

**Growing biofuel outdoors in Seattle: effects of natural light level and temperature on marine microalgal culture density and lipid content in contained natural exposure systems**

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## **Abstract**

The effects of natural light and temperature on the growth rate, culture density, and lipid content of marine micro algae species *Pavlova lutheri* and *Pavlova* sp. were investigated to potentially lend precedent for further algae growth in the Pacific Northwest. Culture cycles grown in the laboratory and an engineered hybrid outdoor growth system CNES (Contained Natural Exposure System), were analyzed using a flow cytometer to find culture density (cell/mL), and relative neutral lipid content. The lipid analysis illustrated that the highly variable outdoor light conditions did not have a profound effect on neutral lipid content nor did temperature. Natural variation of light intensity by day as well as natural light variation by larger seasonal changes proved to have an insignificant relationship to peak cell density, and growth rate. Differences in the lab growth rates indicate an unknown light saturation point that likely lies below the lowest of natural light intensity in the natural environment. Thermoregulation provided from the bay water surrounding the CNES maintained algal cultures within ideal growth temperatures. Thus, Seattle and the Pacific Northwest have the potential for large scale algal growth operations with the use of a CNES.

## Introduction

It is now commonly recognized that the future of energy must look toward sustainability in order to avoid environmental catastrophe (Jefferson 2006). With a growing need for renewable energy to fuel the daily lives of millions of people worldwide, scientists are looking toward the use of algae as a source for the production of biofuel (Jefferson 2006; Mata et al. 2010; Demirbas 2010). Microalgae can potentially be utilized for the production of biofuels in an economically effective and environmentally sustainable manner. Although it has its challenges, algae grown for biofuel appears to be the only source capable of meeting global demands for transport fuels. Second generation biofuels, those produced from lingo-cellulosic biomass, are being sought after to replace first generation crops which are primarily used as a food source (Sims et al. 2010). Compared with other second generation biofuels, algal fuels have a higher yield: they can produce 30–100 times more energy per hectare compared to terrestrial crops (Demirbas 2010). There are two primary energy and cost constraints to large scale production of single cell microalgae for biofuels, expensive culture systems including high capital costs and high energy requirements for mixing and gas exchange; and the cost of harvesting using high-speed continuous centrifugation for dewatering (Schlesinger et al. 2012).

The term microalgae covers a fairly broad spectrum of marine organisms, including both prokaryotic organisms, such as cyanobacteria, along with unicellular eukaryotic organisms. In this study we will examine two species of microalgae: *Pavlova lutheri* and *Pavlova* sp. Both of these species are unicellular eukaryotes, under the division Haptophyta. They are both salt water strains of these species and similar strains of *Pavlova* are found in Puget Sound, among various other habitats across the globe. The original mother cultures have been provided by the Cattolico Laboratory of the University of Washington. *Pavlova lutheri* is very commonly grown for

aquaculture and thus extensive research on growth rates and lipid content are readily available (Meireles et al. 2003). Previous studies have formulated a postulate in which predicting the optimal light and temperature can be attained for the growth of *Pavlova lutheri*, however the postulate is compared only to data measured within a controlled light and temperature laboratory (Carvalho et al, 2003). *Pavlova* sp. has become the subject of several studies at the University of Washington, suggesting it be comparable as a standard algae of choice for biofuel productions (Bigelow et al, 2013).

Light and temperature, in that order of importance, are said to be the key limiting factors for culturing algae in both closed and open outdoor systems (Mata et al. 2010). Effects of temperature in a laboratory are documented, however, the effects on outdoor biomass production is relatively unknown (Chalup and Laws 1990). The two major types of technology used for culturing microalgae are open and closed systems: open systems are most common in the form of raceway ponds and closed in the form of PBRs (Photo-Bioreactors). Closed systems offer their advantages in having higher degrees of control over the growth conditions, and protecting the culture from contamination. The tradeoff is high cost of production and maintenance, as well as low net energy gain.(Mata et al. 2010). Open systems boast low cost of production and maintenance, from natural sunlight and ability to produce larger amounts of algae. However because of their exposed nature, ponds are more susceptible to contaminants and weather conditions, not allowing control of water temperature, evaporation and lighting (Mata et al. 2010). Along with these issues, raceway ponds take up far greater areas of land thus competing with potential agriculture.

A superior design would look to utilize resources, maintain as much control of culture conditions as possible and be as cost effective as possible. Along with the construction, the

location chosen for the placement of the system will play a key role in natural resource abundance, as well as conditional regulation.

To date, no experiments have been done on the selected two species of algae under natural light and temperature or in outdoor floating tanks. Although some studies suggest optimal light and temperature conditions for the two species, none have established a clear boundary in which they cannot grow efficiently and effectively (Carvalho et al, 2003). This study will investigate the use of new technology, in the form of large scale algal incubation tanks, to grow algae in the naturally low and varying light levels of the Pacific Northwest. It was hypothesized, based on the levels of light and temperatures in which the cultures were previously maintained (12/12 light dark cycle of  $25\mu\text{mol m}^{-2} \text{s}^{-1}$  at  $10^{\circ}\text{C}$ ), that if the average light exposure during the growth cycles of the outdoor cultures was higher than those in lab, growth rates and peak densities would have reflect those increases.

