PHYSIOLOGICAL CHARACTERIZATION OF LIGHT-ENHANCED GROWTH IN ACTINOBACTERIA

by

Archana Singh

A thesis submitted to the Faculty of University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Winter 2019

PHYSIOLOGICAL CHARACTERIZATION OF LIGHT-ENHANCED GROWTH IN ACTINOBACTERIA

by

Archana Singh

Approved: _____

Julia A. Maresca, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

E. Fidelma Boyd , Ph.D.
Interim Chair of the Department of Biological Sciences

Approved: _____

John A. Pelesko, Ph.D.
Interim Dean of the College of Arts and Sciences

Approved: _____

Douglas Doren, Ph.D.
Interim Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I am extremely grateful and express my deep sense of gratitude to my advisor, Dr Julia Maresca, for her guidance, constant supervision, keen interest, support and steady encouragement throughout the course of preparation and completion of my academic project and thesis. Her constant encouragement and advice has helped me transform into a better student, writer and research assistant.

I would like to thank all the present and past Maresca lab members- Dr Jessica Keffer for her patience while training me ; Erik Kiledal, Priscilla Hempel, Christie Chapman, Emma Smith for their patience and valuable suggestions which has helped me improve my presentation skills. Additionally, I would like to thank my committee members- Dr Carlton Cooper and Dr Karl Schmitz for their guidance and scientific expertise.

I would also like to thank my roommates for always lending an ear and being a great support system. At last but not the least, my words run few to express my gratitude to my parents and my grandmother, whose guidance, blessings and support is the reason I stand here.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Sunlight is a source of energy for phototrophic organisms and the ecosystems they are a part of. The concept of light utilization in primary producers is well understood in the scientific community. How sunlight acts as a signal and regulates processes in heterotrophs is a concept that is not well characterized in most species. Two heterotrophic actinobacterial strains lacking functional photosystems exhibit enhanced growth in the light. The aim of this study is to characterize the physiological effects light has on these strains. We discovered that the strains of actinobacteria- namely *Rhl. lacicola* and *Aurantimicrobium* sp strain MWH-Mo1, grow significantly better in the light than in the dark due to upregulation of sugar transport and metabolism genes in the light and protein synthesis in the dark. These actinobacterial strains have the ability to sense light and possibly utilize the byproducts of photosynthesis released by primary producers for growth. Since they are ubiquitous in freshwater ecosystems, this makes them model organisms to study the energy flow from primary producers to consumers and study the physiological effect sunlight has on these heterotrophic bacteria.

Chapter 1

INTRODUCTION

Sunlight is a consistent stimulus in the environment– it is known that prokaryotes and eukaryotes depend on sunlight as a source of energy or have evolved biological clocks, which organize their physiological activities in accordance with the solar cycle [1,2].

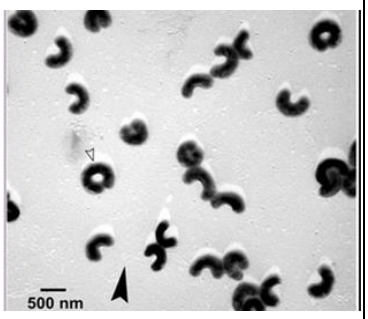
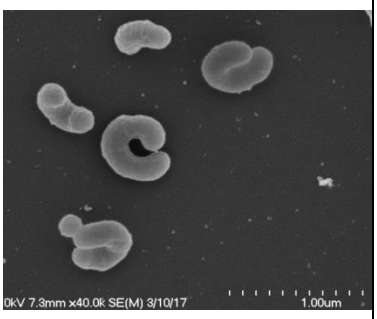
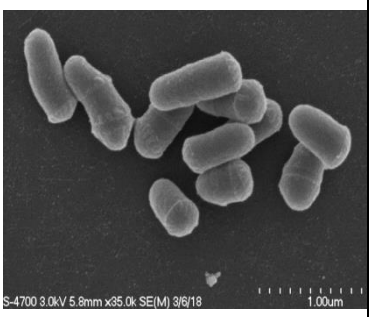
In a nutrient-limited environment, sunlight is an unlimited resource. The conversion of sunlight to energy is well understood in photoautotrophs and photoheterotrophs. Light regulates the circadian rhythm in plants, animals and fungi, informing the organisms about the location and time. Therefore, light acts both as a source of energy and information. Here, we study three heterotrophic actinobacterial strains and their response to light. Actinobacteria are a phylum of Gram-positive bacteria, ubiquitous in freshwater. Three strains of Actinobacteria were isolated from different parts of the world: *Rhodoluna(Rhl). lacicola* strain, Ta8 from Lake Taihu in China; *Aurantimicrobium* sp. strain MWH-Mo1 from Lake Mondsee Austria and *Microbacterium* sp. strain 10M3C3 from Lake Matano in Indonesia [3]. *Rhl*. *lacicola* and MWH-Mo1 are heterotrophs lacking a functional photosystem yet displayed a unique growth phenotype during the initial growth experiments: *Rhl*. *lacicola* and Mo1 exhibited increased growth in the light as compared to growth in the dark whereas 10M3C3 exhibited similar growth rates in the light and in the dark. This enhanced growth in the light was initially thought to be a result of existing photosystems. However, further experimentation[4] revealed that both *Rhl*. *lacicola* and

MWH-Mo1 lack a functional photosystem required for enhanced growth in light. We hypothesize that in *Rhl. lacicola* and MWH-Mo1 that lack a functional photosystem to convert light to chemical energy, light acts as a cue resulting in changes in growth and transcription activities.

1.1 Background Information of the Actinobacterial Strains

The three strains of Actinobacteria were isolated from different parts of the world: *Rhodoluna(Rhl). lacicola* strain, Ta8 from Lake Taihu in China; *Aurantimicrobium* sp. strain MWH-Mo1 from Lake Mondsee Austria and *Microbacterium* sp. strain 10M3C3 from Lake Matano in Indonesia (Table 1.1)

Table 1.1: Bacterial strains.

		
Rhl. lacicola	MWH-Mo1	10M3C3
Red, curved vibrio.	Yellow curvedvibrio.	Yellow, coryneform.
Faster growth in light.	Faster growth in light.	Does not exhibit faster growth in light.

In previous work conducted in the Maresca lab, RNA-seq analysis was performed on the actinobacterial strains *Rhl. lacicola* and MWH-Mo1 to learn more about the transcriptional changes that result in the unique growth phenotype. The

actinobacterial strains were grown till late exponential phase in continuous light and dark conditions and after quantifying the number of transcripts per gene, statistical data analysis was performed to learn if there existed a significant difference in the gene expression levels in the light and dark.

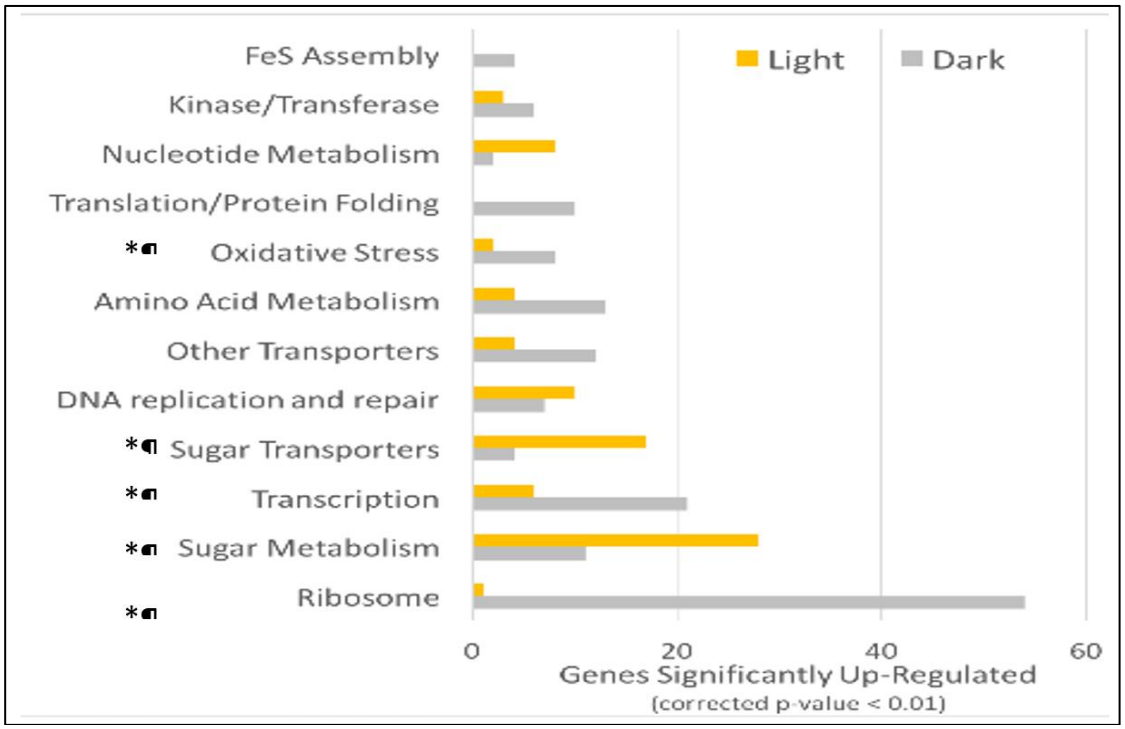


Figure 1.2 RNA-seq results of *Rhl. lacicola*. *Rhl. lacicola* was grown in constant light and dark until late exponential phase. The number of differentially expressed genes in each category (light-yellow, dark-grey) is plotted here. Asterisks (*) indicate the difference between light and dark is statistically significant. (Fischer's Exact test, $p<0.05$)

Figure 1.2 represents the RNA-seq data results for *Rhl. lacicola*, and the results for MWH-Mo1 are similar. These are the results of four biological replicates, the results indicate significant differences in gene expression in the light and the dark-

genes involved in protein synthesis and oxidative stress are upregulated in the dark, genes involved in sugar transport and metabolism are upregulated in the light [5].

