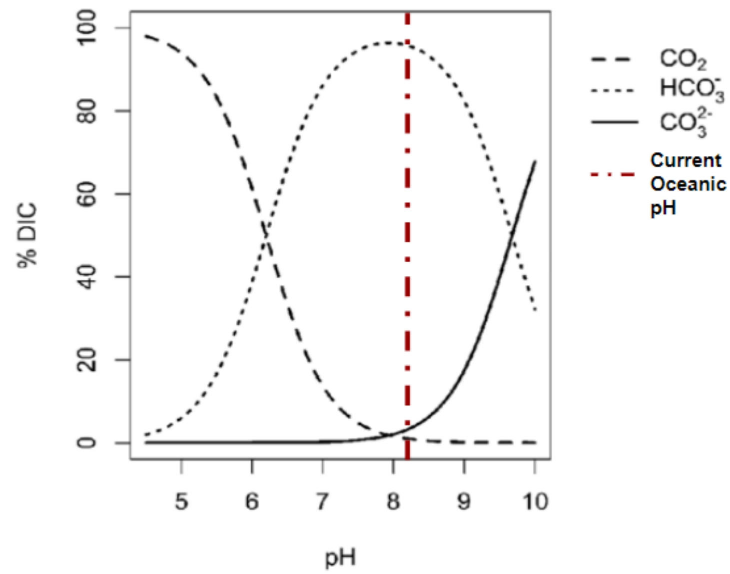


Figure 1: Relative ratio of CO_2, HCO_3^-, and CO_3^{2-} to total DIC under differing pH values of seawater. Modified from (Takolander & Others, 2018).

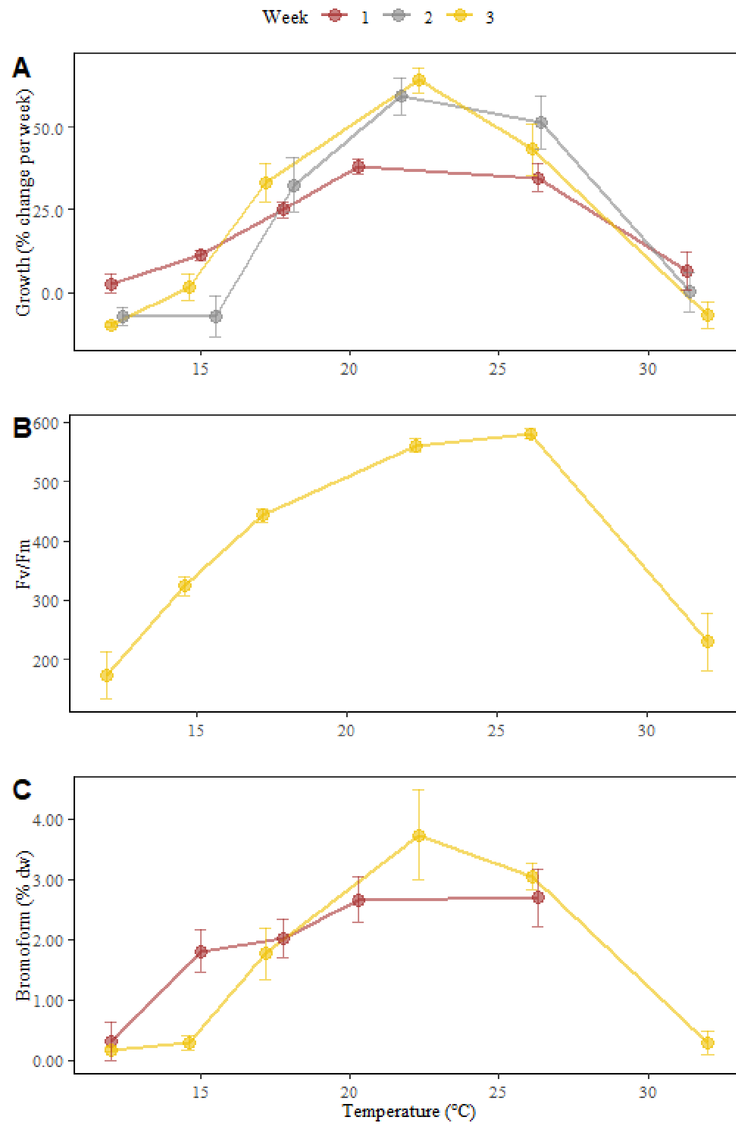


Figure 2: Experiment 1, effect of temperature (°C) (x-axis) on a) the growth rate, measured by % change in weight per week, b) average dark-adapted yield (Fv/Fm) at end of week 3, c) bromoform concentration (% dw) within the biomass at the end of week 1 and 3. Color represents time (week). Error bars represent the standard error of the replicates n = 3.

