Pioreactors at Sea: Assessing Microbial Community Response to Added Dissolved Inorganic Nutrients

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Abstract

This study aims to understand interactions within the microbial community of the oligotrophic (nutrient-poor) equatorial Pacific. This experiment attempts to provide a more realistic representation of in-situ conditions, overcoming previous difficulties capturing the dynamic behavior of microbial communities in the field. A novel methodology utilizing a continuous media supply to incubate natural microbial communities from the field accomplishes this objective. Three growth chambers were employed, one as a continuous incubation control (no nutrients added), one as a continuous incubation with added inorganic nutrients (nitrate, phosphate, and silicate), and one as a batch incubation with the same added nutrients. A continuous incubation constantly exchanges a small volume of external media from outside the growth chamber with volume from inside the growth chamber. A batch incubation does not have any media exchanged during its incubation period. All incubations followed a 16:8 light/dark period using LEDs, simulating environmental day-night cycles. Community response was assessed by monitoring the turbidity of the incubation with continuous optical density measurements, with beginning and endpoint subsamples analyzed for total bacterial abundance and DNA content using flow cytometry. There was a distinct day-night turbidity oscillation in all incubations, with the nutrient-enriched continuous incubation being the most pronounced (average normalized optical density 10% greater during simulated daytime). At the conclusion of the experiment, the continuous incubation without added nutrients had about double the bacterial cell concentration and a greater bacterial DNA content, yet similar optical density values compared to its nutrient-added counterpart. This combination of results suggests that the added nutrients selected for larger, slower growing bacteria, such as the picophytoplankton *Synechococcus*, which would align with previous studies. Additionally, the different turbidity

oscillations between incubation methods suggest that nutrient replenishment results in a greater microbial response to diel variability. This study not only advances our understanding of microbial community dynamics in the oligotrophic equatorial Pacific but also introduces a novel experimental method that can be applied across a diversity of marine and aquatic environments.

Plain Language Summary

This study investigated the interactions among microorganisms in the nutrient-poor equatorial Pacific Ocean. To better simulate conditions these microbes experience in nature, a new experimental approach was used, involving a continuous media supply to simulate ocean mixing. All incubations mimicked day and night cycles with built-in LEDs. Community responses were measured by continuously monitoring optical density, a proxy for microbial biomass, and beginning and endpoint samples that were analyzed for bacteria cell concentration and size (estimated by DNA content). Three incubations were used, one as a continuous incubation (no added nutrients), one as continuous incubation with added inorganic nutrients, and one as a batch incubation with added inorganic nutrients. The results showed distinct day-night optical density oscillations in all incubations, with the nutrient-enriched continuous system having the most significant variation. This implies that the nutrient replenishment from simulated mixing allows for a greater microbial response to day-night cycles. Bacterial abundance varied across all systems, indicating different responses from the bacteria in each incubation. This study not only enhances our understanding of microbial communities in the nutrient-poor equatorial Pacific but also introduces a new experimental method applicable to various marine and aquatic environments.

1 Introduction

1.1 Heterotrophic bacteria and phytoplankton

Heterotrophic bacteria serve as integral components of the marine ecosystem and the movement of nutrients across biota within the ocean. By consuming dissolved organic carbon generated by marine autotrophs (Azam et al., 1983; le B. Williams, 1998) and recycling the associated nutrients, heterotrophs are a key influence in controlling marine nutrient fluxes (Azam et al., 1994).

There is a deep, two-way connection between heterotrophic bacteria and phytoplankton, the predominant marine autotrophs (Fouilland & Mostajir, 2010). Stimulation of phytoplankton blooms results in more organic nutrients for heterotrophic bacteria, stimulating their growth in turn (Gomes et al., 2015). Additionally, phytoplankton rely on heterotrophs to recycle organic nutrients back into inorganic nutrients. Furthermore, heterotrophic bacteria produce and release vitamins into the water columns that are required by but not produced by phytoplankton (Bratbak & Thingstad, 1986).

