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RESEARCH ARTICLE

The effects of temperature and CO_2 enrichment on the red seaweed *Asparagopsis taxiformis* from Southern California with implications for aquaculture

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Abstract

The red alga *Asparagopsis taxiformis* has recently been recognized for its unique ability to significantly reduce methane emissions from ruminant animals when fed in small quantities. The main obstacle in using this seaweed as a methane-mitigating feed supplement is the lack of commercially available biomass. Little is known about how best to grow this red alga on a commercial scale, as there are few published studies that have investigated the factors that influence growth, physiology, and overall performance. This study examined the effects of temperature and CO_2 enrichment on the growth, photophysiology, and concentration of bromoform, the secondary metabolite largely responsible for methane reduction in *A. taxiformis*. A series of single and multifactor closed culture experiments were conducted on *A. taxiformis* collected, isolated, and cultured from populations in Southern California. We identified the optimal temperature range to be between 22 and 26°C, with significant short-term stress observed below 15°C and above 26°C. Carbon dioxide addition resulted in increased performance, when accounting for growth per CO_2 use. In general, we observed the highest bromoform concentrations in algae with the highest growth rates, but these results varied among experiments. These findings indicate that through environmental control and by addressing limiting resources, significant increases in biomass production and quality can be achieved.

KEYWORDS
Asparagopsis, bromoform, climate change, methane, thermal tolerance

INTRODUCTION

Methane (CH_4) is the second most abundant greenhouse gas in the atmosphere but is 28 times more potent than CO_2 over a 10-year time span. However, because it remains in the atmosphere for a relatively short period of time (12 years) in comparison to the more abundant CO_2 (100s of years), it is a key target for greenhouse gas (GHG) emissions reductions (Montzka et al., 2011). With 17% of global methane production coming from ruminant animals via enteric fermentation, a significant reduction could have rapid mitigation

Abbreviations: CA, carbonic anhydrase; CCM, carbon concentrating mechanism; CH_4, methane; CI, Santa Catalina Island; CO_3^{-2}, carbonate; DIC, dissolved inorganic carbon; DW, dry weight; *Fv*/*Fm*, dark-adapted photosynthetic yield; GHG, greenhouse gas; HCO_3^-, bicarbonate; IPCC, International Panel on Climate Change; MB, Mission Bay; OA, ocean acidification; PAM, pulse amplitude modulation; PAR, photosynthetically available radiation; PFD, photon flux density; psi, pound-force per square inch; ROS, reactive oxygen species; SIO, Scripps Institution of Oceanography; WW, wet weight.

effects on climate change (Montzka et al., 2011; Moss et al., 2000; Patra et al., 2017). Scientists have been trying to find solutions to reduce methane emissions in cattle for decades, and many natural products have been investigated. During a screening of multiple species of algae, it was observed that *Asparagopsis taxiformis*, a cosmopolitan red seaweed also known as limu kohu or red sea plume, could lead to a 90% reduction in methane production from livestock when they were fed it in small quantities (Abbott, 1996; Andreakis et al., 2004; Kinley et al., 2016). Subsequent in vivo and in vitro studies have observed that *A. taxiformis* has the largest and most consistent patterns of methane reduction in ruminant animals compared to other algae and commercially available inhibitors (Lopes et al., 2016; Machado et al., 2014). Further, no negative effects on health, metabolism, and/or the quality of the meat and dairy produced have been recorded in livestock fed *A. taxiformis* (Brooke et al., 2020; Kinley et al., 2016; Machado et al., 2016a; Roque et al., 2021).

The effectiveness of *Asparagopsis* at reducing methane in ruminants has been attributed to secondary metabolites including bromoform and other brominated compounds (dibromoacetic acid, bromochloroacetic acid, etc.) that are produced and stored in *A. taxiformis* tissue (Kinley et al., 2016; Machado et al., 2016b). These compounds are believed to intervene in two pathways of methane production: they block an enzyme necessary for the production of methane in methanogenic microbes, and they act as competitive terminal electron acceptors in the reduction of H_2, blocking the byproduct of methane (Patra et al., 2017). Further, there is a positive correlation between the concentration of bromoform fed (% of tissue biomass and grams of seaweed) and the amount of methane reduction achieved (Brooke et al., 2020; Chagas et al., 2019; Kinley et al., 2020; Machado et al., 2016b, 2018; Min et al., 2021; Roque et al., 2021; Vucko et al., 2017). At some of the higher concentrations of seaweed fed to cows (0.2% of daily intake, at a bromoform concentration of 6.55 mg · g^{-1}), up to 95% reduction in methane production has been observed (Kinley et al., 2020). However, in its pure form, bromoform is considered an animal carcinogen, making delivery via seaweed biomass an attractive option. As such, there is significant interest in developing large-scale cultivation of *A. taxiformis* and in determining the factors that influence bromoform production, with the goal of producing large quantities of high-quality seaweed for use as a methane-mitigating supplement in ruminant feed.

The viability of *Asparagopsis taxiformis* to mitigate livestock-derived methane production will depend on our ability to commercialize this species and mass produce it sustainably. As a Florideophycean member of the phylum Rhodophyta, *A. taxiformis* has a triphasic life cycle with an alternation of heteromorphic generations that has yet to be fully completed in captivity (Feldmann, 1939; Zanolla et al., 2015). The tetrasporophyte phase, originally known as *Falkenbergia hillenbrandii*, grows as filamentous tufts made up of branches with three pericentral cells. This diploid stage is capable of propagation via asexual reproduction or fragmentation, (Abbott & Hollenberg, 1992; Paul et al., 2006), making it an ideal candidate for land-based cultivation in tumble culture (Zhu et al., 2021).

Asparagopsis taxiformis is known to produce and store bromoform at concentrations up to 10 times higher than other algae, with reported in-tissue concentrations ranging from 0.1% to 5% dry weight (1–50 mg · g^{-1} DW) depending on lineage, location, and environment (Marshall et al., 1999; Mata et al., 2012, 2017; Paul et al., 2006; Thapa et al., 2020). *Asparagopsis taxiformis* has a dedicated bromoform biosynthetic enzyme, Mbb1, which converts hydrogen peroxide to secondary metabolites such as bromoform, via a peroxidase reaction (Thapa et al., 2020). However, more research is needed to better understand both the genetic and environmental drivers of bromoform production.

Variability in temperature is known to affect algae in many important ways, such as altering enzyme activity, cellular metabolism, nutrient uptake and photosynthetic rates, which are all key factors that influence the growth, distribution, and cultivation of algae around the globe (Roleda & Hurd, 2012, 2019). Specifically, Zanolla et al. (2017) observed that temperature was the single best predictor of the global geographic distribution of the six known lineages of *Asparagopsis taxiformis*. As such, it is not surprising that the growth, photophysiology, and chemical responses of *A. taxiformis* to different temperatures have been shown to vary widely depending on the context in which the seaweed is collected (e.g., life stage, season, location, etc.; Mata et al., 2017; Padilla-Gamiño & Carpenter, 2007; Zanolla et al., 2015). Due to this variability and the high economic and environmental cost of controlling temperature within an aquaculture system, it is important to determine the location-specific thermal tolerance range and optimum needed to achieve the maximum growth rates to model the cost–benefit analysis of various production systems (Davison & Piedrahita, 2015; Mata et al., 2017). Further, little is known about the influence of temperature on bromoform production in seaweeds in general.

Another important factor to consider when seeking to optimize the growth of seaweed, especially in land-based cultivation systems, is the potential for carbon limitation. Although there are three different forms of dissolved inorganic carbon (DIC: CO_2, HCO_3^-, CO_3^{-2}) in seawater, CO_2 is the only form of DIC that algae can use passively, as it diffuses directly through cell walls; however, CO_2 is also the least abundant form of DIC in surface seawater. At a pH of 8.1, the current open-ocean average pH, CO_2 makes up less than 1% of the available DIC (Mata et al., 2012). As pH increases, the

availability of CO_2 decreases exponentially until reaching nearly zero at a pH of 9. Carbon dioxide is often limiting in algal cultivation systems because of this and because the rate at which algae utilize CO_2 surpasses the rate at which CO_2 diffuses into seawater (Mata et al., 2007). Many algae have evolved carbon concentrating mechanisms (CCMs) to allow the use of other forms of DIC (Cornwall et al., 2015), such as HCO_3^-, which is readily available in seawater. Carbon concentrating mechanisms work by either actively transporting other forms of DIC into cells or by transforming HCO_3^- into CO_2 via the enzyme carbonic anhydrase (CA) externally to allow for passive uptake (Hurd et al., 2009; Mondal et al., 2016). Although CCMs are incredibly helpful for algae in CO_2-limited environments, they can require excess energy, with the efficiency of the CCM dependent on the affinity for HCO_3^- (Cornwall et al., 2015). Thus, the presence of a CCM does not necessarily indicate that an alga is replete with CO_2 (van der Loos et al., 2019). Increased CO_2 availability can also lead to the downregulation of CCMs, which can allow more energy and resources to be directed toward growth and other metabolic processes (Mercado et al., 1999).

