CLUSTER FIVE

AQUACULTURE

The gem is depicted as a blue-colored aquatic animal that represents aquatic life, both as an important part of the environment and as an important resource. Blue is also commonly associated with water, our planet's most precious resource and a major part of life and our ecosystem. As such, it is important to maintain our aquatic resources and find solutions to the existing problems that aquaculture faces. These research studies aim to address such problems and provide an avenue of locally-based research in aquaculture-centered fields of science.

These studies also fall under the Aquatic, Agriculture, and Natural Resources (AANR) Research and Development Agenda. They are in line with the priorities and agendas set for aquaculture research moving forward.

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Sodium lactate as a potential preservative to green mussel meat

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Abstract

The short shelf life of green mussels could limit its consumption and wider distribution to the market and thus, proper storage of this product is necessary. In this study, the effects of different concentrations (1%, 2%, 3%, and 4%) of sodium lactate on the preservation of green mussels during chilled storage of nine days were determined. Changes in pH and weight loss of the mussel meat were recorded every three days. Results showed that the pH values of the treated samples are around the neutral pH (6.7 to 7.1) and are significantly higher than the untreated samples throughout the duration of storage. No significant difference was observed in the weight loss between the control and treatment groups. Thus, the results of the parameters showed that sodium lactate has the potential to be utilized as a preservative agent for meat.

Introduction. - Green mussel (*Perna viridis*) is a type of shellfish that is commonly sold in local markets. This bivalve is widely consumed, especially by people living in coastal areas, as a cheap protein source [1]. However, the process of transporting these products from mussel farms to different markets is too laborious due to the small amount of meat produced per kilogram of the green mussels. Consequently, the immediate consumption or proper storage of this product is necessary since it could only be stored for two (2) days at ambient temperature [2]. The process of product deterioration occurs due to the growth of bacteria in the product over time [3]. The development of a processing method is important for extending the shelf life of mussels [4] since this could ensure that the product is still safe for consumption after a period of time.

Various methods on the preservation of green mussel meat, including pre-treatment with organic acids and modified atmosphere packaging, have already been studied. Organic acids are commonly used in food preservation since they have the ability to inhibit the growth of microorganisms, and they also occur naturally in food (i.e. lactic acid from corn and citric acid from oranges) [5]. Preservation occurs when the molecules of these acids dissociate inside bacterial cells due to low pH, resulting in the release of toxic charged anions and protons that inhibit the metabolic reactions of the bacteria [6,7]. Organic salts of these acids, such as sodium acetate, sodium lactate, and sodium citrate, are also used for food preservation.

Sodium lactate is the organic salt of lactic acid that is generally produced from natural lactic acid that is reacted with sodium hydroxide [8], and is reported to be a very prominent flavor enhancer with few negative effects [9]. The addition of this organic salt to meat products delays the development of sour and off-flavors by binding to free radicals in the meat to prevent lipid oxidation [9].

One of the qualities that are analyzed in food preservation is weight loss. This property is an important indicator because it is attributed to the loss of water in the meat [10]. High water retention in food may serve as a nutrient and contribute to the microbial proliferation in the product [4]. Sodium lactate is known for its ability to improve the moisture retention of materials [11]. The addition of sodium lactate to meat products has shown improvement in the cooking yield of the meat due to its humectant properties that contribute to the water-holding capacity of the product [12]. There are three proposed mechanisms by which sodium lactate can have an antimicrobial effect: (1) It has the ability to lower the water activity of the meat and thereby slowing the bacterial growth; (2) Sodium lactate passes through the cell membrane and lowers intracellular pH; and (3) It affects the cellular metabolism by inhibiting ATP^2 generation [13]. The lactic acid portion of sodium lactate and the sodium ion has antimicrobial effects, which slows down the normal metabolic process that generates energy in the cell [13].

Additionally, pH values are usually measured as a quality indicator for seafood products. The ideal pH of green mussels ranges from 6.00-6.85 [14].

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According to Miller (2010) [13], sodium lactate addition is associated with increasing the meat pH, increasing the water-holding capacity, and reducing cook losses which results in the increase in the tenderness of meat. Sodium lactate has been proven to be an effective additive in preserving seafood products such as refrigerated sliced salmon [15], and refrigerated rainbow trout [16]. It is also hypothesized that sodium lactate has the ability to preserve green mussel meat. Therefore in this study, sodium lactate was utilized as an additive for the preservation of green mussel meat.

This study aims to determine the effects of different concentrations of sodium lactate on the preservation of green mussel meat during chilled storage. Specifically, this study aims to:

(i) measure the weight loss of the green mussel meat after treatment with different concentrations (1%, 2%, 3%, 4%) of sodium lactate and without treatment for 9 days.

(ii) determine the change in pH of the green mussel meat after treatment with different concentrations (1%, 2%, 3%, 4%) of sodium lactate and without treatment for 9 days

Methods. - The methods for the conduct of this study were designed to be doable at home. Two parameters were analyzed, namely the change in pH and weight loss. Each member of the work unit prepared a set-up of the experiment. However, the same materials were utilized for the conduct of the experiment. The experiment performed was the same for both set-ups. Green mussels were obtained from a mussel farm in Dangula-an, Anilao, Iloilo. Aqueous solutions of sodium lactate with different concentrations (1%, 2%, 3%, 4%) were prepared for the treatment of the shucked mussel meat.

The study was conducted for 9 days, and analyses of weight loss and pH were performed every 3 days during the duration of the study. Samples were stored in a storage condition of 3 ± 1°C [4].

Sample collection. Samples of green mussels were collected in Dangula-an, Anilao, Iloilo. An icebox filled with seawater was used to store the mussels. The samples were then transported back to Iloilo City, with the duration of the travel being approximately one hour from the collection site.

