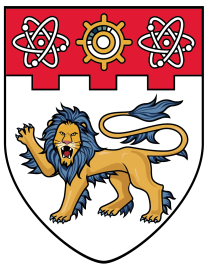

NANYANG TECHNOLOGICAL UNIVERSITY
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Fightoplankton: A low-cost automatic sampler for the study of phytoplankton cell cycle

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Abstract

The latitudinal shift in phytoplankton range due to climate change can have important repercussions for bloom dynamics and assemblages. The effect of seasonal variations in day lengths on the growth and division of phytoplankton is little known. This study investigates how growth and division in a tropical strain of *Picochlorum sp.*, responds to varying light regimes using high resolution sampling data collected through the creation of a low-cost automatic water sampler – the Fightoplankton.

The automatic sampler was designed to sample cultures at regular user-programmed intervals, store samples at 4°C in the dark, and prevent cross contamination through purging systems. Three experimental conditions of 16:8, 12:12, and 8:16 light:dark cycles were set up with at least three replicates per group. Each culture was maintained for a period of 4 - 7 days and sampled hourly over a period of 24 h. Cellular parameters were analysed through flow cytometry and processed using R.

The results of this study indicate that the timing of division is unaffected by light regime conditions, but that growth is coupled directly to light availability. Validation of the Fightoplankton's sampling accuracy and preservation of samples at 4°C suggests that low-cost do-it-yourself equipment could help research in this field. In this study alone, over 200 man-hours were saved through the use of the Fightoplankton.

Keywords: Automatic Sampler, Periodicity, Growth and Division, *Picochlorum*, Circadian Rhythm, Diel Cycle

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Introduction

1.1 Research Motivation

Phytoplankton are a highly diverse taxonomic group of mostly single celled, photosynthetic aquatic organisms (Falkowski et al., 2003). They represent a mere 2% of global photosynthetic biomass, but contribute up to 45% of annual global net primary production (Falkowski, 1994; Falkowski et al., 2003; Field, 1998). Phytoplankton blooms — a qualitative term to describe high biomass events — are predictably recurrent in high latitudinal regions (Behrenfeld & Boss, 2018). These annual blooms play a critical role in the life cycle and migratory behaviours of organisms ranging from birds to whales (Behrenfeld & Boss, 2018). In marine systems, the spatial and temporal distribution of phytoplankton is largely governed by primary limitations in light, nutrients, and temperature (Field, 1998). In addition, most species are short-lived, resulting in a tight coupling between phytoplankton dynamics and environmental change (Hays et al., 2005).

Climate change induced range shifts of terrestrial and marine biota are widely documented, though this tracking of isotherm variations is more pronounced in marine organisms (Gregory et al., 2009; Hays et al., 2005; Sorte et al., 2010). Changes in phytoplankton community assemblages have also consistently followed temperature changes (Jonkers et al., 2019). In fact, Taylor *et al.* (2002) showed that phytoplankton could even be better indicators than environmental variables themselves. The latitudinal range shifts of some phytoplankton could have large implications for the recurrence, productivity, and dynamics of marine food webs. The optimal growth conditions allowing blooms to occur could cause amplifications in climate-induced interannual variations in community assemblages (Behrenfeld & Boss, 2018). This could result in huge impacts on energy flow pathways in the oceans (Friedland et al., 2018).

Despite this understanding of the large-scale potential for change, it is unclear how temperate or tropical phytoplankton strains will respond to extreme seasonal variations in day length, or photoperiods, in higher latitudinal regions. Presently, studies regarding the effect of photoperiod lengths on patterns of growth and division of phytoplankton are mostly restricted to laboratory experiments. This is due to the high logistical costs of sampling in remote *in situ* study sites (Chapin, 2015). Even within controllable laboratory settings, experiments typically use daily sampling to perform growth rate analysis, as higher temporal resolutions quickly become unpractical as several people are required for day and night sampling.

Autosampling of phytoplankton cultures reduces human error, the potential for contamination, and improves experimental reproducibility (Efromson et al., 2021). The greatest benefit of autosampling is that samples can be collected around the clock, with intervals ranging from minutes to days. Commercial samplers available are typically built for the purposes of waste or storm water monitoring and are often expensive, unwieldy, and offer little customisability (Efromson et al., 2021). While low-cost and open-source alternatives are available, many do not fulfil the requirements for phytoplankton sampling, or are outdated given the improvements in electronics (Jacquet et al., 1998; Nelke et al., 2017). As such, this study aims to address some knowledge gaps surrounding the response of phytoplankton growth and division to varying photoperiods using high resolution sampling through the design and application of a low-cost automatic water sampler.

1.2 Literature Review

1.2.1 Phytoplankton Periodicity and Controls

Periodicity in phytoplankton refers to the recurrent cycle of cellular processes at regular intervals (Prézelin, 1992). This period can take the form of circadian periodicity ($\tau \sim 24h$), ultradian periodicity ($\tau < 24h$), or infradian periodicity ($\tau > 24h$) (Chisholm, 1981; Mittag, 2001). On timescales in the order of days, periodicity can arise due to external oscillations in environmental conditions, or internal controls such as the cell division cycle (CDC) or the endogenous circadian rhythm (CR) of a biological clock (Prézelin, 1992; Sournia, 1975).

The CDC is comprised of 4 discrete sequential phases: G1, S, G2, M (Fig 1-1). The S and M phases correspond to well defined and precise biochemical events of DNA synthesis and Mitosis respectively (Chisholm, 1981; Vaulot & Chisholm, 1987). Cells have to pass through tightly controlled regulatory checkpoints to ensure the cell is capable of dividing before undergoing DNA replication or mitosis (Dagenais-Bellefeuille et al., 2008). At the G1/S–phase boundary, the minimum size of a cell is verified, while at the G2/M–phase boundary, the completion of DNA synthesis is verified (Dagenais-Bellefeuille et al., 2008).

Figure 1-1: The Cell Division Phases

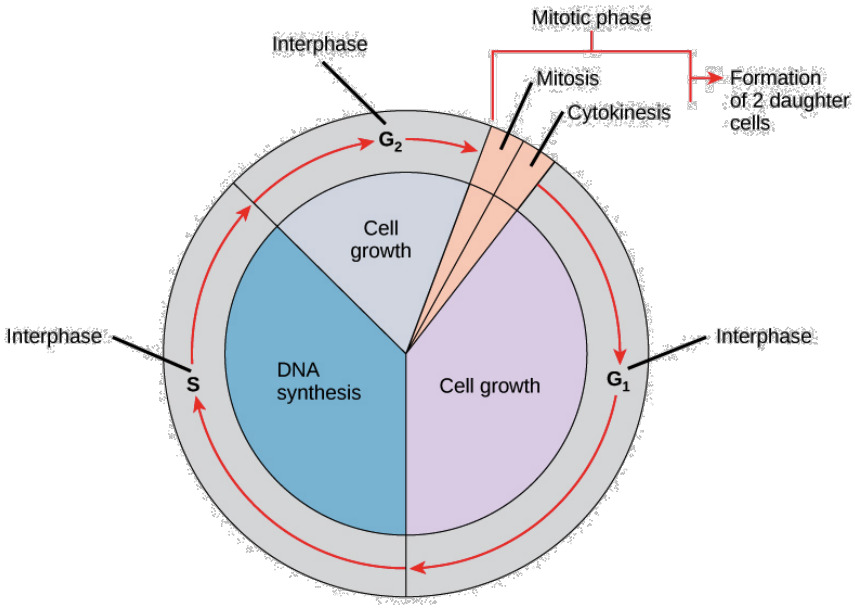


Image credit: OpenStax College, Biology (OpenStax College, 2021)

The other internal control is the CR. The presence of an endogenous biological clock controlling temporal variability of cellular processes in phytoplankton has been explored since the 1960s (Hastings et al., 1961; Palmer et al., 1964; Vaulot & Chisholm, 1987). This time keeping mechanism is vital to the maintenance of temporal order in eukaroytic organisms (Chisholm, 1981). While there are many terms to describe this "clock", such as endogenous clock, biological clock, circadian clock, and innate oscillator, for purposes of consistency we will refer to this clock as the Circadian Rhythm (CR). The CR can function to create temporal order in cellular processes, properly phase these processes to external periodicities, and to measure the passage of time (Chisholm, 1981).

Oscillations in environmental conditions can also exhibit control over biological periodicity in phytoplankton (Prézelin, 1992; Sournia, 1975). In addition to this, environmental variables are able to act as *Zeitgebers* (synchronisers) for the entrainment (synchronisation) of the CR (Chisholm, 1981; Mittag, 2001; Sournia, 1975). Entrainment has been hypothesised to optimise the CR-synchronised cell biology with environmental oscillations for the survival of the organism (Chisholm, 1981; Chisholm & Brand, 1981; Prézelin, 1992). In fact, period changes in environmental oscillations have been shown to influence CR periods. Experimental setups have entrained populations of phytoplankton to periods ranging from 20 h to 40 h (Chisholm, 1981; Edmunds & Funch, 1969).

Of the many environmental variables, the light:dark (LD) cycle is perhaps the most significant due to its direct regulation of photosynthesis and other light-dependent reactions (Edmunds, 1988; Prézelin, 1992). Chisholm (1981) goes so far as to say that the natural LD cycle moulded the evolution of the CR. However, the mechanisms of light control over the CR are still unclear. Vaulot (1995) posed the question of whether the entrainment of division periods of the cell cycle resulted directly from light, or if the light signal is relayed through the CR. The experimental findings of Dagenais-Bellefeuille *et al.* (2008) determined that cell division phase timings are independent of circadian clock control. This indicates that light entrainment of cell division could occur directly rather than through the CR. Even so, recent molecular techniques to analyse the CR – CDC coupling mechanisms 25 years later have yet to fully answer the question (Chakrabarti & Michor, 2020).

As for other environmental variables, it was noted by Chisholm (1981) that while the entrainment, initiation, and amplitudes of processes controlled by the CR are influenced by temperature, the period length is temperature independent. This is remarkable as "chronometers" should be insensitive to temperature for proper time keeping, yet many if not most physiological processes of cells are quite temperature dependent (Chisholm, 1981). The link to temperature as a parameter will be further explored later.

Of all the cellular rhythms, attributing the initiation of cell division periodicity has perhaps proved the most contentious. This is due to the complexities in the interactions between the CDC and the CR. For cells in circadian or infradian growth periods exhibiting generation (cell doubling) times (g) of more than or equal to 24 h ($g \geq 24h$), cell division is restricted to a defined interval relative to the start of the LD cycle, indicating coupling to the CR (Chisholm,

1981; Edmunds, 1988; Prézelin, 1992; Vaulot & Chisholm, 1987). This interval is such that within the period (τ), division can only occur within this gate, hence the term circadian-gated division interval (Chakrabarti & Michor, 2020; Chisholm, 1981; Dagenais-Bellefeuille et al., 2008). However, cells in ultradian growth periods with generation times of less than 24 h ($g < 24h$) no longer divide solely within a CR-defined temporal gate (Chisholm, 1981; Chisholm & Brand, 1981; Harding et al., 1981; Prézelin, 1992; Sournia, 1975). Instead the cell cycle is tightly coupled to the CDC as some cells divide more than once in a period (Chisholm, 1981; Edmunds, 1988; Mittag, 2001; Prézelin, 1992). As CDC and cell cycles are largely temperature dependent physiological processes, temperature plays a key role in cells experiencing ultradian growth periods (Chisholm, 1981). However, when division is slowed down such that the cells exhibit infradian growth, the cell cycle couples with the CR again and exhibits circadian-gated division patterns (Chisholm, 1981; Prézelin, 1992). As mentioned, the mechanisms and even theories, of CR – CDC coupling and uncoupling are still poorly understood even with advances in molecular and genomics research (Chakrabarti & Michor, 2020). Today, circadian-gating is still a leading hypothesis for the divisional control of eukaryotic phytoplankton (Chakrabarti & Michor, 2020).