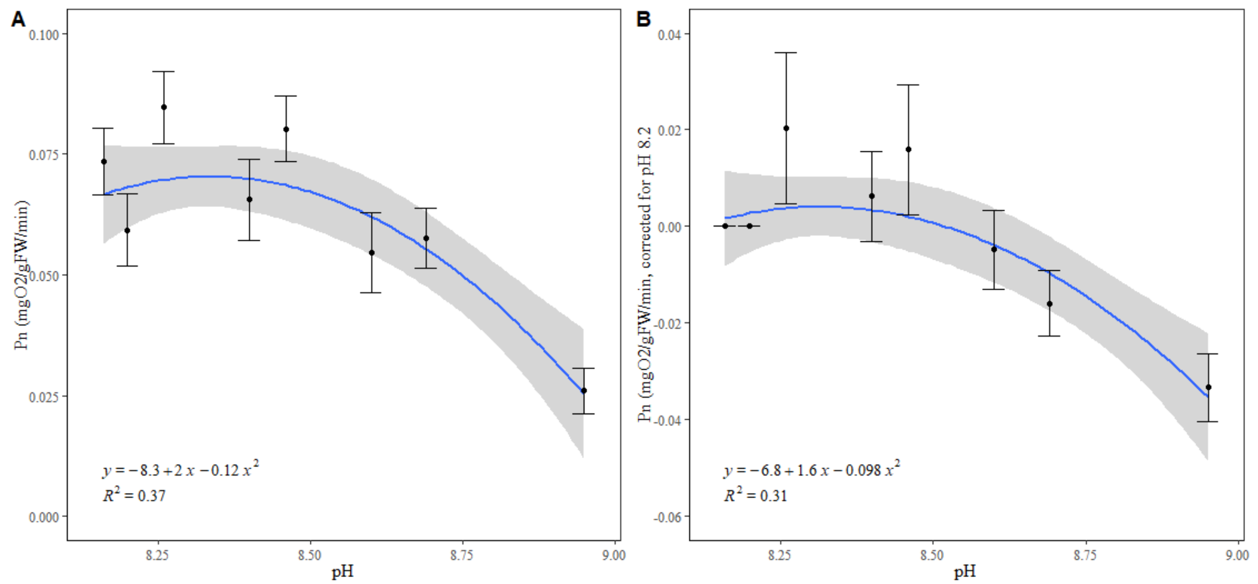


Figure 3: Oxygen evolution experiment, the effect of pH on the a) net photosynthetic rate (Pn) and b) corrected Pn, normalizes for an initial pH of 8.2. Error bars represent standard error of replicates. n = 8