1.2 Hypothesis and Experimental Aims

Rhl. lacicola and MWH-Mo1 grow faster in the light than in the dark. Since they lack a functional photosystem, we predict that light acts as a cue, resulting in changes in transcriptional activities resulting in enhanced growth in the light. We predict enhanced organic carbon uptake in the light enables faster growth. Since carbon catabolism is associated with ATP production, we predict that increased uptake of carbon in the light will result in increased ATP production. Results of RNA-seq analysis states that genes involved in oxidative stress are upregulated in the dark, we predict that this would result in increased oxidative stress[6]in the dark. RNA-seq data results also state that genes involved in protein production are upregulated in the dark, resulting in increased protein production in the dark. To confirm the predictions, I accomplished the following specific aims:

1. Quantify sugar uptake in the light and dark.
2. Quantify ATP production in the light and dark.
3. Quantify oxidative stress in the light and dark.
4. Quantify protein production in the light and dark.

Chapter 2

MATERIALS AND METHODS

2.1 Strains and Growth Conditions

Freshwater Actinobacteria strains –*Rhl. lacicola* strain Ta8, *Aurantimicrobium* sp. strain MWH-Mo1 and *Microbacterium* sp. strain 10M3C3 , were grown in continuous light (~40 µmol photons m^{-2} s^{-1}) where the light source is a 13 W cool fluorescent bulb or dark conditions at 28 °C in 4 mL of 0.3 % nutrient broth-soytone-yeast extract (NSY) medium (Table A.1)[1]. Dark conditions were maintained by wrapping culture tubes in paper and aluminum foil. Growth was monitored by measuring optical density at 660 nm and growth rates were calculated from optical densities during exponential growth phase.

The three actinobacterial strains were grown in a twelve hour light-dark cycle or in continuous dark in NSY media. To achieve this experimental condition, a timer was attached to the power socket of the light source which turned off the light every twelve hours. Continuous dark conditions were maintained by wrapping culture tubes in aluminum and paper foil.

To verify that light does not have any effect on the temperature of the media, two beakers of 0.3 % NSY media were placed in a 28 °C incubator, in continuous light (13 W cool fluorescent bulb) or dark conditions and the temperature of the media was measured using an analog thermometer after four hours. Continuous dark condition was maintained by wrapping the beaker in paper and aluminum foil.

2.2 Aged Media Experiments

To confirm that light was not degrading the media, making the organic carbon more bioavailable to actinobacterial strains, NSY media was aged for 4 days in continuous white light or dark. Actinobacteria *Rhl. lacicola* and MWH-Mo1were inoculated into the fresh and aged media and grown in continuous light and dark conditions as described above for forty-eight hours. Growth rates were calculated using optical density values at 660 nm (OD_{660nm}) during exponential phase.

2.3 Reducing Sugar Assay

Rhl. lacicola, MWH-Mo1 and 10M3C3 were inoculated in 6 mL of minimal media (Table A.2) with a known carbon source of 0.1% fructose. The culture tubes were placed in a 28 °C incubator, in continuous light or dark conditions. 600 μL was removed from the culture tubes at a few time points at mid-exponential phase, the subsamples were then centrifuged at 10,000 rpm for fifteen minutes. The supernatant was incubated in a 37°C water bath for ten minutes, then cooled to room temperature. This was followed by addition of 900μL dinitrosalicylic acid (DNS) reagent to the supernatant, solutions were incubated at 95°C for 5 minutes, and $A_{540\ nm}$ was measured.

To verify that increased fructose uptake in the light is a result of upregulation of sugar transporters and not due to the increased number of cells in light, an overnight culture of *Rhl. lacicola* was suspended in 6 mL IBM(Inorganic Basal Medium, Table A.6) to get final OD_{660nm} values of 0.1, 0.05, 0.01. The culture tubes were placed in a 28°C incubator in continuous light or dark conditions. 1mL subsamples were removed from the test tubes after ten minutes, and the subsamples were centrifuged at 10,000

rpm for twenty minutes to remove the cells. Fructose present in the supernatant was quantified using the reducing sugar assay.

2.4 Quantification of ATP Production in the Light and Dark

ATP pools in light- and dark-grown cells were quantified using Promega BacTiterGlo kit, which is a luciferase-based assay. For ATP quantification, *Rhl. lacicola* was grown in 6 mL of NSY media in continuous light or dark in a 28°C incubator. 100μL of the samples, at multiple time points in the mid-exponential phase, were transferred to an optically clear 96-well plate, followed by 100μL of the reagent. The contents of the well was mixed briefly (0.5s integration 0.5 min shaking at medium speed) on the orbital shaker of GloMax 96 Microplate Luminometer followed by recording the luminescence.

2.5 Quantification of Protein Production

To quantify the protein produced by the cells, *Rhl. lacicola* was inoculated in 6mL of NSY media and were grown in continuous light or dark conditions, where the dark conditions were maintained as described above. The samples were centrifuged at 10,000 rpm for 15 minutes after which the pellet was resuspended in autoclaved distilled water. Following this, the cells were lysed using sonication (10 pulses, twice, 100 Hz), the cell debris was removed by centrifugation and the supernatant was used for soluble protein quantification. Thermoscience Pierce BCA Protein assay kit was used to quantify protein production by *Rhl. lacicola* in the light and dark following the manufacturer's instructions.

2.6 Quantification of Reactive Oxygen Species in the Light and Dark

Preliminary RNA-seq data suggests that genes involved in oxidative stress in *Rhl. lacicola* are upregulated in the dark. To confirm this prediction, we quantified reactive oxygen species (ROS) in cells, *Rhl. lacicola* was grown in NSY media in continuous light overnight. The cells were diluted in 6 mL of NSY media to obtain final OD_{660nm} of 0.1, and further incubated at 28°C for 6 hours. The cells for positive control were exposed to 1 M of H_2O_2 for 1 hour, then washed with PBS and stained with 10μM CM-H_2DCFDA. CM-H_2DCFDA is a fluorescent dye that measures hydroxyl, peroxyl, oxide reactive oxygen species activity within the cell. Fluorescence was measured with GloMax 96 Microplate Luminometer and fluorometer where the excitation wavelength for the fluorescent indicator, CM-H_2DCFDA is 492-495 nm and the emission wavelength range, 517-527 nm. The excitation wavelength for the blue filter utilized for this protocol is 490 nm and it collects data through 510-570 nm wavelengths.

Chapter 3

RESULTS

3.1 Light Enhances Growth in Some Actinobacteria

Actinobacteria *Rhl. lacicola* strain MWH-Ta8, *Aurantimicrobium* sp. strain MWH-Mo1, *Microbacterium* sp. strain 10M3C3 were grown in constant light (intensity ~40 µmol photons m^{-2} s^{-1}) or dark. *Rhl. lacicola* and MWH-Mo1 reach higher cell densities in the light than in the dark as shown in Figure 3.1 A and 3.1 B and 10M3C3 grows at the same rate in both constant light and dark conditions (Figure 3.1 C), indicating that light-enhanced growth is not a phenotype common to all Actinobacteria.

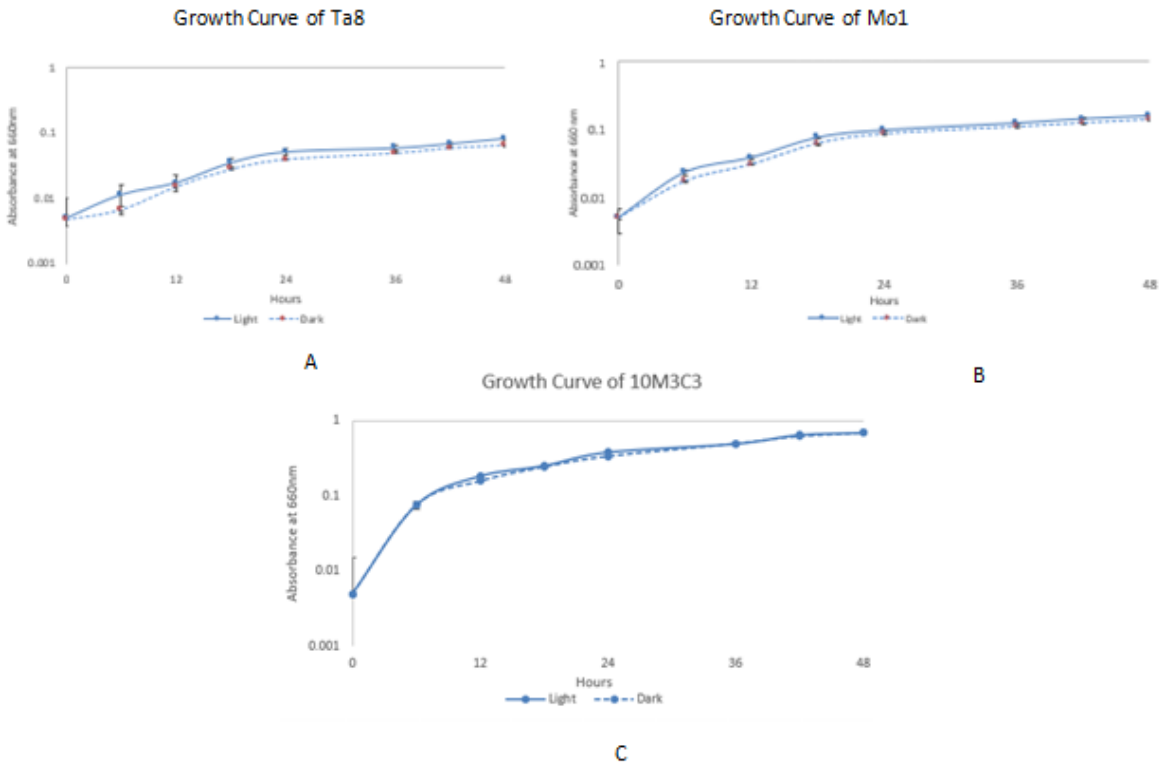


Figure 3.1 Growth rate of Actinobacteria in white light: *Rhl. lacicola* (A) and *Aurantimicrobium* sp. strain MWH-Mo1 (B)*Microbacterium* sp. strain 10M3C3 (C) in constant dark (tubes wrapped in paper and foil) or light (13W cool fluorescent bulb). Growth was monitored by measuring optical density at 660 nm. Each point on the graph is an average of four biological replicates. A two-sample t-test was conducted, comparing the growth rates in the light and dark and it was discovered that both Ta8 and MWH-Mo1 grow significantly better in the light than in the dark($p<0.05$), 10M3C3 does not exhibit significant difference in growth rates in the light and the dark ($p=0.09$).