In populations as a whole, circadian rhythms of individual cells are entrained primarily by alternating LD cycles resulting in synchronous or phased division timings (Chisholm, 1981; Vaulot, 1995). A matter of terminology, "synchronous" is used when the cell division cycles are aligned such that all cells divide once a day ($g \sim 24h$), while "phased" is used when only a portion of the population divides over the period (Chisholm, 1981; Chisholm & Brand, 1981).

1.2.2 Climate Change Induced Migration Patterns

Spatial and temporal distribution of phytoplankton populations on regional and global scales are largely dependent on the primary cellular limitations in light, nutrients, and temperature (Falkowski, 1994; Field, 1998). Light irradiance in particular, is limiting during the winter months near the poles (Holm-Hansen & Greg Mitchell, 1991; Lee et al., 2012). In conjunction with upper ocean physics (Behrenfeld & Boss, 2018; Falkowski, 1994), warming temperatures due to climate change have large implications on phytoplankton distributions both spatially and temporally (Hays et al., 2005; Sorte et al., 2010).

Climate change has caused the pole-ward migration of many terrestrial and marine organisms (Hays et al., 2005; Sorte et al., 2010). For phytoplankton, this range shift has resulted in alterations in community assemblages (Sorte et al., 2010), taxonomic composition (Winder & Sommer, 2012), and seasonal bloom dynamics (Behrenfeld & Boss, 2018; Winder & Sommer, 2012). At high latitudes, warming temperatures have caused phytoplankton blooms to start earlier and be dominated by smaller sized fractions (Winder & Sommer, 2012). These changes can have cascading ecosystem effects and alter important phytoplankton functions of primary production (Falkowski et al., 2003), biogeochemical cycling (Falkowski, 1994), and energy transfer (Winder & Sommer, 2012).

As phytoplankton distribution for different species shift polewards, the effects of seasonal variations in photoperiods, characteristic of high latitude regions, are largely unexplored. Taken in context, it is important to understand how these extreme photoperiods will affect temperate and tropical phytoplankton species that could potentially migrate towards polar regions and influence bloom dynamics.

1.2.3 Existing Experimental Setups

As mentioned in the introduction, field studies (*in situ*) regarding the effect of photoperiod on phytoplankton are largely nonexistent. The large amount of data needed both spatially and temporally to characterise differences in cellular periodicities result in prohibitively high costs (Chapin, 2015). Laboratory experiments (*ex situ*) are much more common due to ability to manipulate primary limitations in light, temperature, and nutrients in a controlled setting (Field, 1998). In studying the effects of periodicity, a wealth of literature exists on experimental setups studying photosynthesis (Edmunds, 1988; Harding et al., 1981; Legendre et al., 1988), nutrient uptake (Chisholm, 1981; Meseck et al., 2005), biomass and productivity (Prézelin, 1992), gene expression (Liu et al., 1995), phototaxis (Edmunds, 1988), and cell division (Chisholm, 1981; Chisholm & Brand, 1981; Edmunds, 1988; Edmunds & Funch, 1969; Jacquet et al., 2001; Vaulot & Chisholm, 1987).

While many studies on cell division periodicity placed a great deal of emphasis on the CR and CDC coupling effects, there are few examples of varying photoperiods on cell division patterns with the exception of Edmunds (1966) and Edmunds and Funch (1969). Since then,

improvements in technology capable of analysing cell division patterns such as flow cytometry have greatly improved the accuracy and reproducibility of such experiments (Marie et al., 2005; Prézelin, 1992). While there are more recent studies on cell division for the purpose of algal aquaculture feed, these studies have focused more on changes in biomass and growth rates as compared to the temporal variation in cell division periodicity (Bouterfas & Belkoura, 2006; Meseck et al., 2005). Additionally, the data of many of these studies were collected manually, increasing error and reducing the reproducibility of the results (Chapin, 2015; Efromson et al., 2021; Meseck et al., 2005). The high cost of manual sampling also resulted in low temporal resolution in the data, with 24 h intervals between data points being the norm.

Many cellular patterns of growth and division in marine phytoplankton are characterised by short time scale variability, which increases the need for high frequency sampling to capture these changes (Edmunds, 1965; Jacquet et al., 1998). Consistent time series data is required for many rate of change analysis projects (Wood et al., 2005). To address the need for consistent high resolution temporal data, as well as reduce error and increase reproducibility of sampling, automatic samplers can be used (Edmunds, 1966; Efromson et al., 2021; Jacquet et al., 1998; Nelke et al., 2017).

1.2.4 Autosampler Background, History, and Comparisons

The necessity of automatic sampling was such that its history is intricately tied to that of the study of periodicity in cells. The earliest records of automatic sampling for the explicit purposes of studying periodicity in phytoplankton was by Petropulus (1964). The sampler was built for the pioneer in cell periodicity and CR studies, Edmunds (1965, 1966; 1969), who conducted high frequency sampling of *Euglena* cultures.

Since then, a wide variety of commercial automatic samplers have been developed, often for the purposes of storm water monitoring, waste water effluent sampling, or aseptic bioreactor sampling (Carvalho & Murray, 2018). However, these commercial machines are often expensive, unwieldy, and not customisable to the exact needs of the experiment (Carvalho & Murray, 2018; Efromson et al., 2021; Nelke et al., 2017). In fact, despite the significant advances in technology, the prohibitively high cost of commercial autosamplers have limited their adoptions in many labs (Efromson et al., 2021).

In 1998, Jacquet *et al.* (1998) built a low-cost alternative for the purposes of phytoplankton growth rate sampling. A number of open-source solutions have also been designed and made publicly available such as the OPEnSampler (Nelke et al., 2017), Osmar (Carvalho & Murray, 2018), and the BioSamplr (Efromson et al., 2021) to name a few. As the applications for autosamplers are not limited to cell cycle studies, but extend to biogeochemical sampling (Hartmann et al., 2018; Nelke et al., 2017) and other bioreactor sampling (Efromson et al., 2021), a plethora of designs have emerged across the various fields. While this new generation of automatic samplers utilise newer, more reliable, and more precise components than Petropulus (1964) or Jacquet *et al.* (1998), many of them were not designed with live cell sampling in mind.

1.2.5 Design Considerations

Flow cytometry is a key tool in the analysis of cell cycle progression and periodicity (Marie et al., 2005; Prézelin, 1992; Vaulot, 1995; Wood et al., 2005). Although continuous flow cytometric monitoring exists (Swalwell et al., 2011; Thyssen et al., 2008), the elucidation of specific parameters, such as DNA, using dyes still requires samples to be prepared before flow cytometric analysis (Marie et al., 2005). Hence, as much as possible, automatic samplers should sample and preserve pure volumes for further analysis.

The basic function of an automatic sampler is to collect samples at fixed temporal intervals. As such, the first design consideration is that an automatic sampler must be able to operate around the clock at regular intervals. These intervals should be programmable by the user, and sampling should not require the presence of an operator.

The second function as noted above is the preservation of samples until they can be analysed. As noted by Jacquet *et al.* (2001), storage at 4°C for up to 10 h had minimal effects on cell parameters measured by flow cytometry. To prevent any unwanted influences of light, samples should also be stored in a dark environment. Hence, the second design consideration would be the ability to store samples at a fixed temperature of 4°C in the dark for at least 10 h until collection by the user.

Finally, an implicit design consideration for samplers is the ability to prevent cross-contamination between samples. Even as early as Petropulus (1964), automatic samplers have been designed to prevent cross-contamination through the "purging" of tubes and valves between samples. As such, the third and final design consideration is that an automatic sampler must be able to purge waste fluid between sampling events. Table 1-1 gives a breakdown of the various do-it-yourself (DIY) sampler designs and their fulfilment of the design considerations relevant to optimal phytoplankton sampling.

Table 1-1: Comparison of existing publicly available Do-it-Yourself Automatic Samplers

	Author	Year	Name	Cost (US$)	Vials	Sample Volume (ml)	Programmable	Temperature Control	Purging	Reference
1	Petropulus	1964	Automatic Sampling Device	Unknown	12	<100	Yes	No	Yes	(Petropulos, 1964)
2	Jacquet	1998	Compact Automatic Sea Water Sampler	300	24	4.5	Yes	Yes	Yes	(Jacquet et al., 1998)
3	Moreira & de Paiva	2010	Low-Cost Automatic Water Sampler	1620	4	1950	Yes	No	Yes	(Moreira & De Paiva, 2010)
4	De Winter	2016	Automatic Fluid Sampler	360	24	1000	Yes	No	Yes	(De Winter, 2016)
5	Nelke et al.	2017	OPEnSampler	450	24	250	Yes	No	No	(Nelke et al., 2017)
6	Hartmann et al.	2018	GUARD	1000	160	12	Yes	No	No	(Hartmann et al., 2018)
7	Carvalho & Murray	2018	Osmar	540	96	<5	Yes	No	No	(Carvalho & Murray, 2018)
8	Efromson et al.	2021	BioSamplr	700	10	1.5	Yes	Yes	Yes	(Efromson et al., 2021)
9	Mah & Ang	2021	Fightoplankton	250	10	15	Yes	Yes	Yes	This Study

While all the DIY autosamplers have programmable time intervals for sampling, some lack the "purge" mechanisms to prevent cross-contamination and few have temperature control capabilities (Table 1-1). Only the BioSamplr (Efromson et al., 2021), published as this study was in progress, and the original sampler designed by Jacquet *et al.* (Jacquet et al., 1998), fulfil all the design considerations for automatic sampling of phytoplankton. While it seems that Efromson *et al.* (2021) may have beaten me to the punch in designing an updated low-cost automatic sampler for phytoplankton cultures, the BioSamplr has sample volume limitations that influence the ability to collect replicates.

1.3 Objectives

This study aims to fulfil two objectives in parallel. The first objective is to further the understanding of the effects of varying photoperiods on the periodicity of cellular processes of phytoplankton acclimated to low latitude consistent light regimes. I aim to investigate this through high resolution growth ratesampling and analysis of *Picochlorum sp.* under varying LD cycles. This is aided by the second objective, which is to design and build an automatic

water sampler to reduce the time-cost of high frequency sampling of phytoplankton cultures. As the experiment is largely exploratory in nature, the results will be mainly observational. The methods employed will also be largely experimental, for the purposes of improving techniques for future time-sensitive studies on phytoplankton.