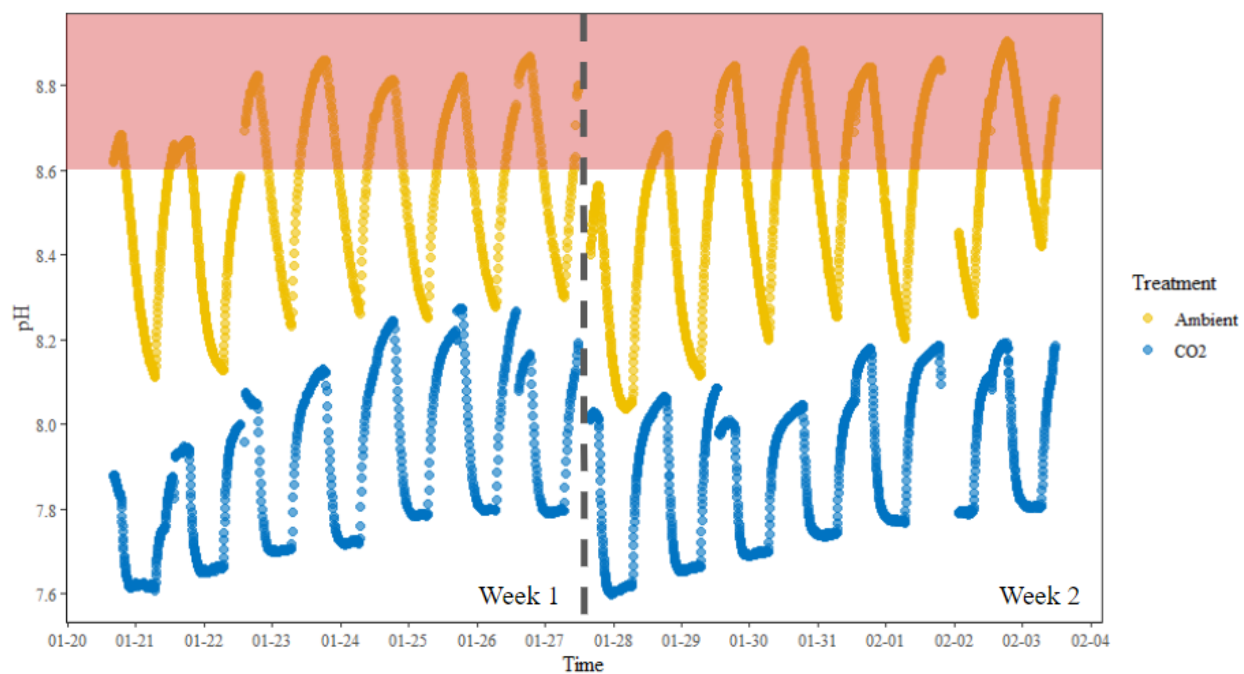


Figure 4: Daily fluctuation in pH of seawater of flasks bubbled with ambient air (300 ppm CO_2) and CO_2 enhanced air (1600 ppm CO_2). Flasks contained A.taxiformis at a density of 1g/l. Shaded area represents the suboptimal conditions for maximal photosynthetic rate. Line represents water replacement between week one and two.

