The Effect of Salinity on the Growth of the Red Alga, *Gracilaria epihippisora*

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1. Abstract

Gracilaria is a diverse genus of red algae that has commercial and cultural significance in Hawai'i (Nelson et al., 2001). *Gracilaria epihippisora* is a rare species that was first described in 1977 by Hoyle and is possibly endemic to Hilo, Hawaii. This species has been observed growing in low salinity waters, making it potentially applicable for aquaculture purposes. This experiment will attempt to determine the optimal salinity for *G. epihippisora* growth by cultivating samples in flasks in a range of salinities. Thalli grew fastest in flasks containing full strength seawater and successively slower in flasks of lower salinity seawater.

2. Introduction

Specimen description

Gracilaria epihippisora is a marine alga endemic to Hawaii with specimens repeatedly recorded from Liliuokalani gardens in Hilo, Hawai'i Island (Hoyle, 1977; Abbott, 1999). This species has growth forms that are similar to other *Gracilaria* species; however, it is unique in that all sexual phases are exhibited in its preferred brackish habitat (Abbott, 1999). Plants are 3-18 cm tall, axes are more than 3 mm in diameter, and thalli exhibit sub-dichotomous branching to 2-3 orders. Similar species include: *G. salicornia,* which grows in low tangled clumps and has different male reproductive morphology; *G. coronopifolia* has more orders of branching, does not tolerate brackish water and is thinner; *G. parviospora* is more flexible, larger, and noticeably thinner towards the apices (Abbott, 1999). Gracilaria *epihippisora* and other native Hawaiian *Gracilaria* species were studied for their agar composition by Santos and Doty (1983). *Gracilaria epihippisora* was found to have fair gelling, palatability, and overall agar quality when collected from the wild.

Salinity

Salinity was the variable tested in this study because of natural fluctuations in anchialine pools, replicable research done on other species, and the applicability of information for research or commercial purposes. Salinity is the dissolved salt content of water, and is a key factor in the survival of marine plants (Kumar et al., 2010). Salinity is often the major constraint on habitat with gradients marking the boundaries between various ecosystems (Maciolek and Timbol, 1981). Salinity causes osmotic pressure in and outside of plant and animals cells, causing them to swell or lost turgor pressure (Kumar et al., 2010). These conditions put constraints on the growth of species.

Background nutrients in groundwater

Pore water of the Hawaiian Islands is seen along the coasts as fresh water emerging from rocks and sand along the coastline. These freshwater inputs are the results of rain and mist on the mountain making its way for decades, in some cases, through the rock along underground channels, accumulating any pollutants that seep into the subterranean stream (Knee et al., 2010). Along the Kona coast of Hawai'i Island, nutrients such as nitrates and phosphates have been detected in ground water and in Anchialine pools. Although beneficial to plant growth, these nutrients can sometimes be in extremely high quantities and have been shown to be detrimental to coral growth and spur macro-algal blooms on reefs adjacent to fresh submarine freshwater discharge (Knee et al., 2010). These nutrients are subject to fluctuations temporally and can alter the ecology of the anchialine pools they are introduced to. These nutrients have to be taken into consideration when designing experiments involving species that live in anchialine pool environments.

Habitat

Anchialine pools are bodies of water that have connections to both the sea and fresh groundwater, but lack a surface connection to the sea (Knee et al, 2010). These pools are the evidence of subterranean seawater intrusion into land and are habitat to a wide variety of native flora and fauna (Marrack, 2015). Salinity between pools can be highly variable due to distance from the ocean, porosity of the rock, flow rate of pore water, and presence of surface seawater input (Knee et al., 2010). Liliuokalani Gardens is not a true anchialine pool because it has at high tide connections to the ocean, but is similar because the connected pools can become isolated. Salinities range from close to full strength seawater at high tide in pools adjacent to ocean entry to almost pure freshwater at low tides where freshwater input is strongest.