Carbon metabolism has not been widely studied for *Asparagopsis taxiformis*. Raven et al. (2002) conducted an extensive survey of δ13C values in algae, including *A. taxiformis*, as a means of predicting CCM presence; they observed that algae with values of δ13C above –10‰ likely have a CCM while those below –30‰ should not. In their study, they examined a single sample of *A. taxiformis* from Southern California that had a δ13C value of –28‰, suggesting that this species likely lacked a strong CCM. This result was further supported by Mata et al. (2007) who investigated the carbon acquisition mechanism in the sister species *A. armata* and observed a CCM with a low affinity for HCO_3^-. The CCM was identified as an external CA mechanism that dehydrates HCO_3^- to CO_2 at pH values between 7.6 and 8.0. This CCM can aid in the acquisition of CO_2 at low pH, but *A. armata* is likely limited by CO_2 availability above a pH value of 8.0 (Mata et al., 2007; Zhu et al., 2021). Based on these findings, it seems probable that *A. taxiformis* would become CO_2 limited as pH approaches 9.0, suggesting that CO_2 supplementation in aquaculture settings will likely increase production. Studies have shown that doubling the amount of available CO_2 for species without a strong CCM can result in up to a 52% increase in growth (Hurd et al., 2009). Although CO_2 addition can be a powerful tool to increase algal growth rates, the associated acidification of seawater can increase an alga's sensitivity to other stressors. This has been observed in studies that focused on the interaction between ocean warming and ocean acidification (OA), where OA caused a shift in the optimum temperature range of certain algae (Koch et al., 2013). Because of this, it has been hypothesized that high CO_2 conditions could narrow the optimal temperature range of *A. taxiformis*.

The goals of this study were to explore the effects of temperature variability and CO_2 concentration on growth, photophysiology, and bromoform concentration in *Asparagopsis taxiformis* collected from multiple sites around Southern California. Limited research has been conducted on *A. taxiformis* from California, and as such, this study explores the independent and combined effects of these factors on *A. taxiformis* performance with relevance to potential commercialization.

MATERIALS AND METHODS

Isolation and cultivation

Over the course of a year, tetrasporophytes of *Asparagopsis taxiformis* were collected from three different locations around Southern California including the seawater system inside of Hubbs Hall at Scripps Institution of Oceanography (SIO), University of California San Diego, a floating dock in Mission Bay (MB), and the shallow subtidal between 1 and 2 m at Chalk Cove, Santa Catalina Island (CI). Samples were returned to the Smith Lab at SIO and were sorted using a stereomicroscope. Very small tips (3–5 pericentral cells from the apical cell) were cut from freshly collected material using scalpels. Tips were individually placed in well plates and were cultured in sterile seawater at 20°C in temperature-controlled rooms with 10 μmol photons · m^{-2} · s^{-1} PAR on a 12:12 h light:dark cycle; water was changed weekly. Cultures were then monitored for biofouling, and if/when detected, cultures were terminated. Establishing stable cultures took between 2 and 4 months. Once stable, the cultures were grown out of well plates and gradually moved up to 500-mL glass vessels. Once cultures were moved from well plates to flasks, filtered air was added to gently agitate seaweeds using 5-mL sterile pipettes that were held in place with foam stoppers. The culture medium consisted of seawater pumped from the end of the SIO pier that was filtered (0.2 μm filter), autoclaved (20 min at 120°C, at 15 psi), and amended with 400 μL · L^{-1} of commercially available F/2 fertilizer solution (Proline F/2 Algae Food). All flasks with seaweed were placed on a bench that was illuminated with a Giesemann light source supplying photon flux density (PFD) of 40–50 μmol photons · m^{-2} · s^{-1} (measured by a hand-held PAR sensor—APOGEE MQ-510 underwater quantum flux meter) programmed for a 12:12 h light:dark cycle. The room was held at 22.5°C, and culture media was changed weekly. Cultures were kept at a density between 1 and 3 g wet weight (WW) · L^{-1} by harvesting the excess biomass during weekly maintenance. Cultures remained in these common garden conditions for 6 months prior to use in experiments, to reduce the impact of original

environmental conditions on the cultures and to ensure there was no biofouling.

Closed culture experiments

Three closed culture experiments were conducted: a thermal tolerance experiment, a CO_2-addition experiment, and a factorial temperature and CO_2-enrichment experiment on *Asparagopsis taxiformis* performance. Experiments lasted between 3 and 4 weeks, with the response variables measured at the end of each week. The response variables included growth rate (% change in wet weight per week), bromoform concentration (% bromoform per gram dry weight), and photosynthetic efficiency (dark-adapted quantum yield). The cultures were inoculated at a density of 1 g WW · L^{-1} into culture media as described above. Culture isolates were selected based on the quality of biomass within the common garden cultures at that time. Each week, cultures were weighed to assess change in biomass. At that time, culture media was replaced, and the biomass was reset to 1 g WW · L^{-1}. The excess biomass was harvested and used to measure in-tissue bromoform concentration and *F*v/*F*m throughout the experiment(s). For samples with a growth rate of less than 10%, bromoform concentration and *F*v/*F*m measurements were solely taken at the end of the experiment due to a lack of biomass. All other culture conditions remained as described above aside from the variables that were manipulated (see below).

Independent variables

Temperature experiment

A 3-week temperature experiment was conducted with six treatments ranging from 12 to 31°C. This temperature range included both the average minimum (15°C) and maximum (22°C) seawater temperature recorded along the coast of Southern California as well as anomalies recorded in the recent past associated with marine heat waves and El Niño events (Fumo et al., 2020). The maximum temperature for the experiment was 31°C, to account for possible future temperature anomalies and to identify the potential thermal maximum for *Asparagopsis taxiformis* in California. Water baths were used to control the temperature in the flasks in an aquarium facility at SIO. The facility is plumbed with running seawater at three temperatures—ambient (current ocean temperature, which was 19°C during the experiment), chilled (12.0°C), and warmed (26.0°C)—using an on-demand industrial chiller and heater. By controlling the ratio of seawater from these three sources, five water baths were created at the following temperatures: 12.0, 15.0, 19.0, 22.5, and 26.0°C. An Exo Terra heat cable was laid across the bottom of the sixth water bath to achieve the highest temperature at 31°C. HOBO temperature loggers (Onset HOBO UA-002-08 Pendant Light and Temperature Data Logger) were placed in flasks without biomass within each water bath, and they logged temperature every 15 min. Photosynthetically active radiation was controlled by a Giesemann light source supplying PFD of 40–50 μmol photons · m^{-2} · s^{-1} (measured by a hand-held PAR sensor APOGEE MQ-510 underwater quantum flux meter) programmed for a 12:12 h light:dark cycle. Each treatment had four replicates for a total of twenty-four 1-L Erlenmeyer flasks with 1 L of culture media containing 200 μL of F/2. Each flask was inoculated with 1 g of biomass from a culture originally collected at SIO. Every 7 days, growth rates were measured by filtering the seaweed through a sieve and gently pressing the biomass through paper towels until no more excess water was removed, then weights were taken, culture media was replaced, and the density of the flask returned to 1 g · L^{-1} by harvesting excess biomass. Weights were measured weekly to explore potential acclimatization to temperature treatments. Tissue samples to measure bromoform concentration were taken at the end of week 1 and week 3. *F*v/*F*m measurements were taken at the end of week 3.