Sample preparation. Upon arrival, the mussels were shucked, washed with distilled water, and drained. Mussels having a foul odor or open shells were removed. A total of 2 kg of mussel meat was obtained. The mussels were divided into five (5) groups: (a) 1% sodium lactate, (b) 2% sodium lactate, (c) 3% sodium lactate, (d) 4% sodium lactate, and (e) negative control, which are the samples without treatment. Sodium lactate (USP grade) was purchased online through the website of Dalkem Corporation. The formula $C_1V_1 = C_2V_2$ was used to calculate the different concentrations of sodium lactate. The concentrations 1%, 2%, 3%, and 4% were obtained by diluting 60% sodium lactate solution with distilled water. The solutions were stored in clean, plastic bottles. The calculations were as follows:

Calculation for 1%:

$$C_1V_1 = C_2V_2$$
$$60\% \cdot V_1 = 1\% \cdot 1000\,mL\ water$$
$$V_1 = 16.67\,mL$$

Calculation for 2%:

$$C_1V_1 = C_2V_2$$
$$60\% \cdot V_1 = 2\% \cdot 1000\,mL\ water$$
$$V_1 = 33.33\,mL$$

Calculation for 3%:

$$C_1V_1 = C_2V_2$$
$$60\% \cdot V_1 = 3\% \cdot 1000\,mL\ water$$
$$V_1 = 50.00\,mL$$

Calculation for 4%:

$$C_1V_1 = C_2V_2$$
$$60\% \cdot V_1 = 4\% \cdot 1000\,mL\ water$$
$$V_1 = 66.67\,mL$$

For the sample storage, 20 plastic resealable bags were utilized. A different bag for each treatment was used for every sampling interval. The ratio of the weight of the mussels to the solution is 1:2. Each pack of samples contains 100 g of green mussel meat. The samples were treated with 200 ml sodium lactate solution. The solutions were then poured into the resealable plastic bags according to the labels. The samples were stored inside the refrigerator for nine (9) days with a storage condition of 3 ± 1°C [4]. A thermometer was utilized in order to monitor the temperature inside the refrigerator.

pH determination. The pH of the control and treated samples was measured using a pen-type pH meter (Milwaukee, PH600AQ Digital pH Pen). This device was calibrated by measuring the pH of distilled water in every sampling interval before analysis. The pH of each group was determined by following the standard method of measuring the pH of solid-liquid mixtures. The samples were drained using a strainer to separate the mussel meat from the solution. The liquid solution was transferred to a beaker, and then its pH value was measured. Then, the mussels were blended into a homogenous paste and the pH measurement was taken. After that, the liquid solution and paste were combined, and pH was measured. The measurements were done in triplicates.

Weight loss. For the weight loss determination, the samples were weighed before and after storage. An analytical balance (Shimadzu, BL3200H) was utilized to determine the weight of the samples. This analysis was done in triplicates for every sampling interval. Percentage of weight loss was determined using the formula [17]:

$$Weight\ loss\ (\%) = \frac{initial\ weight - final\ weight}{initial\ weight} \times 100$$

Data Analysis. The data gathered was analyzed using One-way ANOVA, and post-hoc analysis was evaluated using Duncan's multiple range test. Differences between the means of the control and treated samples were examined with the level of significance set at $\alpha < 0.05$. This analysis was performed through the SPSS software.

Safety Procedure. The use of safety equipment such as laboratory gowns, gloves, and surgical masks was observed at every sampling interval. Different types of waste were segregated into different bins, and liquid wastes were collected in empty plastic bottles.

Results and Discussion. - The study aimed to determine the effects of different concentrations of sodium lactate on the preservation of green mussel meat. To this end, the pH change and weight loss of treated and untreated samples were monitored for 9 days.

Weight loss analysis. Weight loss is attributed to the loss of water in meat products [4]. High water retention is linked to the deterioration of meat since it might serve as a nutrient, which contributes to the microbial proliferation in food products [18]. However, the ability of a product to retain water is also integral to its quality in terms of juiciness and tenderness [19].

All samples showed a decrease in weight at the end of the storage. Results of the statistical analysis showed no significant difference in the weight loss between the control and treatment groups (Figure 1). This indicates that adding sodium lactate has no significant effect on the water retention of the mussel meat. The ability of myofibrillar proteins and myofibrils to entrap water is directly affected by pH and ionic strength [20]. This may explain the absence of significant difference in the weight loss between the treated and untreated samples since the pH values recorded are near neutral.

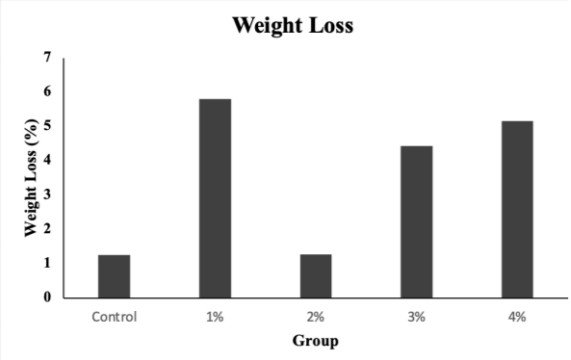

Figure 1. Percent weight loss of treated (1%, 2%, 3%, or 4% sodium lactate) and untreated samples after treatment (n=3). No significant difference observed between the treated samples and control.

In the second set-up, the analysis also showed no significant difference between the weight of the control and the treated samples. The mussels had high water retention since the weight loss percentage was low. Sodium lactate exhibits high water holding capacity which may explain the low weight loss of the samples [21].

Changes in pH. One of the physical qualities that are frequently analyzed for food quality control is pH value [4]. This factor is examined along with the Total Volatile Basic Nitrogen (TVB-N), Trimethylamine Nitrogen (TMA-N), and Thiobarbituric acid reactive substances (TBARS) for seafood quality assessment [22]. It indicates the degradation of muscle components and post-mortem change of glycogen to lactic acid during long storage [23].

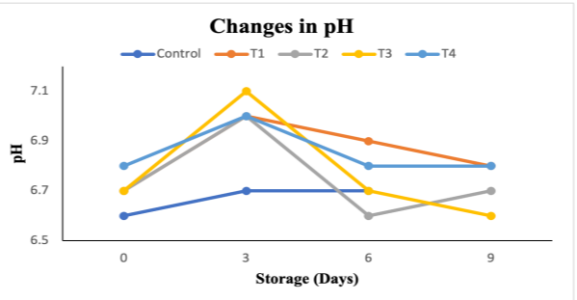

Figure 2. The trend of change in pH of the homogenized mussel meat observed per treatment throughout the nine-day period (n=3).