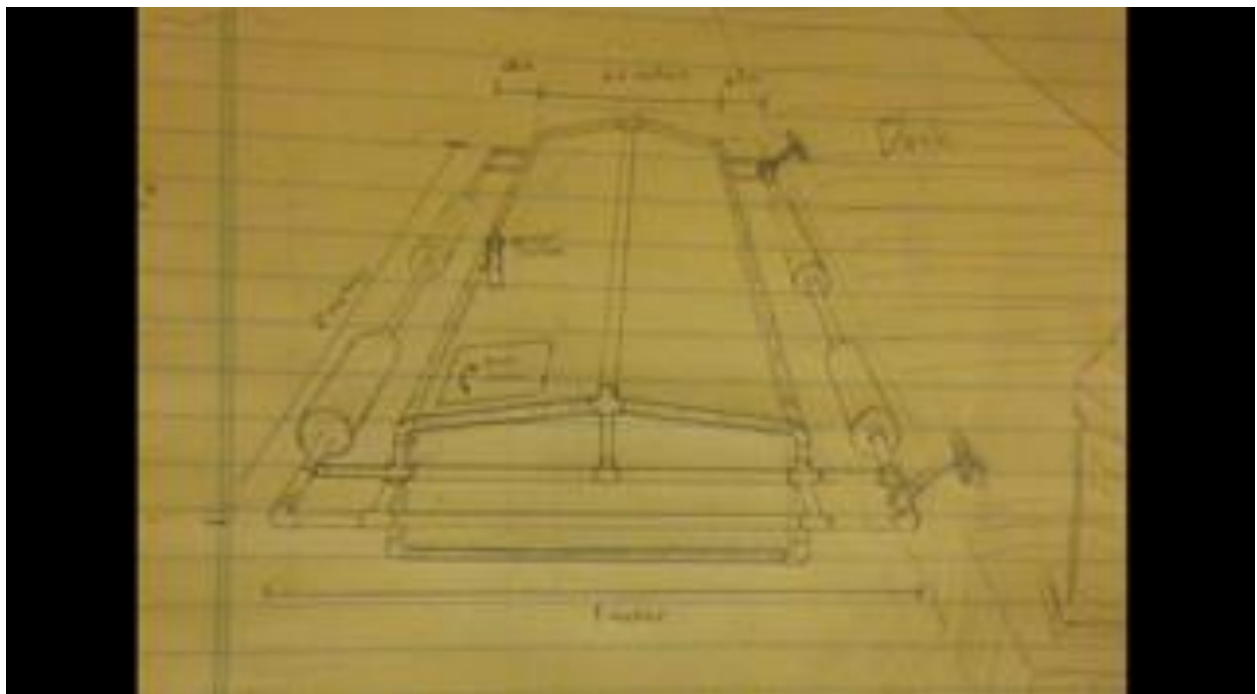
## **Methods**

### Design Goals

The overall goal of the design process was to find the most efficient and effective hybrid of the two major algal growth systems, Open (Raceway Ponds) and Closed (PBRs). The first major decision was to use natural lighting as done in an open system, thus eliminating the use of electricity to move toward a lower carbon foot print and reduce costs. The second major decision was to grow the algae in closed tanks that would be deployed in the marine environment to reduce evaporation, protect from contamination, and to keep conditions far more regulated than those of a Raceway pond. The third major decision was to grow marine algae thus the use of seawater could be implemented providing us with a less costly medium that doesn't compete

with agriculture. The hybrid system will be referred to as a Contained Natural Exposure System or CNES.

The initial design for the CNES was a simple cubic PVC pipe framework that supports two internal acrylic tanks to keep them secured and floating in the water (Fig. 1). PVC is a relatively cheap material that can withstand long durations of water contact without degrading, unlike untreated wood or metal alternatives. Acrylic is optimal for durability, strength, and creating a fully sealed, closed system environment, without limiting light irradiance, as opposed to glass or other plastic alternatives. The PVC pipe frame work had an outer frame where the floatation buoys were secured around the pipes. This frame was 1.5 meters by 1.3 meters in size and attached to the inner frame level with the top of the inner frame. The inner frame was 1.2 meters by 1.15 meters by 0.2 meters. This part secured snugly around the acrylic tanks rising about half way up the height of the tanks' sides, in order to allow the tanks to be semi