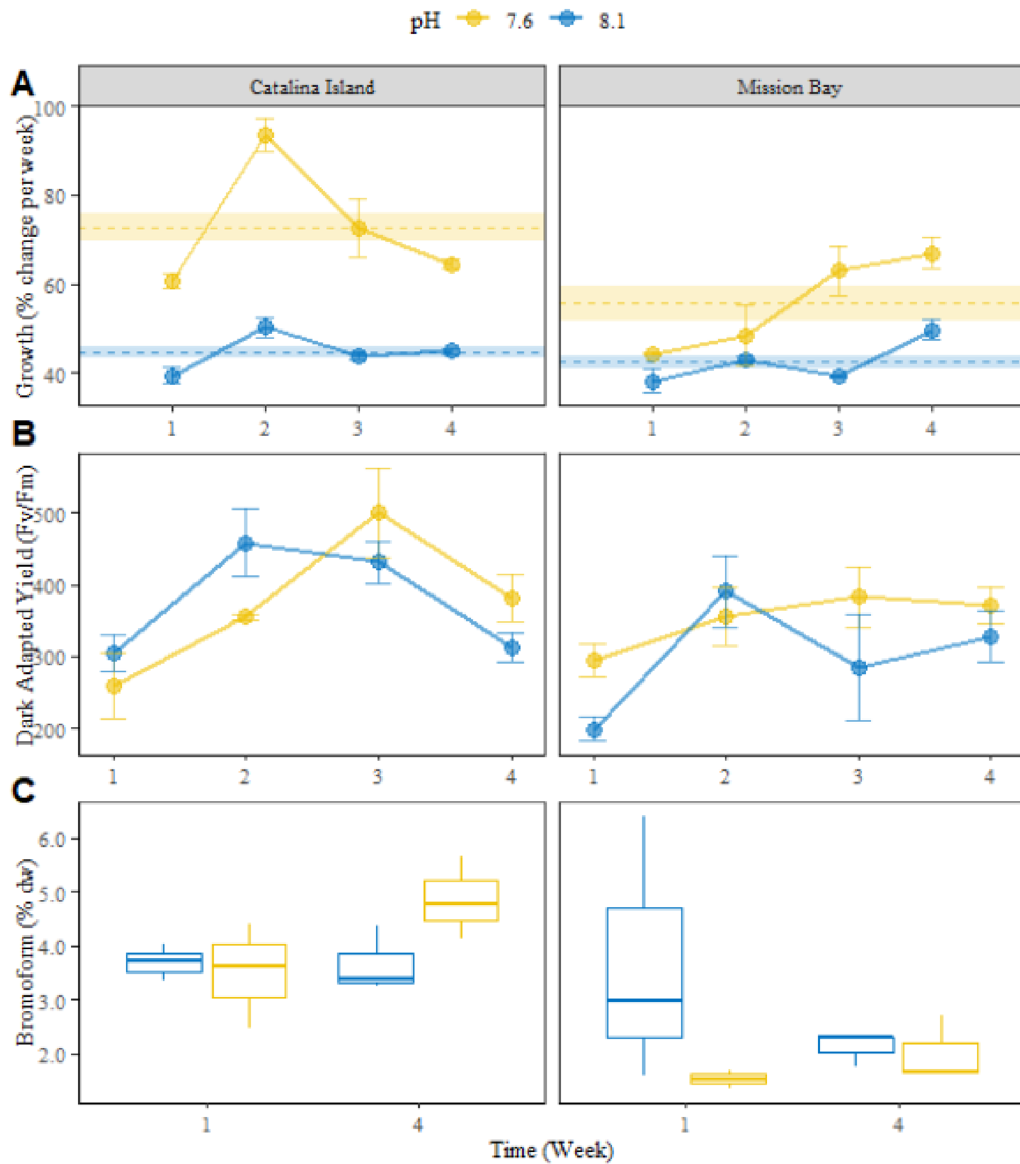


Figure 5: Experiment 2, effect of CO_2 availability on a) growth rate, measured by % change in weight per week across the four weeks. The dashed line represents the average growth rate across the four weeks, with the shaded area representing standard error, n = 3 b) Average dark-adapted yield (Fv/Fm) across time, measured by the PAM. c)Bromoform concentration within the tissue of the biomass, measured at the end of weeks 1 and 4. Color represents pH of seawater, as a result of CO_2 enhanced air. n = 3.