It is observed that all groups have yielded a pH value that is near neutral. The pH values of the mussel meat for the last day of storage were lower compared to the initial pH values. Figure 2 shows the trend of the pH change per group throughout the storage period. Based on the initial pH values of the treated and untreated samples, an increase in the pH of the treated samples could be observed on the third day of storage. After this, the pH value of the samples eventually decreased in the succeeding days except for treatment 4, where an increase in pH was observed on the final day of storage. Slight changes in the pH value upon addition of sodium lactate were also observed in other studies on meat products such as ground beef [24], cig kofte [25], poultry sausage [26], and sliced salmon [14]. Sallam and Samejima [24] indicated that sodium lactate has the ability to stabilize the pH of most meat products during storage. The recorded pH of the treated samples in this study verified an almost constant pH since all treatment groups have maintained pH values that are near neutral (6.7-7.1) throughout the duration of storage. The results of this analysis contradict the data reported in the study conducted by Eckert et al. [27], and Tan and Shelef [28], where it was stated that sodium lactate had no significant effects on the initial pH of ground meat products.

After the 9-day period, One-way ANOVA showed that the green mussels treated with sodium lactate have pH values significantly higher than the control group which indicates that there is a significant difference between the groups of samples. However, no significant difference was found to exist between the treatment groups, which implies that these four (4) treatments are not significantly different in terms of their effect on the pH of green mussel meat during chilled storage. This further indicates that 1% sodium lactate is enough to significantly increase the pH value of green mussels during storage. Having a pH value near neutral indicates that the green mussels are still safe for consumption. A decline in pH values could be due to factors such as post-mortem changes, muscle component degradation, and the fermentative conversion of glycogen [18,29]. It could also be attributed to increasing microbial count, which could be considered as the deterioration stage of the product [30]. A pH value of 5.9 for mussels is an indicator of deterioration according to Hardey [31].

However, no significant difference has been found between the control and treated groups during

the second set-up. The result may have been affected due to an error in data gathering during the first sampling interval. The pH was not correctly measured because the mussels were not blended and there was no separate measurement for the liquid part. Throughout the storage, the pH of the control has declined. This was also observed in the study conducted by Arcales and Nacional (2019) [21] that showed that the control samples had a near-neutral pH value at the start of the study and had decreased during storage.

The results of the analysis conducted on the weight loss and pH in the untreated and treated samples showed that the effect of adding sodium lactate is only evident on the change in pH and not on the percent (%) weight loss. An increase in the pH value of the samples was only observed on the third day of sampling. Based on these data alone, it could be inferred that the addition of 1% sodium lactate is already sufficient in increasing the pH level of green mussel meat that makes it suitable for consumption after longer storage. Although the pH value of the control group is significantly lower, it still falls in the range of suitable pH values for green mussels. Therefore, the data collected in this study only shows that sodium lactate has the potential to be used as a preservative agent as evidenced by its ability to increase the pH value of green mussel meat. Further analysis on the microbial proliferation and chemical reactions taking place in green mussel meat treated with sodium lactate shall be made to further determine the effectiveness of sodium lactate as a preservative for green mussels.

Limitations. A power outage occurred during the storage which lasted for 10-15 minutes (during the first set-up) and 30 minutes (during the second set-up). This circumstance may have affected the storage condition of the samples. Slight differences in the draining time of the samples may have affected the recorded weight of the samples in every sampling interval.

Conclusion. - The results obtained from each parameter showed that there is a significant difference in the pH between the treated and untreated samples. However, no significant difference was observed in the weight loss of the control and treatment groups. These results indicate that sodium lactate has the potential to be used as a preservative to green mussel meat. However, further analysis on the microbial proliferation and chemical reaction taking place in the mussel meat must be performed to determine the effectiveness of sodium lactate as a preservative and to further prove this claim.

Recommendations. - A study analyzing the bacterial load and chemical reactions taking place in green mussel meat after subjecting to sodium lactate preservation must be conducted to determine the quality of green mussel meat after the addition of the organic salt. Other recommendations include the setting of a specific duration for the draining time and provision of a backup power source in case of power outages. The addition of parameters to be analyzed such as TVB-N and TMA-N determination and sensorial evaluation is also recommended. Another recommendation is the addition of a positive control group such as using other organic substances such as sodium acetate, lactic acid, and sodium citrate, which already have established concentrations for green mussel meat preservation, in order to have a comparison for the efficacy of the organic salt that is utilized in the study.

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DNA barcoding of freshwater gastropods found in the upstream of Jalaur River in Barangay Garangan, Calinog, Central Philippines

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Abstract

Multiple species of gastropods can be found in the Jalaur River which encompasses almost the entire province of Iloilo. However, the identification of these species can be challenging with their complex morphology. DNA barcoding using the cytochrome oxidase I (COI) gene was performed to accurately identify and classify the organisms of Class Gastropoda. Through phylogenetic analysis, one sample was identified to be *Pomacea canaliculata* while the remaining four samples remained unidentified due to unrepresented taxa in the GenBank. This shows a lack of available information about the organisms found in the Jalaur River as nucleotide sequences of these species have not been provided to public databases. With this, further research must be done to know more about the species found in Jalaur River and their conservation status. It is recommended to maintain the storage condition of samples to prevent DNA degradation. PCR conditions should also be adjusted to achieve optimal results.

Introduction. - Systematics and taxonomies are the basis of all biology as it ensures the quality of life of future generations [1]. However, there are still a vast number of unknown species that have not yet been identified. Studies suggest that the Earth is home to about 8.7 million species [2]; meanwhile, only 14% had been identified as of 2011 [3]. The Philippines is home to about 22,000 mollusks species [4]. Gastropods, in particular, have a wide range of habitats and are prone to evolve as they are sensitive to slight changes in the environment [5]. In addition to this, gastropods have one of the highest numbers of documented extinctions among the major taxonomic groups in the world making its identification necessary [6].

Moreover, gastropods are also ecologically important as some of their species can be used as indicators to assess the condition of the aquatic habitat along with the quality of any water impoundments [7]. Gastropods may also serve as pests to agriculture with the potential to invade and alter the ecosystem [8]. Additionally, some gastropods species may be intermediate hosts of infections despite being a source of food for fishes, birds, and humans [7,9]. Despite this, the understanding of its systematics is still incomplete [10] and the phylogenetics among its family is largely unresolved [11].