Table 3.1 Growth rates of Actinobacteria in white light

Growth Rate	***Rhl. lacicola***	**MWH-Mo1**	**10M3C3**
Continuous Light	0.062 +/- 0.002	0.075 +/- 0.0002	0.108+/- 1.7E-05
Continuous Dark	0.055 +/- 0.0002	0.073+/- 0.0001	0.108+/- 1.6E-05

The three strains of Actinobacteria were grown in constant light or dark conditions in NSY media. Growth was monitored by measuring optical density at 660 nm, growth rates were calculated using the optical density values at mid-exponential phase, the growth rate values are presented in table 3.1. The values in each cell of Table 3.1 are an average of four biological replicates along with their standard deviations.

On performing a two-tailed t-test on the difference in the growth rates between the light and the dark, we observed that the difference in the growth rates is not statistically significant for 10M3C3 but is statistically significant for *Rhl. lacicola* and MWH-Mo1, with a p-value of less than 0.05.

To determine if *Rhl. lacicola* and MWH-Mo1 exhibit enhanced growth in environmentally relevant conditions (i.e. alternate periods of light and dark), the strains were grown in a twelve hour light- twelve hour dark cycle. This experimental condition was achieved by attaching a timer to the power socket of the light source which turned off the light source every twelve hours.

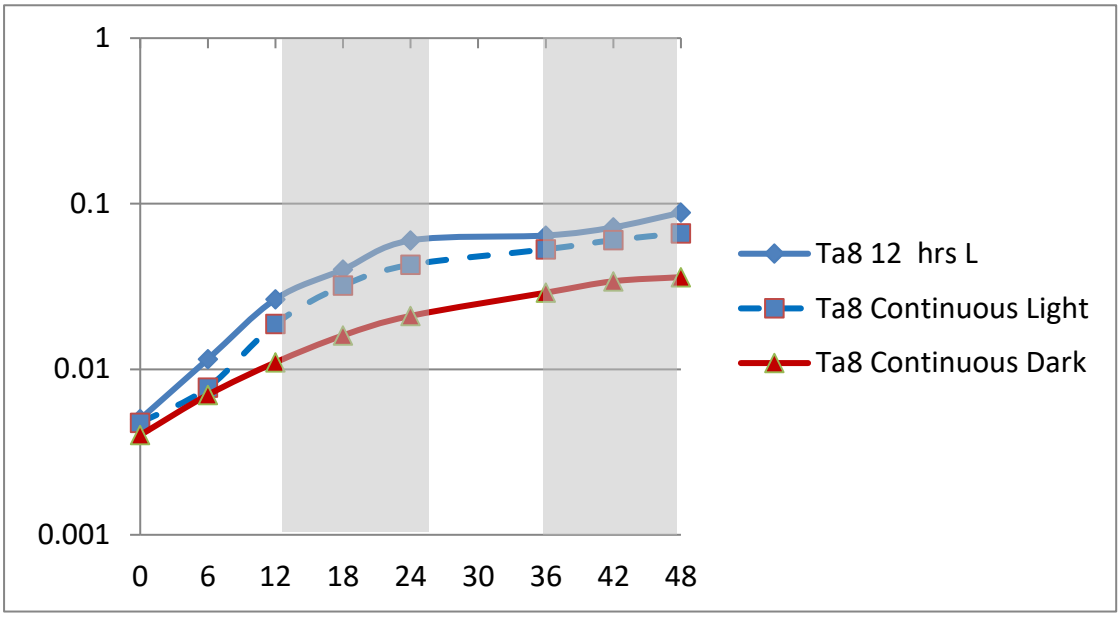


Figure 3.2 Growth of actinobacteria in light/dark cycle
Rhl. lacicola was grown in a twelve hour light-dark cycle, continuous light or dark. A timer was attached to the power socket of the light source which turned off the light every twelve hours to maintain the 12 hour light/dark experimental condition. Each point on the graph is an average of four biological replicates. The shaded region on the graph represents the time during which the light was turned off to maintain the 12 hour dark conditions.

Table 3.2 Growth Rate and Statistical results for Light/dark cycle

Condition	**Time**	**Growth Rate**	**p-value**
Continuous Dark	12-24 Hours	0.055	**x**
Continuous Dark	12-24 Hours	0.072	p=0.005
12 hour Light/Dark Cycle(Light)	6-12 Hours	0.119	p=0.0003
12 Hours Light/Dark Cycle (Dark)	18-24 Hours	0.0494	x

Rhl. lacicola exhibits higher growth rate and growth yield in a 12 hour light-dark cycle when compared to continuous exposure to the light as depicted in the growth curve (Figure 3.2, Table 3.2). However, the growth phenotype of faster growth in the light is maintained throughout the growth cycle.

On performing a two sample t-tail test between the difference in the growth rates in light(continuous light, 12 hours of light) and dark conditions, we observed that the difference in the growth rates between the light and dark conditions is statistically significant ($p<0.05$ in both cases, Table 3.2. The optical density for this experiment was recorded every six hours, growth rates were calculated when the cells were in exponential phase as demonstrated in Table 3.2. Figure 3.2 exhibits the growth of *Rhl. lacicola* in the light/dark growth cycle.

3.2 Abiotic Changes in Media do not Affect Growth

To verify that abiotic changes in media due to continuous exposure to light do not affect the growth phenotype making the organic carbon more bioavailable to actinobacterial strains, NSY media was aged for 4 days in continuous white light or dark.

Table 3.3 Growth of Actinobacteria in Fresh vs Aged Media.

Growth rate	***Rhl. lacicola***	**MWH-Mo1**
Fresh media, continuous Light	0.103 +/- 0.07	0.051 +/- 0.01
Fresh media, continuous Dark	0.081+/- 0.02	0.046 +/- 0.01
Aged media, continuous Light	0.072 +/- 0.01	0.047 +/- 0.02
Aged media, continuous Dark	0.068+/- 0.02	0.039 +/- 0.01

Rhl. lacicola and MWH-Mo1 were grown in continuous light or darkness in fresh and aged media. The values in each cell in table 3.3 represents the average of four biological replicates along with the standard deviation. Even though a difference in the growth rates in fresh and aged media was observed, the growth phenotype remains unchanged i.e. *Rhl. lacicola* and MWH-Mo1 exhibit higher growth rate and growth yield in the light than in the dark.

On performing a two-sample two-tail t-test on the difference in the growth rates between the different conditions, we observed that the difference in the growth rates is statistically significant for growth rates in light conditions, for fresh vs aged media with a p-value of less than 0.05, as indicated in table 3.4.

Table 3.4 Statistical output for growth experiments in fresh and aged media.

Growth rate	***Rhl. lacicola***	**MWH-Mo1**
Fresh media, continuous light vs continuous dark	0.038	0.035
Aged media, continuous light vs continuous dark	0.039	0.036

To eliminate the question that continuous exposure to light results in changes in temperature of the media resulting in the enhanced growth phenotype, the temperature of NSY media exposed to constant light and dark was recorded using an analog thermometer. The results as shown in table 3.5 confirm that continuous exposure to light, does not result in an increase in temperature of the media and that

enhanced growth phenotype of *Rhl. lacicola* and MWH-Mo1 in the light is not due to abiotic changes in media(table 3.5).

Table 3.5 Temperature of NSY Media in light and dark

Temperature of NSY media in the Light	**Temperature of NSY media in the Dark**
28.1 °C	28 °C

3.3 Enhanced Sugar Uptake in the Light

The results from RNA-seq identified genes of *Rhl. lacicola* that were upregulated in the light and the dark. The results suggest that genes involved in sugar transport and metabolism are upregulated in the light, which led us to our first hypothesis that since growth is enhanced in the light and growth involves carbon utilization in the form of sugar uptake from the media, sugar uptake is enhanced in the light. To test our hypothesis, a reducing sugar assay was performed to quantify the sugar uptake by *Rhl. lacicola* during exponential phase.