Material and Methods

2.1 Sampler Design and Build

The Fightoplankton was designed to reduce the time-cost and lower barriers to performing high frequency sampling. To be effective, it must fulfil the following 3 design considerations:

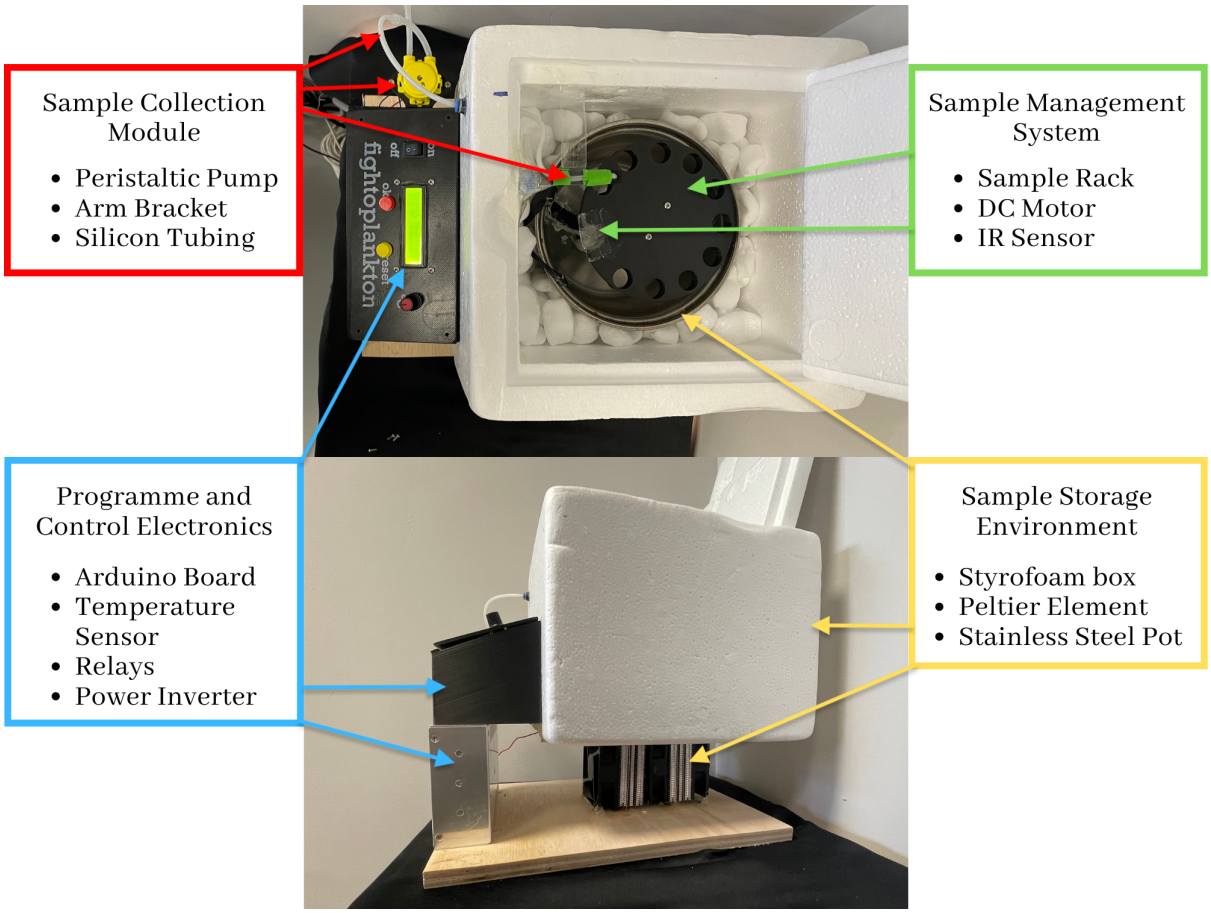
1. Samples to be collected at regular user-programmed time intervals
2. Samples are to be stored in a dark environment at 4°C
3. No cross-contamination between samples

In order to accomplish these 3 objectives, the Fightoplankton can be broken down into 4 main subsystems i) program and control electronics, ii) sample collection module, iii) sample management system, and iv) sample storage environment (Fig 2-1).

2.1.1 Program and Control Electronics

The electronics, and a programmable Arduino Uno microcontroller are stored in a 3D printed housing at the front of the storage unit. The housing also has an inbuilt user interface for sampling parameter input. The code used for the project with detailed comments, as well as the designs of all 3D printed components, can be found on Github at https://github.com/dmah002/Automatic-Water-Sampler. A short excerpt of the code can be found below, in which the user can modify sampling parameters such as collection timing, collection intervals, motor speed, and temperature control.

Figure 2-1: Final Fightoplankton Automatic Sampler



2.1.2 Sample Collection Module

The sample collection module consists of a 3V Kamoer NKP peristaltic pump, 2×4 mm silicon tubing, taper shaped tubing connectors, and 3D printed mounts for the pump and tubing. As with all other components mentioned in this report, the price and supplier information can be found in the bill of materials in Appendix Table A-1. When sampling occurs, a relay activates the peristaltic pump, which first purges the tube of previous sample remnants followed by drawing a predetermined volume of sample into a storage tube. Based on the length of sampling tube used (l) and the radius of the tube (r), the time required for purging (T) can be described in the following equation:

$$T = \frac{\pi r^2 \times l}{0.00223881} \tag{2.1}$$

Figure 2-2: Extract of definable variables in david_FYP.ino progamme file

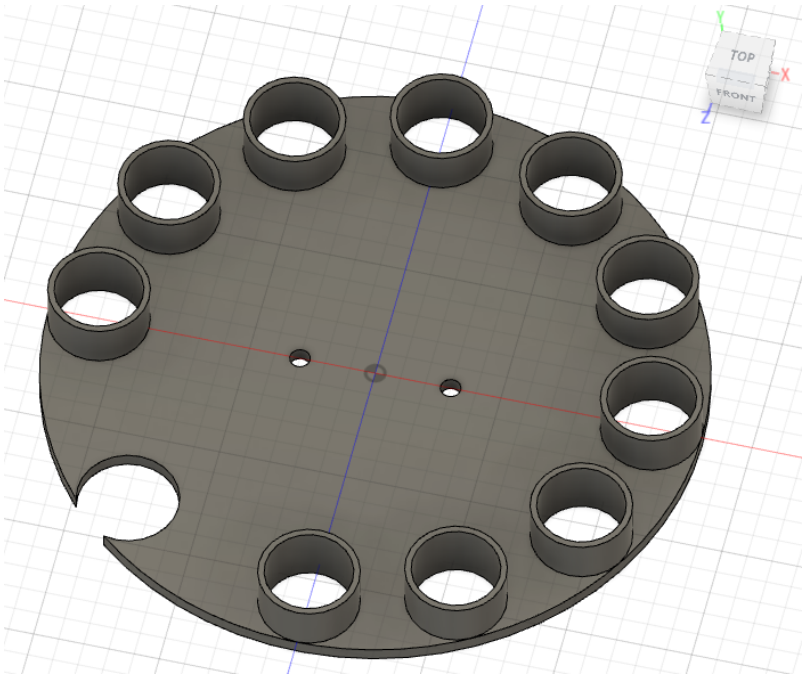
```
1       float mode_read;
        String MODE = "Norm" ; // "Norm": normal mode, sample once over hour.  "3in3":
    sample 3 times every 3 hours
3       float temperature_set;
        float range = 0.3 ;
5       float temp_now ;
        long sampling_interval ; //3600000; MODIFIED IN select_mode() function
7       int   Peltier_STATE = HIGH;
        int   SAMPLE_COUNT = 0 ;
9       volatile int   front = HIGH;
        volatile int   back = LOW;
11      long start ;
        long timeleft ;
13      int button_state = 1;          // current state of the button
        int last_button_state = 1;
15      int OK = 0;
        int reset_counter = 0;
17
        // Variables will change:
19      int TUBE_COUNT = 0;   // counter for the number of button presses
        // for IR sensor, "black" is logic 1, white is logic 0
21      int IR_state = 1;          // current state of the button
        int last_IR_state = 1;      // previous state of the button
23      int last_flakey_state = 1;
        unsigned long lastDebounceTime = 0;  // the last time the output pin was toggled
25      const int DEBOUNCE_DELAY = 10;
        //duration to turn on the pump for
27      int   WASTE = 6000;
        int   COLLECT = 3000;
29      int   MOTOR_SPEED = 175;


31
```

2.1.3 Sample Management System

The purpose of this sub-system is to organise and manage the sample storage tubes within the storage module of the machine. It consists of a 3D printed rack (Fig 2-3) capable of holding 10 15 ml glass Pyrex storage tubes, a 5V DC motor, and an infrared sensor. During each sampling event, the motor rotates the rack until the infrared sensor determines that the assigned storage tube is directly below the output of the sample collection module. Once in position, the motor stops and waits for samples to be drawn before rotating back to the starting purge position.

Figure 2-3: 3D render of Sample Management Rack



2.1.4 Sample Storage Environment

Proper storage of the samples after collection is critical to obtaining accurate results after sampling. In order to preserve the samples, they should be stored at 4°C and kept in a dark environment. Due to this, the storage environment occupies the largest portion of the entire sampler. It consists of a large Styrofoam box, a 240W 12V TEC1-12715 Peltier chip with accompanying heat sinks, a DS18B20 waterproof digital temperature probe with an accuracy of $\pm$0.5°C, and a stainless steel pot.

The temperature is defined by the user at the beginning of the experiment. Temperature feedback from the probe allows for bang-bang control (Artstein, 1980) to ensure the water bath in the pot is maintained at the set temperature. This is done through the on/off control of the Peltier chip as the temperature deviates from the set temperature. The Styrofoam box prevents excess heat from entering the storage area, maintaining the temperature gradient between the storage area and the external environment. This ensures that the temperature of the storage area never deviates more than 1°C from the set temperature.

2.1.5 Assembly

Assembly of the Fightoplankton should take no longer than 60 man-hours. First, the structural components such as the pot, Styrofoam box, plywood base, 3D printed housings, and Peltier elements are fixed together through the use of screws, hot glue, silicon, thermal paste, and super glue. Next, the internal electronics such as the DC motor, infrared sensor, and temperature probe are connected and routed out of the Styrofoam box. Finally, the Arduino control unit as well as other relays and converters are connected and soldered together.

After the Fightoplankton is assembled, calibration of the infrared sensors, DC motor speed, peristaltic pump duration, and alignment of glass tubes should be carried out through the loading of the test software, which runs through all the various tube positions. Before starting a sampling effort, a test run should be conducted at least once to ensure that the system is working correctly. While most issues can be solved with a little troubleshooting, some potential malfunctions and remedies are listed below:

Table 2-1: Common Fightoplankton Malfunctions and Remedies

Malfunction	Remedy
Storage rack miscounts during sampling	Adjust the infrared sensitivity through the blue potentiometer
Sampling collection output not aligned to storage tube	Adjust the output bracket to align and run position test
System restarts intermittently	Power issue, check power cables
Display or UI inputs flicker or become nonfunctional	Connectivity issue, check if cables are properly connected
Temperature control no longer working	Connectivity issue, check relays and cables

2.2 Sampling Operation

Sampling operation can be broken down into three distinct phases: initial setup, sample collection, and counter reset. While the operation procedures are dependent on the user-defined program loaded onto the arduino microcontroller, we will be explaining the operations of a general program of hourly sampling intervals and sample storage at 4°C.

2.2.1 Set-up

The Fightoplankton is powered on by toggling the power switch on the User Interface (UI) board. The LCD screen will then display the available user-loaded programmes for selection

using the rotary encoder. After selecting “Norm” for the hourly sampling routine and setting the temperature at 4°C, the Fightoplankton will begin to cool the water bath within the storage area to the set temperature. While the Peltier chip will eventually cool the water bath to the desired temperature, the process can be rather slow and energy intensive. To avoid this delay, a small volume of water can be cooled in advance.

Once the water bath has attained the set temperature, the Fightoplankton will prompt the user that the experiment is ready to begin. The first sampling period will occur 1 h from the moment the user begins the experiment. Every hour from that moment, the Fightoplankton will perform a sampling event up until the 10^{th} sample is collected. After all storage tubes are fully utilised, the Fightoplankton will cease to collect additional samples. However, a timer on the UI will continue to display the time elapsed since the last sampling event.

2.2.2 Sample Collection

As periodicity experiments typically last longer than 10h, often stretching to a minimum of 24h to capture the entire diel period, the storage tubes will need to be collected and replaced with a clean set of storage tubes. This can be done at any point within the 10h and does not necessarily need to be at the end. Samples collected can then be immediately analysed, or fixed with a solution of gluteraldehyde and stored at -80°C for future processing.

2.2.3 Counter Reset

After collecting the storage tubes, the user should then replace these with a clean set of storage tubes. Once replaced, the “reset” button on the UI should be pressed and held for 3s to trigger a reset of the sample counter. Once reset, the Fightoplankton will restart the sampling process from the first storage tube in the rack. The timing for the next sample will continue uninterrupted from the previous sampling cycle. In order to maintain the hourly interval, the tube replacement and reset should be done before an hour has elapsed from the collection of the 10^{th} sample.