CO_2-addition experiment

A 4-week CO_2-enrichment experiment was conducted using cultures originating from two collection locations, CI and MB, which had been in the common garden for over 6 months. There was no inherent expectation for samples from these different locations to respond differently to CO_2 enrichment. 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 via a 10-mL serological pipet. pH was used as a real-time proxy for CO_2 availability within the cultures. Large bubbles were required to ensure the continued circulation of biomass through the flasks, which caused low diffusion rates of CO_2. As such, a significant amount of CO_2 was required to reach the desired reduction in pH. The flasks bubbled with atmospheric air (300 ppm CO_2) were held at a pH of 8.1, and the flasks with CO_2-enriched air (1500 ppm CO_2) from a pre-mixed cylinder were held at a pH of 7.65. The pH of a control flask without biomass was measured using Durafett sensors (Honeywell 51453503-501 Durafet III pH Electrode) attached to a Honeywell UDA2182 Dual Input Analyzer, which recorded pH and temperature every 5 min using LabView software. Photosynetically active radiation was provided with a Giesemann light source supplying PFDof 40–50 μmol photons · m^{-2} · s^{-1} (measured as described above) programmed for a 12:12 h light:dark cycle. The experiment consisted of

six replicates per treatment for a total of twelve 500-mL Erlenmeyer flasks with culture media containing 100 μL of F/2. Every 7 days the cultures were assessed, growth rate data and *F*v/*F*m measurements collected, and the density of each flask returned to 1 g · L^{-1} by removing excess biomass as described above. Tissue samples to measure bromoform concentration were taken at the end of week 1 and week 4.

CO_2-availability and temperature experiment

A 3-week factorial experiment was designed to provide insight into how temperature affects the performance of *Asparagopsis taxiformis* under different CO_2 concentrations. This experiment was fully factorial with three levels of each treatment. The pH treatments included pH levels of 8.1, 7.9, and 7.7, which required bubbling with CO_2-enriched air at 300 ppm (atmospheric), 800 ppm, and 1200 ppm, respectively. To control the concentration of CO_2 within the air, three mass flow controllers mixed ambient air with pure CO_2. A LI-COR CO_2 Gas Analyzer measured the ppm of CO_2 within the atmospheric and two mixed-air treatments in real-time. The LI-COR was calibrated at the beginning of each experiment using certified pre-mixed CO_2 gas and nitrogen. 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. To account for the effect of off-gassing due to the aeration and the effect of the algae on the pH of the seawater, pH was monitored in flasks without algae for 3 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 Analyzer, which recorded pH and temperature every 5 min. The Durafetts were calibrated using certified pH calibration solutions (pH of 7 and 10) at the beginning and end of each experiment. Flasks were kept at three temperatures, 19.7 ± 0.3°C, 22.4.0 ± 1.0°C, and 26.0 ± 0.3°C to represent the current average summer ocean temperature in Southern California, as well as predicted future temperatures put forth by the Intergovernmental Panel on Climate Change (IPCC, 2019). The temperature was controlled using water baths as described above. HOBO temperature loggers (Onset HOBO UA-002-08 Pendant Light and Temperature Data Logger) were deployed in a flask in each water bath to record the temperature every 15 min. The pH and temperature conditions were crossed, resulting in nine different environmental treatments for this experiment. Photosynthetically active radiation was controlled with Giesemann light source supplying PFD of 40–50 μmol photons · m^{-2} · s^{-1}, programmed for a 12:12 h light:dark cycle. The experiment consisted of three replicates per treatment combination for a total of twenty-seven 1-L flasks with culture media containing 600 μL of F/2. The biomass used in this experiment was originally collected from CI. Every 7 days, growth rate and *F*v/*F*m measurements were collected, and the density was returned to 1 g · L^{-1} by removing excess biomass. Tissue samples to measure bromoform concentration were taken at the end of week 3.

Short-term response to variability in pH

A short-term incubation experiment was conducted where photosynthesis and respiration were measured across a gradient of pH values (Dubinsky et al., 1987). pH values were chosen based on the assumption that *Asparagopsis taxiformis* does not have an active CCM, so the rate of photosynthesis was expected to decrease with an increase in pH (Mata et al., 2007; Raven et al., 2002). Seawater was titrated with 0.1 M NaOH to raise the pH of the seawater to 8.2, 8.4, 8.6, and 8.9. For each pH treatment, 2 L of seawater was prepared, with 100 μL · L^{-1} of F/2 added. A dissolved oxygen optode (HACH LDO10101 Intellical LDO Lab Probe) was placed in a 265-mL clear, water-tight container with a magnetic stir bar and 265 mg of *A. taxiformis* for a density of 1 g · L^{-1}. The container was placed in a temperature-controlled water bath with built-in magnetic stir bars. The temperature was held at 21.0°C, and containers were exposed to 100 μmol photons · m^{-2} · s^{-1} PAR with programmable LED lights (Hydra 64 HD series). There were four replicates per pH treatment, and the seawater within each container was replaced between each run. To measure photosynthesis, oxygen was measured every 30 s for 10 min, and the rates were calculated as the change in O_2 per g WW of *A. taxiformis* per minute. Respiration was measured as a decrease in O_2 in the dark, during an initial 20-min dark incubation. The experiment was repeated twice. The goal of the experiment was to identify the specific point or pH range where CO_2 limitation had a significant effect on photosynthesis.

Utilization of multiple forms of DIC

An observational study was used to measure how much *Asparagopsis taxiformis* was able to raise the pH of seawater in a closed environment as a proxy for use of different forms of DIC, otherwise known as the pH drift technique (Maberly, 1990). As algae photosynthesize, they remove DIC from seawater; CO_2 is removed first, leading to an exponential rise in the pH of seawater until there is no remaining available CO_2, which typically occurs around a pH of 9. Algae with CCMs can utilize multiple forms of DIC and are able to continue drawing DIC from seawater

after the CO_2 has been depleted; thus, they are able to raise the pH above 9.0. Immediately following the CO_2 and temperature experiment, biomass from each of the nine environmental conditions were placed in 500-mL flasks at densities of 1 g · L^{-1}, with seawater reaching the top of the flasks. A magnetic stir bar was placed in the bottom of each flask to create flow within the flasks. A rubber stopper was placed in the top of each flask to prevent CO_2 exchange with the surrounding air. The flasks were placed in a temperature-controlled environment held at 22.5°C, with illumination of 150 μmol photons · m^{-2} · s^{-1} PAR (Hydra 64 HD series). Biomass was left in the light for 24 h. A pH electrode (HACH PHC101 IntelliCAL Lab pH Probe, calibrated with certified pH buffers of 7 and 10) was used to measure pH at time 0 and 24 h to determine initial and final pH within the closed vessel. The probe was left in each flask for 1.5 min to ensure stable readings were made. A maximum of nine samples were run at a time and the experiment was repeated three times (total $n = 27$).

Response variables

Growth rate

Algal growth rates were measured as the percent change in wet weight (WW) per week. To measure WW, the biomass was strained through a 100-micron mesh bag, gently squeezed, and pressed dry with paper towels until no more water could be extracted. The biomass was weighed to ±0.01 g using an analytical balance (METTLER Toledo AB 265-S/Fact Classic Plus). The growth rate (% increase in weight per week) was calculated using the following equation:

$$\text{Growth rate (\% increase in weight per week)} = \left(\frac{(W_f - W_i)}{W_i}\right) \times \left(\frac{7 \text{ days}}{L}\right) \times 100,$$

where W_f was the final weight (WW), W_i was the initial weight (WW), and L was the length of the experiment in days.

Bromoform concentrations

After WWs were recorded, 0.10 g of *Asparagopsis taxiformis* were placed in a 1.5-mL Eppendorf tube and immediately put into the deep freezer (−80°C). At the end of the experiment, the samples were packed in a shipping container with dry ice and sent to the Georgia Institute of Technology where the in-tissue bromoform concentrations were analyzed using standard techniques (Thapa et al., 2020). The samples were first freeze-dried and then soaked in MeOH for several hours before extraction with vigorous agitation on a vortex mixer. Samples were then centrifuged at 16,000 *g* for 30 min to remove debris, and an aliquot of supernatant was analyzed by gas chromatography/mass spectrometry (GC-MS; 1260G with 7890a MS; Agilent Technologies) in electron ionization (70 eV) mode using a DF-5 ms ultra-inert GC column (30 m length, 0.25 mm width, and 0.5 μM film thickness). The concentration of bromoform in *A. taxiformis* tissue was quantified based on calibration curves generated from a bromoform standard. The column temperature conditions were as follows: 40°C for 3 min, increased to 200°C at 10°C · min^{-1}, and held for 1 min with a total run time of 20 min. Injection port, interface, and ion sources were kept at 250, 300, and 230°C, respectively. Helium was used as carrier gas at a flow rate of 0.9 mL · min^{-1} (Thapa et al., 2020).