Studies had been done assessing freshwater gastropods in the Philippines which was identified as *P. canaliculata*, *V. costata*, *L. natalensis*, *M. tuberculata*, *M. turricula*, *T. granifera*, *G. ladacensis*, *L. accuminata*, *L. caperata*, and Planorbis sp. [7, 12];. Alcala et al. [13] were also able to taxonomically identify *N. polita, T. granifera, P. porcellana, T. scabra, P. canaliculata, L. scabra, C. cucullate, C. manillensis, C. plicata,* and Ostrea sp. in Jalaur River. Despite their findings, morphological identification may not always be accurate due to the existence of cryptic species having similar morphology but different DNA sequences [14, 15].

To address this, past research recommends the use of DNA barcoding as an effective tool to authenticate and accurately identify organisms [14, 15]. DNA barcoding is a process that involves sequencing a short fragment of the COI gene taken from the unidentified organisms and comparing their DNA barcodes to existing sequences [16]. It can reveal possible misidentified organisms, discover overlooked species, and identify new or evolved species promptly and accurately as complete data can be collected from a single specimen irrespective of its morphological features or its stage in life [1, 17]. The DNA barcodes can be used to generate a phylogenetic tree for the evaluation of each organism, along with the phylogeny, diversity, and relationship among the organisms. With the data,

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actions can be taken for an organism's conservation and management [18]. The significance of DNA barcoding makes it a prominent research topic as it can authenticate the organisms to assess their safety, conserve genetics, and detect possible invasive alien species [14].

The Jalaur River encompasses almost the entire Iloilo with its upstream located at Barangay Garangan, Calinog, Iloilo. Previous studies mentioned that freshwater gastropods, specifically stream snails, are known to exhibit upstream migration in temperate and tropical regions [19,20]. Additionally, this area is also home to the Panay Bukidnon indigenous people thus with the identity of the freshwater gastropods, awareness will be given to the locals regarding the availability of certain gastropods in their area. Monitoring and assessment of the species could also be done along with its conservation and management. Additionally, the result of this research can be used as a baseline for further taxonomic research.

This study aimed to identify selected freshwater gastropods in the Jalaur River located in Barangay Garangan, Calinog, Iloilo, Philippines by analyzing gene sequences to establish their relationship and conservation status. Specifically, this study aimed to:

(i) identify selected species of freshwater gastropods collected from Jalaur River in Barangay Garangan, Calinog, Iloilo using phylogenetic analysis;

(ii) determine the relationship among the collected gastropod species by interpreting the phylogenetic tree; and

(iii) query the conservation status of each identified gastropod species on the International Union for Conservation of Nature (IUCN) Red List Index.

Methods. - This research is a descriptive study on the phylogenetics of freshwater gastropods. The methodology includes sample collection, DNA extraction, DNA amplification, agarose gel electrophoresis, DNA sequencing and alignment, species identification, and phylogenetic analysis.

Sample Collection and Preparation. To collect samples, a permit was requested from the National Commission on Indigenous Peoples (NCIP) since the sampling site is part of the Panay-Bukidnon's ancestral domain. Freshwater gastropods in shallow waters were collected through handpicking [7]. A total of 11 samples were collected from upstream of the Jalaur River in Barangay Garangan, Calinog, Iloilo, Philippines with the geographic coordinate 11°11'29" N 122°27'13" E at 367.0 meters of elevation using opportunistic sampling with the help of the indigenous people.

The collected samples were then stored in separate airtight bags labeled according to their vernacular names that were provided by the indigenous people and were placed in a cooler for transportation. The samples' foot muscle was then extracted and cut into small pieces then submerged in 70% ethanol to prevent the degradation of DNA [20].

DNA Extraction. DNA extraction was performed following the standard protocol for animal tissue according to the NucleoSpin Tissue Genomic DNA Purification User Manual. It was then tested in the Thermo Scientific™ Multiskan™ GO to check the quality of the extracted DNA.

DNA Amplification. DNA amplification was done through polymerase chain reaction (PCR). The universal primers for the amplification of the (COI) gene, LCO1490 (*5′* -GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (*5′* -TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) were used [21]. The PCR was performed following the thermal regime: 3 min at 94 ℃, then 25 cycles of 20 sec at 94 ℃, 30 sec at 50 ℃, and 1 min at 72 ℃, followed by extension for *5* min at 72 ℃ set for seashells at the West Visayas State University laboratory.

Agarose Gel Electrophoresis. The amplified DNA was subjected to gel electrophoresis using 1% agarose gel and stained with Invitrogen 10X BlueJuice to check the integrity of the samples. The result was then viewed under the UV transilluminator.

DNA Sequencing and Alignment. The PCR products were sent to Macrogen Inc., Seoul, South Korea for sequencing. The sequences were then assembled using their forward and reverse sequences with DNABaser.

Species Identification. Each assembled DNA was queried using Basic Local Alignment Search Tool (BLAST) to compare the sequences to the available sequences in GenBank. The species with the lowest E-value and the highest bit score was determined for each sample. From the family, sequences of multiple species were downloaded to be included in the phylogenetic tree.

Phylogenetic Analysis. Using MEGA X, the sequences were aligned using ClustalW. This software also generated the best model for DNA substitution to be the Tamura 3-parameter model. Then a Neighbor-Joining tree from 1000 replicates was constructed using the maximum likelihood statistical method. Clades with bootstrap values higher than 70 were considered well-supported [22]. These were used as the basis to verify the identity of the sample. After verifying each sample's species, it was queried in the IUCN Red List Index for the conservation status.

Safety Procedure. Proper protective equipment was utilized at all times. Lab gowns and nitrile gloves were used during DNA extraction, DNA amplification, and gel electrophoresis to avoid exposure to hazardous chemicals. After the collection of DNA, the waste materials and the samples were autoclaved and disposed of properly as medical/biohazard waste.

Results and Discussion. - Freshwater gastropods in the upstream of Jalaur River were identified through DNA barcoding. The process includes sample collection, DNA extraction, DNA amplification, agarose gel electrophoresis, DNA sequencing and alignment, species identification, and phylogenetic analysis. From the 11 samples that were collected, only *5* were successfully barcoded due to possible DNA degradation and non-optimal conditions set for the

PCR amplification. Furthermore, only one of the five was identified to its species-level namely *Pomacea canaliculata* while the other 4 barcoded samples remained unidentified due to unrepresented taxa.