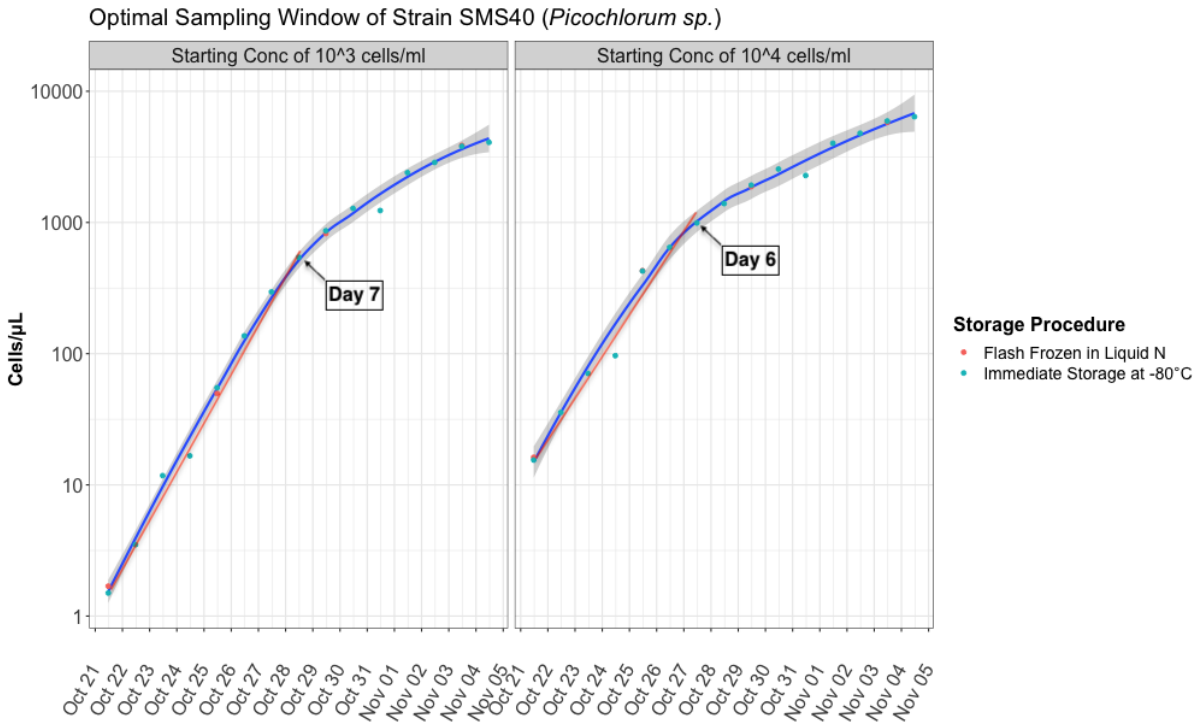
2.3 Fightoplankton Validation

2.3.1 Strain and Sampling Information

A laboratory culture of *Picochlorum sp.*, SMS40, from the Singapore Marine Strains (SMS) collection was grown in L1 Artificial Sea Water (ASW) media (Guillard & Hargraves, 1993) at 22°C and maintained under a 12:12 LD cycle. SMS40 was chosen due to its suitability for testing the Fightoplankton as well as to investigate the effects of varying photoperiods on the periodicity of cellular processes of phytoplankton. SMS40 was isolated by Rae Chua in November 2019 from marine coastal waters off Pulau Ubin (1.4126°N, 103.9577°E) (Lopes dos Santos & Chua, 2020). Members of the *Picochlorum* genus have been extensively studied for a vast array of potential applications in biomass production, aquaculture feed, and wastewater remediation (Foflonker et al., 2018). They are unicellular organisms, coccoid in shape, and each contain a single mitochondrion and chloroplast (Foflonker et al., 2018). Many members of *Picochlorum* are considered "polyextremotolerant", due to their ability to withstand hypervariable environments (Foflonker et al., 2016). As they are small, unicellular, and robust, this will allow us to test the Fightoplankton with relative ease. Also, as a strain isolated in tropical waters off Singapore, it is unlikely the strain has been exposed to extremely variable photoperiods in its recent evolutionary history, making it amenable to variable photoperiod studies.

To accurately study periodicity in cellular processes, sampling should occur after at least 3 days of acclimation and during exponential growth of the population (Wood et al., 2005). An earlier experiment regarding the growth rate of SMS40 was conducted to calibrate the optimal initial cell concentration and to identify the timing of the exponential growth phase (Appendix A). The results of this experiment showed that exponential growth was achieved within the first 7 days of culturing given an initial cell concentration to the order of 10^3 cells/ml, or within the first 6 days for initial concentrations of 10^4 cells/ml (Fig 2-4). Hence, it was decided that future experimental setups would utilise starting cell concentrations in the order of 10^3 cells/ml for an optimal sampling window of 4 - 7 days after inoculation (Wood et al., 2005).

Figure 2-4: Results of Exponential Growth and Cell Concentration Test



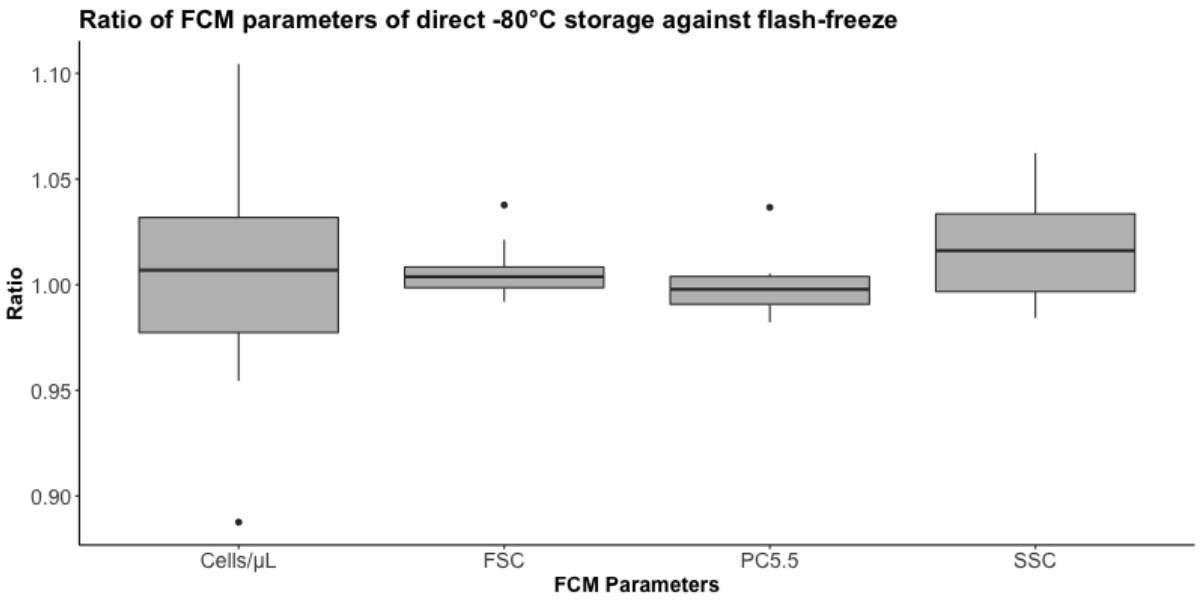
2.3.2 Pre-Experiment Tests and Setup

While experimental procedures dictate that samples for flow cytometry are flash frozen in liquid nitrogen (N_2) before long term storage at -80°C, material constraints surrounding the use of cryotubes and N_2 availability meant skipping the flash freeze process and immediately storing samples at -80°C. Another pre-experimental test was conducted in parallel to determine the effect of skipping the flash freeze process on parameters measured by flow cytometry (Appendix A).

The results of the test indicate that skipping the flash freeze process had minimal effects on cellular parameters measured (Fig 2-5). This is likely due to the microtubes only capable of storing small volumes of 1.5 ml, resulting in a relatively fast freezing process at -80°C with similar effects to flash freezing in N_2.

Another obstacle to using the Fightoplankton for sampling was the space constraints regarding areas where light and temperature could be controlled. To facilitate the experiment, a temporary culture shelf was assembled in the GEEK lab such that the Fightoplankton had full

Figure 2-5: Results of Storage Procedure Test



access to the cultures (Fig 2-6). This was done through the installation of 2 units of 3000K OMLOPP LED lights from IKEA (IKEA, 2021) controlled by a mechanical timer and covered on all sides with black-out curtains. Within the shelf, 2 magnetic stirrers were used to agitate any growing cultures. The temperature in the room was monitored over the entire period and typically fluctuated between 24-26°C. The temperature dropped to 21-23°C when a change in air-conditioning temperature was implemented on 16 February (Fig 2-7).

2.3.3 Fightoplankton Test Experimental Setup

To validate the reliability of the Fightoplankton, a test of sample preservation and the accuracy of sampling intervals was conducted. A 800 ml culture of SMS40 was set up in a conical flask under 12:12 LD conditions starting at 0805 h and ending at 2005 h each day at a temperature of 24 - 26°C. The culture was inoculated to an initial concentration of 5.52 cells/µL. On days 6 - 7 after inoculation, during the optimal sampling window, sampling was performed hourly by the Fightoplankton over a period of 24 h. Storage tubes were changed on 16/12/20 at 0700 h and 1700 h, with the experiment being stopped at 2050 h after the 24^{th} sample was collected. Duplicate samples of 1000 µL were pipetted from the storage tubes into 1.5 ml microtubes pre-filled with 10 µL of 25% gluteraldehyde solution. Sample microtubes were left to incubate at room temperature for 10 mins followed by long term storage at -80°C.

Figure 2-6: Culture Shelf Setup

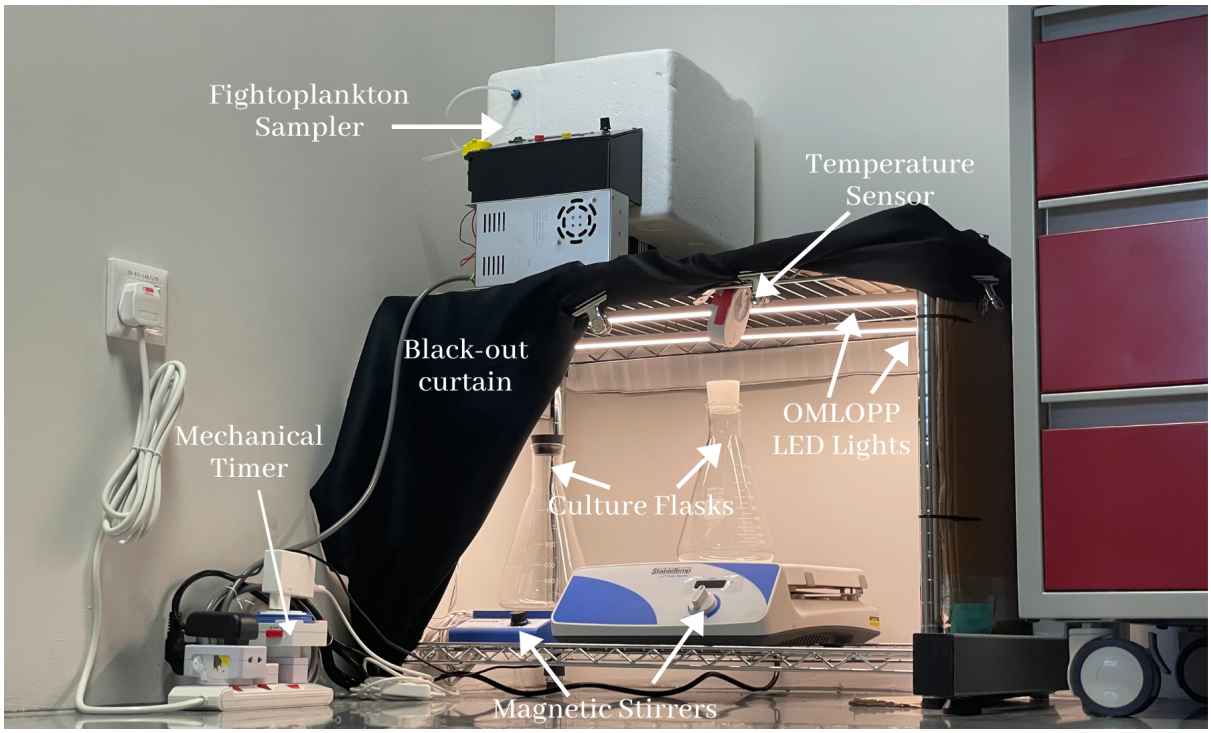
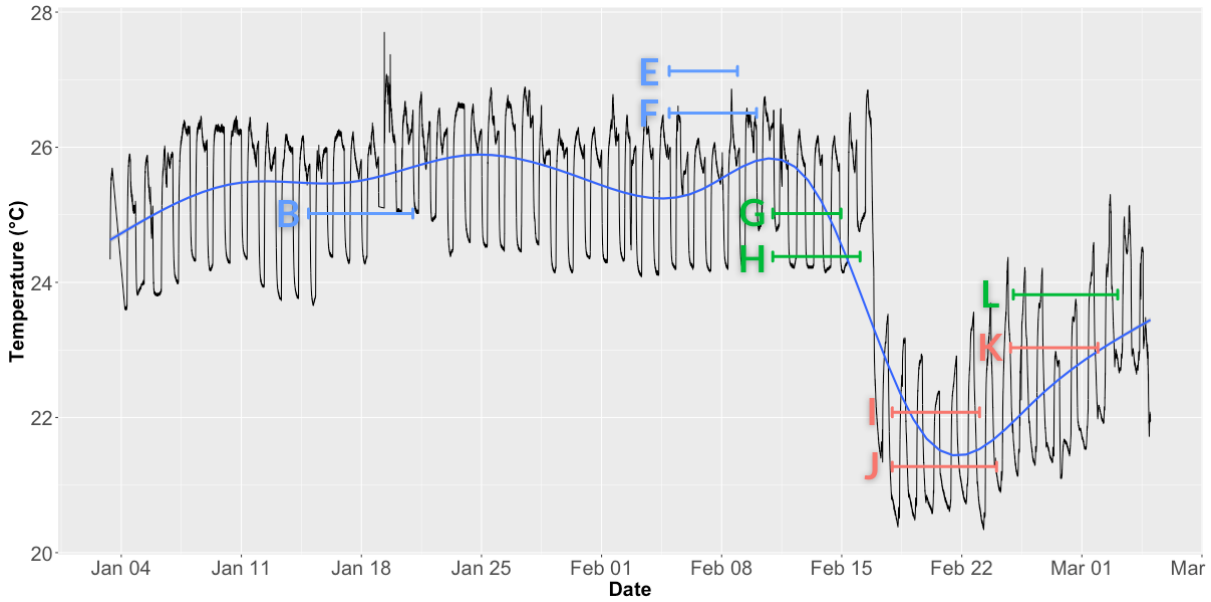