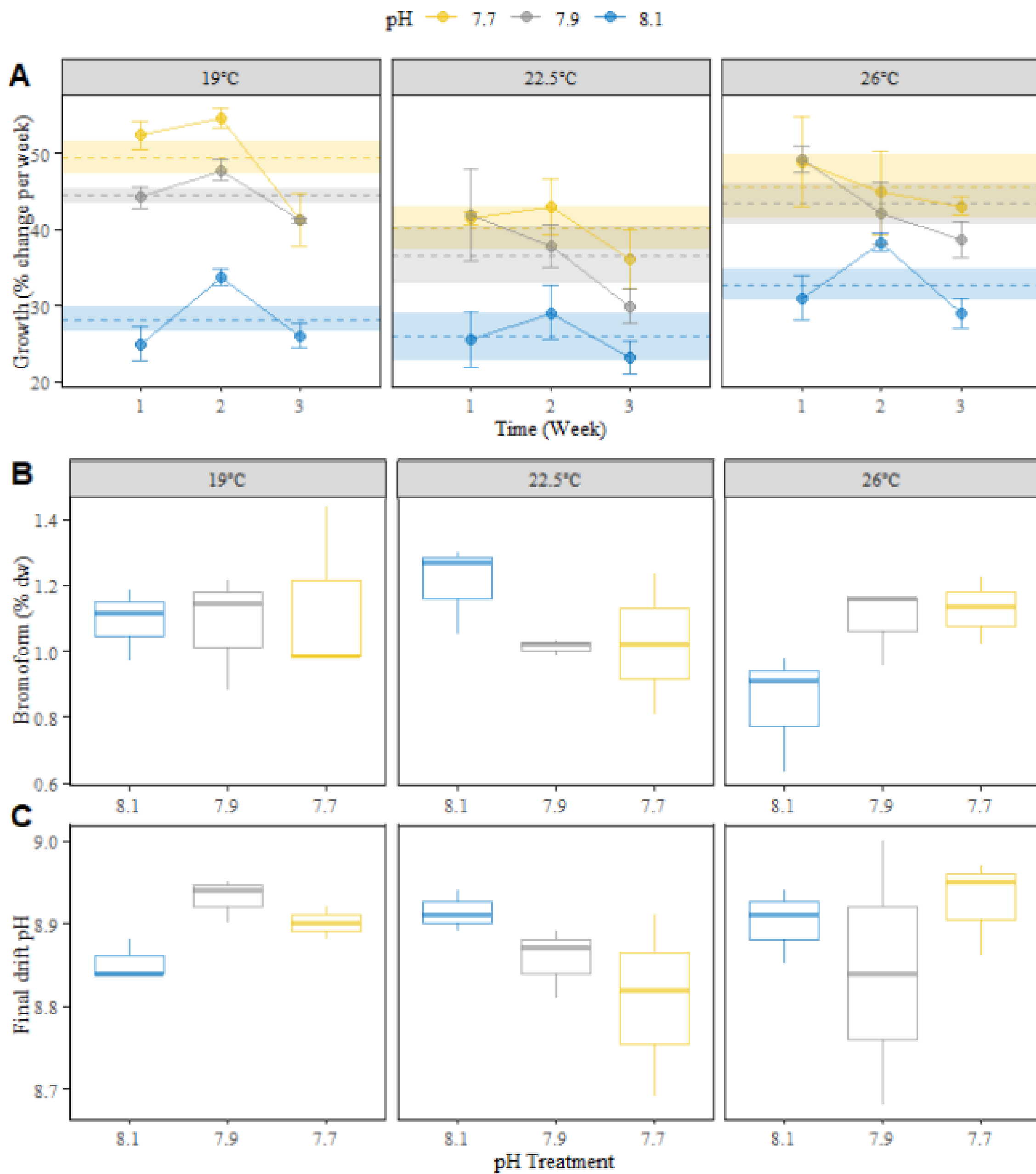


Figure 6: Experiment 4, interaction of CO_2 availability and Temperature on a) growth rate measured by the % change in weight per week across three weeks, b)Bromoform concentration within the tissue of the biomass. Measured at the end of each week, c) final pH after 24hr in high light, as a result of the drift experiment. Color represents pH levels, as a result of CO_2 enhanced air. The dashed line represents the average of all measurements across the four weeks, with the shaded area representing standard error. n = 3.

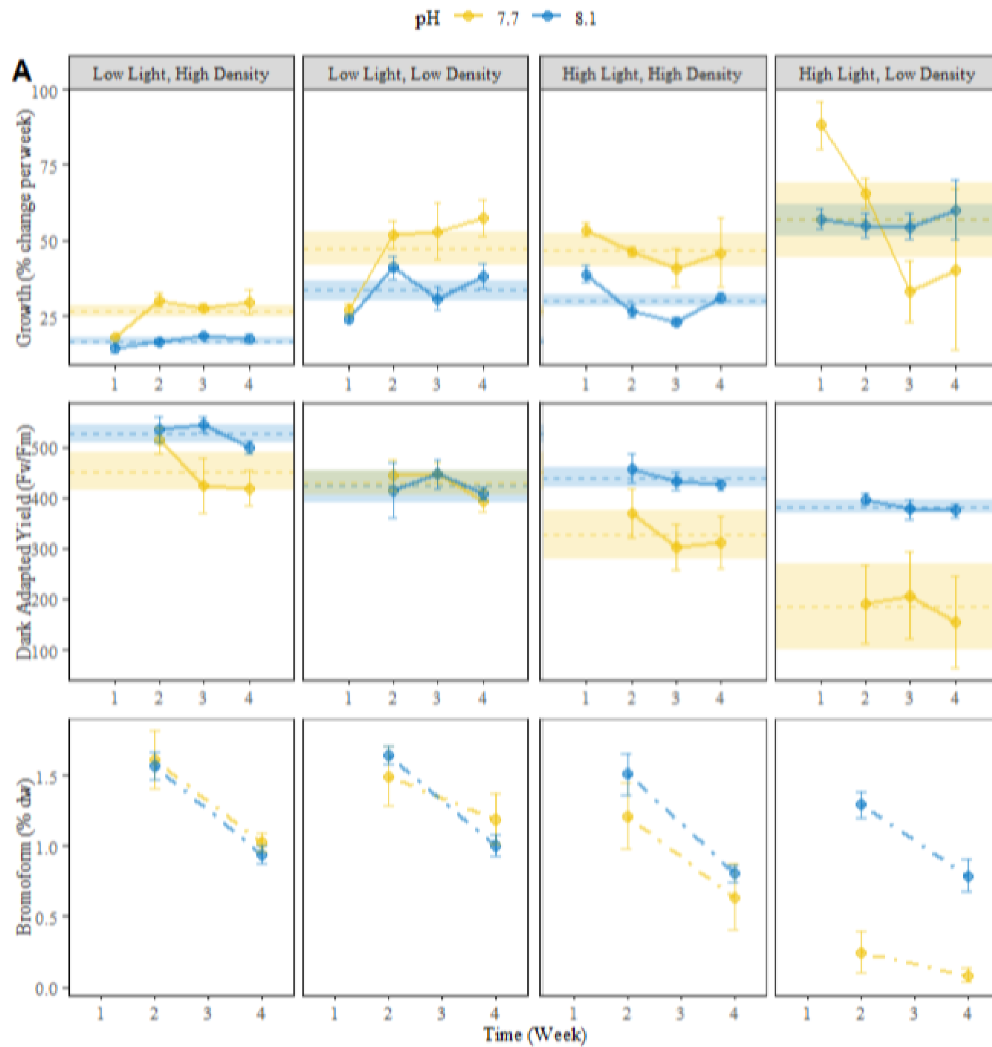


Figure 7: Experiment 3, interaction between CO_2 availability, light intensity, and density on a) growth rate, measured by % change in weight per week across four weeks, b) Average dark-adapted yield (Fv/Fm) across time, measured by the PAM, c)Bromoform concentration within the tissue of the biomass. Measured at the end of weeks 2 and 4. Color represents pH levels, as a result of CO_2 enhanced air. The dashed line represents the average of all measurements across the four weeks, with the shaded area representing standard error. n = 4.