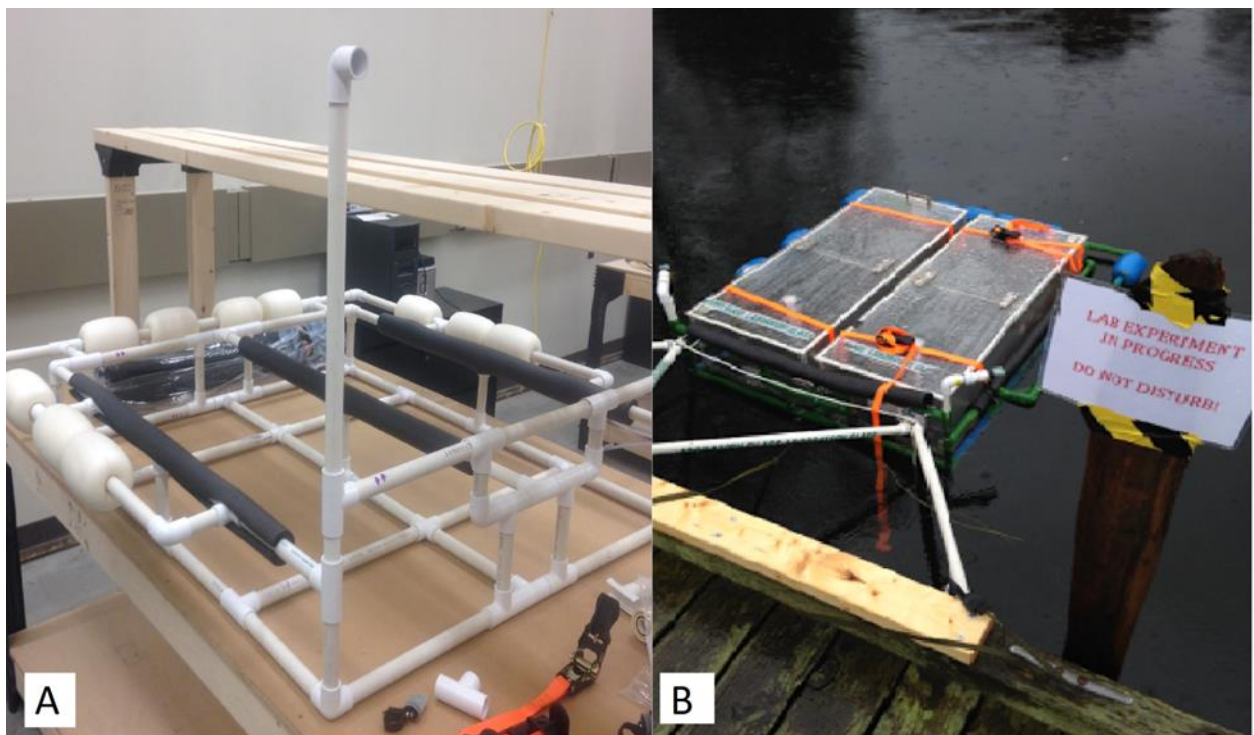
*Fig 1. Rough Sketch of initial CNES design.*

submerged, with the medium even with the external water level. This was an import design specification in that it aimed to use the external water to thermoregulate the algae growth medium. The acrylic tanks were 1.14 meters long by 0.45 meters wide by 0.32 meters deep. The volume of medium in each tank was 50 liters. On the dockside edge of the external frame two pieces of PVC piping extended horizontally from the frame 0.1 meters where a 90 degree elbow extend two more pipes vertically 1.0 meter. These vertical pipes slid into slightly two larger PVC pipes that secured directly to the dock. The two pipe interlocking system was to allow vertical movement with tides but keep the tanks from being battered around by waves. The two tanks will hold each type of algae, *Pavlova lutheri* and *Pavlova sp.* Each tank was equipped with a removable lid for sampling, and three HOBO pendant temp/light data loggers: one inside the tank and outside the water, one in the surface of the water and one on the bottom of the tank (See Fig 3). The first location for the sensor allows us to record light data unaffected by changing cell concentrations and records air temperature. The second sensor gives us an idea of the higher range light intensity the cells are experiencing with increased cell concentration and an idea of temperature that is being thermoregulated by the water. The third sensor gives us the lower range values for light intensity that the cells will be experiencing along with another temperature reading most strongly affected by the thermoregulation of the surrounding water.

## Trial

A trial of the initial tank was run from January 29 to February 3, 2015. The results varied from one parameter to another with some meeting their design criteria and some failing to deliver the desired results. The first component being tested was the light sensor readings to see if the CNES was allowing light like that of an “Open” system. The individual light sensors were calibrated with a separate light sensor that was shown to be accurate. The sensors displayed that

the tanks were receiving ample amounts of light from natural sunlight and based on the multiple sensor positions the acrylic tank was not hindering light exposure. The second parameter being checked was the tanks effectiveness in maintaining a “Closed” system type environment. In order to do this, the tank needed to remain sealed to protect from external contaminants. After the trial it was determined that no contaminants had penetrated the CNES. The third parameter being examined in the trial was the CNES effect on temperature. The bottom sensor within the algal medium showed a major difference in thermal changes as compared to the external air temperature readings; indicating the tanks were conducting heat enough to allow the external body of water to help thermoregulate, thus seeing smaller fluctuations in temperature.



*Fig 2 A. An image of the initial CNES framework used in the trial run. B. An image of the final CNES during experimentation.*

## Final Design

The final design for the CNES was very similar to the initial build with the exception of a large adjustment to the CNES dock attachment features (Fig. 2B). In the first design we looked to reduce movement to maintain more control of the algal environment. However the wave intensity endured during the trial run caused the external frame and attachment points to break from too much angular torque (Fig. 2A). The re-design looked to reduce all torque on the frame while maintaining security to the dock, and allowing the wave motion to help with natural mixing. The new feature came in the form of a two-to-three attachment point system which formed two triangles made of PCV pipes. At the apex of these triangle were the two points that attached to the CNES frame via eye hooks and carabineers, and at the base of the two triangles was the three dock attachment points. The system kept the tanks safely away from the dock, while still secured, allowing the tanks to move freely with any incoming waves. These final product tanks were used for the first culture batch as well as the second.