Figure 2-7: Temperature over Sampling Period



To determine the preservative effects of sample storage at 4°C in the dark, samples were also manually collected and fixed immediately using 25% gluteraldehyde solution. These were then compared with samples stored for 10h in the Fightoplankton storage tubes before fixation and storage at -80°C. The samples, both manually and automatically collected, were then analysed

using flow cytometry at a later date. The results of sampling accuracy and sample preservation were determined.

2.4 Test of Varying Photoperiods on Cellular Periodicity

2.4.1 Photoperiod Experimental Setup

An experiment was conducted to gain insights regarding the effects of varying photoperiods on the periodicity of cellular processes of phytoplankton acclimated to low latitude consistent light regimes. Seed cultures of SMS40 were grown under 3 different light regimes through the adjustment of mechanical timers controlling LED lights. Light regimes of 16:8 and 8:16 LD cycles, were chosen to mimic the long and short photoperiods characteristic of high latitude summers and winters respectively. The 12:12 LD cycle was also used as a control group. After at least 1 week of acclimation, these seed cultures were used to inoculate sample flasks.

Sample flasks were set up in conical flasks filled up with 800 ml of L1 ASW. Once inoculated to a starting cell concentration to the order of 10^3 cells/ml, the flasks were maintained in the temporary culture shelf (Fig 2-6) and agitated with a magnetic stirrer set at 550 rpm. Flasks were then sampled hourly for a period of 24 h within the optimal sampling window of 4 - 7 days after inoculation, using the initiation of the light as a universal starting point. Table 2-2 shows the break down of the conditions and timings for each of the culture flasks sampled.

During tube resets, 1300 µL of sample was pipetted from each storage tube into a 1.5 ml microtube pre-filled with 13 µL of 25% gluteraldehyde solution. Following 10 minutes of incubation at room temperature, the samples were stored at -80°C. The samples were then analysed using flow cytometry at a later date and the results of varying light regimes on cellular periodicities was investigated.

Table 2-2: Summary of Flask Conditions

Flask	Light Regime	Light Start Time (h)	Dark Start Time (h)	Culture Start Date	Culture Start Time	Sampling Date	Sampling Start Time	Days After Innoculation	Temp Range (°C)
B	16:8	0755	0800	2021/01/15	1855	2021/01/21	0820	6	24-26
E	16:8	0850	0048	2021/02/05	1615	2021/02/09	0929	4	24-26
F	16:8	0850	0048	2021/02/05	1615	2021/02/10	0830	5	24-26
G	12:12	0850	2050	2021/02/11	1215	2021/02/15	0900	4	24-26
H	12:12	0850	2050	2021/02/11	1215	2021/02/16	0850	5	21-23
I	8:16	0845	1641	2021/02/18	1300	2021/02/23	0905	5	21-23
J	8:16	0845	1641	2021/02/18	1300	2021/02/24	0907	6	21-23
K	8:16	0845	1641	2021/02/25	1108	2021/03/02	0838	5	22-24
L	12:12	0845	2104	2021/02/25	1108	2021/03/03	0838	6	22-24

2.4.2 Flow Cytometry and Data Analysis

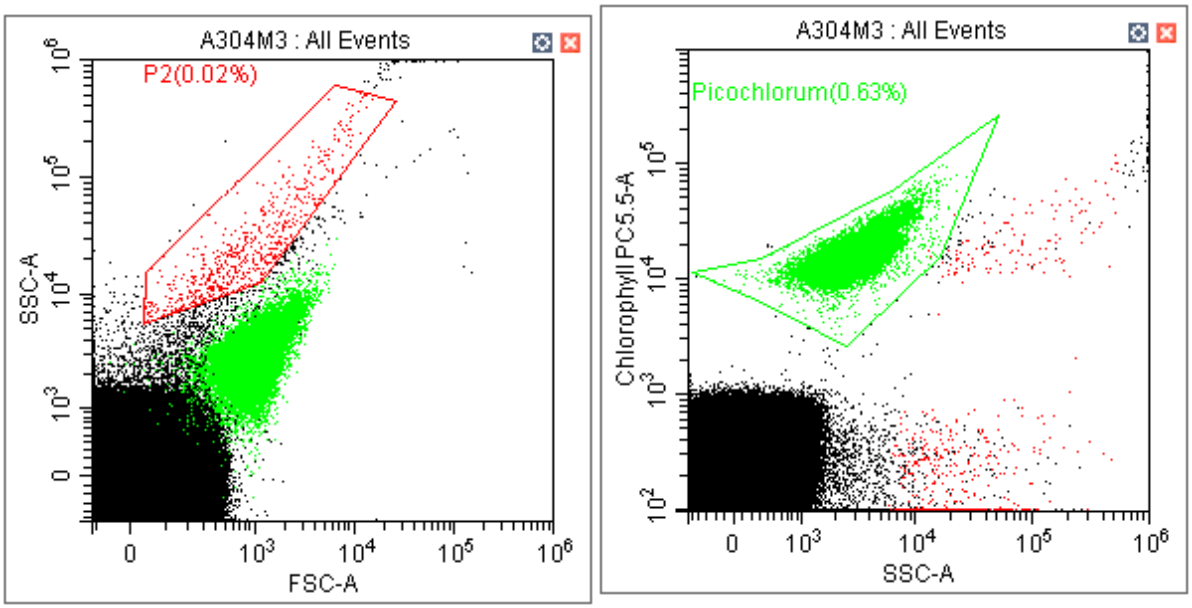
Used since the 1970s for cell counts, flow cytometry (FCM) is a method of single-cell analysis in which cells in a solution are drawn through a small aperture and successively pass through a laser beam (Marie et al., 2005; Trask et al., 2005). As cells pass through the laser, the angle and distribution of light scattering is measured. Light scattered at small angles results in a more forward direction, hence the name forward scatter (FSC). FSC can give us information regarding a cells size, albeit with caution (Shapiro, 2003). Light scattered at large angles typically fall to the side, hence the name side scatter (SSC). SSC can give us a better idea of the cells internal granularity (Shapiro, 2003). On top of light scatter, the fluorescence of photosynthetic pigments such as phycobiliproteins can also be measured. In a simplified manner, PC5.5 measurements on the FCM coincide with the excitation and emission spectrum of photosynthetic pigments and hence can be used as a proxy for chlorophyll pigmentation (Shapiro, 2003). Shapiro (2003) puts it more poetically in his textbook *Practical Flow Cytometry*:

> Though forward scattering gives us a smattering
> Of data related to particle size,
> Change in refraction comes into action,
> Decreasing signals, when a cell dies.
>
> Light scattered wider gives us insider
> Information about cells' detail,
> Irregularity and granularity,
> Which we can use and still stay out of jail.

But to learn most, we measure fluorescence,
Which is now flow cytometry's essence,
Much as tumescence is to male adolescence

Samples from the Fightoplankton validation and variable photoperiods experiment were analysed using FCM. For Fightoplankton validation, 300 µL of sample from each microtube was loaded into a 96 well flat bottom plate. For the variable photoperiod experiment, triplicates of 300 µL were taken from each microtube and loaded into a 96 well flat bottom plate. Milli-Q water filtered through a 0.2 micron filter was used as sheath fluid. Each well was sampled at a flow rate of 30 µL/min for 180 seconds and the data recorded. SMS40 cells were distinguished from other debris particles through the "gating" of cells exhibiting similar values for cellular parameters measured. Figure 2-8 shows how SMS40 cells are identified and grouped together through the use of a "gate". As *Picochlorum sp.* cells will display similar ratios in their cellular parameters, be it FSC against SSC, or PC5.5 against SSC, we can qualitatively cluster cells according to their position in scatter plots of 2 parameters against each other. In the case of Figure 2-8, a green "gate" is drawn around cells exhibiting similar PC5.5 to SSC ratios, illustrating the *Picochlorum sp.* cells.

Figure 2-8: Example of Gating of FCM data



While understanding how the FCM measurements translate into cellular properties is much more complex than the poem describes, we are more interested in the periodicity of the

parameters over time than the actual amplitudes in the changes. The FCM data acquired was processed and visualised on R. Instantaneous growth rates of cultures were determined through the estimated derivative of the nonparametric regression of cell concentration mean across time for each culture flask (Sestelo et al., 2017). Maximum instantaneous growth rates were compared using a one-way Analysis of Variance (ANOVA) to determine if changing photoperiods had significant influences on the periodicity of cell division. Additionally, variations in the timing and periodicity of the maxima of other cell parameters such as FSC, SSC, and PC5.5 were similarly investigated. Significant ANOVA results were pursued with pairwise T tests to determine finer details regarding the deviations. From the results and existing scientific literature on the topic, some observations are discussed.

Results and Discussion

3.1 Fightoplankton Validation

3.1.1 Results of sampling accuracy

Over the course of the 24 h sampling period, the Fightoplankton consistently sampled at the appropriate time intervals, as well as deposited each sampling event in the correct storage tube without spillage. While some calibration was required in the days leading up to the sampling process, all the functions of the Fightoplankton performed consistently and with precision.

As the Fightoplankton was used to sample hourly for the variable photoperiod experiment, comments on the accuracy and reliability of the Fightoplankton can be made on its performance throughout that period as well. While Table 2-1 in the previous chapter indicates possible malfunctions and solutions, a timeline of actual issues faced and remedy actions performed can be found in the Appendix in Table A-2.