All microorganisms, but especially autotrophs, respond to changes in inorganic nutrients. Three key dissolved inorganic nutrients (DIN) limit marine autotrophic productivity at the surface: nitrate, phosphate, and silicate (Mackey et al., 2002). Within the central equatorial Pacific, the predominant two autotrophs are the picophytoplankton *Prochlorococcus* and *Synechococcus*. More specifically, within nutrient-depleted surface waters of the equatorial Pacific, *Synechococcus* is the most dominant, while *Prochlorococcus* is most dominant at depths of 100–125 meters (Mackey et al., 2002). Both picophytoplankton and heterotrophic bacteria co-exist in this area at typical cell densities on the order of 10^5 cells mL^{-1} (Mackey et al., 2002; Lim, 2023). This concentration is relatively low in the global ocean. For example, the northeast Atlantic Ocean has typical concentrations of these organisms on the order of 10^6 cells mL^{-1} (Lim, 2023).

1.2 El Niño Southern Oscillation

Phytoplankton in equatorial Pacific waters are limited by DIN availability (Mackey et al., 2002). Therefore, an addition of DIN into the system should result in an increase in biological productivity. DIN are found in lower concentrations during El Niño (Mackey et al., 2002). El Niño events occur every few years due to the weakening of equatorial trade winds by decreased surface warming. This weakening can occur for various reasons, but this process oscillates with stronger equatorial winds with increased surface warming, known as La Niña (Timmermann et al., 2018). This cycle is known as the El Niño Southern Oscillation. Due to decreased easterly trade winds during El Niño, upwelling also decreases across the equatorial Pacific. Decreased upwelling results in lower nutrient concentrations, specifically nutrients that are found in greater concentration below the mixed layer (Mackey et al., 2002). This process can result in even greater oligotrophic conditions at the surface (Ducklow et al., 1996). Oligotrophic environments are classified by both low nutrient flux and low ambient nutrient concentrations (Schut et al., 1997). Conversely, during La Niña seasons, upwelling is increased at the equator, increasing nutrient concentrations that can drive phytoplankton blooms and overall biological productivity (Ryan et al., 2006). The hypothesis motivating this study is that an increased supply of inorganic nutrients will stimulate the growth of *Synechococcus,* the larger of the two picophytoplankton in this region.

1.3 Comparing microbial culturing methodologies

1.3.1 Culturing versus incubation

Given the importance of understanding the microbial communities that inhabit the ocean, there have been countless different culturing and incubation methods utilized over the years to evaluate their potential behaviors in situ. In this study, use of the term “culture” refers predominantly to a pure culture grown from an isolated strain and thus to culturing it in the lab.

While there are mixed cultures, or cultures containing more than one microbe, used in lab experiments, these cultures are unable to mimic an entire microbial community from nature. Therefore, this experiment uses the term “incubations” to refer to the facilitated growth of a natural microbial community. However, many of the techniques this experiment used to analyze the community response during an incubation were inspired by pre-existing culturing methodologies.

1.3.2 Batch culture

A batch culture is one of the simplest methods to grow microbes in a controlled environment. In a batch culture, a single strain is inoculated into its growth media, such as a bottle of defined, nutrient-rich media. Growth is then observed over time through various methods (flow cytometry, microscopy, optical density, etc.) to calculate a growth rate of the organism. Multiple batches can easily be run in parallel to obtain replicates, test different growth media, test different microbes, etc. (MacIntyre & Cullen, 2009)

1.3.3 Fed batch culture

A fed batch culture is very similar to a standard batch culture, except that it receives continuous addition of media. No media is removed from the system, only added. The rate at which media is supplied controls the nutrient content within the growth chamber, therefore controlling microbial growth (Minihane & Brown, 1986). However, due to no media being removed, this method requires a growth chamber large enough to accommodate all the added media, rendering it difficult to use for long term experiments, or experiments that are small scale. This method is very common in the culturing of baker’s yeast, where a large final quantity is desirable, and allows for constant fine tuning of the nutrient content within the growth chamber.