Salinity in anchialine pools is highly variable by location, thus salinity was sampled in the pool were *G. epihippisora* growth was the found. Salinity ranged from 30ppt at high tide to 4ppt at low tides, and was even exposed to the air during extreme low tides. Exposed areas were also observed to have natural springs of very cold fresh water seeping from the rocks adjacent to algae suggesting a tolerance for fresh water for at least short periods of time. Freshwater input into Liliuokalani Gardens has been observed to fluctuate over time, with activity of groundwater markedly higher in the days after large rainfall events.

Species distribution

Liliuokalani Garden's low salinity pools represent the only known habitat of *G. epihippisora* due to the connection to the ocean and unique water characteristics. It is crucial to know the acceptable habitat for this species, in the case the only two known locations it is found are threatened, the species could be transplanted to preserve the species. Due to the limited range of species distribution, it may be necessary in the face of sea level rise to find other suitable habitat for *G. epihippisora* to ensure the continuation of the species. In the future, the salinity of Liliuokalani Park is predicted become progressively more saline along with other coastal pools (Marrack, 2015). Anchialine pools are also threatened by development throughout Hawaii due to their location near coastlines (Knee et al, 2010). Future growth along the coastlines of Hilo is expected and may alter ground water and anchialine pools to the detriment of endemic organisms.

Aquaculture potential

Aquaculture of seaweeds provides relief for some of the growing concerns about land and water use for agriculture globally. Research involved with farming seaweeds is crucial to meet future needs. *Gracilaria* is among the most cultured seaweeds in the world and is economically important to countries in Southeast Asia (Redmond et al., 2014). With the emerging techniques of hybridizing algae, all species must be carefully considered for aquaculture potential. Species such as *G. epihippisora* that can thrive in low salinity, high nutrient waters may have significance to the aquaculture and wastewater treatment industries. Growing interest in natural methods of waste processing has lead commercial interests to growing macro-algae tolerant to wide varieties of water quality (Zhou et al., 1998).

3. Objectives

The objectives of this experiment are to increase the basic biological knowledge for the relatively unknown species *G. epihippisora*. This project tests the effects of salinity on *G. epihippisora* growth and morphology. Parameters of salinity tolerance are crucial if this species is to be cultured or transplanted.

4. Methods & Materials

Study site

Liliuokalani Gardens in Hilo are a sprawling 30-acre, Japanese garden with pools, rock gardens, traditional teahouses and exotic trees. The park was commissioned a century ago to commemorate the life of Queen Liliuokalani. The park is separated from the ocean by a wall surmounted by a road and sidewalk. At two places, tunnels have been installed to allow the passage of seawater with the tides. The wall is porous in many other sections, and seawater can be seen entering the pond on the rising tide. The ponds has strong input of ground water (on the northern border of the park in particular) that can be seen at low tide, moving across the muddy, exposed bottom of the pond or bubbling from cracks in the basalt. The small isolated ponds on the northern edge of the park are the only place where *G. epihippisora* can be found. These northern ponds have irregular, basaltic edges that entrain fine sediments and prevent proper circulation. Areas of low water movement are where the density of algae is the greatest. In the shadow of a mango tree, there is one pond in particular that hosts to the majority of the population. This shaded pond it is near a saltwater inlet for a series of three connected pools, a large basalt rock in the middle pool greatly reduces circulation and clogs the area with fallen leaves. It is in here, tangled amongst the deteriorating vegetation and anaerobic sediments that *G. epihippisora* is the most prolific.

Experimental design

The project was designed to test the tolerance of different salinities by *G. epihippisora*. Salinities tested were chosen to mimic the natural conditions that the algae would experience in the natural habitat. Culture flasks were stocked with a known quantity of algae that was weighed and observed for qualitative characteristics including color, growth, etc. Each salinity had three replicates that were measured for growth weekly by draining the flasks removing dead material, blotting dry the thalli, and weighing their mass. Every three weeks, all material was replaced was replaced with new algae that had been held in 25ppt seawater mixture. The new algae was used to return to the starting weight of 5.0-5.5g.