The initial identification was done based on their vernacular names. After obtaining the assembled DNA, initial species identification was performed using BLAST to classify them into specific species. The program presents the e-value and bit score of the top matches for each sequence. The e-value shows the statistical significance of a match while the bit score gives an indication of the quality of the alignment [22]. A low E-value and bit score higher than 950 are suggested for assigning species identity; thus, the sequence with the lowest E-value and the highest bit score was chosen as the BLAST identification [23]. These identifications are summarized in Table 1.

Table 1. The barcoded samples are presented with their corresponding vernacular name and BLAST Identification with the lowest E-value and the highest bit score.

Sample	Vernacular Name	BLAST Identification	E-value	Bit Score
A1	Awis	*Stenomelania* sp.	0	1158
A2	Awis	*Stenomelania* sp.	0	1122
K2	Kuol	*Pomacea canaliculata*	0	1179
TG	Tamburuko (gurob-gurob)	*Tarebia granifera*	0	1210
TM1	Tamburuko (mugot)	*Stenomelania denisoniensis*	0	1031

While BLAST was able to identify the samples with E-value = 0 and bit score > 1000, the study conducted by Ross et al. [24] raises major concerns as many taxa are unrepresented. In addition to this, it cannot give accurate identification of species because top hits are often not the closest phylogenetic relatives of the organisms [24, 25]. In a study by Hillis and Bull [26], clades having bootstrap values ≥70 correspond to a ≥95% probability of it being real. Thus, to verify the initial identity of each sample, phylogenetic analysis was performed where clades with bootstrap values of seventy (70) or higher were considered well-supported to be of the same species after undergoing one thousand (1000) replicates. Figure 1 shows the bootstrap consensus tree of the five (5) samples along with representative sequences from the family of their BLAST identities with the outgroup *Paludomus siamensis*.

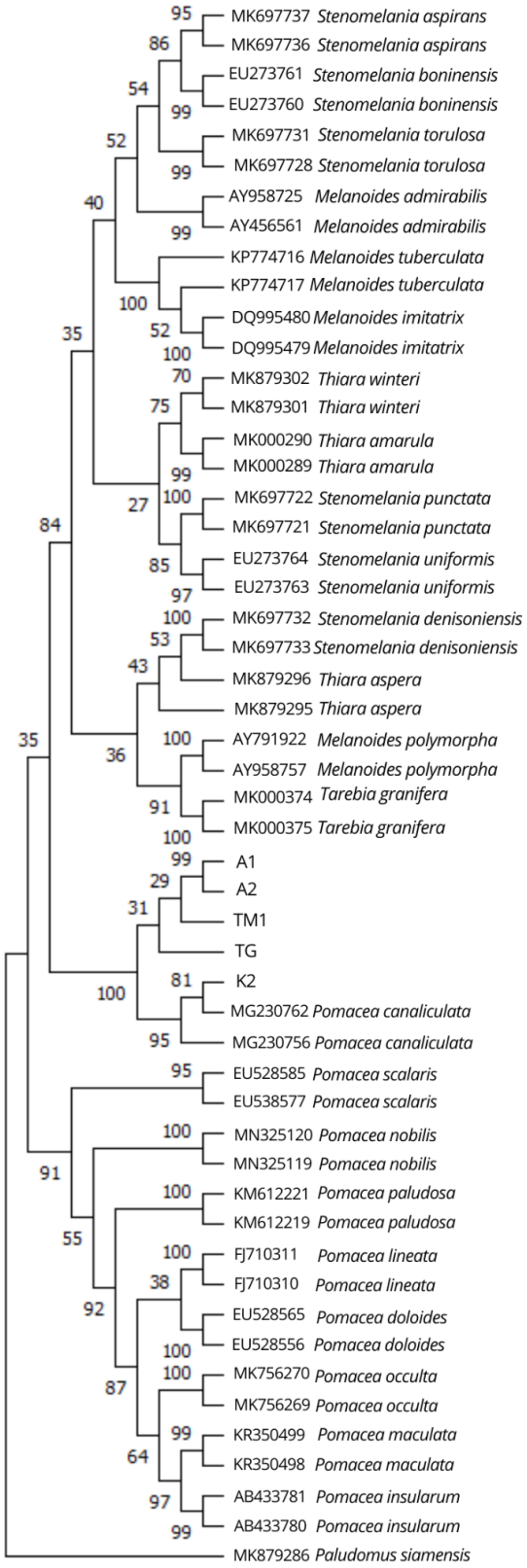

Figure 1. Bootstrap consensus tree of the samples is shown along with representative sequences from the family of their BLAST identification with *Paludomus siamensis* as the outgroup.

The phylogenetic tree shows that K2 can be identified to be *Pomacea canaliculata* with a bootstrap value of 81. This is the same species of gastropods that was identified by Alcala et al. [13] in the same river in

2010. Aside from this, it was also found in other parts of the Philippines such as Bukidnon and Agusan del Sur [7,12].

The identified species for K2 was queried in the IUCN Red List of Threatened Species for the identification of their conservation status. *Pomacea canaliculata* is categorized as Least Concern. Though it is not a focus of species conservation, its management is still necessary as it can serve as pests to rice crops [27].

Additionally, *Pomacea canaliculat*a was found to be an invasive species in the Global Invasive Species Database (GISD) [28]. With its ability to adapt to harsh environmental conditions along with its high reproductive rate, it can colonize and invade natural habitats [27, 29] which results in alterations in the ecosystem [8].

The remaining four samples belong to the same clade. It can be interpreted that A1 and A2 that have the same vernacular name, "Awis", are of the same species based on the phylogenetic analysis having a bootstrap value of 99. Collectively, the samples were more closely related to each other than their BLAST identification. This may be due to more common ancestors shared by the samples. The inaccuracy of the BLAST identification can be attributed to unrepresented taxa in the GenBank [24]. This shows a lack of available information about the organisms found in the Jalaur River as nucleotide sequences of these species have not been provided to public databases.

Limitations. The study barcoded less than the actual number of samples collected due to possible degradation of DNA and non-optimal conditions during the DNA amplification. Moreover, some samples were unrepresented species of gastropods in the GenBank, thus out of the five barcoded samples, only one organism was identified.