## Experimental Procedures

Strains received from the Cattolico algae culture library were used to inoculate two 250 mL Erlenmeyer flasks with 100 ml of standard F/2 no Si medium (Nano et al. 1975). These new cultures were grown in a 10 degree Celsius light room on a 12/12 light/dark cycle at  $25\mu\text{mol m}^{-2} \text{s}^{-1}$ . The cell density and mean BODIPY fluorescence was measured every other day. The initial in lab cultures went through two batch cycles of growth, the first being the aforementioned, and the second grown in 1 liter of medium in 2.8 liter Erlenmeyer flasks, concurrently with 100mL medium grown in 250 mL flasks. This second batch was also grown in a 10 degree Celsius light room on a 12/12 light cycle, but under slightly more light at  $75\mu\text{mol m}^{-2} \text{s}^{-1}$ . The 1 liter cultures were then inoculated into the outside tanks which held 50 liters of medium per strain of algae.

The medium was made from seawater taken from Bellingham Bay and filtered through a 5 $\mu$ m filter and then again through a 0.2 $\mu$ m filter, and then mixed with F/2 no Si nutrient mix (Nano et al. 1975). The tanks were located directly off of the salmon pond dock in Portage Bay, on the University of Washington campus. The location was chosen for its convenience and accessibility while also displaying weather conditions very typical of the Pacific Northwest. The cultures were measured every other day for cell concentration and lipid content using a flow cytometer. One .495 mL sample per culture was extracted using a 1 mL pipetter into glass test tubes. Once in the lab, .005 mL of a BODIPY dye solution was added to the samples. The BODIPY attaches to lipid bodies and then fluoresces under the flow cytometer allowing for a rough idea of lipid content within the cell. Along with these measurements, temperature and light intensity was measured over the course of each day, in 15 minute intervals, in order to see, the daily fluctuation and the total intensity per day, using a HOBO pendant temp/light logger. In order to attain temporal data the experiments were run twice.

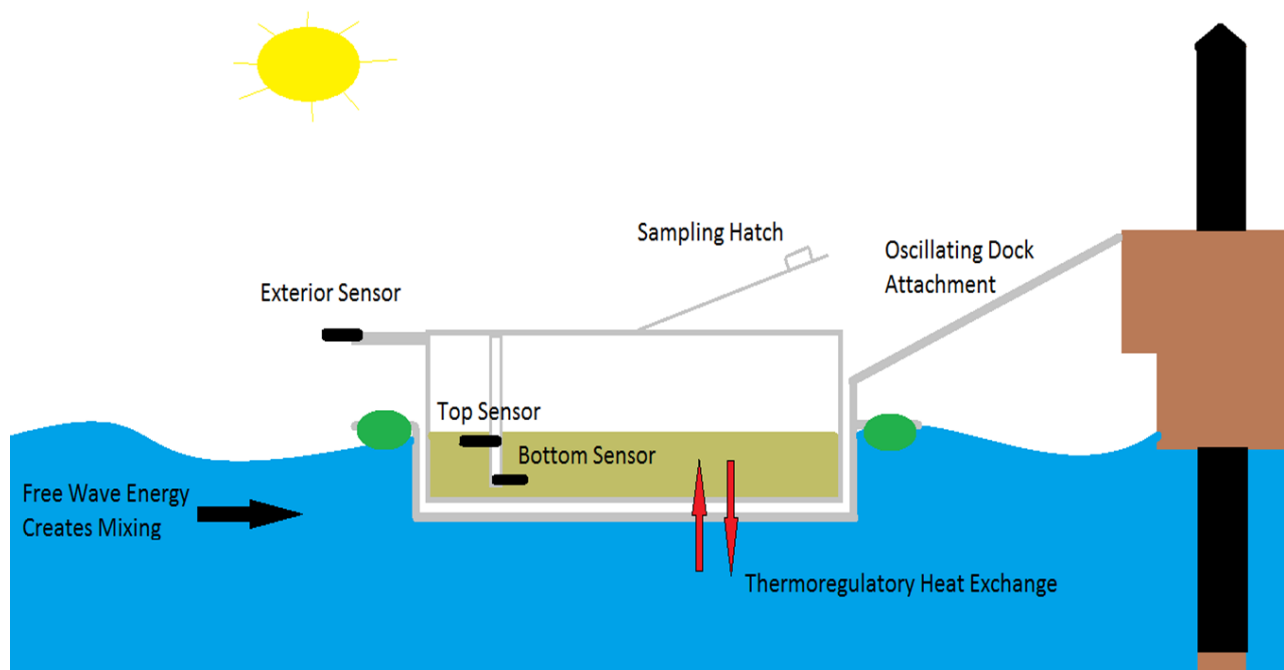


Figure 3. Is a schematic illustrating the experimental setup of the CNES.