Dark adapted quantum yield

A diving-PAM fluorometer (Walz, Germany) was used to measure dark-adapted photosynthetic yield (*Fv*/*Fm*) as a proxy for photosynthetic efficiency. To do this, 0.2 g of biomass from each replicate were placed into a well of a clear 24-well culture plate (Kemtec™ 24-Well Microplates) with autoclaved seawater. Well plates were placed in the dark for 60 min, to allow for dark acclimation before measurements were taken. The diving-PAM was set to a saturation intensity of 8, pulse width of 0.8, measuring intensity of 8, damping of 2, and gain of 2. A special attachment on the end of the fiber optic cord allowed the tip to sit flush with the bottom of the well plate. All measurements were taken in the dark. The *Fv*/*Fm* measurement was taken with a background fluorescence value between 130 and 400.

Data analysis

A series of statistical analyses were used to determine the effect of each treatment across time on the different variables including growth rates, bromoform concentration, and photosynthetic efficiency. For those in which multiple measurements were taken from the same individual, a repeated measures multifactor analysis of variance (ANOVA) was used. Prior to analysis, Shapiro–Wilk's tests were used to test for normality; Cocran's test was used for variance homogeneity; and Mauchly's test was used to test for sphericity. Transformations were used when assumptions were not met as described below. All analyses were performed in R, using the rstatix package. The repeated measures multifactor ANOVA was run with the aov function. When significant effects were detected, a paired *t*-test or a one-way analysis of variance (ANOVA)-followed by a Tukey–Kramer

post hoc test was performed using the pairwise_t_test, aov, and HSD.test to explore function, respectively, to calculate the statistical significance between levels within one treatment type.

Temperature experiment

A two-way repeated measures ANOVA was used with temperature and time as fixed variables and sample ID as a random variable. A repeated measures ANOVA was chosen because the same cultures were tracked across multiple time points, with the density being reset at the end of each week. Growth rates were examined weekly over 3 weeks, and bromoform was measured at two time points. When a significant interaction term between time and temperature was identified, the data were subset by each time point, and a one-way ANOVA was used to test for temperature differences. These were both followed by a Tukey–Kramer post hoc test. We sought to identify the optimal temperature per week and determine how the cultures reacted to each temperature over time. A one-way ANOVA was used to analyze *F*v/*F*m, as this was only measured at the end of the experiment with temperature as a fixed effect.

CO_2 addition experiment

A multifactor repeated measures ANOVA was used with location, CO_2 use, and time as fixed effects and culture ID as a random effect. Due to interaction terms, the data were then separated by location and a two-way repeated measures ANOVA was run for each location separately, with time and CO_2 as the fixed effects. A paired *t*-test was used to identify in which week there was a significant CO_2 effect.

CO_2-availability and temperature experiment

A multifactor repeated measures ANOVA was run with CO_2, temperature, and time as fixed effects and culture ID as a random effect for growth rate and *F*v/*F*m. A two-way ANOVA was used to analyze the bromoform data, as these data were only collected at the end of the experiment. Significant effects were followed by a Tukey–Kramer post hoc test.

For the oxygen evolution experiment, a one-way ANOVA was used to identify the statistical significance of the different pH on rates of photosynthesis. This was followed by a Tukey–Kramer post hoc test. A two-way ANOVA was used to analyze if the environmental conditions in which the cultures were kept prior to the drift experiment had a significant effect on the final pH of the seawater.

RESULTS

Experiment 1: Temperature

Temperature had a significant effect on growth rate, photosynthetic efficiency, and bromoform concentration in *Asparagopsis taxiformis*. On average, the highest growth rates (64.25% increase in weight per week) were measured in the 21.4°C treatment. There were slightly lower growth rates in the 17.7 and 26.3°C treatments, followed by significantly lower growth in the extreme cold and warm treatments of 12.1, 15.0, and 31.6°C (Figure 1a).

There was a significant interaction between time and temperature on *Asparagopsis taxiformis* growth rates (Table 1). Between weeks 1 and 3, there were increases in the growth rates of cultures held at 17.7, 21.4, and 26.3°C and decreases in cultures held at 12.1, 15.0, and 31.6°C (Figure 1a). The 21.4°C treatment resulted in a statistically significant increase in growth of 68% between weeks 1 and 3 (Table S1 in the Supporting information). The 17.7 and 26.3°C treatments had slight non-significant increases of 32.0% and 24.5%, respectively. There were significant reductions in growth over time for both the 12.1 and 15.0°C (Table S1).

There was a significant effect of temperature on *F*v/*F*m, measured at the end of week 3 (Figure 1b, Table 1). The highest yield was recorded in the 26.3°C treatment, followed by the 21.4°C treatment (Figure 1b, Table S2 in the Supporting Information). *F*v/*F*m values were significantly lower at 12.1, 15.0, and 31.6°C than at the other temperatures (Table S2).

Bromoform concentrations in *Asparagopsis taxiformis* varied by over an order of magnitude from 0.18% to 3.74% DW, with the highest concentration measured in the 21.4°C treatment. Like growth rates, bromoform concentrations decreased at temperatures above and below 21.4°C, reaching a minimum concentration of 0.18% at 12.0°C. There was also a significant statistical interaction between time and temperature on bromoform concentration (Table 1). Although there was no significant difference in bromoform concentration at the end of week 1, changes in bromoform resulted in a statistically significant difference at the end of week 3 (Table S2). Bromoform concentrations in the 21.4°C treatment increased by 40% between week 1 and week 3, from 2.66% to 3.73% DW, while the bromoform concentration in the 15.0°C treatment, decreased from 1.81% DW to 0.28% DW between weeks 1 and 3 (Figure 1c, Table S1).

Experiment 2: CO_2 addition

The pH in the experimental flasks varied between 8.0 and 8.9 for the ambient conditions and between 7.6 and 8.3 for the CO_2-enhanced conditions (Figure S1 in the

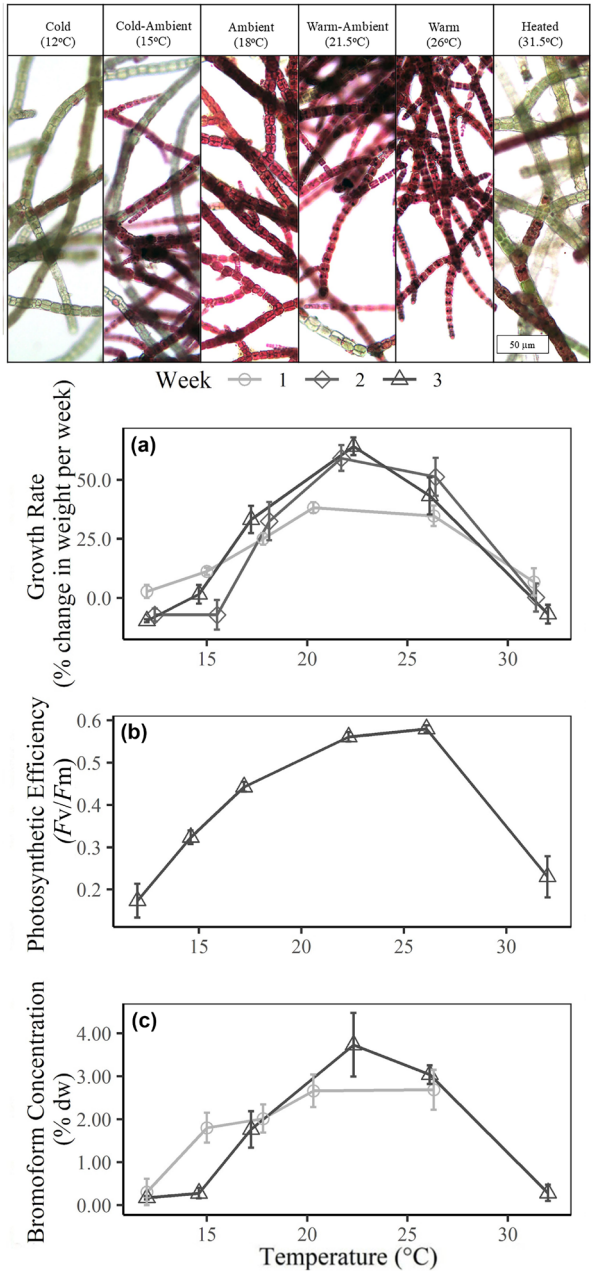

FIGURE 1 Results of the experiment exploring the effects of temperature (°C; *x*-axis) on (a) growth rate, measured by % change in wet weight per week, (b) average dark-adapted yield (*Fv*/*Fm*) at end of week 3, (c) bromoform concentration (% DW) in *Asparagopsis taxiformis* at the end of weeks 1 and 3. Shapes represent unique time points (weeks). Error bars represent standard error, $n = 3$. The photographs on the top show representative samples after 3 weeks of exposure to the different treatments.