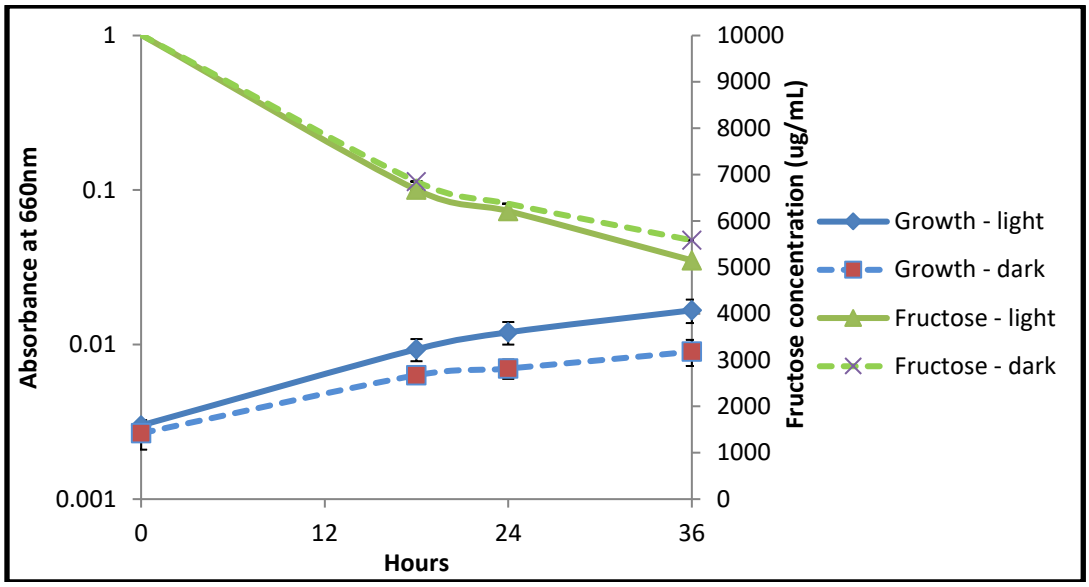


Figure 3.3a Optical density at 660 nm and fructose concentration of *Rhl. lacicola* as a function of time**.** *Rhl. lacicola* was grown in continuous light and dark in minimal media, where the only source of carbon is fructose. Growth was monitored by measuring optical density at 660 nm and the fructose remaining in the supernatant was quantified by the reducing sugar assay. Each point on the graph is an average of four biological replicates, the error bars represent the standard deviation.

Figure 3.3.1 indicates that exists a correlation between growth of the cells and the fructose consumed. An increase in the number of cells of *Rhl. lacicola* due to enhanced growth in the light, results in an increase in the fructose uptake in the light relative to the dark. This results in a decrease in the fructose content in the media.

To confirm the correlation between fructose consumed and the growth of *Rhl. lacicola*, Pearson test of correlation was performed, comparing the optical density and concentration of fructose over 36 hours. A p-value of less than 0.05 confirms the positive correlation between the growth of *Rhl. lacicola* over time and the fructose consumed by it.

To confirm that enhanced sugar uptake in the light is a result of upregulation of sugar transporters and metabolism genes and not just due to an increase in fructose uptake due to the increased cell density in the light, *Rhl. lacicola* was transferred to IBM solution and sugar uptake was quantified using the reducing sugar assay.

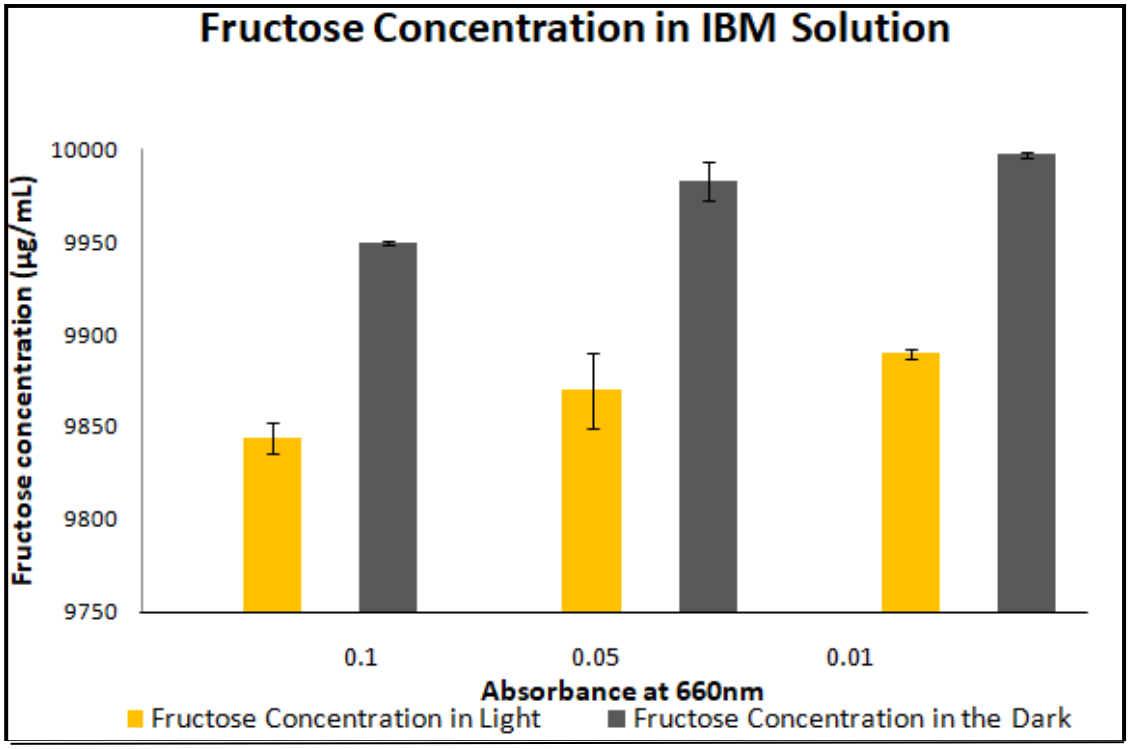


Figure 3.3b Fructose concentration as a function of OD. Each bar on the graph represents the mean of four biological replicates, the error bars represent the standard deviation. This experiment verifies that the enhanced consumption of fructose in the light is not a result of the increased cell density, but the result of upregulation of sugar transport and metabolism genes in the light.

A two-tailed t-test performed on the results of fructose concentration displayed in figure 3.3.2 indicate that the difference in fructose uptake in the light and the dark is statistically significant, with a p-value of less than 0.05.

CONCLUSION: The experiments supports our initial hypothesis that light induces an upregulation of sugar transport and metabolism genes, which results in increased sugar uptake in the light.

3.4 Increased ATP Production in the Light

Sugar uptake is enhanced in the light and breakdown of sugar is associated with production of ATP, we hypothesize than an enhanced sugar uptake in the light will also result in an increase in ATP production in the light. To confirm our hypothesis, ATP produced by the cells was quantified using the Luciferase assay.

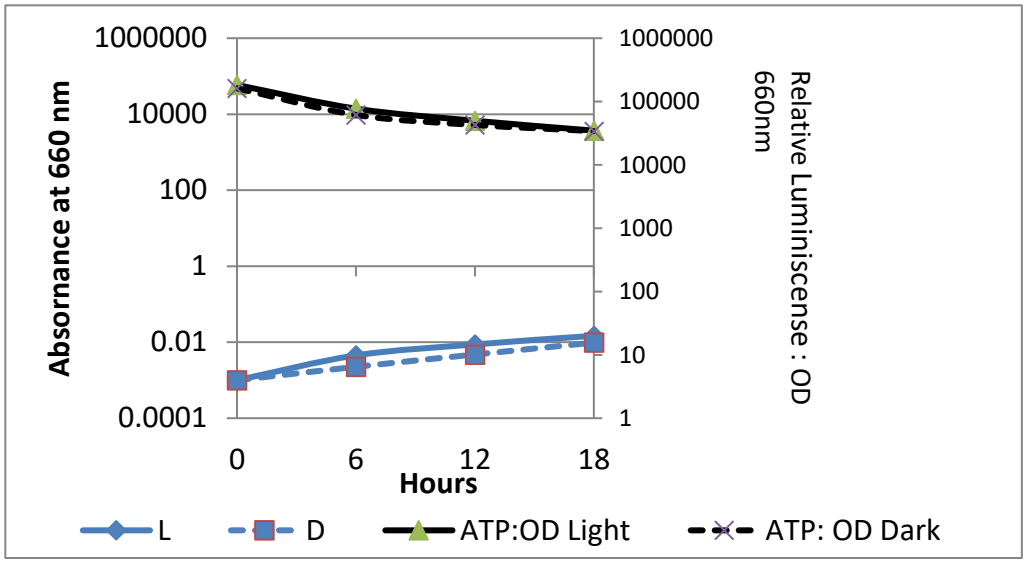


Figure 3.4 Growth and ATP concentration in *Rhl. lacicola*. Growth of *Rhl. lacicola* in the light and dark was monitored by measuring optical density at 660 nm, ATP produced by the cells was quantified by the luminescent luciferase assay and the ratio of relative luminescence and optical density has been plotted. Results indicate that the ATP levels in the light and dark grown cells are similar, contrary to our hypothesis. On performing a two-tailed t-test on the difference in relative luminescence between the different conditions, p-value of greater than 0.05 indicates that the difference is not statistically significant.

CONCLUSION: The experiment does not support our initial prediction that light induces an upregulation of sugar transport and metabolism genes, which results in increased ATP production in the light.

3.5 Increased Protein Production in the Dark

Results from the RNA-seq data reveal that genes involved in protein production are upregulated in the dark. To confirm the prediction, soluble protein concentration in the cells was quantified. The results indicate that protein production is greater in the dark than in the light and increases with time as exhibited by figure 3.5.