## Results

The results for the first in lab cultures (grown at 10°C under 25  $\mu\text{mol m}^{-2} \text{s}^{-1}$  on a 12/12hour light dark cycle) show that both species grow at similar rates throughout the entire cycle, hitting peak densities between  $1 \times 10^6$  and  $5 \times 10^6$ . The results for the laboratory cultures (grown at 10°C under 75  $\mu\text{mol m}^{-2} \text{s}^{-1}$  on a 12/12hour light dark cycle) show that both strains in both culture sizes immediately upon inoculation begin to enter a logarithmic growth phase (see Fig 4). In this log phase, the cultures reach densities of nearly 100 times more than initial densities within the first ten days. After day 15, the cultures move into a stationary phase in which their rate slows and densities plateau. Peak densities in all cultures were in the range of  $5.0 \times 10^6$ – $9.0 \times 10^6$  cells  $\text{mL}^{-1}$ . During log phase, the relative amount of neutral lipids in all cultures

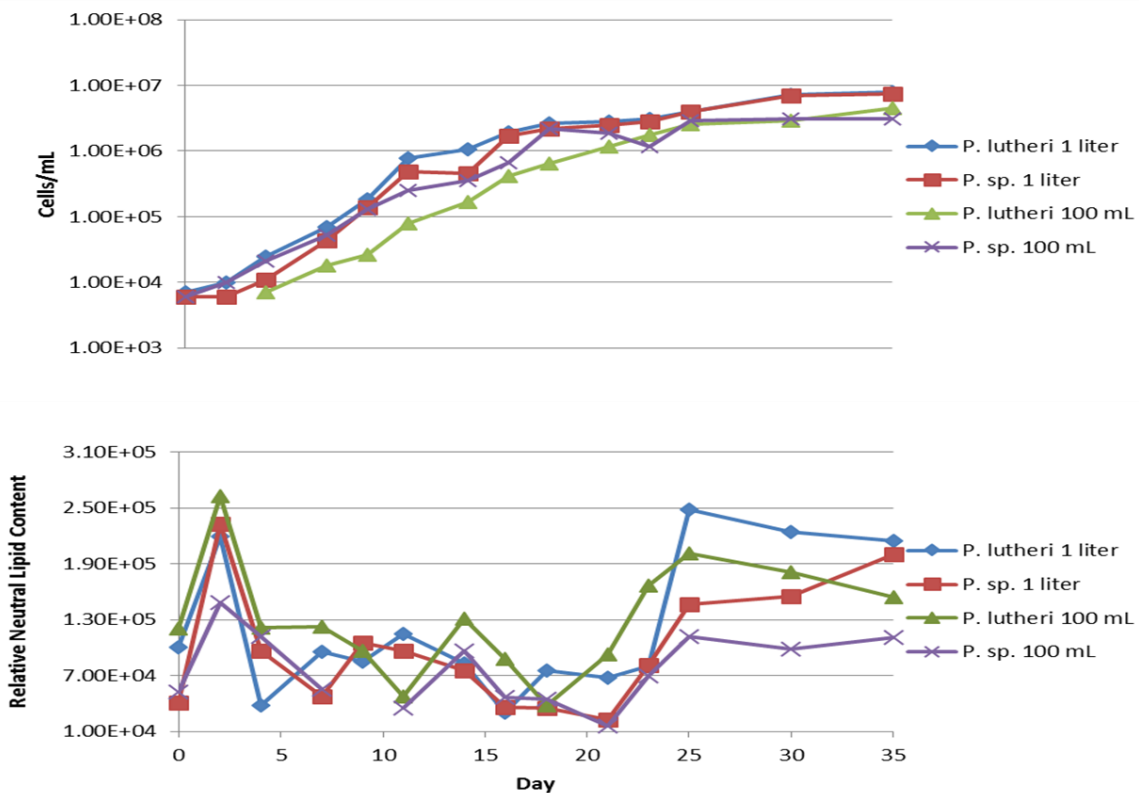


Figure 4. Cultures were grown in the Laboratory at 10°C under a 12/12 light dark cycle. Graph A represents the culture density over the time. Graph B is the relative Neutral Lipid content on average per cell over time.

drops off significantly, then stays in a generally lower range until the algae have gone into full stationary phase where the lipids increase again to higher concentrations. Both cultures grow at very similar rates throughout the entire culturing process.