Table 1: Mixed Factor Repeated Measures ANOVA identifying the interaction terms and significance of temperature and time on Growth, Fv/Fm, and Bromoform Concentration in Experiment 1.

Variable	Effect	DFn	DFd	F	p	p<0.05
Growth	Temperature	5	18	65.962	6.12E-11	*
	Time	2	36	0.183	8.34E-01	
	Temperature: Time	10	36	3.797	1.00E-03	*
Fv/Fm	Temperature	5	18	38.379	5.49E-09	*
Bromoform	Temperature	5	17	11.126	7.01E-05	*
	Time	1	17	4.026	6.10E-02	
	Temperature: Time	5	17	7.899	5.27E-04	*

Table 2: One Way ANOVA on isolated variables from Experiment 1, followed by a Tukey- Kramer post hoc test, to identify significance between treatments of the same variable. Identifying the effect of temperature within each week of experiment 1.

Variable	Time (week)	Variable	Df	Sum sq	Mean Sq	F value	p	Significance	Post-Hoc: Tukey Kramer					
									12.1°C	15.0°C	17.7°C	21.4°C	26.3°C	31.6°C
Growth	1	Treatment	5	4500	900	18.54	1.55E-06	***	c	bc	ab	a	a	c
		Residuals	18	874	48.5									
	2	Treatment	5	18156	3631	22.5	3.65E-07	***	b	b	a	a	a	b
		Residuals	18	2904	1610									
	3	Treatment	5	18446	3689	39.45	4.39E-09	***	c	c	b	a	ab	c
		Residuals	18	1684	94									
Fv/Fm	1	Treatment	5	579233	115847	38.38	5.49E-09	***	e	cd	bc	ab	a	de
		Residuals	18	54332	3018									
Bromoform Concentration	1	Treatment	5	15.89	3.177	4.865	6.05E-03	**	b	ab	ab	a	a	b
		Residuals	17	11.1	0.653									
	3	Treatment	5	48.55	9.71	17.56	2.30E-06	***	c	c	bc	a	ab	c
		Residuals	17	18	9.95	0.553								

Table 3: Mixed Factor Repeated Measures ANOVA identifying the interaction terms and significance of CO_2 Availability, Location, and time, on Growth, Fv/Fm, and Bromoform Concentration in Experiment 2.

Variables	Effect	DFn	DFd	F value	p	p<0.05
Growth	CO_2 Availability	1	8	245.655	2.74E-07	*
	Location	1	8	53.406	8.32E-05	*
	Time	3	24	11.473	7.34E-05	*
	CO_2 Availability:Location	1	8	32.336	4.62E-04	*
	CO_2 Availability:Time	3	24	2.862	5.80E-02	
	Location:Time	3	24	12.95	3.12E-05	*
	CO_2 Availability:Location:Time	3	24	5.587	5.00E-03	*
Fv/Fm	CO_2 Availability	1	8	1.816	2.15E-01	
	Location	1	8	7.753	2.40E-02	*
	Time	3	24	8.992	3.59E-04	*
	CO_2 Availability :Location	1	8	2.337	1.65E-01	
	CO_2 Availability :Time	3	24	2.6	7.60E-02	
	Location:Time	3	24	1.968	1.46E-01	
	CO_2 Availability :Location:Time	3	24	0.729	5.45E-01	
Bromoform	CO_2 Availability	1	8	0.747	0.412	
	Location	1	8	19.61	0.002	*
	Time	1	8	0.018	0.896	
	CO_2 Availability :Location	1	8	5.105	0.054	
	CO_2 Availability :Time	1	8	3.162	0.113	
	Location:Time	1	8	1.539	0.25	
	CO_2 Availability :Location:Time	1	8	0.106	0.753	

Table 4: Pair-t-test analyzing the statistical difference in growth between treatments with and without CO_2 addition in different locations and across time, in Experiment 2.