1.3.4 Turbidostat culture

A turbidostat culture is a semicontinuous culturing method that controls microbial growth by diluting the growth media. It relies on a sensor to measure the optical density within the growth chamber and automatically dilute the culture when that turbidity reaches a certain value (MacIntyre & Cullen, 2009). This technique can estimate growth rate in pure cultures where the average cell size does not dramatically change throughout the experiment.

1.3.5 Chemostat culture

Every microbial growth rate is determined by its environment. A chemostat culture is a continuous culturing method where microbial growth is restricted by the frequency at which limiting nutrients are added to the system (MacIntyre & Cullen, 2009). Two main factors influence how quickly microbes can grow in a continuous culture: media exchange rate and nutrient concentration. These two variables can be scaled together and allow for variable growth rates in a microbial community. For example, a highly nutrient rich media typically can be in equilibrium with a culture using a faster exchange rate than a lower nutrient media and will therefore have a greater microbial growth rate. This method allows for the experimenter set the nutrient content and exchange rate of the culture. Over a culturing period, the culture may adapt to its environment, allowing it to grow out of an equilibrium state.

1.3.6 Continuous incubation

This experiment uses a continuous incubation methodology. As defined here, a continuous incubation starts with a natural microbial community and all the organisms present in it. There is no isolation of any culture, pure or mixed, as the goal is to work with a natural community provided with an environment as close to in-situ as possible. Second, instead of nutrients being

added according to the response of the microbial community, as in the turbidostat, media is added and removed from the growth chamber (like a chemostat) consistently throughout the entire experiment. Because the incubation includes the natural microbial community, it cannot be classified as a chemostat due to the microbial interactions constantly occurring never allowing the incubation to be in equilibrium.

1.3.7 Scientific need for continuous incubations

The environmental variables discussed in Section 1.2 are tightly intertwined with one another, and understanding how they covary has proven difficult. For example, oligotrophic marine heterotrophic bacteria have been notoriously difficult to isolate in culture, even when provided both optimal temperature and nutrient conditions. Research has been conducted on organic carbon fluxes and mineralization, interactions between marine autotrophs and heterotrophic bacteria, productivity of heterotrophic bacteria, heterotrophic bacterial concentration with depth, etc. (Schut et al., 1997; Ducklow & Kirchman, 2000; Lim, 2023). However, no prior experiment has analyzed the effects of added DIN on a natural microbial community in a continuous incubation.

This study analyzes how the equatorial Pacific microbial community responds to the addition of nitrate, phosphate, and silicate in different incubation methods. The addition of DIN attempts to simulate a change in environmental conditions that microbes experience during a shift from El Niño to La Niña.

1.4 The Pioreactor system

This experiment utilized 3 Pioreactors (Pioreactor, Waterloo, ON, Canada) to conduct the incubations. A Pioreactor is a small, 3D printed bioreactor that is controlled by a custom user

interface on a Raspberry Pi. Its small footprint (9 x 15 x 15 cm with pumps) and reasonable price (~$250 per unit) make it incredibly useful for field deployments when compared to larger incubators that could be similarly customized. Features include a built-in and customizable optical density sensor, controllable mixing with a magnetic stir bar, temperature control, and the ability to power multiple external loads, such as peristaltic pumps and additional LEDs. The growth chamber has a working volume of 15 mL, and the complete chamber and tubing system can be easily autoclaved. During incubation, the system can automatically calculate optical density normalized to starting density and estimate growth rate. Multiple Pioreactors can be linked together into a cluster and controlled through one "leader." The entire system uses Wi-Fi for communication between units and allows for a browser-based control interface. It also has the capability for its own local network, allowing for operation without an external Wi-Fi source. There are some limitations of the system, namely the small growth chamber and occasional software difficulties. However, when compared to traditional culturing and incubation systems, the Pioreactor system allows for much greater customizability and control across multiple variables, with real-time growth measurements.