<u>Culture vessel</u>

Cultures were kept in 1 L Erlenmeyer flasks filled with filtered seawater and filtered freshwater. Flasks were kept on a windowsill-facing east, allowing for at least four hours of direct sunlight, a condition similar to the natural habitat. A 1.8 L flask stocked with 25ppt seawater served as a reservoir of seaweed in the event that more biomass was needed. Flasks had their mouths covered with Parafilm to limit evaporation and contain spray from added aeration. Parafilm coverings were perforated with two holes, one to allow for insertion of ¼" vinyl airline tubing and one to allow for gas escape.

<u>Water source</u>

Water was supplied from saltwater wells located at the Pacific Aquaculture and Coastal Resources Center (PACRC). Salinity of well water was 32ppt and was diluted with deionized freshwater to create media with salinities of 32, 20, 15, and 10ppt.

<u>Aeration</u>

Air was supplied with a Tetra Whisper 60-aquarium air pump. Air was fed into a closed loop from each of the two outlets on the pump. From the closed loop "T" junctions were placed at intervals that allowed for an airline for each flask. Air loading was controlled using plastic needle valves on each flask's airline; loading rate was significant enough to induce circulation in the flasks but not enough to agitate the samples.

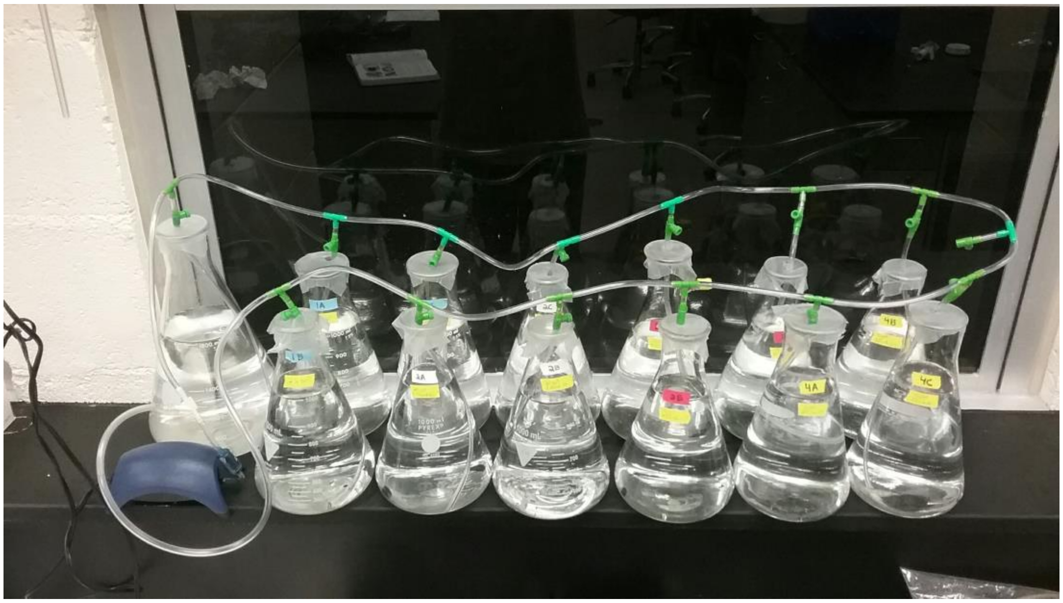

Figure 1. Algae culture system, aerator on left supplies air in a closed loop. Branches from the loop supply a controlled amount of air to each individual flask.

Weighing

Weight was taken from samples prior to stocking with an Ohaus Adventurer balance (sensitive to 0.01g). Samples prior to growth trials were taken from plants that had been sitting in full strength seawater for at least a day. Plants were separated to individual thallus in order choose similar size thalli for each replicate. Material was collected from a holding container with a small net and placed gently into dry paper towels. The towels were then balled loosely around the plants and shaken vigorously for several seconds to remove excess water. Plants were combined for a mass of 5.0-5.5 g per 1 L flask and then gently pushed into flasks. At the end of each week, water was drained from flasks through a small net to catch algal fragments. Larger pieces of algae were removed with a large pair of tweezers. Blotting procedure was repeated and algae placed in a weigh boat on the balance. This procedure was repeated weekly to ascertain change in mass.