Variable	Location	Week, p value			
		1, n=3	2, n=3	3, n=3	4, n=3
Growth	Catalina Island	9.96E-04	5.71E-04	1.20E-02	5.45E-05
	Mission Bay	7.35E-02	0.455	1.24E-02	1.38E-02
* <0.05	** <0.01	*** <0.001			

Table 5: Mixed Factor Repeated Measures ANOVA identifying the interaction terms and significance of Temperature, CO_2 Availability, and Time, on Growth and Fv/Fm, as well as significance of CO_2 availability and Temperature on Bromoform Concentration and drift pH in Experiment 3.

Variable	Effect	DFn	DFd	F	p	p<.05
Growth	Temp	2	18	19.923	2.74E-05	*
	CO_2 Availability	2	18	104.51	1.24E-10	*
	Time	2	36	11.694	1.22E-04	*
	Temp:CO_2 Availability	4	18	2.62	6.90E-02	
	Temp:Time	4	36	0.776	5.48E-01	
	CO_2 Availability:Time	4	36	1.852	1.40E-01	
	Temp:CO_2 Availability:Time	8	36	0.625	7.51E-01	
Fv/Fm	CO_2 Availability	2	18	2.573	1.04E-01	
	Temperature	2	18	0.061	9.41E-01	
	Time	2	36	4.186	2.30E-02	*
	CO_2 Availability:Temperature	4	18	0.224	9.21E-01	
	CO_2 Availability:Time	4	36	1.265	3.02E-01	
	Temperature:Time	4	36	1.017	4.11E-01	
	CO_2 Availability:Temperature:Time	8	36	0.974	4.71E-01	
Bromoform Concentration	CO_2 Availability	2	18	0.218	8.06E-01	
	Temperature	2	18	0.636	5.41E-01	
	CO_2 Availability:Temperature	4	18	2.061	1.29E-01	
Drift pH	CO_2 Availability	2	18	0.087	9.17E-01	
	Temperature	2	18	0.626	5.46E-01	
	CO_2 Availability:Temperature	4	18	1.755	1.82E-01	

Table 6: One-Way ANOVA on isolated variables from Experiment 3, followed by a Tukey-Kramer post hoc test, to identify significance between treatments of the same variable. Identifying the effect of pH, temperature, and Time throughout experiment 3.

Variables	Treatment		Df	Sum sq	Mean Sq	F value	p	Significance	Post-Hoc: Tukey Kramer		
Growth	pH	pH	2	3837	1918.4	41.1	6.44E-13	***	pH: 7.7	pH: 7.9	pH: 8.1
		Residuals	78	3640	46.7				a	a	b
	Temperature	Temp	2	731	365.7	4.229	1.80E-02	*	19.0°C	22.5°C	26.0°C
		Residuals	78	6746	86.5				a	b	a
	Time	Time	2	745	372.5	4.316	1.67E-02	*	Week 1	Week 2	Week 3
		Residuals	78	6732	86.3				ab	a	b
Fv/Fm	Time	Time	2	26871	13435	3.643	3.07E-02	*	Week 1	Week 2	Week 3
		Residuals	78	287684	3688				a	ab	b

Table 7:Mixed Factor Repeated Measures ANOVA identifying the interaction terms and significance of CO_2 Availability, Density, Light, and Time, on Growth and Fv/Fm, and Bromoform Concentration in Experiment 4.

Variable	Effect	DFn	DFd	F value	p	p<0.05
Growth	CO_2 Availability	1	24	9.748	0.005	*
	Light Level	1	24	26.141	3.12E-05	*
	Density	1	24	33.169	6.18E-06	*
	Time	3	72	1.95	0.129	
	CO_2 Availability :Light Level	1	24	0.287	0.597	
	CO_2 Availability :Density	1	24	0.914	0.349	
	Light Level:Density	1	24	0.006	0.937	
	CO_2 Availability :Time	3	72	0.9	0.446	
	Light Level:Time	3	72	13.928	2.97E-07	*
	Density:Time	3	72	0.741	0.531	
	CO_2 Availability :Light Level:Density	1	24	2.557	0.123	
	CO_2 Availability :Light Level:Time	3	72	5.043	0.003	*
	CO_2 Availability :Density:Time	3	72	1.531	0.214	
	Light Level:Density:Time	3	72	2.546	0.063	
	CO_2 Availability :Light Level:Density:Time	3	72	3.943	0.012	*