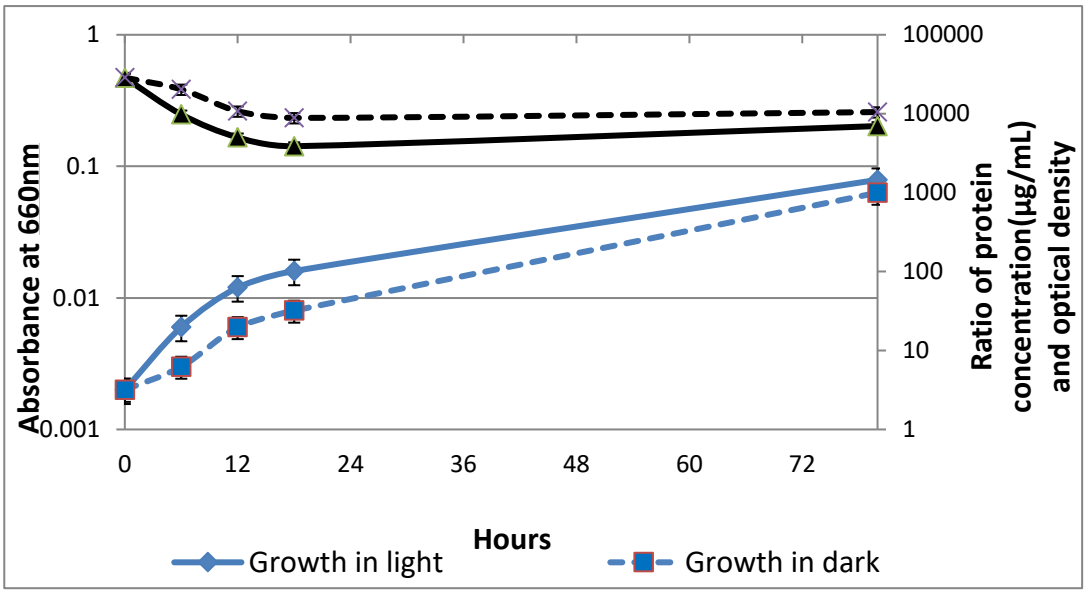


Figure 3.5 Growth of *Rhl. lacicola* and ratio of protein concentration and optical density. Growth of *Rhl. lacicola* in the light and dark was monitored by measuring optical density at 660 nm. The soluble protein concentration was quantified using the Pierce BCA assay kit. Each point on the graph is an average of four biological replicates and the error bars represent standard deviation. The results indicate that the soluble protein concentration in cells increases in the dark.

To confirm the hypothesis of increased protein production in the dark, a two sample t-test was conducted between the soluble protein concentrations in the light and dark at mid-exponential phase, a p-value of less than 0.05 denotes that the difference is statistically significant.

CONCLUSION: The experiments supports our initial hypothesis that genes involved in protein production are upregulated in the dark, resulting in increased protein production in the dark.

3.6 Increased Oxidative Stress in the Dark

Gene expression results from the initial RNA-seq analysis reveal that genes involved in oxidative stress are upregulated in the dark. This data suggests that *Rhl. lacicola* could use periods of darkness to repair oxidative damage. To confirm this prediction, reactive oxidative species in the cells grown in continuous light and dark were quantified by using a fluorescent stain, CM-H_2DCFDA which interacts with the reactive oxygen radicals (peroxides, oxides and hydroxyl species). The fluorescence as a result of this reaction is proportional to the concentration of the reactive oxygen radicals.

The results confirm the prediction since *Rhl. lacicola* cells grown in the dark show more oxidative stress that the light grown cells, as presented in figure 3.6.

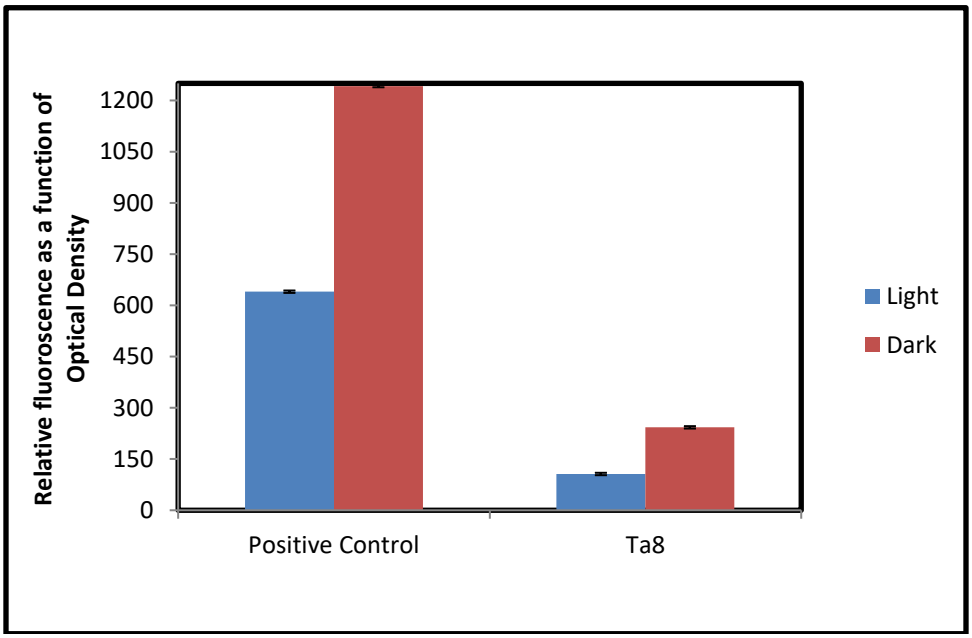


Figure 3.6 Fluorescent detection of intracellular reactive oxygen species in *Rhl. lacicola.*

Positive control are cells of *Rhl. lacicola* exposed to 1 M of H_2O_2 and stained with 10μM CM-H_2DCFDA and they therefore exhibit high oxidative stress. Negative control are cells that were not exposed to H_2O_2 or the fluorescent dye and the negative control values were deducted before plotting. Each bar graph is an average of four biological replicates and its ratio to the optical density, the error bars represent standard deviation. The bars indicate that for both the experimental and the control condition, *Rhl. lacicola* undergoes greater oxidative stress in the dark when compared to the light.

A p-value of less than 0.05, when comparing the oxidative stress measured by the cells in the light and dark using a two sample t-test confirms that the difference is statistically significant.

CONCLUSION: The experiment confirms our initial hypothesis that *Rhl. lacicola* undergoes increased oxidative stress in the dark when compared to cells grown in the light.

Chapter 4

DISCUSSION AND FUTURE DIRECTIONS

Two heterotrophic strains of Actinobacteria- *Rhl. lacicola* and MWH-Mo1 exhibit a unique growth phenotype where they grow significantly faster in the light than in the dark. Based on the experimental results, we have shown the physiological effect of light on these strains: light results in a significant increase in the uptake and utilization of organic carbon whereas processes involved in protein production and in oxidative stress exhibit significant increase in the dark.

We predicted that this increased uptake of carbon in the light is utilized in biomass production. This was quantified by measuring the growth rate, sugar uptake and ATP produced, as depicted in the results section above. Enhanced sugar uptake in the light corresponds to sugar catabolism and we predicted that this would result in increased ATP production. However, when the ATP levels in the cells grown in the light and dark were quantified, we observed no difference in the standing pools of ATP .We speculate that the cells utilize dark growth conditions to synthesize new proteins and possibly repair any damage due to oxidative stress.

Aerobic bacteria face the inevitable consequence of dealing with reactive oxygen species (ROS) generated during metabolism. This production of ROS is detrimental for macromolecules such as DNA, proteins, and lipids due to the high reactivity of the ROS[6]. The production of ROS activates a downstream mechanism resulting in the upregulation of genes involved in oxidative stress to detoxify the cells from ROS[9]. Oxidative stress occurs when the cells are incapable of detoxifying

existing ROS. Upregulation of oxidative stress genes in the dark in response to the production of ROS species raises a lot of interesting questions that needs further research- What is the subset of ROS the cells are responding to? Would the quantified ROS levels change if the cells are grown in alternate light and dark cycles?

Determination of carbon use efficiency (CUE) using these Actinobacteria as model organisms would help determine the flow of carbon and energy in aquatic ecosystems[8]. CUE is defined as the amount of carbon incorporated into biomass relative to the total carbon uptake ($C_{biomass}/[C_{biomass} + CO_2]$) [10,11]. We predict that as growth rate increases, CUE also increases[10]. This will result in increased ATP flux as light grown cells would require energy for increased biomass production. If light improves the efficiency of microbial growth, these results will have consequences on carbon cycling in nutrient-rich and nutrient-poor natural environments- CUE will decrease in a nutrient-poor environment. It will also be interesting to observe if light at different intensities and different wavelengths has an effect on CUE. Since *Rhl. lacicola* and MWH-Mo1 exhibit enhanced growth in blue light, we should observe increased CUE in blue and near UV-light when compared to other wavelengths.

Rhl. lacicola and MWH-Mo1 exhibit enhanced growth in the light to possibly coordinate carbon uptake and utilization processes with the time of maximum production of photosynthates by primary producers. The ability to increase metabolic machinery as a response to light-sensing acts as an advantage for *Rhl. lacicola* and MWH-Mo1 over strains that upregulate organic carbon after detecting substrates by decreasing competition for substrates. The carbon-dioxide produced by *Rhl. lacicola* and MWH-Mo1 as a byproduct of metabolism is possibly utilized by primary producers for photosynthesis. Hence, in a freshwater ecosystem, there exists a

synergistic relationship between primary producers and heterotrophs like *Rhl. lacicola* or MWH-Mo1.

Based on the experimental results, we know that light in *Rhl. lacicola* and MWH-Mo1 is sensed by light sensing proteins, triggering changes in transcription resulting in enhanced growth. When growth of *Rhl. lacicola* and MWH-Mo1 was monitored in different wavelengths of light, it was observed that both *Rhl. lacicola* and MWH-Mo1 grow significantly better in UV (375 nm) and near UV (425 nm) light than in the dark. Genome comparisons of *Rhl. lacicola* and MWH-Mo1indicate that a DNA photolyase and a CryB-type cryptochrome may possibly be the potential blue-light photoreceptors shared by the two strains. We suggest that CryB absorbs blue light, initiating a signal cascade informing the cells to upregulate carbon and other processes, resulting in faster growth.