Supporting Information). A slight increase in the daily maximum and minimum pH values was observed over the course of each week before returning to the initial values after a full water replacement and subsequent reduction in density.

Carbon dioxide availability within seawater had a significant effect on the growth rate of *Asparagopsis taxiformis*. Overall, cultures aerated with CO_2-enriched air had higher growth rates than those with atmospheric air (Figure 2a). The extent of this increase was initially dependent on the collection location of the algae (Table 2). On average, cultures from CI experienced 65% higher growth rates when CO_2 was supplemented, with the growth rate increasing from 44.6% per week without additional CO_2 to 72.8% with (Figure 2; Table S3 in the Supporting Information). Although there was a significant effect of CO_2 availability on the growth rates overall, the culture from MB showed no significant differences in growth between flasks with and without CO_2 addition during the first 2 weeks, with an average growth rate of 43.5% (Table S4 in the Supporting Information). This shifted during the third and fourth weeks when the growth rates of cultures with CO_2-enriched air increased by 61.1% compared with cultures with atmospheric air, which only increased by 34.6% (Figure 2). The growth rates at week 4 with CO_2 addition were similar between the MB and CI (61.1% vs. 66.9%, respectively). The growth rates in the ambient conditions did not vary between locations.

Unlike growth rates, the photosynthetic efficiency and bromoform concentration of *Asparagopsis taxiformis* did not change significantly with increased CO_2 availability (Figure 2b, Table 2). Bromoform concentration in *A. taxiformis* tissue varied from 1.5% to 4.85% DW across the treatments in this experiment. There was a significant effect of location on the bromoform concentration (Figure 2c, Table 2) where cultures from MB had, on average, significantly lower bromoform concentrations, at 2.32% ± 0.40% than the CI cultures, at 3.92% ± 0.24%.

Experiment 3: CO_2 addition and temperature

In the factorial experiment, both temperature and CO_2 availability had significant effects on the growth rates of *Asparagopsis taxiformis* (Table 3, Figure 3a), but there was no significant interaction. For temperature, the 19 and 26°C treatments resulted in similar average growth rates of 40% increased growth in weight per week, and the 22.5°C treatment had a lower growth rate of 34% (Table S5 in the Supporting Information). Cultures with ambient air had an average growth rate of 29.0%, whereas cultures aerated with CO_2 (pH of 7.9 and 7.7) had average growth rates of 41.4% and 45.1%, respectively, when averaged across all three temperatures (Figure 3). Although the addition of CO_2 resulted in a statistically significant increase in growth, there was no

TABLE 1 Mixed factor repeated measures ANOVA identifying the independent and combined effects of temperature and time on growth, *Fv*/*Fm*, and bromoform concentration in *Asparagopsis taxiformis* cultures from the temperature experiment.

Response variables		Variable	*df*	SS	MS	*F*	*p*-Value
Growth rate	Between subject	Temperature	5	37,500	7500	65.96	**<0.001**
		Error	18	2047	114		
	Within subject	Time	2	35	17.4	0.18	0.834
		Temperature: Time	10	3602	360	3.80	**0.001**
		Error	36	3415	94.9		
Fv/*Fm*		Temperature	5	579,233	115,847	38.38	**<0.001**
		Residuals	18	54,332	3018		
Bromoform	Between subject	Temperature	4	48.72	12.179	12.14	**<0.001**
		Error	15	15.05	1.003		
	Within subject	Time	1	0.094	0.0941	0.52	0.481
		Temperature: Time	4	7.263	1.8159	10.09	**<0.001**
		Error	15	2.699	0.1799		

Note: *p* values in bold identify significant effects at the $p < 0.05$ level.

statistically significant difference between the two CO_2 enrichment treatments (Table S5).

Although there was no significant effect of temperature on the *Fv*/*Fm* measurements, there was a significant effect of CO_2 availability, with a decrease in *Fv*/*Fm* with CO_2 addition (Figure 3b, Table 3). There was also a significant effect of time, with an average increase of 12.5% in *Fv*/*Fm* measured between the end of weeks 1 and 3 (Table S5). Bromoform concentrations in *Asparagopsis taxiformis* tissue ranged from 0.63% to 1.44%, with an average concentration of 1.07% at the end of week 3. However, there were no significant effects of CO_2 availability or temperature on the bromoform concentration in this experiment (Figure 3c, Table 3).

Short-term response to pH variability

Short-term exposure to pH variability had a significant effect on photosynthesis. Specifically, there was a significant reduction in the rate of oxygen production as pH increased (Table S6 in the Supporting Information). The maximum recorded O_2 production, at 0.085 mg $O_2 \cdot g^{-1}$ WW $\cdot$ min^{-1} occurred at a pH of 8.25. There was a significant reduction in O_2 production at pH values above 8.6. At the highest pH of 8.95, there was a 70% reduction in O_2 production compared to the rate at a pH of 8.25 (Figure S2 in the Supporting Information).

Utilization of multiple forms of DIC

After exposing *Asparagopsis taxiformis* biomass to light for 24 h in sealed chambers, we found that pH in the tanks changed from 8.0 to final pH values of between 8.8 and 8.9. There was no significant effect of prolonged CO_2 addition or temperature exposure on the final pH of the closed cultures (Table S6).

DISCUSSION

There is increasing need to better understand the biology and physiology of the red seaweed *Asparagopsis taxiformis* due to the growing interest in large-scale farming of this species as a methane-mitigating supplement for the livestock industry. Understanding how key factors such as temperature and CO_2 availability affect performance are the first steps toward developing successful cultivation practices. In addition to exploring how environmental conditions affect growth, there is a unique opportunity to determine what, if any, cultivation conditions influence the production of the methane-mitigating bioactive compound bromoform. Here we have reported on the independent and combined effects of temperature and CO_2 availability on *A. taxiformis* performance from Southern California. Manipulation of environmental conditions within our experiments resulted in significant changes in growth rates (% change in weight per week) from 10%–88% and bromoform concentrations (% DW), which ranged from 0.18% to 4.85% and which fall within, if not on the high side of, bromoform concentrations previously reported in the literature (Marshall et al., 1999; Mata et al., 2012, 2017; Paul et al., 2006; Thapa et al., 2020). These results may help identify thermal thresholds and optimal temperatures for growth of this seaweed in California as well as provide some new insights into how bromoform concentrations may be manipulated in cultivation settings to produce high-quality seaweed biomass.