2 Methods

2.1 Sampling procedure

Samples were collected aboard the R.V. *Thomas G. Thompson* on 6 January 2024, at 5°N, 167°W (Figure 1). The collected samples were collected by 10 L Niskin bottles from a depth of 40 m and transferred into sample-rinsed 1 L Nalgene bottles. Each sample was prefiltered through a 47 mm, 3 µm filter into a 1 L beaker using a vacuum pump (Figure 2a). This step aimed to eliminate large grazers and other organisms that could introduce variability among smaller vial sizes.

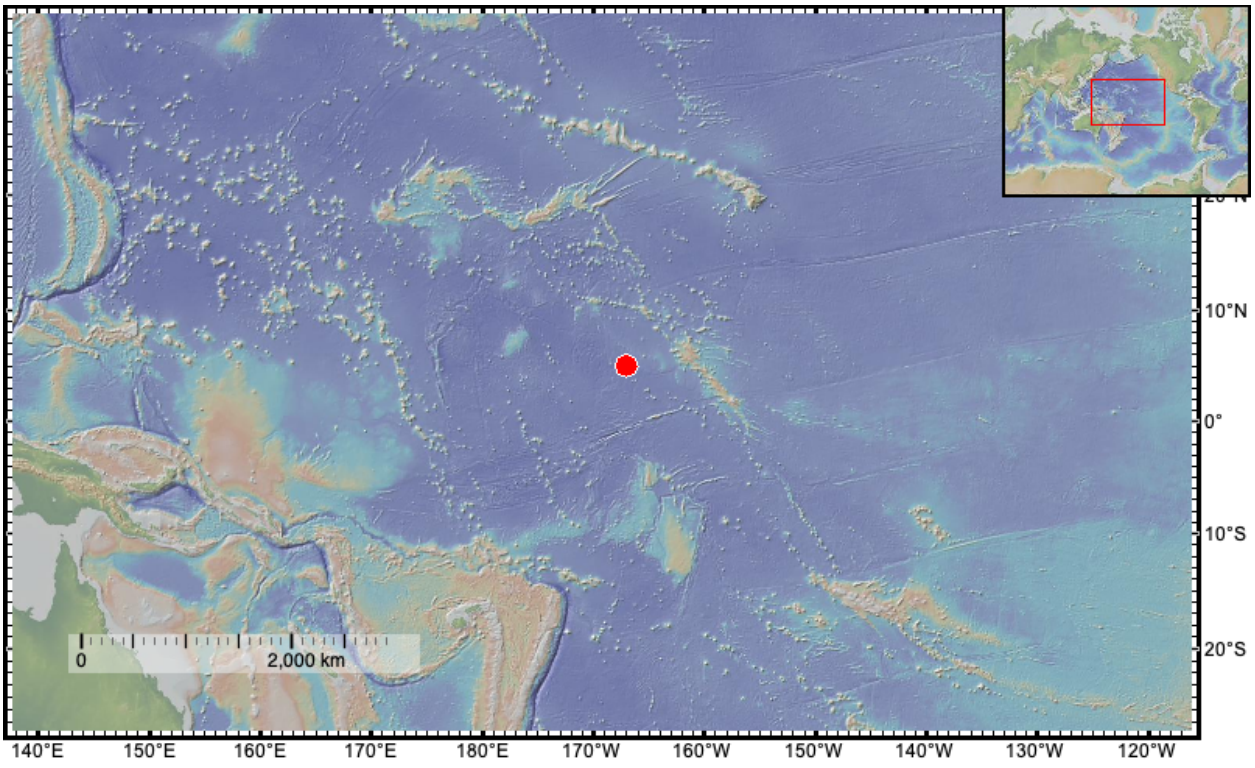


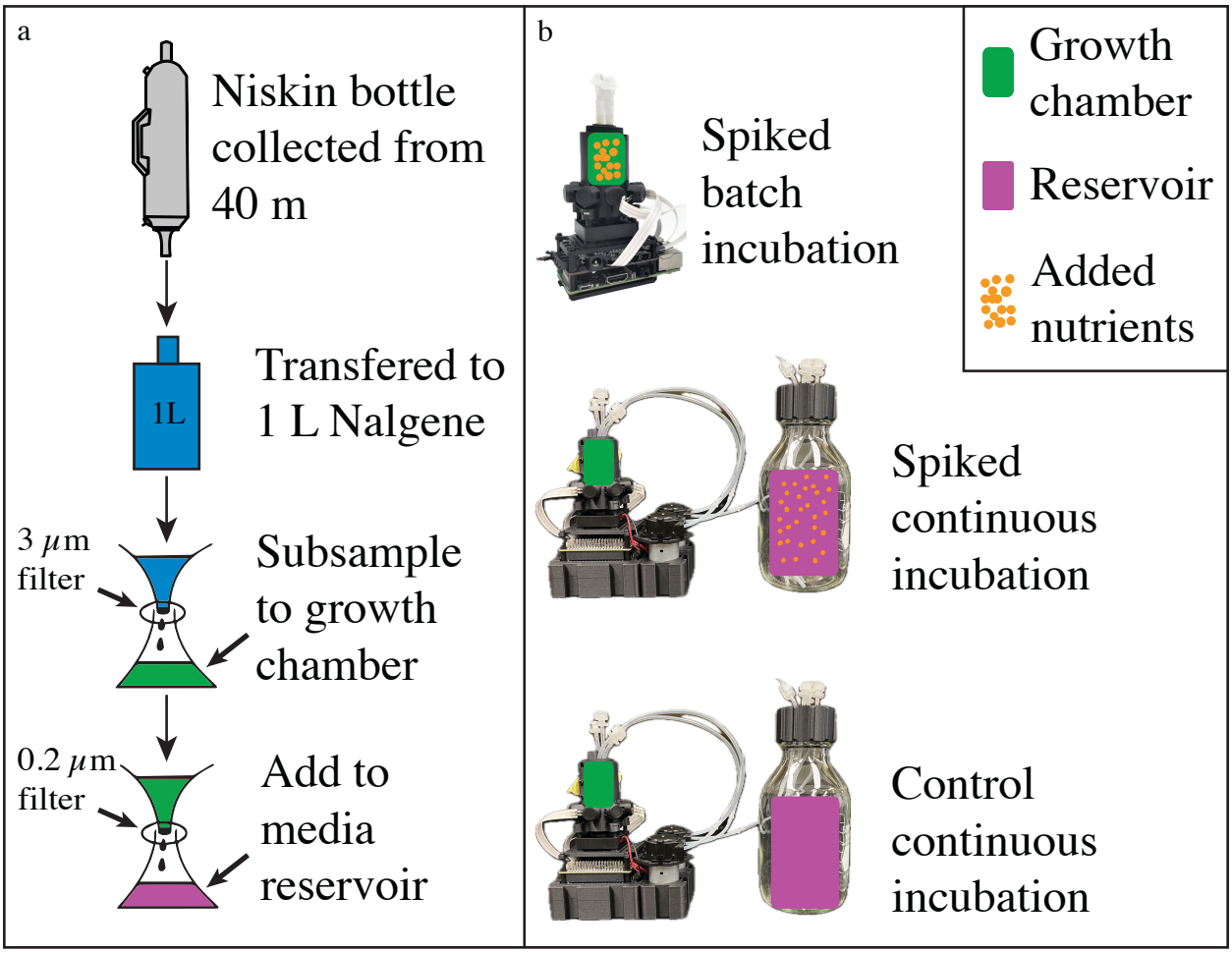
Figure 1. Location of sampling site. Sample sight is the red point (5°N, 167°W). Samples were collected at a depth of 40 m.