Conclusion. - In conclusion, "Kuol", identified to be *Pomacea canaliculata* or Golden Apple Snail, can be found in the Jalaur River along with four other unidentified gastropods. The identified species was not found to be a focus of species conservation since it is under the Least Concern categorization; however, its management is necessary as it is an invasive species and pests to rice crops. Aside from the Jalaur River in Iloilo, *Pomacea canaliculata* was also found in other areas in the Philippines. The four other organisms were not identified due to the limited sequences recorded in GenBank or the possibility of evolution.

Recommendations. - Further research must be done to identify the gastropods found in Jalaur River for their proper management. It is also recommended to perform all procedures in one laboratory with complete equipment for DNA barcoding to maintain a constant storage temperature of -20 ℃ and prevent DNA degradation [30]. More time should also be dedicated in the study to allow adjustments in PCR conditions to achieve optimal results which may vary depending on the organism being amplified.

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Effect of different irradiance levels on the growth of the cyanobacterium *Lyngbya majuscula*

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Abstract

Lyngbya majuscula is a prolific producer of secondary metabolites that are used in the pharmaceutical industry. As a photosynthetic organism, the effect of irradiance on its cultivation was studied to maximize algal growth for mass production. The cyanobacterium was subjected to different irradiances of 20, 45, 110, 180, and 320 µmol photons $m^{-2}s^{-1}$ to determine its specific growth rate (SGR) in each treatment. Results showed the highest SGR under 20 and 45 µmol photons $m^{-2}s^{-1}$ during the exponential phase, showing a significant difference ($p < 0.05$) among all other treatments. Minimal growth rates were obtained under 110, 180, and 320 µmol photons $m^{-2}s^{-1}$ and results showed that the SGRs under these treatments have no significant difference ($p > 0.05$) with the negative control; however, color changes were observed at these irradiances. These showed that *L. majuscula* prefers lower irradiances to maximize its growth, while higher irradiances are unideal.

Introduction. - *Lyngbya majuscula* is a filamentous marine alga that is a prolific producer of 196 novel and diverse secondary metabolites, whose genus is responsible for over 40% of all marine cyanobacterial secondary metabolites (1,2,3). These metabolites are carotenoids, proteins, and vitamins which are beneficial in the pharmaceutical industry and food technology for their antioxidative and antimicrobial properties [4].

Several factors can be modified to optimize the growth and secondary metabolite production of cyanobacteria. For instance, Burja et al. [2] investigated the effect of culture vessel configuration, growth conditions, and media composition and determined that culture conditions have the greatest effect on secondary metabolite production. One important culture condition [3,5,6] that can be modified is light availability. Studies [7,8] reported that algal growth is better under continuous lighting since growth is directly proportional to the length of the light exposure.

Although most studies investigate the relationship of light received by the algae to its growth, little research has been done regarding the optimization of the yield under continuous light exposure. There are claims that *L. majuscula* would yield highest growth under various light–dark cycles and among different levels of irradiation from ranges of 20 µmol photons $m^{-2}s^{-1}$ to 120 µmol photons $m^{-2}s^{-1}$ [3,9,10].

Previous research would suggest that the optimum growth of the algae *L. majuscula,* would be sustained under higher light intensities within the ranges of 180 µmol photons $m^{-2}s^{-1}$ to 320 µmol photons $m^{-2}s^{-1}$. This is due to the property of *L. majuscula* being a surface cyanobacterium; hence, it readily produces more carotenoids as a mechanism against photoinhibition [9]. However, Loogman [10] also observed general cyanobacterial death at 320 µmol photons $m^{-2}s^{-1}$. Low light irradiances have also been claimed as the optimum irradiance for other *Lyngbya* species such as *Lyngbya kuetzingii* and *Lyngbya stagnina,* where the irradiance values found were 20 µmol photons $m^{-2}s^{-1}$ and 45 µmol photons $m^{-2}s^{-1}$, respectively [11,12].

Realizing the need to optimize the growth of the algae for future use in the pharmaceutical industry, *L. majuscula* was chosen to be subjected to stress or bioprocess intensification through light irradiance. Mass cultivation of the algae was targeted because most drug candidates do not reach the pharmaceutical market due to the low availability or small yield of bioactive compounds [13].

This study aimed to determine at which irradiance between 20, 45, 110, 180, and 320 µmol photons $m^{-2}s^{-1}$ the algal growth of *Lyngbya majuscula* is at a maximum. Specifically, it aimed to:

(i) assess changes in the color, pH, and temperature of *L. majuscula* during each treatment;

(ii) analyze the trend of algal growth in terms of dry weight and specific growth rate (SGR); and

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For supplementary data, contact: publiscience@wvc.pshs.edu.ph.

(iii) determine the highest specific growth rate by comparing the SGR values during each treatment using Planned Comparisons after One-Way Analysis of Variance (ANOVA)

Methods. - The methods were divided into 5 steps: (1) preparation of the alga, (2) culture of the alga in different irradiance levels, (3) determination of dry weight using gravimetry, (4) computation of specific growth rate, and (5) analysis of data.

Sample Collection. *L. majuscula* was purchased from the Southeast Asian Fisheries Development Center/Aquaculture Department (SEAFDEC/AQD) in Tigbauan, Iloilo. Samples were washed to remove residues and were subjected to a scale-up of 2 L after purification based on the standard protocol from the Handbook of Phycological methods [14].

Culture Set-up. Twenty-four (24) containers were each filled with 5 L algal cultures composed of 500 mL alga mixed with 4500 mL Ozonated Seawater-Conwy solution [15]. Five (5) lightboxes (92 cm x 31 cm x 66.5 cm) were made to contain four replicate containers each. The lightboxes had different irradiance levels: 20, 45, 110, 180, and 320 µmol photons $m^{-2}s^{-1}$, which were achieved using LED lamps (1 lamp = 2000 lumens). For the negative control, four replicate containers were not exposed to light.

The setup was irradiated for 24h throughout the 8-day culture. Irradiance was measured using a photometer (resolution: 0.1 µmol photons $m^{-2}s^{-1}$ in the range 0 - 1999 µmol photons $m^{-2}s^{-1}$), and one-point continuous aeration, pre-filtered (5 µm), was supplied by centralized pumps in the laboratory. The temperature and pH of the alga were recorded everyday using a temp/pH meter (resolution: 0.01 pH at range: 0.00 to 14.00 pH and 0.1°C at range: 0.0 to 100.0°C). The color of the alga in each treatment was also monitored everyday.