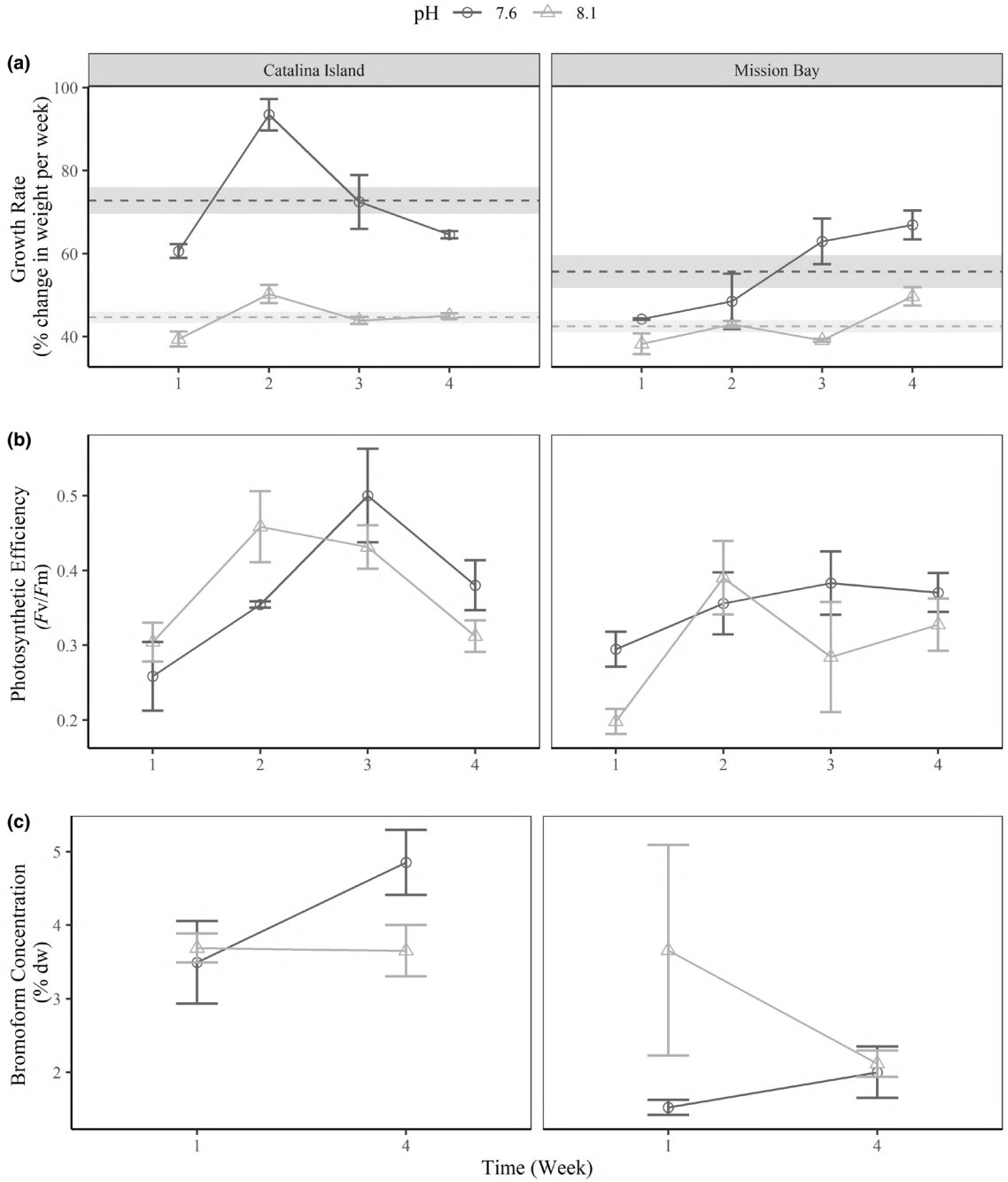

FIGURE 2 Results of the experiment exploring the effect of CO_2 availability on (a) growth rate, measured by % change in wet weight per week, (b) average dark-adapted yield (*Fv*/*Fm*) at the end of each week, (c) bromoform concentration (% DW) in *Asparagopsis taxiformis* at the end of weeks 1 and 4. Shapes represent the pH of seawater. Error bars represent standard error, $n = 3$. The dashed line represents the average growth rate across the 4 weeks, with the shaded area representing the standard error $n = 12$.

Temperature

The response of *Asparagopsis taxiformis* to changing temperatures can be grouped into four categories: optimal, suboptimal, long-term lethal, and short-term lethal. Our results suggest that the optimal temperature range for *A. taxiformis* from Southern California is 22–26°C, which is typical for a warm-temperate species, (Tom Dieck (Bartsch) & de Oliveira, 1993) yet surprising given that average monthly temperatures in Southern California range from 15 to 20°C (Fumo et al., 2020). Cultures kept in these temperatures displayed the highest growth rates, bromoform concentrations, and photosynthetic efficiencies. Further, we saw evidence of acclimation over time: There was a significant increase in growth rates between weeks 1 and 2, followed by a minor increase between weeks 2 and 3. Interestingly, bromoform concentrations showed very similar patterns

TABLE 2 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 the CO_2 experiment.

Response variables		Variable	*df*	SS	MS	*F*	*p*-Value
Growth rate	Between subject	CO_2 Availability	1	5112	5112	245.66	**<0.001**
		Location	1	1111	1111	53.41	**<0.001**
		CO_2 Availability: Location	1	673	673	32.34	**<0.001**
		Error	8	166	21		
	Within subject	Time	3	1201.1	400.4	11.473	**<0.001**
		CO_2 Availability: Time	3	299.6	99.9	2.862	0.058
		Location: Time	3	1355.7	451.9	12.95	**<0.001**
		CO_2 Availability: Location: Time	3	584.9	195	5.587	0.005
		Error	24	837.5	34.9		
Fv/*Fm*	Between subject	CO_2 Availability	1	6840	6840	1.816	0.215
		Location	1	29,205	29,205	7.753	**0.024**
		CO_2 Availability: Location	1	8802	8802	2.337	0.165
		Error	8	30,136	3767		
	Within subject	Time	3	137,470	45,823	8.992	**<0.001**
		CO_2 Availability: Time	3	39,747	13,249	2.6	0.076
		Location: Time	3	30,083	10,028	1.968	0.146
		CO_2 Availability: Location: Time	3	11,147	3716	0.729	0.545
		Error	24	122,301	5096		
Bromoform	Between subject	CO_2 Availability	1	0.583	0.583	0.747	0.412
		Location	1	15.282	15.282	19.61	**0.002**
		CO_2 Availability: Location	1	3.978	3.978	5.105	0.054
		Error	8	6.234	0.779		
	Within subject	Time	1	0.025	0.025	0.018	0.896
		CO_2 Availability: Time	1	4.363	4.363	3.162	0.113
		Location: Time	1	2.124	2.124	1.539	0.250
		CO_2 Availability: Location: Time	1	0.146	0.146	0.106	0.753
		Error	8	11.04	1.38		

Note: *p* values indicated in bold identify significant effects at the $p < 0.05$ level.

to growth responses with the highest concentrations observed in cultures growing at their optimal temperature. The sub-optimal temperatures of 17.7 and 22.0°C had both lower growth rates and bromoform concentrations. This was likely due to a decrease in enzyme activity, which can lead to reductions in photosynthesis and nutrient uptake (Zanolla et al., 2015). The other treatments ultimately lead to mortality and could be categorized as either long- or short-term lethal responses. At around 15°C, the negative effects of temperature stress were apparent only after 1 week of exposure, when the algae gradually began to decline in health, bleach, and die. In the extreme conditions, 12 and 31.0°C, the algae showed significant signs of stress or cell death within the first week of exposure. Signs of stress included a significant reduction in pigmentation, lack of growth, low photosynthetic efficiency, and/or a reduction in bromoform concentration.

In general, our data followed a standard thermal response curve shown by most species of algae where growth and photosynthetic rates increase with increasing temperature until the maximum is reached, at which point values then begin to drop rapidly (Eggert, 2012). The mechanism that causes a decline in algal growth and health differs between hot and cold thermal stress. 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 reactive oxygen species (ROS), which can cause cell damage and death (Allakhverdiev et al., 2008). Stress response to low temperatures can include a decrease in membrane fluidity, reduction in enzyme activity, and damage to the pigment-protein complexes needed for photosynthesis (Eggert, 2012).

The results of our temperature experiment were similar to one by Padilla-Gamiño and Carpenter (2007), who observed that the gametophytes of *Asparagopsis taxiformis* from California experienced high-temperature inhibition above 30°C as well as lethal stress between 10 and 15°C, as measured by a significant reduction in

TABLE 3 Mixed factor repeated measures ANOVA identifying the independent and interactive effects of temperature, CO_2 availability, and time, on growth, *Fv*/*Fm*, and bromoform concentration in Experiment 3.

Response variables		Variable	*df*	SS	MS	*F*	*p*-Value
Growth Rate	Between subject	CO_2 Availability	2	3837	1918.4	104.51	**<0.001**
		Temperature	2	731	365.7	19.92	**<0.001**
		Temperature: CO_2 Availability	4	192	48.1	2.62	0.069
		Error	18	330	18.4		
	Within subject	Time	2	745.1	372.5	11.694	**<0.001**
		CO_2 Availability: Time	4	236	59	1.852	0.140
		Temperature: Time	4	98.9	24.7	0.776	0.548
		Temperature: CO_2 Availability: Time	8	159.2	19.9	0.625	0.751
		Error	36	1146.9	31.9		
Fv/*Fm*	Between subject	CO_2 Availability	2	25,091	12,546	2.573	0.104
		Temperature	2	594	297	0.061	0.941
		Temperature: CO_2 Availability	4	4374	1094	0.224	0.921
		Error	18	87,757	4875		
	Within subject	Time	2	26,871	13,435	4.186	**0.023**
		CO_2 Availability: Time	4	16,241	4060	1.265	0.302
		Temperature: Time	4	13,060	3265	1.017	0.412
		Temperature: CO_2 Availability: Time	8	25,017	3127	0.974	0.471
		Error	36	115,550	3210		
Bromoform	Between subject	CO_2 Availability	2	0.011	0.006	0.218	0.806
		Temperature	2	0.033	0.016	0.636	0.541
		Temperature: CO_2 Availability	4	0.213	0.532	2.061	0.129
		Residual	18	0.465	0.026		

Note: *p* values indicated in bold identify significant effects at the $p < 0.05$ level.