Throughout the duration of the first outdoor cultures light and temperature was taken every 15 minutes from sensors located on three different areas of the tank (Fig. 5). The exterior sensor showed the highest fluctuations in temperature ranging from 4-27°C, a 23 degree change, in a single day. The top sensor showed smaller same day fluctuations ranging from 7-21°C in a

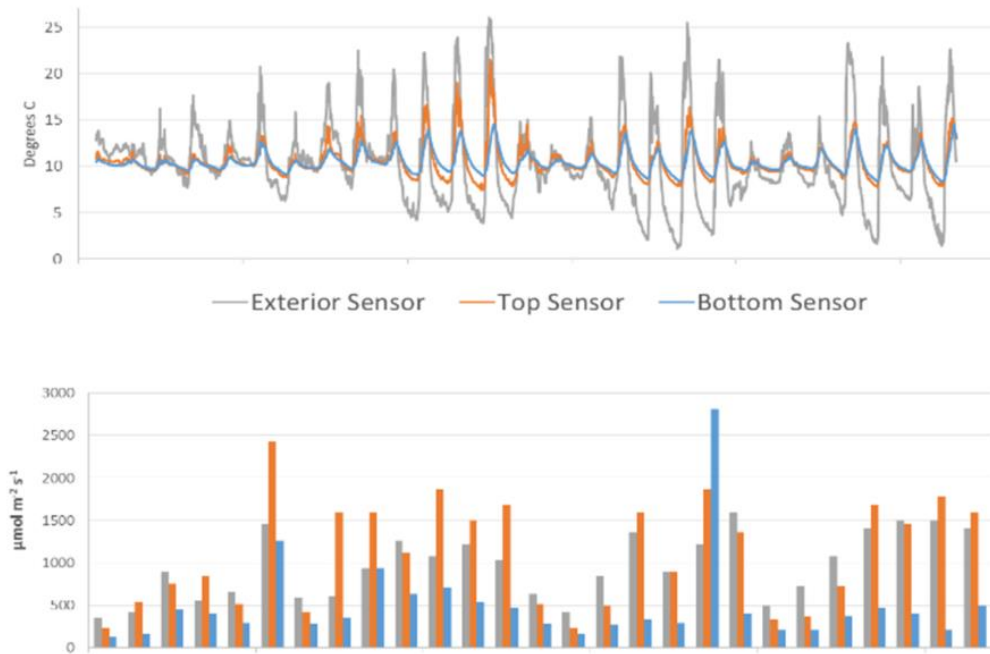


Figure 5. A. The graph represents the temperature taken from the three different tank sensors with data points every 15 mins. day and night during the first cycle. B. The bars show the average light intensity per day over the course of the first out door cycle

single day. The bottom sensor showed significantly less fluctuation through the entire duration never fluctuating more than 4 degrees in one day and staying within the range of 8-14°C.

During the first outdoor growth cycle the cultures do not see as high of initial growth rates as the in lab cultures indoors, but still grow consistently at fairly high rate (Fig.6A). After hitting peak densities around day 12, with *Pavlova sp.* hitting peak densities just over  $1.0 \times 10^6$  cells  $\text{mL}^{-1}$ , both species move into a stationary phase. However there is a large die off of *Pavlova lutheri* before it becomes consistent. The relative lipid content shows similar trends to the indoor cultures dropping off right away and then maintaining for a period of time (Fig.6B). However,

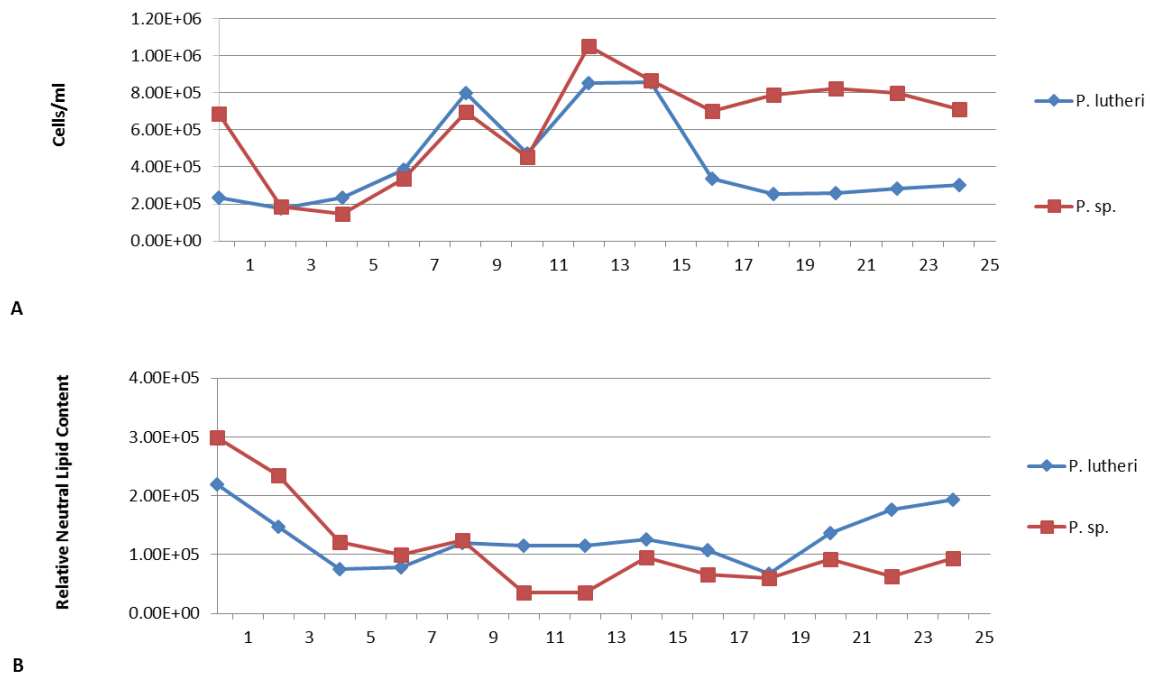


Figure 6. Cultures were grown in CNES tanks located in portage bay from 02/05/2015-02/29/2015. Graph A. Represents the culture density over the time. Graph B. is the relative neutral lipid content on average per cell over time.

unlike the lab cultures, the outside neither show as significant of an increase in lipids when the cultures go back into a stationary phase.