Algal Dry Weight. The biomass of *L majuscula* was measured in terms of dry weight using the Gravimetric method. The standard protocol set by the American Public Health Association (APHA) was followed [16]. One hundred (100) mL of the sample from each treatment replicate was vacuum filtered in pre-tared GF/F glass microfiber filters (0.7 µm pore size). Twenty (20) mL ammonium formate (Sigma Aldrich, 1M concentration with purity >99.0%) was then added to remove salt residues from the sample. The filter papers with alga residue were then oven-dried (Precision Scientific) at 60 °C for 2 hours, desiccated for 30 minutes, and then weighed using an analytical balance. This process was repeated until the net weight of the alga was obtained, with a difference of ± 0.0002 when weighed. Dry weight was calculated using the formula:

$$Dry\ Weight\ = \frac{W_1 - W_2}{mL},$$

Equation 1. Formula for Dry Weight

where W_1 = weight of the filter paper with the dried residue of the sample in mg, W_2 = tare weight of the filter paper in mg, and mL = volume of the sample.

Specific Growth Rate. Specific growth rate was calculated after obtaining the dry weight of the alga to determine the rate of algal biomass increase per day. It was computed using the formula [17]:

$$Specific\ Growth\ Rate\ (SGR) = \frac{ln\ (W_f) - ln(W_i)}{t}\ x\ 100,$$

Equation 2. Formula for Specific Growth Rate

where W_f = final weight in mg, W_i =initial weight in mg, and t = days of culture.

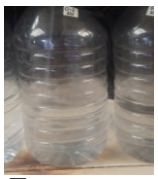
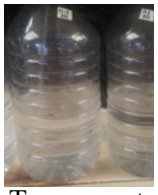
Data Analysis. Qualitative data were in the form of pictures of the treatment replicates each day. Quantitative data were in the form of algal dry weight (mg/mL), specific growth rate (d^{-1}), temperature (°C), and pH, where the mean and standard deviation in each treatment were computed. One-Way ANOVA with Planned Comparisons at 95% Confidence Interval was used to analyze the significant difference between samples. Tests of normality and homogeneity were also conducted to determine the conditions for the conduct of Planned Comparisons as post-hoc analysis.

Results and Discussion. - *Lyngbya majuscula* is a marine cyanobacterium that forms dense mats near the surface of the water, and its growth is dependent on numerous factors, with one of these being light availability [3,5,6]. The growth of the cyanobacterium in 8 days of culture was observed in different irradiances of 20, 45, 110, 180, and 320 µmol photons $m^{-2}s^{-1}$ using LED lamps.

The exponential phase of the alga was first identified before being subjected to the different irradiances. This phase was chosen among all other phases of the cyanobacterial growth due to the rapid and frequent cell division that contributed to the maximum growth rate of the alga. In addition, the higher growth rate or biomass production rate will result in faster production of metabolites which are usually produced in the late exponential phase [18,19]. The alga showed the highest growth during the 4th day of culture, showing that the exponential phase can be observed from Day 0 to Day 4. After the 4th day, disintegration of the alga was observed, indicating that it has entered the stationary phase, leading to the death phase. A similar study on the culture of *L. majuscula* conducted by Mandal et al. [20] also showed the decay of the alga after its exponential phase of 3-4 days.

Morphological Color Changes. Changes in the color of the alga were observed in the different treatments, which indicate the presence of photosynthetic pigments that contribute to the color of the alga such as chlorophyll *a*, phycobilin, and carotenoids (Table 1). At low irradiances, the alga produced a dark-red violet color, possibly due to high concentrations of the photosynthetic pigment, phycoerythrin. In contrast, at high irradiances, the alga exhibited a dull yellowish-brown color, possibly due to the decrease in the phycobilin accessory pigment and chlorophyll *a* levels, and an increase in the carotenoid production [21].

Table 1. The color variation of the algae on day 0, 4, and 8 with color observations below the pictures.

Day	Irradiance (μmol photons $m^{-2}s^{-1}$)					
	0	20	45	110	180	320
0	 Transparent	 Transparent	 Transparent	 Transparent	 Transparent	 Transparent
4	 Transparent	 Dark-red	 Red	 Yellowish-brown	 Brown	 Light brown
8	 Transparent	 Dark-red	 Red	 Brown	 Brown	 Greenish-brown

The increase in the amount of carotenoids in the alga at high irradiances is necessary to protect cyanobacteria from photoinhibition. Carotenoids absorb excessive light energy that would damage the chlorophyll molecules. Excess absorption of irradiation of the cyanobacterium can lead to the formation of harmful reactive oxygen molecules through interaction with oxygen which would result in further damage of the photosystem II from photoinhibition [22]. These observations indicate that exposure to higher irradiances is closely linked to marked changes in the morphology of an alga.

A similar study by Mandal et al. [20] showed that *L. majuscula* under long exposure to high UV-B radiation formed yellowish sheaths. The alga under the negative control (0 μmol photons $m^{-2}s^{-1}$) experienced a color change from light red to transparent, showing no growth due to the inability of cyanobacterium to photosynthesize under the absence of light. These indicate that the alga exhibits color changes depending on the irradiance level.

Data Analysis. The dry weight and SGR of each of the treatments during the exponential phase were compared and analyzed. A significant difference existed among the groups at 95% confidence interval. After conducting Planned Comparisons, it was found that a significant difference exists between 20 μmol photons $m^{-2}s^{-1}$ and all other treatments, and 45 μmol photons $m^{-2}s^{-1}$ and all other treatments. However, no significant difference was found between the two treatments. Moreover, the treatments 110, 180, and 320 μmol photons $m^{-2}s^{-1}$ also showed no significant difference with each other and the negative control.

Table 2. The dry weight obtained at each irradiance level during the exponential phase.

Irradiance	Dry Weight (mg/mL)	
(μmol photons $m^{-2}s^{-1}$)	Day 0	Day 4
0	0.018 ± 0.005	0.024 ± 0.003[b]
20	0.018 ± 0.005	0.10 ± 0.04[a]
45	0.018 ± 0.005	0.08 ± 0.02[a]
110	0.018 ± 0.005	0.07 ± 0.04[b]
180	0.018 ± 0.005	0.04 ± 0.01[b]
320	0.018 ± 0.005	0.039 ± 0.003[b]

*where a and b indicate the groupings based on their significant differences.