Cryptochromes are blue-light sensing proteins founds in the three domains of life, some of them are involved in regulation of circadian rhythm[12,13]. We propose that the mechanism of light sensing of CryB is similar to the one found in *Rhodobacter sphaeroides.* The signal transduction, however, might differ due to the fact that the homologs to proteins in the pathway are not found in either *Rhl. lacicola* or MWH-Mo1[5]. In *Rhodobacter sphaeroides,* the N-terminus of AppA which has the FAD blue-light sensing domain senses blue light, transfers signal to C-terminus by a process not yet known[12,13,14]. We speculate that the mechanism of light sensing in *Rhl. lacicola* and MWH-Mo1 involves a light sensing cryptochrome, informing the cells to upregulate carbon and other processes resulting in enhanced growth in blue light.

This study is significant as it informs us of how limited our knowledge is regarding light sensing in heterotrophs. A few potential light sensors heterotrophs

posses are- carotenoids, rhodopsin, cryptochrome, BLUF domain [14,15]. However, previous work in the Maresca laboratory [5] establishes that in *Rhl. lacicola* and MWH-Mo1, carotenoids, and rhodopsin are not the light sensors. We speculate that *Rhl. lacicola* and MWH-Mo1 have crpytochrome light sensors which convert light to information, informing the cells to upregulate sugar metabolism, resulting in enhanced growth in the light. This increased upregualtion of sugar, after sensing of light is utilized in increased biomass production which lines up with the increased growth rate. Upregulation of genes involved in oxidative stress in the dark results in increased oxidative stress. We observe an increase in soluble protein production in cells grown in the dark indicating that while the cells are involved in biomass production in the light, they utilize dark conditions for cell maintenance activities. The reason we observed similar standing pool of ATP in the light and dark grown cells is that ATP concentration is regulated by various cell maintenance processes besides sugar catabolism[18]. The results to our experiments, while informing us about the physiological effects of light on *Rhl. lacicola* and MWH-Mo1 do raise additional questions that need further research- how would the ATP concentration, ROS levels and soluble protein concentration change if the cells were grown in a light-dark cycle instead of in continuous light or dark. Comparing the RNA-seq data and the experimental results for the two subsets of data (cells grown in continuous light and dark, cells grown in twelve hour light-dark cycle) will help understand the physiological effect of light on these unconventional strains of heterotrophic bacteria better.

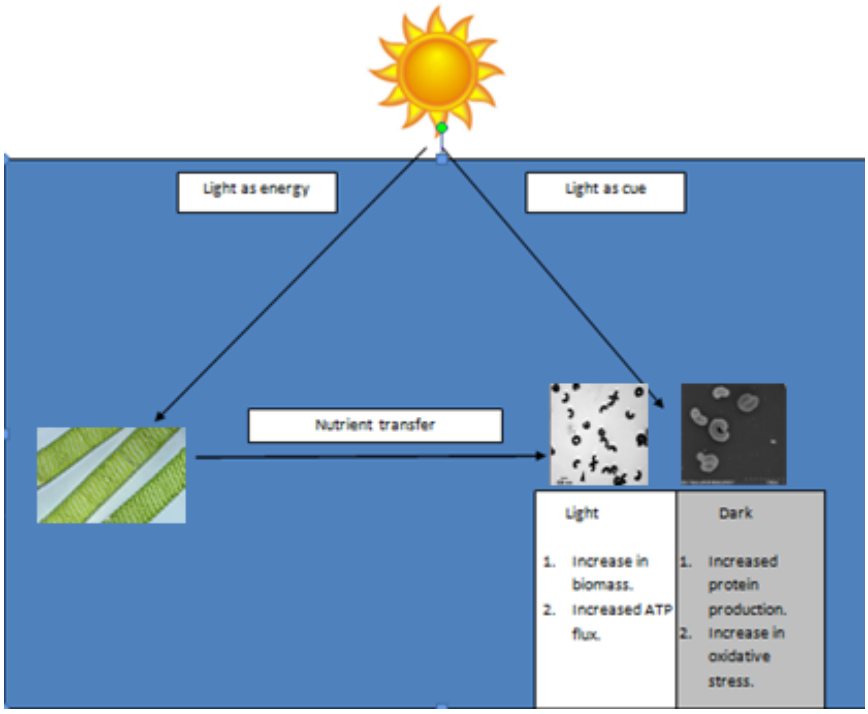


Figure 4.1 Energy flow in Freshwater ecosystems. This diagram depicts how sunlight acts as a source of energy for primary producers such as green algae, resulting in production of sugar and nutrients. These nutrients are utilized by the heterotrophic strains of actinobacteria, *Rhl. lacicola* and MWH-Mo1 which sense light as information, informing the cells to uptake the nutrients released by the primary producers. The actinobacterial strains exhibit enhanced growth in the light and perform maintenance related activities in the dark i.e. they exhibit increase in biomass and increased ATP flux in the light and increased protein production and oxidative stress in the dark.

REFERENCES

1. Cohen, S. E., & Golden, S. S. (2015). Circadian Rhythms in Cyanobacteria. Microbiology and Molecular Biology Reviews. https://doi.org/10.1128/MMBR.00036-15.

2. Nowicka, B., & Kruk, J. (2016). Powered by light: Phototrophy and photosynthesis in prokaryotes and its evolution. Microbiological Research. https://doi.org/10.1016/j.micres.2016.04.001

3. Hahn, M. W., Stadler, P., Wu, Q. L., &Pöckl, M. (2004). The filtration-acclimatization method for isolation of an important fraction of the not readily cultivable bacteria. Journal of Microbiological Methods. https://doi.org/10.1016/j.mimet.2004.02.004

4. Keffer, J. L., Hahn, M. W., & Maresca, J. A. (2015). Characterization of an Unconventional Rhodopsin from the Freshwater Actinobacterium *Rhodolunalacicola.* Journal of Bacteriology, 197(16), 2704 LP-2712. https://doi.org/10.1128/JB.00386-15

5. Maresca, J.A., Keffer, J.L, Hempel, P., Polson, S.W., Shevchenko, O., Bhavsar, J., Powell, H.D., Miller, K.J., Singh, A., Hahn, M.W. (In Press) Light modulates the physiology of non-phototrophic Actinobacteria.

6. Imlay, J. A. (2008). Cellular Defenses against Superoxide and Hydrogen Peroxide. Annual Review of Biochemistry. https://doi.org/10.1146/annurev.biochem.77.061606.161055

7. Mempin, R., Tran, H., Chen, C., Gong, H., Kim Ho, K., & Lu, S. (2013). Release of extracellular ATP by bacteria during growth. *BMC Microbiology*. https://doi.org/10.1186/1471-2180-13-301

8. Oh, E., McMullen, L., & Jeon, B. (2015). Impact of oxidative stress defense on bacterial survival and morphological change in Campylobacter jejuni under aerobic conditions. Frontiers in Microbiology.https://doi.org/10.3389/fmicb.2015.00295

9. Zhao, X., &Drlica, K. (2014). Reactive oxygen species and the bacterial response to lethal stress. Current Opinion in Microbiology. https://doi.org/10.1016/j.mib.2014.06.008

10. Sinsabaugh, R. L., Manzoni, S., Moorhead, D. L., & Richter, A. (2013). Carbon use efficiency of microbial communities: stoichiometry, methodology and modelling. Ecology Letters, 16(7), 930–939.https://doi.org/10.1111/ele.12113

11. Keiblinger, K. M., Hall, E. K., Wanek, W., Szukics, U., Hämmerle, I., Ellersdorfer, G., Zechmeister-Boltenstern, S. (2010). The effect of resource quantity and resource stoichiometry on microbial carbon-use-efficiency. FEMS Microbiology Ecology, 73(3), 430–440. https://doi.org/10.1111/j.1574-6941.2010.00912.x

12. Todo, T. (1999). Functional diversity of the DNA photolyase/blue light receptor family. Mutation Research - DNA Repair, *434*(2), 89–97. https://doi.org/10.1002/biof.552210116

13. Hendrischk, A.-K., Frühwirth, S. W., Moldt, J., Pokorny, R., Metz, S., Kaiser, G., Klug, G. (2009). A cryptochrome-like protein is involved in the regulation of photosynthesis genes in Rhodobactersphaeroides. MolMicrobiol. https://doi.org/10.1111/j.1365-2958.2009.06912.x

14. Masuda, S., & Bauer, C. E. (2002). AppA is a blue light photoreceptor that antirepresses photosynthesis gene expression in *Rhodobactersphaeroides*. Cell. https://doi.org/10.1016/S0092-8674(02)00876-0

15. Braatsch, S., Gomelsky, M., Kuphal, S., & Klug, G. (2002). A single flavoprotein, AppA, integrates both redox and light signals in Rhodobactersphaeroides. Molecular Microbiology. https://doi.org/10.1046/j.1365-2958.2002.03058.