Fv/Fm	CO_2 Availability	1	24	14.158	9.58E-04	*
	Light Level	1	24	24.318	4.95E-05	*
	Density	1	24	10.448	4.00E-03	*
	Time	2	48	4.809	1.20E-02	*
	CO_2 Availability :Light Level	1	24	5.753	2.50E-02	*
	CO_2 Availability :Density	1	24	0.006	9.40E-01	
	Light Level:Density	1	24	0.559	4.62E-01	
	CO_2 Availability :Time	2	48	1.078	3.48E-01	
	Light Level:Time	2	48	0.358	7.01E-01	
	Density:Time	2	48	1.797	1.77E-01	
	CO_2 Availability :Light Level:Density	1	24	2.753	1.10E-01	
	CO_2 Availability :Light Level:Time	2	48	0.652	5.25E-01	
	CO_2 Availability :Density:Time	2	48	1.121	3.34E-01	
	Light Level:Density:Time	2	48	0.08	9.23E-01	
	CO_2 Availability :Light Level:Density:Time	2	48	0.003	9.97E-01	
Bromoform	CO_2 Availability	1	24	7.738	1.00E-02	*
	Light Level	1	24	27.941	2.02E-05	*
	Density	1	24	4.435	4.60E-02	*
	Time	1	24	143.768	1.27E-11	*
	CO_2 Availability :Light Level	1	24	10.448	4.00E-03	*
	CO_2 Availability :Density	1	24	3.504	7.30E-02	
	Light Level:Density	1	24	6.925	1.50E-02	*
	CO_2 Availability :Time	1	24	6.238	2.00E-02	*
	Light Level:Time	1	24	0.4	5.33E-01	
	Density:Time	1	24	6.83	1.50E-02	*
	CO_2 Availability :Light Level:Density	1	24	2.586	1.21E-01	
	CO_2 Availability :Light Level:Time	1	24	0.054	8.18E-01	
	CO_2 Availability :Density:Time	1	24	2.112	1.59E-01	
	Light Level:Density:Time	1	24	0.981	3.32E-01	
	CO_2 Availability :Light Level:Density:Time	1	24	0.065	8.02E-01	

Table 8: Pair-t-test analyzing the statistical difference in Growth, Fv/Fm, and Bromoform concentration between treatments with and without CO_2 addition in light and density combinations across time, in Experiment 4.

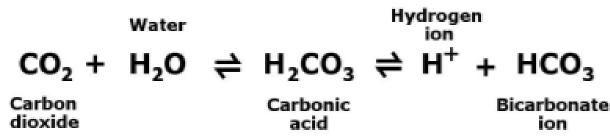
Variable	Light	Density	Week, p-value				
Growth			**1, n=4**	**2, n=4**	**3, n=4**	**4, n=4**	**Average, n=16**
	Low	High	3.36E-02	1.15E-03	6.94E-04	2.71E-02	5.11E-06
	Low	Low	0.235	0.113	6.96E-02	3.89E-02	5.97E-03
	High	High	6.52E-03	4.30E-04	3.47E-02	0.239	7.71E-05
	High	Low	1.05E-02	0.159	0.1	0.51	0.991
Fv/Fm				**2, n=4**	**3, n=4**	**4, n=4**	**Average, n=12**
	Low	High		0.567	7.94E-02	7.91E-02	1.29E-02
	Low	Low		0.645	0.956	0.543	0.824
	High	High		0.172	3.27E-02	7.71E-02	9.92E-04
	High	Low		3.74E-02	0.103	5.19E-02	2.40E-04
Bromoform				**2, n = 4**		**4, n=4**	**Average, n=8**
	Low	High		0.859		0.345	0.744
	Low	Low		0.516		0.371	0.929
	High	High		0.323		0.514	0.352
	High	Low		1.17E-03		1.59E-03	2.68E-05
* <0.05	** <0.01	*** <0.001					

Table 9: Pair-t-test analyzing the statistical difference in bromoform concentration between weeks 2 and 4 in Experiment 4.

Variable	Light	Density	With CO_2		Without CO_2	
			p	Significance	p	Significance
Bromoform Concentration	Low	High	3.30E-02	*	1.55E-03	**
		Low	3.19E-01		6.49E-04	***
	High	High	1.33E-01		4.64E-03	**
		Low	3.47E-01		1.73E-02	*

Equations

Equation 1:



Appendix:

Appendix 1:

Variable	Location	Effect	DFn	DFd	F value	p	p<.05
Growth	Catalina Island	CO_2_Availability	1	4	580.296	1.76E-05	*
		Time	1.72	6.86	16.692	0.003	*
		CO_2_Availability: Time	1.72	6.86	5.506	0.041	*
	Mission Bay	CO_2_Availability	1	4	31.034	0.005	*
		Time	3	12	8.482	0.003	*
		CO2_Availability: Time	3	12	3.158	0.064	
* <0.05	** <0.01	*** <0.001					

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