Algal Dry Weight. Noticeable increase in average dry weight from Day 0 to Day 4 can be observed in each treatment as seen in Table 2. The highest biomass of *L. majuscula* during the exponential phase was observed under 20 μmol photons $m^{-2}s^{-1}$ among all treatments. The alga under 45 μmol photons $m^{-2}s^{-1}$ exhibited the second highest biomass. Previous studies have also identified both irradiance values as optimum algal growth values [6,22,23].

Specific Growth Rate. The alga under the treatment of 20 µmol photons $m^{-2}s^{-1}$ exhibited the highest SGR of 42.3 % d^{-1} during the exponential phase, while the alga under 45 µmol photons $m^{-2}s^{-1}$ exhibited the second highest SGR of 36.9% d^{-1} as shown in Figure 1. Minimal growth rate was also observed in treatments under 110, 180, and 320 µmol photons $m^{-2}s^{-1}$ (Figure 1).

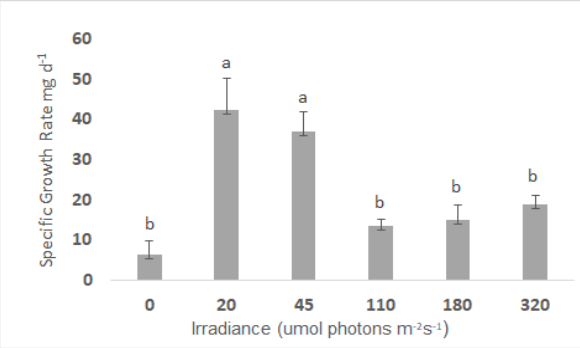


Figure 1. The Specific Growth Rate (SGR) of *L. majuscula* during the exponential phase (Day 0 to Day 4), where a and b indicate the groupings in which they are significantly different using Planned Comparisons after One-Way ANOVA ($p<0.05$).

pH and Temperature. Differences in irradiance caused small changes in the temperature and pH of each setup as shown in Figure 2.

As the irradiance increased, the temperature also increased. The excess energy formed from the reaction during the absorption of chlorophyll *a* photons is turned into heat; thus, the higher the irradiance, the more heat is transferred. According to Ras et al. [24], increasing temperature, above optimal conditions, in the outdoor production of algae may result in the decrease in the growth of the alga.

As shown in Figure 2, the pH in each setup was ≈ 8 which indicated growth and efficient CO_2 retention of the algae. A pH level closer to 8 corresponds to normal CO_2 concentration in a saltwater environment [25].

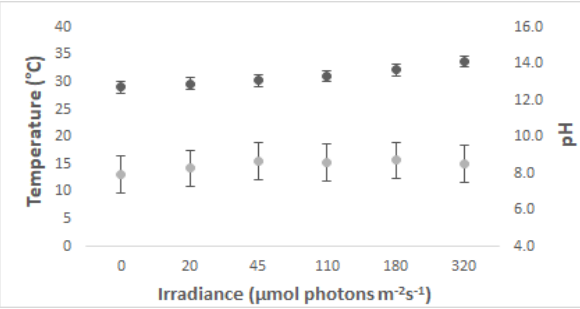


Figure 2. The average temperature and pH obtained in each treatment for 8 days of culture.

Effect of irradiance on growth. Individual dry weights and specific growth rates of the alga were significantly affected by the irradiance they were exposed to. For both 20 and 45 µmol photons $m^{-2}s^{-1}$, the alga exhibited higher dry weight and SGR values than the other treatments. This indicates that *L. majuscula* prefers low irradiances possibly because of the low maintenance rate of cyanobacteria - requiring only little energy to maintain their cell structure and function. Van Liere & Mur [9] compared the maintenance energy requirements in *Oscillatoria agardhii*, a cyanobacteria, with those of eukaryotes [22]. It was shown that maintenance requirements in cyanobacteria are much smaller than that of eukaryotes under limiting light. In addition, cyanobacteria at low irradiances have the capability to broaden the overall absorption band in order to balance the two antenna pigments responsible for their photosynthesis [6]. A study conducted by Yin et al. [26] on *Lyngbya wollei*, a close relative of *L. majuscula*, exhibited optimum growth at 22 µmol photons $m^{-2}s^{-1}$. Similarly, in a study conducted by Zhang et al. [12] on *Lyngbya kuetzingii,* the alga had its optimum growth under 20 µmol photons $m^{-2}s^{-1}$ while the study on *Lyngbya stagnina* by Jindal et al. [11] resulted in the highest exopolysaccharides and protein production under 45 µmol photons $m^{-2}s^{-1}$ in continuous light [11,12].

The minimal growth exhibited at irradiance levels of 110, 180, and 320 µmol photons $m^{-2}s^{-1}$ indicates that higher irradiances limit the growth of the alga. This is a minimizing response of the alga to the effects of photoinhibition caused by the excessive formation of Reactive Oxygen Species (ROS) [26]. Consequently, the results in the data analysis showed that the SGR values at high irradiances do not have a significant difference with each other and the negative control due to the cyanobacterium not being able to grow in the absence and at high light intensities.

Limitations. Due to time constraints, sampling was done only on days 0, 4, and 8; hence, daily growth rate of the alga was not assessed. No repetitions of the experiment were also made to further narrow down the irradiance values.

Conclusion. - *L. majuscula* exhibited maximum growth at 20 µmol photons $m^{-2}s^{-1}$ (29.7 °C, 8.28 pH) and 45 µmol photons $m^{-2}s^{-1}$ (30.25 °C, 8.67 pH). It can therefore be concluded that the species prefers low irradiance to maximize its growth. High irradiance, on the other hand, limits its growth.

Recommendations. - Information in this paper can be used as a basis for future studies in determining the irradiance that will result in the maximum growth of *L. majuscula*. The researchers recommend further studies between the irradiance levels of 20 µmol photons $m^{-2}s^{-1}$ and 45 µmol photons $m^{-2}s^{-1}$, with equal intervals, to exactly identify the most ideal irradiance level of maximum growth of the alga. Lastly, the researchers would also like to recommend daily sampling to determine the daily growth rate of the alga which is necessary to model a cyanobacterial growth curve.

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