Fv/*Fm*. However, that study 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-Gamiño and Carpenter's (2007) study was freshly harvested, the *A. taxiformis* was likely less affected by culture conditions. The seaweed used in our experiments had been isolated in common garden conditions for over 6 months prior to the experiment, as described above. Since tolerance to variability in temperature can depend on local conditions and gradual acclimation to those conditions, it is possible the mere act of cultivation in controlled laboratory conditions could limit the optimum temperature range of *A. taxiformis* (Nejrup et al., 2013). Perhaps this is why we observed the long-term stress response in cultures kept at 15°C, a temperature that falls within the annual temperature range of San Diego, CA where our samples were collected. The extent to which lab acclimated conditions affect the ability of *A. taxiformis* to survive at different temperatures in the wild is unknown but is an important question for better understanding how thermal tolerance will change over time in an aquaculture setting.

Interestingly the temperature and CO_2-enrichment factorial experiment identified the importance of short-term temperature variability on the growth of *Asparagopsis taxiformis*. It was hypothesized that the highest growth would be at 22.5°C, as that was within the optimum temperature range identified in the first experiment. However, the 22.5°C treatment resulted in the lowest average growth rate of the three temperature treatments. The temperature data from the three treatments showed that the 22.5°C treatment had the largest variability in temperature over the course of the experiment due to the nature of mixing the ambient and warmed seawater sources in the experimental water bath (Figure S3 in the Supporting Information). Although the temperature did not get above 26°C or below 19°C, i.e., temperatures previously shown to result in higher productivity, there were several large fluctuations during the first week of the experiment. As such, it is possible that this variation in temperature resulted in stress and a reduction in growth rates. Further research is needed to disentangle the effects of temperature variability on algal stress and growth rates.

Although little is known about the factors that induce or enhance the production of the bioactive compound bromoform in seaweed, here we observed a clear positive correlation between growth rate and bromoform production. Specifically, we observed the highest concentrations of bromoform in the fastest-growing

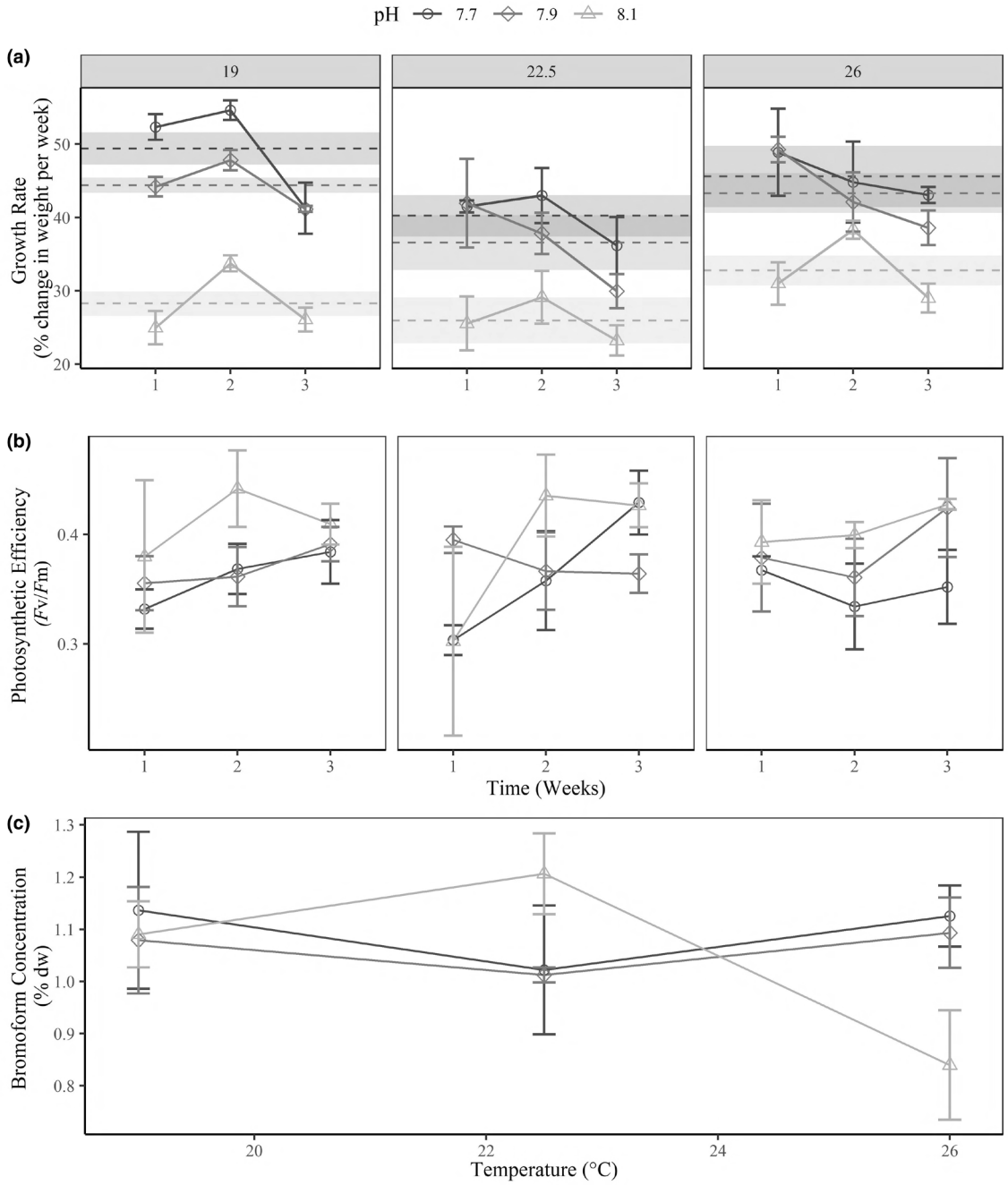

FIGURE 3 Results of factorial experiment exploring the interaction between CO_2 availability and temperature on (a) growth rate measured by the % change in wet weight per week (b) average dark-adapted yield (*F*v/*F*m) at the end of each week, (c) bromoform concentration (% DW) in *Asparagopsis taxiformis* at the end of week 3. Shapes represent the pH of the seawater. Error bars represent standard error, $n = 3$. The dashed line represents the average growth rate across the 3 weeks, with the shaded area representing the standard error, $n = 9$.

seaweeds cultivated under the optimal temperature range. There was also a significant increase in bromoform across time in the optimal temperature range and a significant reduction in bromoform in algae grown within the lethal ranges. Since bromoform concentrations were only measured twice, it is unclear if concentrations within the optimal temperature range had reached their maximum or were still increasing. Past research has documented a correlation between temperature and bromoform in *Asparagopsis taxiformis* from Townsville and Caloundra, Australia, with the highest bromoform concentration measured at 20.2°C and the lowest at 28.1°C (Mata et al., 2017). Although that study reported a linear relationship between bromoform content and temperature, it did not explore the more extreme temperatures tested in our study. It remains unknown if temperature has a direct effect on bromoform production (or release) in seaweed, but our

results suggest that for *A. taxiformis*, growth and bromoform concentration are correlated and are highest when the seaweed is cultivated under optimal temperature conditions.

CO_2 addition

We explored the potential impacts of CO_2 enrichment on *Asparagopsis taxiformis* when grown under ambient conditions and observed a significant increase in growth of cultures with CO_2-enriched air as opposed to cultures exposed to atmospheric air. During the CO_2-enrichment experiment, pH was measured continually within the flasks with and without CO_2-enriched air. These data show large daily variation, with increasing pH during the light as the CO_2 was used via photosynthesis and a decrease during the dark as the stores of CO_2 were replenished due to respiration and flask aeration. The daily variation in pH within the ambient conditions, which stabilized at 8.1 without biomass, resulted in the cultures inhabiting seawater with a pH above 8.6 for 29.4% of the daytime photoperiod. As seen with the O_2-evolution data, 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 algae was likely limited by CO_2 availability for up to 3.6 h per day when grown at a density of 1 g · L^{-1}. This period of limitation increased with increased density. 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 CO_2-enriched air allowed for at least 30% more growth than in the ambient conditions.

The results of the temperature and CO_2-factorial experiment suggest that *Asparagopsis taxiformis* used in this study lacked an efficient CCM, as increasing the CO_2 concentration resulted in a significant increase in growth rate. This was supported by the pH drift measurements, which showed that *A. taxiformis* was unable to raise the pH of surrounding seawater above 8.9, thus indicating that *A. taxiformis* is likely unable to utilize alternative forms of DIC. It is important to remember that while the results suggest the lack of a CCM mechanism, Mata et al. (2007) identified the presence of a CCM with low affinity for HCO_3 in *A. armata*, which helped to supplement CO_2 at pH values between 7.5 and 8.00; however above 8.00 the CCM could not provide enough CO_2 to meet the organism's demand, causing the alga to become carbon limited. Although understanding the CCM in *A. armata* may provide insight into the carbon uptake strategy of *A. taxiformis*, CCMs can vary between species and even within morphologies of the same species (Wang et al., 2019). Since a low-affinity CCM would not have been identified in the pH drift measurements used here, more research is needed to confirm the presence and identification of a CCM in *A. taxiformis*.