3.1.2 Results of sample preservation

The results support Jacquet *et al.* (2001), in which storage at 4°C for up to 10 h showed minimal effects on the results of cell parameters attained through FCM analysis. No significant differences in FCM measured cell parameters were detected between samples kept at 4°C for 10 h in the Fightoplanktopn followed by long-term storage, and samples immediately placed in long-term storage (Table 3-1).

In addition to the single test conducted during the Fightoplankton validation experiment, manual samples were collected during the variable photoperiod experiment when convenient for comparison with the samples kept at 4°C in the Fightoplankton. Figure 3-1 shows the

Table 3-1: Welch T Test on the Effects of 4°C Preservation for 10 h on FCM Parameters.

Parameter	Group 1	Group 2	statistic	df	p	p.signif
Events/	Fightoplankton	Immediate Storage	0.250	1.506	0.832	ns
FSC	Fightoplankton	Immediate Storage	0.262	1.035	0.836	ns
SSC	Fightoplankton	Immediate Storage	3.149	1.047	0.187	ns
PC5.5	Fightoplankton	Immediate Storage	3.192	1.679	0.107	ns

ratios of cellular parameters (samples preserved in the Fightoplankton over those immediately placed in long-term storage) against the time that Fightoplankton samples were maintained at 4°C for prior to long-term storage. Ratios for FCM parameters of FSC, SSC, and PC5.5 seem to be minimally affected by storage at 4°C for up to 10 h. For cell concentration, the ratio goes as high as 1.4, indicating a 40% difference. These large differences can be attributed to the relatively low cell concentrations in the order of 1 cell/µL in some samples. At those concentrations, even small variations could result in relatively differences in the ratios.

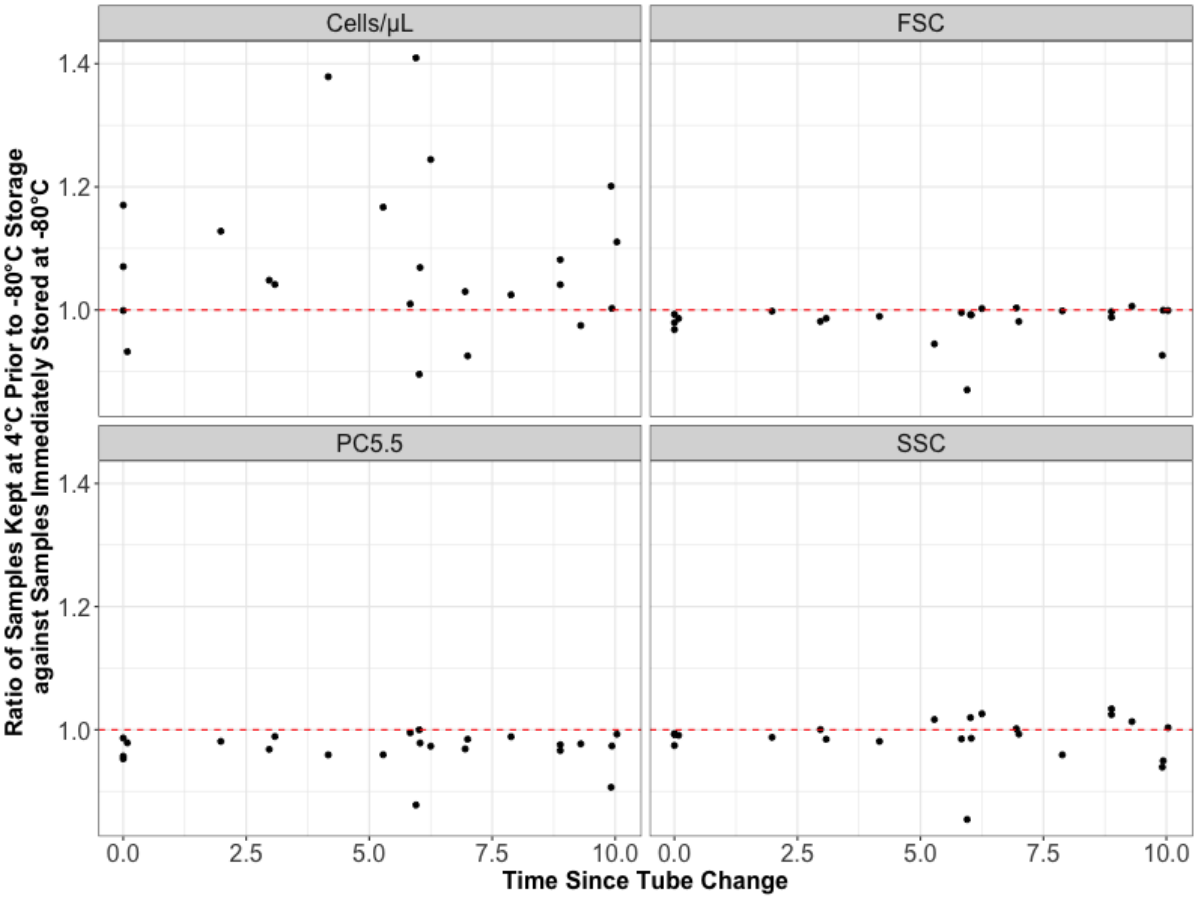
Another observation would be the seemingly random pattern of distribution of the ratios as preservation time increases. If storage at 4°C had any effects on cell parameters measured by the FCM, there would be a trend in the distribution of points the longer a sample is stored. The lack of such a trend seems to indicate that variations between the samples stored at 4°C and those fixed immediately could be due to other factors. As samples in the storage tubes were collected via the Fightoplankton, and samples fixed immediately were collected manually via a pipette, the differences in sampling methods is likely to have had a bigger influence on the differences in cellular patterns observed than preservation at 4°C.

3.1.3 Limitations of the Fightoplankton Automatic Sampler

When carefully maintained and calibrated, the Fightoplankton is able to accurately collect and preserve samples continuously over a period of at least 26 h as shown in the sampling of flask F. However, it should be noted that a number of inherent and consequent limitations arose through the further testing of the Fightoplankton.

The Fightoplankton is limited to sampling 1 flask at a time. There are no capabilities for sampling from multiple flasks or storing multiple sets of samples. This limitations results in

Figure 3-1: Plot of Fightoplankton Preservation Test during the Variable Photoperiod Experiment



the inability to sample true replicates, in which flasks are cultured and sampled simultaneously as compared to successively as is the case for the variable photoperiod experiment. A second inherent limitation is the maximum limit of 10 storage tubes in the Fightoplankton storage area. By utilising a concentric storage management design, a large amount of space is wasted as only the outer edge of the area can be used. In other designs, a Cartesian system using an X-Y plane is utilised, maximising the sample storage area (Carvalho & Murray, 2018; Efromson et al., 2021; Hartmann et al., 2018). This upper limit of 10 samples places an implied restriction on the sampling interval to a minimum of 1 h between samples if the experimenter can only come to the lab every 10 h.

Limitations arising from further testing of the Fightoplankton include both design flaws and performance flaws. The KCD-041 rocker switch used was unable to handle the high current used to power the temperature control system resulting in instability in the connections. Future

designs could incorporate rocker switches with higher electrical ratings. The PLA plastic used to print the storage rack (Fig 2-3) was black with a glossy sheen. This resulted in false positives registered by the infrared sensor as the glossy surface reflected extra light, causing it to miscount storage tubes. This can be remedied by spraying a matt black coating on the base of the storage rack. In terms of reliability, the electrical systems often had glitches due to connectivity issues between the components. This is likely due to the use of jumper cables instead of soldering down each connection. While jumper cables allow for easier maintenance and setup, they are more prone to coming loose than soldered connections.

Finally, one issue that only arose after long term use was the appearance of rust on certain components within the storage environment of the Fightoplankton. This included the Steel Pot, the DC motor shaft, as well as the original infrared sensor. Within the control and electronics box, rust appeared on the data cable connection of the Arduino board after 4 months of use. The sampling of a marine strain meant that high salinity media was entering the storage environment, potentially causing components to rust. To resolve this, better waterproofing techniques should be employed, or waterproof components chosen during the design phase.

3.2 Periodicity Experiment

3.2.1 Results of Variable Photoperiods on Cell Periodicity

Changing light regimes had little effect on the periodicity of cell division and chlorophyll pigmentation of cells. It did however, show some apparent results for discrepancies in growth periods.

Similar to the well studied *Euglena* genus, the division burst occurs around the range of 12 - 13 h after the onset of light (Chisholm, 1981). A comparison between the timings of peaks in instantaneous growth rates relative to the onset of light can be found in Figure 3-2. A one-way Analysis of Variance (ANOVA) test confirms this result, indicating that there are no significant differences between the 3 light regimes tested when it comes to cell division timings (Table 3-3). This result is consistent with scientific literature surrounding the investigation of circadian-gated cell division periodicity (Chakrabarti & Michor, 2020; Chisholm, 1981).