16. Stramski, D., & Kiefer, D. A. (1998). Can heterotrophic bacteria be important to marine light absorption? Journal of Plankton Research. https://doi.org/10.1093/plankt/20.8.1489

17. Montgomery, B. L. (2007). Sensing the light: Photoreceptive systems and signal transduction in cyanobacteria. Molecular Microbiology. https://doi.org/10.1111/j.1365-2958.2007.05622.x

18. Lynch, M., & Marinov, G. K. (2015). The bioenergetic costs of a gene. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.1514974112

Appendix A

TABLES

Table A.1 Recipe of NSY media

Chemical	**Composition in Media**
Nutrient Broth	1 g
Soytone	1 g
Yeast Extract	1 g
10X IBM (see table A.6)	100 mL
Distilled Water	1 L

Table A.2 Recipe of Minimal Media

Chemical	**Composition in Media [L^{-1}]**
100X FW Base table (A.3)	10 mL
10X IBM table (A.6)	100 mL
NH_4Cl	1 g
pH 8.0	
After autoclaving,	
1 M $MgSO_4$	2 mL
1 M $CaCl_2$	0.1 mL
L-asparagine	0.04 g
L-cysteine	0.04 g
1000X 8-Vitamin mix table (A.4)	1 mL
1000X Vitamin B12 table (A.5)	1 mL
Fructose	1 mL

Table A.3 Recipe of 100X FW Base

Chemical	**Composition in Media[g L^{-1}]**

NaCl	100
MgCl2.6H2O	40
CaCl2.2H2O	10
KH_2PO4	20
KCl	50

Table A.4 Recipe of 1000X 8-Vitamin Solution

Chemical	**Composition in Media[L^{-1}]**
10 mM Phosphate Buffer	100 mL
Thiamine HCl	100 mg
D-Ca Pantothenate	100 mg
Folic acid	100 mg
Nicotinic acid	100 mg
4-aminobenzoic acid	100 mg
Pyridoxine-HCl	100 mg
Lipoic Acid	100 mg
Biotin	100 mg
NaOH is added till dissolved, freeze in aliquots	

Table A.5 Recipe of 1000X Vitamin B12 Solution

Chemical	**Composition in Media[mg mL^{-1}]**
Cyanocobalamin	1
HCl is added till dissolved, freeze in aliquots	

Table A.6 Recipe of IBM

Chemical	**Composition in Media[mg L^{-1}]**
$MgSO_4.7H_2O$	750
$Ca(NO_3)_{2.}4H_2O$	430
$NaHCO_3$	160
KCl	50
$K_2HPO_{4.}3H_2O$	37
Na_2EDTA	44
$FeCl_{3.}6H_2O$	32
H_3BO_3	10
$MnCl_{2.}4H_2O$	2
$NiCl_{2.}6H_2O$	1
$ZnSO_{4.}7H_2O$	0.2
$CuSO_{4.}6H_2O$	0.2
$CoCl_{2.}6H_2O$	0.1
$Na_2MoO4.2H_2O$	0.06

Appendix B

IMPROVING GROWTH OF *Rhl. lacicola*

If the culture media is rich in nutrients, bacteria grow faster. Different bacteria have different growth requirements. The maximum growth yield observed by measuring optical density at 660 nm for the strain *Rhl. lacicola* in complex or NSY media is approximately 0.2 and approximately 0.12 in minimal media. *Rhl. lacicola* was isolated from a hypertrophic lake and we wanted to assess if addition of specific nutrients to the media would improve the growth rate and growth yield of the strain. Based on previous literature [1][2], we predict that addition of sulphur and vitamins, increasing the percentage of carbon in the media and adjusting the pH would provide *Rhl. lacicola* with the necessary conditions to achieve a higher growth yield.

B.1 Addition of Sulphur

Cysteine and sodium thiosulphate were added to NSY media in the concentrations 40 μg/mL and 80 μg/mL and growth was measured by recording optical density at 660 nm every 24 hours in continuous light and continuous dark conditions.

Table B.1. The table represents growth rate and growth yield values of *Rhl. lacicola* in NSY media with additional sulphur in it. Each value in the cell is an average of four biological replicates; the cells were grown in continuous light and dark for four days.

Nutrients	Growth Rate Light	Growth Rate Dark	Growth Yield Light	Growth Yield Dark
Control	0.052	0.048	0.192	0.148
Cysteine 40 µg/mL	0.051	0.0373	0.091	0.079
Cysteine 80 µg/mL	0.0624	0.0483	0.111	0.099.
Sodium Thiosulphate 40 µg/mL	0.0774	0.0694	0.25	0.121
Sodium Thiosulphate 80 µg/mL	0.0887	0.072	0.363	0.197

On comparing growth rate and growth yield of *Rhl. lacicola* in normal NSY and media with additional sources of sulphur in varying concentrations, we observed that addition of sodium thiosulphate to NSY media does result in an improvement in the growth rate and growth yield as depicted in table B.1.

B.2 Addition of Vitamins

1000X 8-Vitamin mix (table A.4), Vitamin B-12 (table A.5) and a combination of both in the concentration of 1 g L^{-1} were added to NSY media. Growth was measured by recording the optical density at 660 nm every 24 hours in continuous light and continuous dark conditions.

Table B.2. The table represents growth rate and growth yield values of *Rhl. lacicola* in NSY media with additional vitamins in it. Each value in the cell is an average of four biological replicates; the cells were grown in continuous light and dark for four days.

Nutrients	**Growth Rate Light**	**Growth Rate Dark**	**Growth Yield Light**	**Growth Yield Dark**
Control	0.052	0.048	0.092	0.081
1000X 8-Vitamin mix	0.119517	0.117745	0.843	0.823
Vitamin B-12	0.122719	0.119517	0.929	0.783
Vitamin B-12+ 1000X 8-Vitamin mix	0.123	0.11987	0.894	0.739

On comparing growth rate and growth yield of *Rhl. lacicola* in the control media and media with additional vitamins in varying combination, we observed that addition of Vitamin B-12 to NSY media results in higher growth yield as shown in table B.2.

B.3 pH

The pH of NSY media is 6.56. To investigate the optimum pH for growth of *Rhl. lacicola*, potassium phosphate buffer was added to the media to get a final pH of 5,6, or 8. Growth was measured by recording the optical density at 660 nm every 24 hours in continuous light and continuous dark conditions.

Table B.3. The table represents growth rate and growth yield values of *Rhl. lacicola* in NSY media with varying pH. Each value in the cell is an average of four biological replicates; the cells were grown in continuous light and dark for four days.

Nutrients	Growth Rate Light	Growth Rate Dark	Growth Yield Light	Growth Yield Dark
Control (pH 6.56)	0.05132	0.044	0.099	0.088
pH 5	0.05162	0.042241	0.062	0.079
pH 6	0.17521	0.47183	0.062	0.044
pH 8	0.048115	0.0377	0.1	0.065

On comparing growth rate and growth yield of *Rhl. lacicola* in the control media and media with varying pH, we observed that *Rhl. lacicola* exhibited improved growth rate at pH 6 and maximum growth yield at pH 8 as shown in table B.3.

B.4 Percentage of Carbon

The percentage of carbon in the NSY media for all growth experiments performed is 0.3 %. To investigate the impact percentage of carbon in the media has on the growth rate and growth yield of *Rhl. lacicola*, the cells were inoculated in NSY media with varying carbon percentage of 0.18, 0.9, or 1.8%. Growth was measured by recording the optical density at 660 nm every 24 hours in continuous light and continuous dark conditions.

Table B.4 The table represents growth rate and growth yield values of *Rhl. lacicola* in NSY media with varying percentage of carbon in it. Each value in the cell is an average of four biological replicates; the cells were grown in continuous light and dark for four days.

Percentage of Carbon	**Growth Rate Light**	**Growth Rate Dark**	**Growth Yield Light**	**Growth Yield Dark**
Control (0.3%)	0.034631	0.025226	0.101	0.086
0.18 %	0.046457	0.036398	0.127	0.099
0.9%	0.059245	0.053009	0.483	0.392
1.8 %	0.05145	0.04969	0.714	0.671

Rhl. lacicola does exhibit improved growth rate and yield on increasing the carbon in the media as shown in table B.4. However, the growth rate decreases on increasing the percentage of carbon possible due to an increase in carbon-dioxide and hence an increase in the acidity of the media.

On assessing the results of growth of *Rhl. lacicola* in NSY media modified by addition of nutrients, we predict that the growth rate and growth yield of *Rhl. lacicola* would improve significantly if NSY media had a combination of all the nutrients. We propose to call the modified NSY media, NSY ++.

Table B.5 Recipe for NSY ++

Chemical	**Composition in Media**
Nutrient Broth	3 g
Soytone	3 g
Yeast Extract	3 g
10X IBM (see table 2.6)	100 mL
Sodium Thiosulphate	80 μg/mL
Vitamin B-12(see table A.5)	1 mg mL^{-1}
pH 8	
Distilled Water	1 L

Appendix C

CARBON USE EFFICIENCY (CUE)

Carbon use efficiency (CUE) is defined as the amount of carbon incorporated into biomass comparable with the total carbon uptake ($C_{biomass}/[C_{biomass} + CO_2]$)[3,4]. This can be measured by measuring two of the following parameters: organic carbon lost from the medium, carbon incorporated into biomass or CO_2 released. As growth rate increases, CUE generally increases[4], so we predict that CUE will increase in *Rhl. lacicola* cells grown in the light and CO_2 production will decrease.

In order to quantify CO_2released by *Rhl. lacicola*, the cells were inoculated in 30 mL of minimal media in sealed serum bottles and were grown in continuous light and dark conditions for four days. To convert the carbonate species in the media produced as a byproduct of metabolism to CO_2, gas stripping was performed where 0.1 M HCl was injected to the serum bottles, 60 minutes before analysis[5]. The CO_2in the serum bottles before and after gas stripping was quantified by using a Gas Chromatograph (GC-MS Shimadzu QP-2010) . Headspace samples were manually collected and injected using an airtight syringe. Gas separation was performed on a Carboxen-1006 porous layer open tabular (PLOT) column (30 m by 0.32 mm, 15 μm pore size- Sigma Aldrich) with an injector temperature of 150°C, a carrier gas flow of 15.4 lb/in^2 He. The oven temperature was initially held at 30°C for 2.5 min, with ramping at 40°C/min to 120°C.