Although temperature had a clear effect on photosynthetic efficiency of *Asparagopsis taxiformis*, there were no significant effects of increased CO_2 availability. This is not altogether surprising, given that there is no universal consensus on the effect of CO_2 on photosynthetic efficiency of algae, with one study (Connell & Russell, 2010) having reported a 5% increase in *Fv*/*Fm* under elevated CO_2 conditions and another (Yıldız, 2018) having reported a reduction in *Fv*/*Fm* when grown at a pH of 7.7. Increased growth in algae is not always coupled with increased photosynthesis under elevated CO_2 conditions (Ho et al., 2020). Ho et al. (2020) identified that the photosynthetic efficiency of algae species that rely primarily on direct diffusion of CO_2 from surrounding seawater were less affected by increased CO_2 availability than those with an efficient CCM, similar to our findings. Although no positive effects were observed, there were also no negative effects caused by the decrease in pH on the algae; rather, the addition of CO_2 simply alleviated a limiting resource on growth.

Implications for population distribution

The results generated from this study provide insight into the current and possible future distribution of *Asparagopsis taxiformis* in Southern California and beyond. As interest in cultivation of *A. taxiformis* grows, understanding its natural biogeographic range will aid in identifying optimal locations for land-based aquaculture. Globally, this species has a tropical to subtropical distribution. Although not thoroughly studied, data suggest that Southern California represents the northern limit of its eastern Pacific distribution (Andreakis et al., 2009), with historical herbarium collections reaching as far north as Santa Barbara Island in the Channel Islands (Jepson Herbarium). Here, we have identified cold temperature stress starting at 15°C, a temperature often recorded in the ocean in Southern California between January and March. Thus, it is likely that these colder temperatures have restricted *A. taxiformis* to Southern California and Baja California (Eggert, 2012). Although the seasonal distribution of sporophytes of *A. taxiformis* has not been studied in San Diego, our results suggest that the population likely declines in the winter due to temperature stress and rebounds in the spring before reaching its maximum population size during the summer, when temperatures are more consistently within the identified optimal temperature range (Eggert, 2012).

Our results also provide insight into the potential effects of climate change on the future distribution of *Asparagopsis taxiformis* in Southern California. The conditions used in the factorial experiment simulated

conditions in San Diego under different climate change scenarios, including current conditions, Representative Concentration Pathways (RCP) 6.0, and the RCP 8.5 "business as usual" model (IPCC, 2019). It has been hypothesized that an increase in acidification would result in increased stress at the edges of the optimal thermal range as reported in other algae (Ho et al., 2020). However, this was not observed for any of the response variables measured in our study. Thus, these findings indicate that *A. taxiformis* will likely be unharmed or may even be positively impacted by global change in California. As temperature and OA increase, it is likely that *A. taxiformis*, like other subtropical species (Kübler & Dudgeon, 2015), will migrate farther away from the equator and possibly become more prolific in its current range, as growth rates may increase with warming (to a point). This is especially true with lineage 2, which has been shown to have high thermal plasticity and adaptability (Dijoux et al., 2014; Zanolla et al., 2015). In California, we may expect to see *A. taxiformis* move northward past the southern Channel Islands under expected future climate change resulting in what is considered a "climate change creep."

CONCLUSIONS

The results of this study suggest that optimizing both temperature and CO_2 availability are critical for developing a successful cultivation environment to ensure growth rates and bioactive compound production are optimized for *Asparagopsis taxiformis*. Although the optimal temperature range for *A. taxiformis* from Southern California is between 22 and 26°C, cultures were able to survive in temperatures ranging between 18 and 26°C. Since algae have been shown to recover from short-term temperature stress, *A. taxiformis* would likely survive short periods of exposure to more extreme temperatures, such as 15°C (Eggert et al., 2003). This large thermal range is advantageous for aquaculture, as temperature control can be extremely costly. However, it is likely that thermal optima will differ for *A. taxiformis* in other regions, as more tropical populations likely have narrower ranges of thermal tolerance. Overall, the results of our CO_2-enrichment experiments indicated that although *A. taxiformis* might not be limited under normal open ocean conditions, it will likely be limited by CO_2 in batch culture systems, independent of temperature. To maximize the growth rate of *A. taxiformis* in culture, especially cultures with low seawater exchange rates and grown at high density, CO_2 will need to be supplemented to keep the pH below 8.6 and ensure positive growth rates. The addition of CO_2 can account for a major increase in the operational costs of growing algae, and if it is not done correctly, then it could decrease the sustainability and effectiveness of *A. taxiformis* to mitigate methane emissions in ruminants (Mata et al., 2007). Other strategies to increase CO_2 concentration should be explored, such as reuse or recycling from other industries, such as from power plants, or using effluent from fish farms (Mata et al., 2007). Unlike growth, in-tissue bromoform concentrations appear to be less directly affected by temperature and CO_2 availability. Although there appeared to be a strong correlation between growth and bromoform concentration in the temperature experiment, this did not hold up in the CO_2 or factorial experiment, indicating that bromoform concentration is likely correlated to physiological status rather than to growth rates alone.

This study provides insight into the temperature and carbon requirements of *Asparagopsis taxiformis* in California, but more research is needed to better understand how this species will respond to changing environmental conditions in the wild, as well as to optimize growth conditions for large-scale aquaculture. Understanding the ideal environmental parameters is critical for producing large quantities of *A. taxiformis* with high bromoform concentration. It is still unclear whether the environmental benefits associated with using *A. taxiformis* as a methane-mitigating agent in livestock will outweigh the resources required to mass produce seaweed biomass in aquaculture settings. 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 emissions reductions. Nonetheless, the red alga *A. taxiformis* is on track to be the first commercially available methane-mitigating supplement for ruminants, making it an exciting ocean-based solution for fighting climate change.

AUTHOR CONTRIBUTIONS

Hannah M. Resetarits: Conceptualization (lead); data curation (lead); methodology (lead); project administration (lead); writing – original draft (lead); writing – review and editing (equal). **Gal Dishon:** Conceptualization (supporting); data curation (supporting); methodology (supporting); writing – review and editing (supporting). **Vinayak Agarwal:** Data curation (supporting); methodology (supporting); writing – review and editing (supporting). **Jennifer E. Smith:** Data curation (supporting); formal analysis (supporting); funding acquisition (equal); methodology (supporting); writing – review and editing (equal).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. 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 *Asparagopsis taxiformis* at a density of 1 g · L^{-1}. The shaded area represents the suboptimal conditions for the maximum photosynthetic rate.

Figure S2. Oxygen evolution experiment, the effect of pH on the corrected P_n, normalized for an initial pH of 8.2. Error bars represent the standard error of replicates. Letters represent significant differences, $n = 8$.

Figure S3. Fluctuation of temperature in the three temperature treatments for the factorial experiment. Dashed line represents average temperature, shaded

area represents standard deviation of temperature over the 3-week experiment.

Table S1. One-way ANOVA identifying the effect of temperature within each week of the temperature experiment, with a Tukey–Kramer post hoc test to identify significance between different temperature treatments. *p* values indicated in bold identify significant effects at the $p < 0.05$ level.

Table S2. One-way ANOVA identifying the effect of time within each temperature treatment of the temperature experiment, with a Tukey–Kramer post hoc test to identify significance across time. *p* values indicated in bold identify significant effects at the $p < 0.05$ level.

Table S3. Mixed factor repeated measures ANOVA identifying the independent and interactive effects of CO_2 availability and time on growth, *F*v/*F*m, and bromoform concentration in the CO_2 experiment. *P* values in bold identify significant effects at the $p < 0.05$ level.

Table S4. Paired *t*-test analyzing the statistical difference in growth between treatments with and without CO_2 addition within the different locations at each time point, in the CO_2 experiment.

Table S5. One-way ANOVA on isolated variables from the factorial experiment, followed by a Tukey–Kramer post hoc test, to identify significance between treatments of the same variable. Identifying the effect of CO_2 availability, temperature, and time throughout the experiment.

Table S6. One-way and mixed factor ANOVA identifying the effect of pH on respiration and the effect of temperature and CO_2 availability on the uptake of CO_2, respectively.

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