Sample collection and preparation

Gracilaria epihippisora was identified in the field, gently rinsed with anchialine pool water to remove loose and clumped sediment, then placed into a 4 L jar. The jar was filled with 2 L seaweed and 2 L pond water, and then vigorously shaken. The jar was shaken for 30 second intervals then emptied of water and refilled with clean water, this process was repeated until no further detritus was removed, and the drained water was clear. The cleaned algae were then placed into a chilled cooler lined with moist paper towels and transported back to the lab for processing.

Samples upon arrival were loosely packed into 2 L jars and the rinsing process repeated with fresh water. The algae was then individually untangled and removed of encrusting animals and plants with a pair of tweezers and Q-tips. Algae was then packed into 4 L jars and then filled with 1:1000 bleach to freshwater mixture and allowed to sit for 20 minute intervals. Bleach treatment was repeated 3 times over the course of an hour. It was observed that bleach had a damaging effect on encrusting animals and plants, where bubbles would form along the length of the thallus especially where foreign animals were clearly visible. By the end of the 1hr treatment, areas that were previously discolored by epifauna or encrusting algae turned white. The growing tips of *G. epihippisora* after one hour would also begin to turn white. If this was observed in more than a couple thalli, the treatment was cut short at to not unduly harm the algae of interest. During periods before and after bleach treatment, algae were kept in darkness submerged in 25ppt media supplied with air.

<u>Water change</u>

Water was changed completely with each weekly weighing session. Flasks were scrubbed with fresh water, rinsed thoroughly and refilled with appropriate salinity water.

<u>Analysis</u>

Statistical comparisons were conducted using ANOVA analysis through the computer program Minitab. This test allows for statistical clarification on differences of growth rate in the various concentrations of salinity.

5. Budget and budget narrative

Table 1. Budget

Item	Cost (dollars)	Narrative
Bubblers	20.00	Supplies air to growth media
Vinyl tubing	5.00	Transports air from bubblers
Pyrex 1L flask	120.00	Contain media, samples, and allows light penetration
Pyrex 1.8L flask	14.00	Contains backup samples
Kim Wipes	5.00	Clean microscope slides, dry delicate materials
Bleach	8.00	Used to disinfect and clean beakers and algae
Paper towels	2.50	Used to blot dry algae prior to weighing
1 gal jar	5.00	Used in cleaning process
Parafilm	8.00	Prevents water escape via evaporation
Balance	200.00	Quantifies algal growth
Air valves	20.00	Regulates air loading rates
Air tube fittings	5.00	Used to build air distribution network
Long tweezers	10.00	Used to extract specimens from growing flasks
Q-tips	2.50	Used to clean algae
Camera	80.00	Document changes in algae (color, texture, etc.)
Marking tape	1.50	Used to label flasks
Pens	1.50	Used to label flasks
Isopropyl Alcohol	2.50	Used to prevent contamination

Items associated with the budget were used in the building of growing apparatus, collecting of data, and recording of data. The University of Hawaii at Hilo provided for the most part materials, to the point that only negligible monies were provided out of pocket by those conducting this study.

5. Results

Through the course of this study, biomass increased in higher salinity waters and decreased in lower salinity waters, contradicting the hypothesis of this study. This trend remained consistent throughout the course of the study, indicating that this was not a trend associated with specific thalli.