Figure 3-2: Results of *Picochlorum sp.* Growth Rate against Time

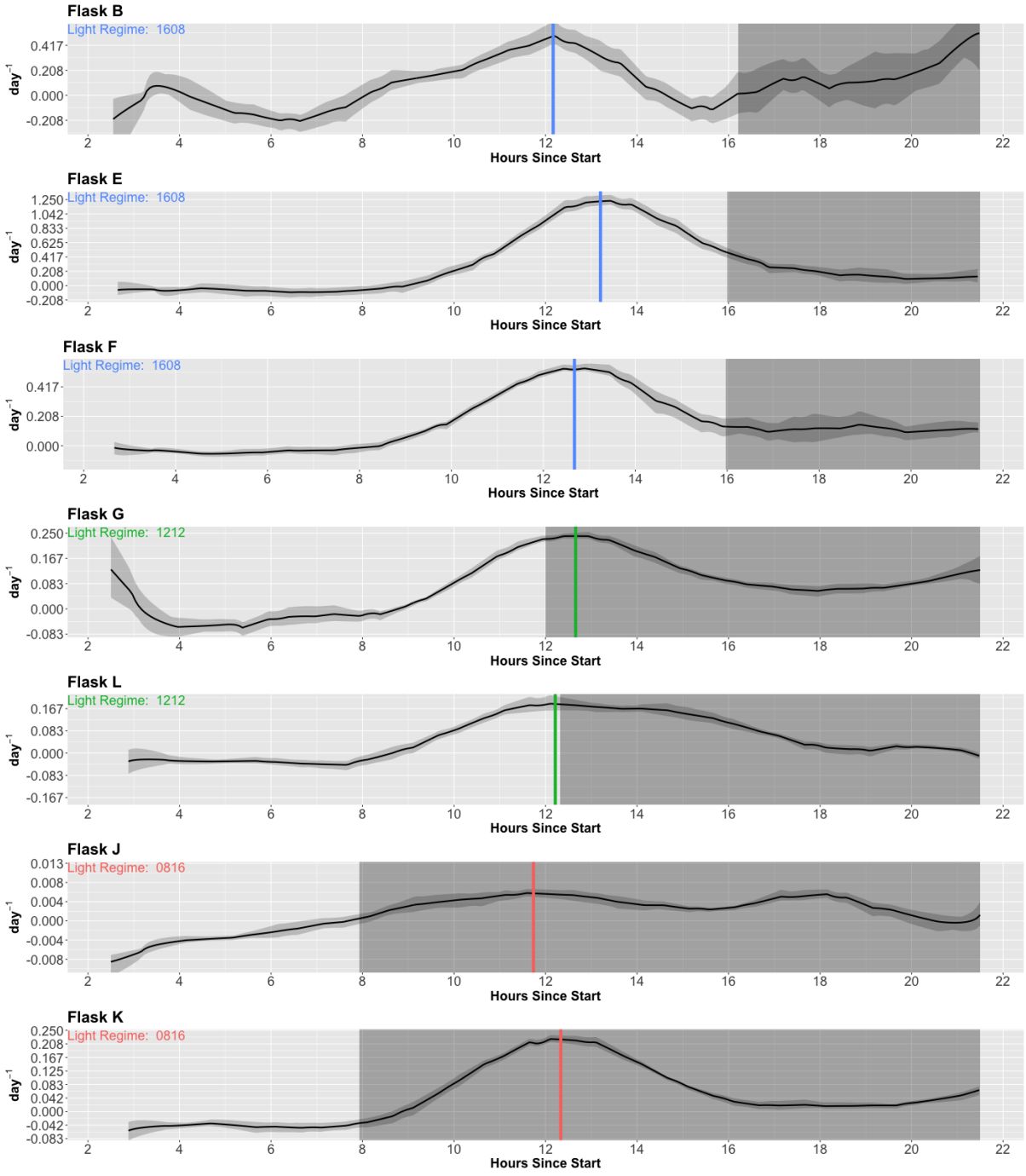
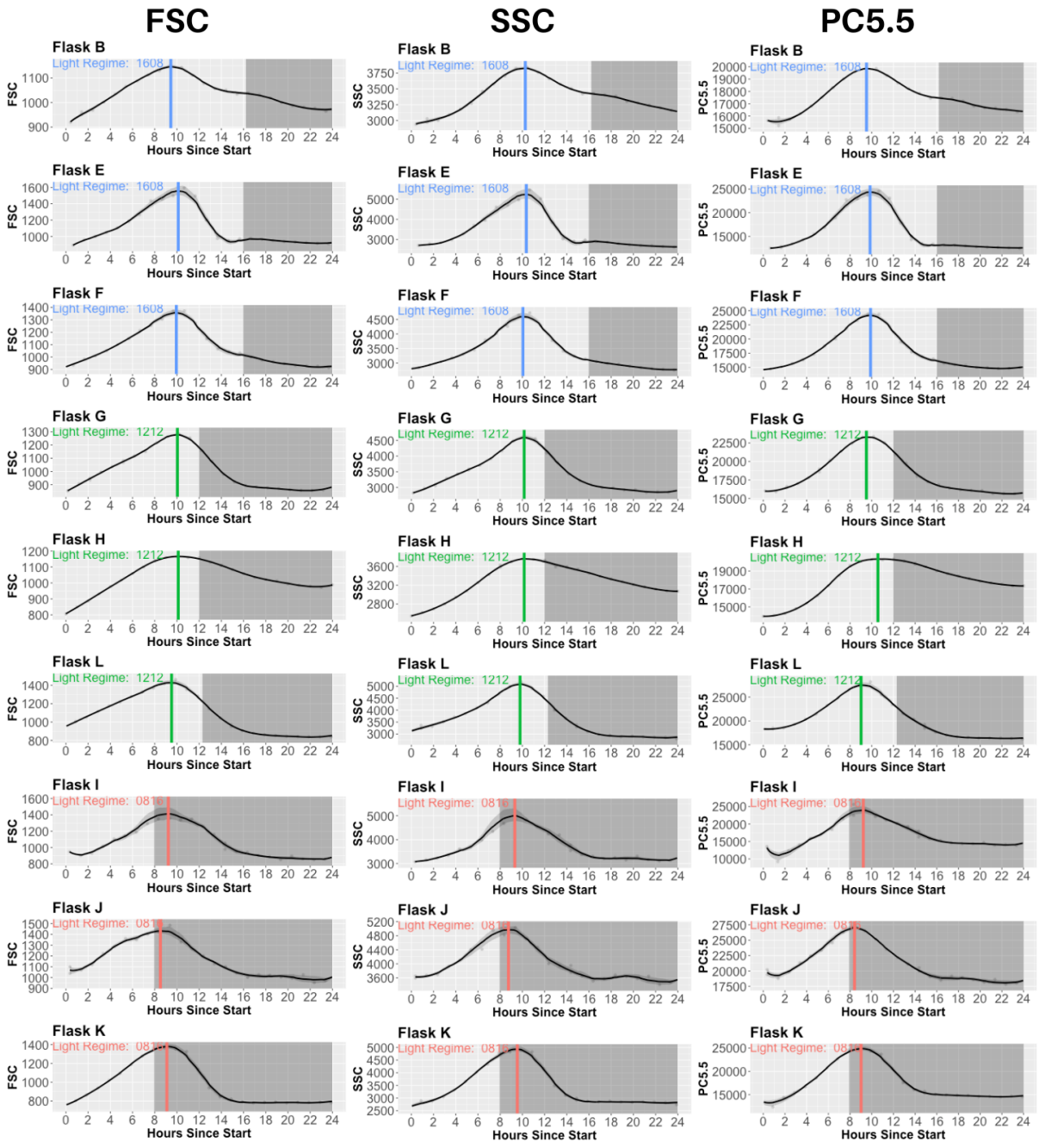


Figure 3-2 excludes flask H and I due to errors in FCM analysis. In particular, it is possible that a clog in the FCM caused large discrepancies in cell concentrations of an order of magnitude. As cell counts should not vary by an order of magnitude within a single 24 h period this data was deemed corrupted and hence excluded from the study. However, other measurements of cell parameters through light scatter or fluorescence such as FSC, SSC, and

PC5.5, could still be used. This is because these parameters are time-independent during FCM analysis and should still be able to provide a representative snapshot of each sample. Hence, for fluctuations in these other cellular parameters, flasks H and I are included. As we are not concerned with the rate of change of these parameters, but rather the point in time at which the maximum or minimum was reached, the absolute values for each of the parameters are plotted against time since onset of light (Fig 3-3).

Figure 3-3: Results of test of Variable Photoperiods on other Cellular Parameters against Time



At first glance, the periodicity in the changes of FSC, SSC, and PC5.5 seem to mimic the patterns in cell division; albeit slightly earlier, peaking at 9 - 10 h after light onset. One striking observation is that the curves of cell parameters against time seem to have a strong resemblance within each flask (Fig 3-3). A one-way ANOVA test across the parameters for each light regime showed that the timing of the peaks after light onset do not differ statistically (Fig 3-2). This means that the timing of the 3 parameters are not statistically different. This leads us to think that they could be coupled to each other, or to an endogenous CR, or each directly entrained by light (Vaulot, 1995).

Table 3-2: One-Way Analysis of Variance of Light Regimes by Cellular Parameters

	Light Regime	Df	Sum Sq	Mean Sq	F value	Pr(>F)
parameter	16:08	2	0.376	0.188	3.032	0.123
Residuals	16:08	6	0.372	0.062		
parameter	12:12	2	0.173	0.086	0.332	0.730
Residuals	12:12	6	1.561	0.260		
parameter	08:16	2	0.174	0.087	0.537	0.610
Residuals	08:16	6	0.973	0.162		
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						

However, although barely perceptible in Fig 3-3, the results of another one-way ANOVA test performed on each of the parameters yielded significant differences in both FSC and SSC parameters (Table 3-3). Variable photoperiods had a significant effect on FSC, $F(2,6) = 6.93$, $p = 0.03$, and SSC, $F(2,6) = 11.25$, $p = 0.01$. A pairwise T test was conducted between each of the groups for FSC and SSC. The results of the pairwise T tests indicate that the 08:16 LD cycle gave statistically significantly different results from the other 2 cycles of 12:12 and 16:08 (Table 3-4). The 12:12 and 16:08 LD cycles on the other hand were not statistically different from each other, indicating that only the 08:16 LD cycle was different. Combined with a visual reference in Figure 3-3 we observe that FSC and SSC parameters in flasks under 08:16 LD cycles peak slightly earlier at around 8.5 - 9.5 h after the onset of light.

One final point to note would be the occurrence of a secondary peak in instantaneous growth rate seen in Flask F at around the 19 h mark after light onset (Fig 3-4). Given the more than doubling in cell concentration over the period from around 60 cells/µL to 130 cells/µL, we note that Flask F was in ultradian growth. The smaller secondary peak was likely a subsequent division of cells that had already undergone cell division once within that period.

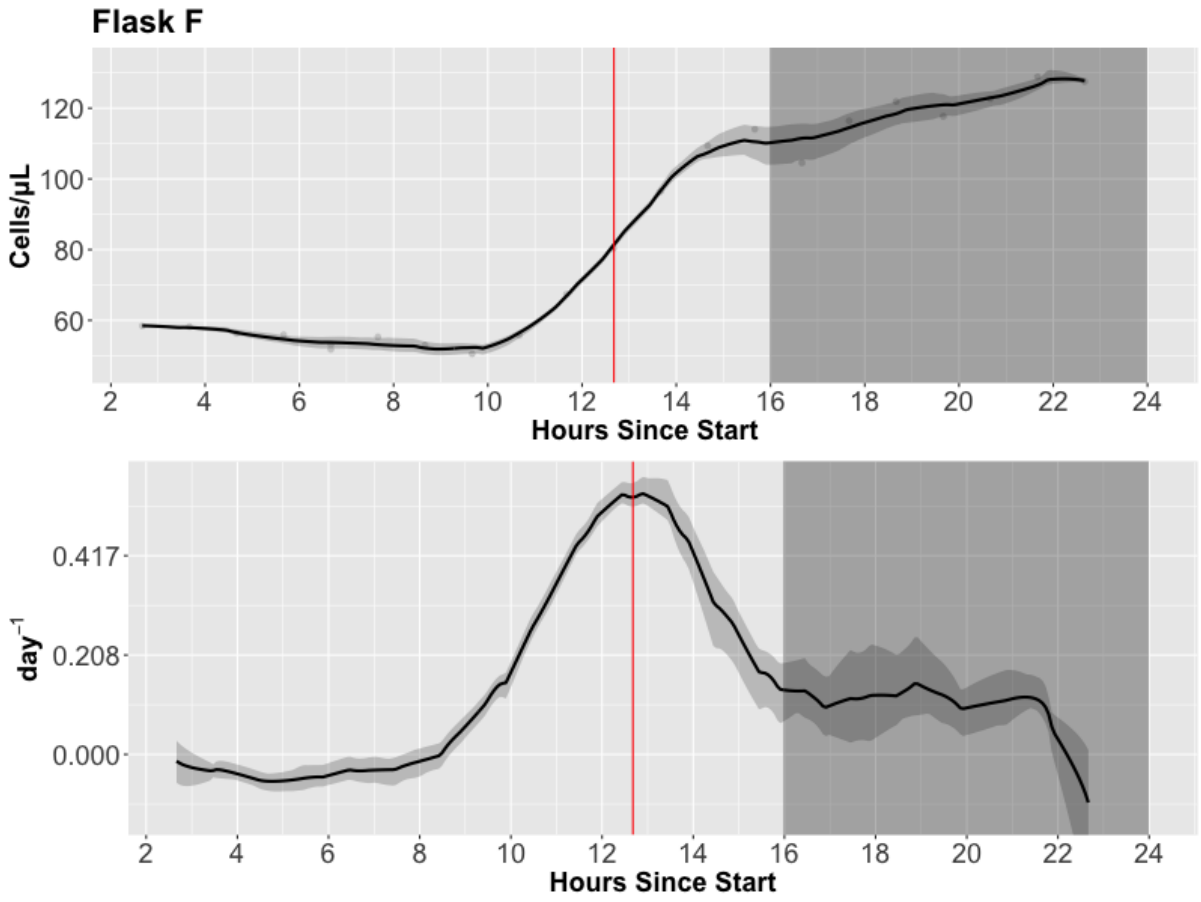
Table 3-3: One-Way Analysis of Variance of Cellular Parameters by Light Regimes

	Parameter	Df	Sum Sq	Mean Sq	F value	Pr(>F)
light regime	Events/µL	2	0.502	0.251	1.238	0.382
Residuals	Events/µL	4	0.812	0.203		
light regime	FSC	2	1.705	0.853	6.932	0.028*
Residuals	FSC	6	0.738	0.123		
light regime	SSC	2	1.772	0.886	11.249	0.009**
Residuals	SSC	6	0.473	0.079		
light regime	PC5.5	2	1.427	0.713	2.525	0.160
Residuals	PC5.5	6	1.695	0.282		
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1						

Table 3-4: Pairwise Results of FSC and SSC

	Parameter	Group 1	Group 2	p	p.signif	p.adj	p.adj.signif
1	FSC	08:16	12:12	0.0161	*	0.0484	*
2	FSC	08:16	16:08	0.0204	*	0.0612	ns
3	FSC	12:12	16:08	0.858	ns	1	ns
4	SSC	08:16	12:12	0.0108	*	0.0324	*
5	SSC	08:16	16:08	0.00432	**	0.013	*
6	SSC	12:12	16:08	0.449	ns	1	ns

Figure 3-4: Plot of Flask F Cell Concentration and Derivative against time



3.2.2 Discussion of Observations

There seems to be no significant differences in periodicity between the different LD cycles for both cell division and chlorophyll pigment. Cells entrained to the 08:16 LD cycle displayed FSC and SSC maxima significantly earlier than the other 2 light regimes tested. Understood in context of other literature, this is not surprising given that FSC and SSC are both related to cell size. As cells require an estimated minimum of 10 - 14 h of saturating light to undergo cell division (Chisholm, 1981), it is possible that the 08:16 LD cycle is light limiting and was insufficient light for continued cell growth past the 9 - 10 h mark. In comparison, flasks under LD cycles with light periods extending past this mark reached their maxima at the 9 - 10 h mark. These observations seem to indicate a coupling between cell division and chlorophyll pigmentation, and the CR. On the other hand, physical growth of cells in terms of the accumulation of size and internal organelles is likely to be entrained to either the CR or

CDC as seen from the consistent 9 - 10 h peak, but also directly limited by light availability as seen in the 08:16 LD light regime.