In the second outdoor growth cycle we see similar growth rates between the two species once again for the first few days (Fig.7). However, after day five you can see the growth rates start to deviate and *Pavlova sp.* outperform *Pavlova lutheri* once again. During the second cycle the relative lipid content drops off at the beginning as we have seen in the previous two cultures, then goes through a stable period. However in this cycle we see a much larger spike in the relative lipid content of *Pavlova lutheri* once it has gone into stationary phase.

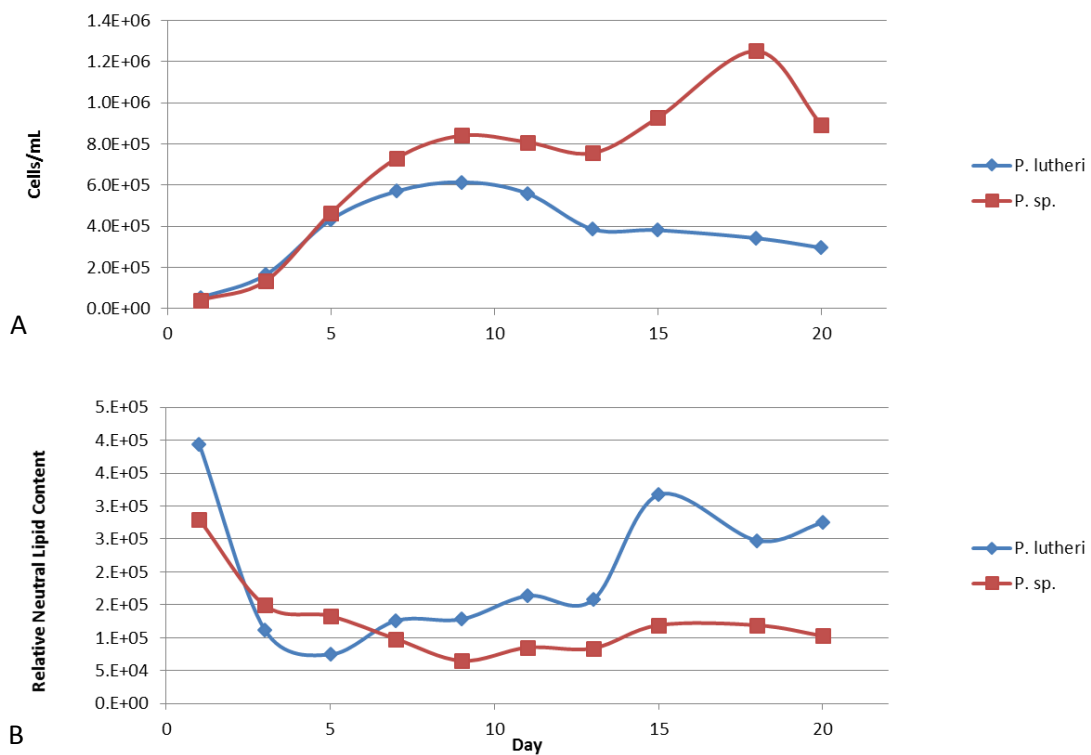


Figure 7. Cultures were grown in CNES tanks located in portage bay from 04/22/2015-05/10/2015. A. Represents the culture density over the time. B. Represents the relative neutral lipid content on average per cell over time.

During the two outdoor growth cycles we saw major changes in average light intensity from the first cycle to the second. In the first cycle we see the light intensity average around 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Fig.8.B). These are somewhat typical conditions of gloomy Pacific Northwest weather conditions. In the second cycle the light intensity average is much higher around 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , which are more typical weather conditions of spring time in the Pacific Northwest (Fig.8.C).