Table 2. Displays from left to right; Salinity average by level over the course of the study, number of value compared (before and after study for a total of two), Tukey's comparison test output for average change in biomass between two four week periods, average starting weight, and average percent change

Factor	N	Mean	Grouping	Start avg	% change
32ppt	2	.5	A	5.3	8.9
20ppt	2	.2	A	5.3	3.7
15ppt	2	.0	A	5.3	.2
10	2	-1.6	B	5.3	-31.3

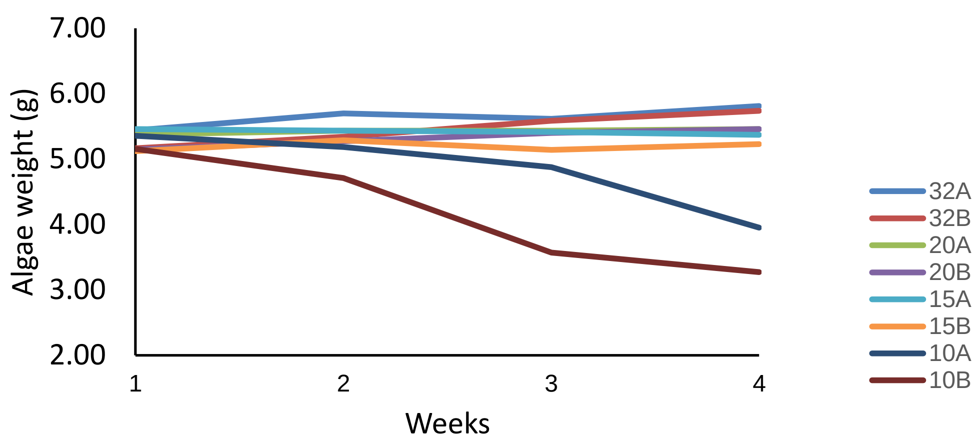

Figure 2. Tracks the change in algal growth over three week intervals (A, B) for the four salinities tested (10, 15, 20, 32ppt).

Qualitative data mirrored biomass loss: lower salinity samples showed signs of deleterious effects. During the experiment, beakers with higher salinity showed growth in the form of new thallus tissue at apices and along the thallus. New growth was lighter in color and tended to be a deep to light red. As salinity decreased, samples began to show white, dead tissue in areas where the thallus had been abraded or damaged. After the second week, the color the thallus began to increase green or brown tinge as opposed deepening in color as was apparent at higher salinities. The texture of the algae was also noticeably different at the end of the three-week replicates. Healthy tissue retained a crisp, stiff texture; whereas, unhealthy tissue in lower saline waters was softer and more flexible by the end of the experiment.

6. Discussion

The alga, *G. epihippisora* was grown in a range of salinities and performed differently that what was expected. It was hypothesized that this alga would grow faster in conditions of lower salinity; however, it grew fastest in full strength seawater. Future studies should include more trials over a longer period of time, and account for variables such as dissolved carbon dioxide and nutrients present naturally in ground water. Genetic analysis should be conducted to determine if *G. epihippisora*'s relative relatedness to other members of Gracilaria. It is perplexing that this alga apparently prefers full strength seawater and yet is restricted to the small pools of Liliuokalani Gardens. Restricted range may indicate that unknown variables play a key role in its reproductive cycle. This alga may have only recently evolved and has yet to incidentally colonize similar habitats around Hawaii Island. Future studies should also assess the quality of agar produced at by plants grown in different salinities. Differences in morphology may be indicative of changes of agar composition, making cultured plants potentially more useful than wild gathered specimens.

7. Conclusion

The endemic algae *Gracilaria epihippisora* presents special interest to the scientific community due to its specialized habitat. This species is able to thrive in the low salinity water prevalent in its only known locality: Liliuokalani Gardens. It has been observed to display all phases of its reproduction in low salinity waters, conditions that inhibit anything other than vegetative reproduction in similar species. Trials were conducted to determine the range of salinity tolerance for this species in order to ascertain optimal growing conditions, information that is critical to commercial culture or conservation efforts. Salinities tested mimicked the natural range that would be experienced by plants in the isolated pools of Liliuokalani Gardens, ranging from 10-32ppt. Higher salinities yielded higher growth contrary to what was hypothesized. Growth ranged from a decrease of almost 32% at the lowest salinity to an increase of almost 9% for normal seawater over the course of three weeks.

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