While it is tempting to use the synchronised peaks in chlorophyll pigmentation to approximate the cell division timings for flasks H and I based on the coupling with the CR, the work of Dagenais-Bellefeuille *et al.* (2008) harkens a warning against trivialising this link as mere time intervals. As discussed in the literature review, exceptions to CR controlled cell division exist such as for cells under ultradian growth (Prézelin, 1992). With respect to flask F, the presence of a second division peak at the 19 h mark after light onset whilst under ultradian growth corroborates the existing literature. It is interesting to note that although Chisholm (1981) claims that the cell is no longer coupled to the CR, the timing of the first cell division peak still falls within the circadian-gated interval. It is possible that the CR still maintains certain influences over the periodicity of cultures under ultradian growth.

Lastly, obtaining such high resolution samples would be highly unfeasible if not for the Fightoplankton automatic water sampler. As seen in the results, the differences in period timings could be as fine as 30 minute intervals, which would not have been captured without this resolution of data. While the Fightoplankton may have its own limitations, and is quite specific to phytoplankton sampling, the availability and creation of more low-cost and open source designs will help in a global movement to drive down the costs of research and education (Zhang et al., 2016).

3.2.3 Limitations of the Variable Photoperiod Experiment

Although the experimental design is very simple in nature with just 3 experimental groups and 3 replicates per group, the high labour and time costs of high frequency sampling place inherent limitations on the study. These limitations are often caused by limitations in equipment and logistics, overlapping with the some of the limitations discussed regarding the Fightoplankton sampler. As only 1 sample can be collected at a time, there are no “true” replicates in a sense, as not all the other variables such as starting culture concentration or temperature could ever be exactly the same.

Another limitation of the one-at-a-time sampling of the Fightoplankton means that there is an incremental time-cost associated with increasing the number of replicates. This meant that only 3 replicates were collected, which is insufficient for making statistical conclusions about the data. As the low sample size violates the ANOVA assumption of normally distributed residuals (Gotelli & Ellison, 2013), the findings of the ANOVA test and pairwise T-tests should be taken with a pinch of salt.

Finally, this experimental design did not truly push the limits of phytoplankton growth under more extreme LD cycles. If light phases had been reduced to 6 h or even 4 h, the changes in periodicity of the physical growth parameters of FSC and SSC could have been more marked. As it stands, the differences in the timings of the FSC and SSC peaks of only 30 mins and the low replicate count means that we have insufficient claim to reject growth coupling to the CR.

Perspectives and Future Work

4.1 Perspectives on Periodicity of strain SMS40 (*Picochlorum sp.*)

Divided into two main objectives, this study has yielded interesting perspectives regarding both. On understanding the effects of varying photoperiods on the periodicity of cellular processes of phytoplankton acclimated to low latitude consistent light regimes, this study showed indications that an endogenous CR was responsible for gating cell division and regulating chlorophyll pigmentation. On the other hand, physical growth attributes such as size and internal granularity of a cell seemed be directly influenced by limitations in light at shorter photoperiods. However, this result is not conclusive given the small sample sizes and small shift in peak timings. The implication of this on a global scale, could mean that given time, phytoplankton acclimated to annually consistent light regimes could potentially adapt their patterns in growth and division to light regimes at high latitudes. As a result, temperate or even tropical strains of phytoplankton could eventually end up as large components of high latitudinal blooms as their range shifts poleward due to climate change.

The second objective was to design and build an automatic water sampler to aid in the capture of high resolution data of phytoplankton cultures. This study showed that this endeavour is entirely possible and is not very expensive, with a price tag of US$250. Over the course of the study, the sampler saved over 200 man-hours in sampling. High resolution data obtained of cell growth rates allowed the periodicity in cellular parameters to be studied in depth.

4.2 Improvements to Fightoplankton

Whilst far from perfect, the Fightoplankton was fully functional during the sampling period. However, the Fightoplankton has much more room for improvement.

One major improvement to the design would be adding the capability to sample from more than one input at a time. While ambitious, this would allow for true replicates to be sampled and hence greatly improve the reliability and reproducibility of data. It will also reduce the incremental time cost of producing replicates, allowing sample sizes to increase. The implementation of this would be easier through the use of a Cartesian storage system instead of a radial layout. While more expensive to implement, a Cartesian system would be also be less prone to sampling errors and allow for more storage within the same area (Carvalho & Murray, 2018; Efromson et al., 2021; Hartmann et al., 2018).

Lastly, to improve the overall reliability of the Fightoplankton, care should be taken in the selection of components and in their assembly. Waterproof components would allow the long-term viability of the Fightoplankton, ensuring that the benefits are realised over a longer time frame. During assembly, components should be soldered together to ensure that there are no loose connections that could impede the samplers functioning.

4.3 Recommendations for Future Works

This study was quite lean, performing the bare necessities to obtain usable data regarding cellular periodicity. There is much room for improvement in terms of experimental design and analysis. First, a greater range of experimental groups could be studied, investigating more extreme photoperiods of 4:20 or 20:4 LD cycles. Next, FCM analysis can include the use of DNA fluorescent stains to accurately elucidate the true cell division timings of the population(Jacquet et al., 2001; Vaulot & Chisholm, 1987). Through FCM with these stains, we can identify the timings of the G1, S, G2, and M phases, comparing these with the CR and checking their responses to different photoperiods (Dagenais-Bellefeuille et al., 2008). Lastly, we can expand the species used to include other temperate strains. In fact, the original ambition of this experiment centred around the use of different strains of *Micromonas sp.*. As

Micromonas sp. is another well studied genus due to its ubiquity in the oceans (Demory et al., 2019), it was a strong candidate for identifying the effects on changing photoperiods on cellular periodicities across geographically distant strains.

Something of interest to study would be the rate of acclimation to new light regimes. This could potentially be of even greater interest than a "static" periodicity, as the dominance of "invading" tropical or temperate strains in high latitude regions would be highly dependent on their rate of acclimation to different photoperiods.

While there are plenty more areas of research that could potentially be carried out in this field, a final area of interest is the periodicity of cells under ultradian growth. As mentioned in section 3.2.2, the uncoupling of the CDC from the CR under ultradian growth presents a unique opportunity to further understand what processes continue to be linked to the CDC or to the CR. Either way, high frequency sampling is becoming all but necessary when it comes to experimental setups regarding temporal variations.

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Appendix

Exponential Growth Phase and Initial Cell Concentration

An experiment was set up to determine the timing of exponential growth rate of SMS40 as well as an ideal starting cell concentration for cultures. Cultures with initial cell concentrations of 10^3 cells/ml and 10^4 cells/ml were maintained under 12:12 LD conditions at 22C for a period of 14 days. Each initial concentration consisted of 3 replicates sampled at 1130am daily. Samples of 350 μL were pipetted into microtubes containing 3.5μL of 25% gluteraldehyde solution, incubated at room temperature for 10 minutes, and stored at -80°C.

Test of Storage Methodology

In tandem with the daily sampling for the exponential growth test, an additional 350 μL of sample was collected on days 0, 4, 8, and 13. These samples were pipetted into cryotubes containing 3.5 μL of 25% gluteraldehyde solution. After 10 minutes of incubation at room temperature, the samples were flash frozen in N_2 and placed in long-term storage at -80°C. This test was conducted to determine if flash freezing samples were necessary for preserving cellular parameters.

Table A-1: Bill of Materials

Item	Cost/Unit	Quantity	Shipping Cost	Total Paid
Sample Storage Environment				
Peltier Chip Thermoelectric Generator	42.63	1	1	43.63
Temperature Probe	9.9	1	0	9.9
Styrofoam Box	8.98	1	1.49	10.47
Styrofoam Box Test	3	1	0	3
Metal Pot	10	1	0	10
TOTAL				77
Sample Extraction System				
Peristaltic Pump	16	1	0	16
Silicon Tubing	4.68	1	0	4.68
Pneumatic Connector Valve	1.99	1	1	2.99
Buck Converter	7	1	0	7
TOTAL				30.67
Multi Sample Management System				
DC motor	32	1	0	32
Servo Motor	18.8	1	0	18.8
Stepper Motor	21	1	0	21
Motor Driver, Photodiodes, Connectors	30	1	0	30
TOTAL				101.8
Miscellaneous				
Power Supply (30A)	38	1	0	38
Power Supply (5A)	5.85	1	1	6.85
4 Channel Relay	18.5	1	0	18.5
Arduino	10	1	0	10
LCD Display	8	1	0	8
Arduino Cable	6.9	1	0	6.9
Rotary Encoder + Knob	2	1	0	2
Buzzer	1	1	0	1
Jumper Cables	1.52	3	1	5.56
PCB Strip boards	1.5	3	0	4.5
3D printer filament	22	1	0	22
Silicon	3.5	1	0	3.5
Thermopaste	8.4	1	0	8.4
18 AWG electrical wires	9	1	0	9
Solder wire	2	1	0	2
Plywood Board Base	3	1	0	3
TOTAL				149.21
TOTAL				**358.68**

Table A-2: Timeline of issues faced throughout sampling

Date	Issue	Remedy Action Taken
2021/01/18	IR sensor not counting tubes correctly	Ensured the bottom of the rack is completely black using a marker Reduced the speed of the motor from 175rpm to 125 rpm
2021/01/19	IR sensor not counting tubes correctly	Ensure height of rack from sensor is correct
2021/01/19	Software froze	Timer and Temperature Control stopped and restarted
2021/01/20	Entire water bath was frozen	Check wires and connection
2021/01/20	IR sensor not counting tubes correctly	Adjusted potentiometer of IR sensor to calibrate distance
2021/01/20	Power Failure due to power switch shorting	Rewire power to bypass switch
2021/01/21	IR sensor not counting tubes correctly	Adjusted potentiometer of IR sensor to calibrate distance
2021/01/26	IR sensor not counting tubes correctly	Adjusted potentiometer of IR sensor to calibrate distance
2021/01/27	IR sensor not counting tubes correctly	Adjusted potentiometer of IR sensor to calibrate distance
2021/01/28	IR sensor completely broken	Replace IR sensor module
2021/02/05	Peltier Plate not responding	Replace Relay
2021/02/07	Wrong voltage replacement relay	Replace Relay
2021/02/09	IR sensor not counting tubes correctly	Added a matte black cardboard beneath rack
2021/03/03	Peltier Plate not responding	Rewire power