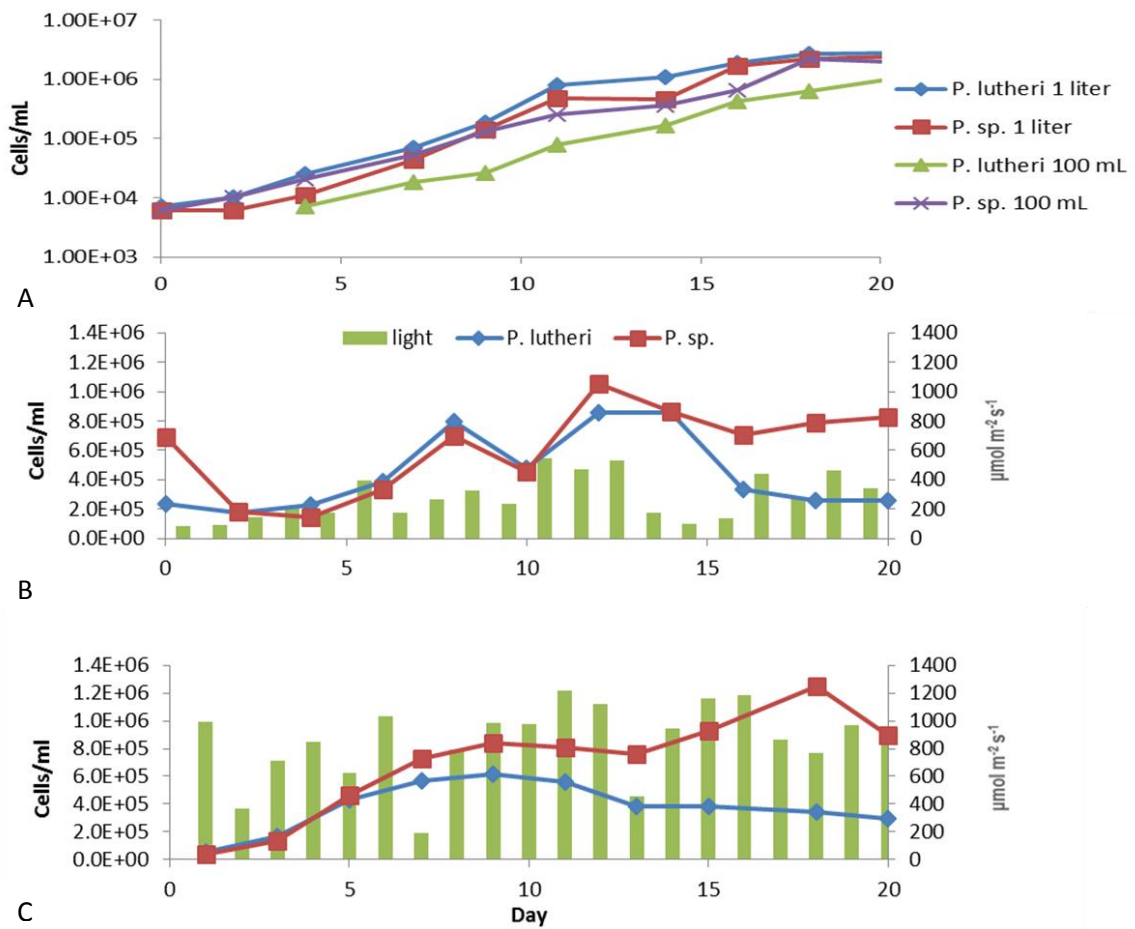


Figure 8. A. represents the culture densities over time grown in the lab. B. Represents the culture density over time during the first outdoor cycle with its corresponding average light intensity. C. Represents the culture density over time during the second outdoor cycle with its corresponding average light intensity.

## Discussion

When the two species, *Pavlova lutheri* and *Pavlova* sp., are subjected to identical light and temperature conditions in the lab, growth curves are nearly the same regardless of culture size. Lipid content of each species also follows a trend that is very similar to one another in the lab. However, when both species are subjected to the same light and temp conditions as one another, but these conditions are variable and uncontrolled each day, they grow at much different rates and one species out performs the other. Their lipids seem to also show slightly different patterns from one another. There is no correlation with cell density or lipid content with light and temperature changes throughout either individual growth culture once grown in the high variability natural conditions. The lipid content however does seem to show more distinguishable changes with each phase of the algae growth cycle. This is supported in a study that shows the amount of lipid content changes significantly during the different growth phases of microalgal cultures (Miller et al. 2012). Suggesting that point of their life cycle has a greater impact on lipid content and best possible harvesting time than the amount of light or temperature they are exposed to under circumstances when light and temperature cannot be controlled. Contrary to what some studies suggest, this conclusion implicates that the harvesting point in a cultures life cycle is more important than the seasonal changes of light and temperature on a cultures lipid content in an outdoor variable environment (Guihéneuf et al. 2015). The broader implications are that many studies which may draw conclusions from work done in a laboratory should not expect that similar outcomes will apply in the field unless the variable being indicated as influential is staying relatively fixed in a natural environment.

During the two outdoor cycles the seasonal changes in weather are seen in the light and temperature graphs. In the first cycle we see conditions typical of winter in the Pacific Northwest

with low average light intensity. In the second cycle we see conditions more representative of spring weather with much higher average light intensity. However, when comparing the two cultures grown outside both cycles show very similar patterns of culture density and growth rate and both species reach similar peak densities from one cycle to another. During these two cycles the average light irradiation changes significantly. As well as surviving the low light conditions of the winter, and higher light conditions of the spring, the cultures reached reasonably high cell densities for large scale algae growth systems, reaching densities of above  $1 \times 10^6$  cells  $\text{mL}^{-1}$  in both cycles. Thus proving that it is possible to grow microalgae that has been shown have high lipid content in a contained environment with natural exposure to Pacific Northwest weather. Further studies will need to be performed to understand the economic feasibility of the overall production of biofuel from a microalgae source in the Pacific Northwest. However, the hope is that this study will lend the precedent needed to gain momentum in that direction.

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