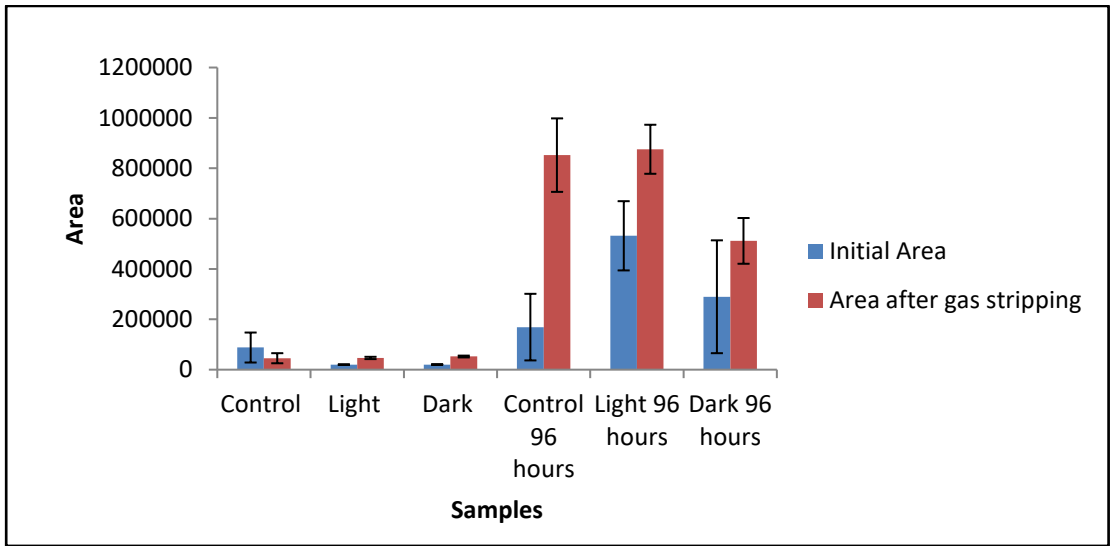


Figure C.1a. The figure depicts GC-MS analysis of carbon-dioxide in the headspace before (blue bars) and after (red bars) gas stripping. The error bars represent standard deviation.

Each bar in the figure C.1a is an average of three biological replicates. CO_2 release by cells inoculated in the media for one hour and for 96 hours or four days was measured before and after gas stripping. The high variation in the control readout for the two time points raises questions on the reliability of our results which is why we decided to opt for another method.

To quantify the total organic carbon content of *Rhl. lacicola***,** a TOC analyzer (9000 HS from Teledyne Tekmar) was used. *Rhl. lacicola* and 10M3C3 were inoculated in NSY media and the analysis was performed 96 hours after inoculation.

The cells were diluted in 15 mL of autoclaved distilled water (dilution factor 1:20) before analysis. The cells were grown in constant light and dark, each bar as shown in the figure C.1b is an average of four biological replicates and the error bars represent standard deviation. In order to calculate the total organic carbon content of

the cells, the TOC analyzer was utilized to quantify carbon content of the media before and after filtering the cells.

Two stacked 0.2 µm polypropylene filters were for filtration purposes.

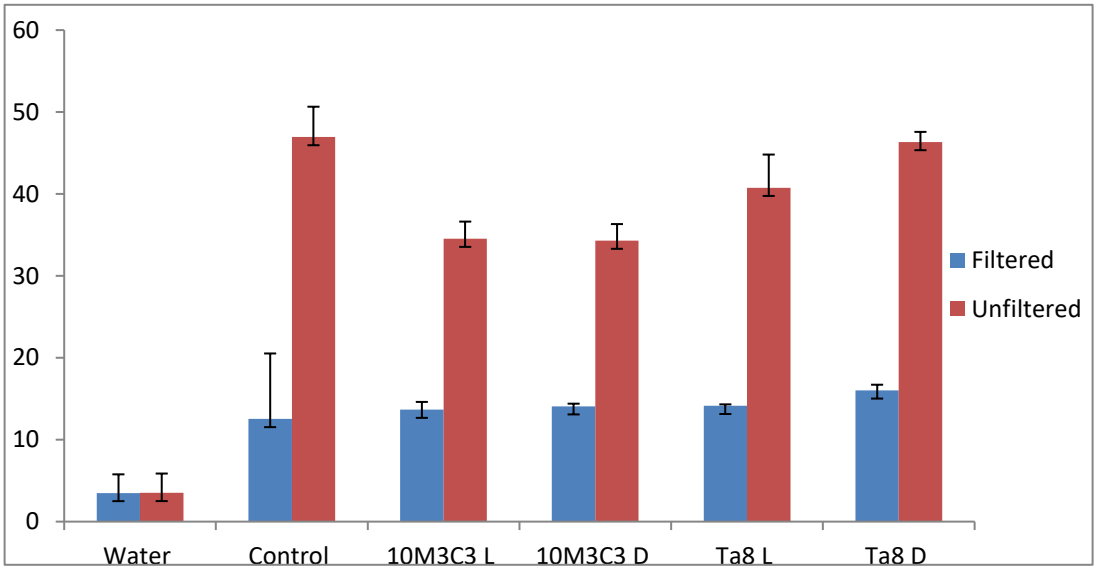


Figure C.1b The figure represents the TOC data for *Rhl. lacicola* and 10M3C3 cells grown in the light and the dark. Each bar is an average of four biological replicates. The cells were diluted in the ratio 1:20 in distilled water before analysis. The readout from the analyzer was multiplied by the dilution factor while plotting the graph.

The total organic carbon content of the control should have been uniform for filtered and unfiltered conditions because the control media has no cells. Also, 10M3C3 has a higher growth yield when compared to *Rhl. lacicola* but that was not quantified during the TOC analysis. We hence conclude that the equipment is not sensitive enough to measure the differences in the organic carbon content of the cells grown in the light and dark.

The CHNS elementar was used to quantify the carbon content in the cells. The cells were inoculated in NSY media and grown in continuous light and dark for 24 and 48 hours. 25 mL of the samples were then placed in a lyophilizer, 24 hours prior analysis to freeze dry the samples. 10 mg of the lyophilized samples were used for analysis. Each bar is an average of three biological replicated, the error bars denote the standard deviation. The high variation recorded among biological replicates led to the realization that the CHNS elementar might not be the best equipment to quantify the total carbon content in cells.

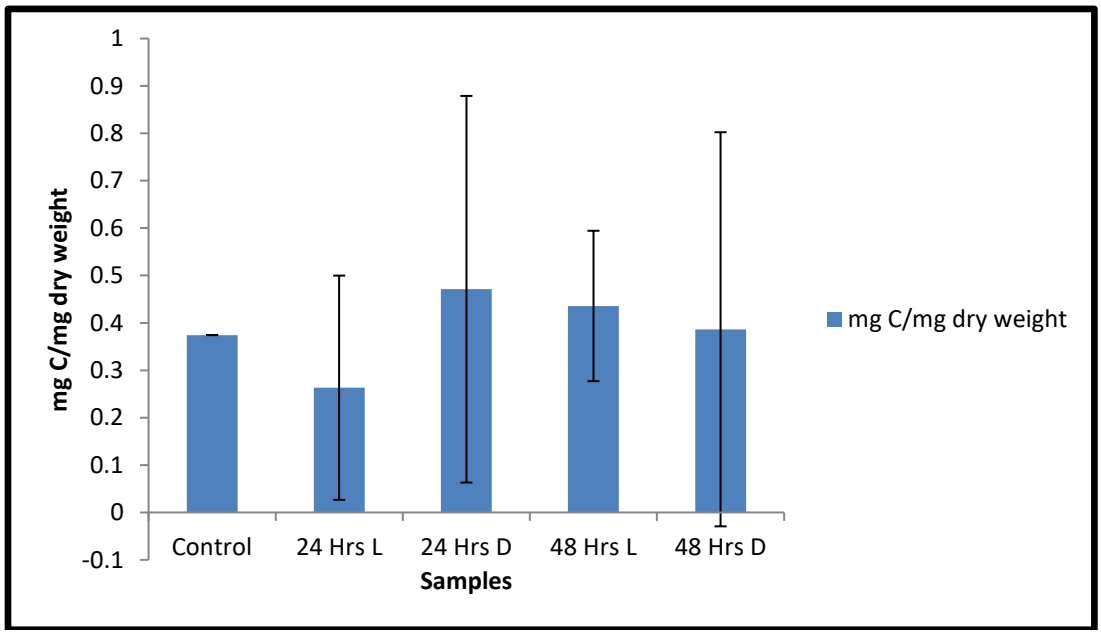


Figure C.1c The figure represents the mg C/ mg dry weight values for *Rhl. lacicola* cells grown in the light and the dark. Each bar is an average of four biological replicates. 10 mg of the lyophilized cells were placed in the Elementar .

Further research needs to be done to assess the best method to quantify the total organic carbon content in these freshwater actinobacteria.

REFERENCES

1. Towbin, B. D. *et al*. Optimality and sub-optimality in a bacterial growth law. *Nat. Commun.* **8,** 14123 doi: 10.1038/ncomms14123 (2017).

2. A. Abdul Hamid, S. Ariffin and S.A. Syed Mohamad. Identification and optimal growth conditions of actinomycetes isolated from mangrove environment (2015) Aug. Malaysian Journal of Analytical Sciences 19(4):904-914.

3. Keiblinger KM, Hall EK, Wanek W, et al. (2010) The effect of resource quantity and resource stoichiometry on microbial carbon-use-efficiency. FEMS Microbiol Ecol 73:430–40. doi: 10.1111/j.1574-6941.2010.00912.x

4. Sinsabaugh RL, Manzoni S, Moorhead DL, Richter A (2013) Carbon use efficiency of microbial communities: stoichiometry, methodology and modelling. Ecol Lett 16:930–9. doi: 10.1111/ele.12113

5. Miyajima, T., Miyajima, Y., Hanba, Y. T., Yoshii, K., Koitabashi, T., & Wada, E. (1995). Determining the stable isotope ratio of total dissolved inorganic carbon in lake water by GC/C/IIRMS. Limnology and Oceanography. https://doi.org/10.4319/lo.1995.40.5.0994