2.2 Media preparation

The remaining seawater from the beaker was filtered through a 47 mm, 0.2 µm filter into a 1 L beaker using a vacuum pump. This filtered seawater served as the reservoir media for the continuous incubations (Figure 2a).

2.3 Preparation of Pioreactors

Three subsamples of 15 mL from the 3 µm filtered seawater were pipetted into the three Pioreactor growth chambers (Figure 2b).



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202 **Figure 2. Diagram of experimental design.** a) Seawater sampling and processing procedure.
203 Water was collected from 40 meters before undergoing a 2-step filtration. After the first filtration
204 (3 µm), 15 mL of the filtrate were transferred into each of the three Pioreactor growth chambers.
205 After the second filtration (0.2 µm), this (microbe-free) filtrate was transferred to the continuous
206 incubation reservoirs to serve as the media. b) Layout of the incubation setup. The spiked batch
207 incubation had no media input, but DIN was added to the 3 µm filtered seawater in the growth
208 chamber. The spiked continuous incubation had DIN added to its media reservoir. No DIN was
209 added to the control continuous incubation.

210

2.4 Nutrient addition

Nitrate, phosphate, and silicate were added to the spiked incubations to double the estimated nutrient content (Table 1). These inorganic nutrients were added to the media reservoir in the spiked continuous incubation and directly to the growth chamber for the spiked batch system (Figure 2b). Initial nutrient concentrations were estimated using the SeaState model (Armbrust Lab) for the day of sampling in the region. Actual concentrations were then updated after the cruise with measured nutrient content.

Table 1. Estimated nutrient concentrations of the ambient seawater. Ambient values calculated from nutrient analysis conducted after the cruise. Spiked values were initially calculated based on assumed ambient concentrations, to which nutrients were added. These values were then updated using the actual ambient concentrations determined after the cruise.

Nutrient	Estimated ambient concentration (µM)	Measured ambient concentration (µM)	Final spiked concentration (µM)
Silicate	3.70	1.03	4.83
Nitrate	1.50	0.20	1.70
Phosphate	0.52	0.27	0.77

2.5 Incubations

All incubations were exposed to a 16:8-hour day-night cycle using two built-in LEDs set to draw 5 mA when on. These LEDs had peak wavelengths of 505 and 630 nm. Temperature was set to

30°C using the built-in heating printed circuit board (PCB), and each growth chamber maintained a stirring rate of 500 rpm using a small (sterile) stir bar. The continuous systems utilized peristaltic pumps to add reservoir media into the growth chamber at a rate of 9 mL day^{-1} (0.6 day^{-1} dilution rate). Volume was removed at the same rate to keep the growth chamber volume the same. Waste tubing ran from the growth chamber into 100 mL waste bottles (Figure 3).

In both types of continuous incubations, media was dispensed manually to fill the tubing between the media reservoir and the growth chamber. This step resulted in unequal initial dosing between the continuous incubations. There was an early spike in optical density for both continuous incubations before they returned to average levels after the first day cycle. To allow for a more direct comparison across samples, this analysis focuses on all optical density values measured after the initial 24-hour period. The spiked batch incubation did not have any early spike in optical density, since there was no manual dosing of media. However, there was no significant change in optical density trends over the first 24 hours and it followed the same trend as the rest of the incubation.

2.6 Optical density

During the entire experiment, the optical density of each incubation was measured every 5 seconds with the built-in infrared LED and reference photodiodes (Figure 3). To prevent LED mixing, the system was set to briefly (about 0.25 seconds) turn off the day-night cycle LEDs whenever an optical density measurement was taken. The angle of the reference photodiodes can be changed to resolve different levels of turbidity. In this experiment, the reference photodiode was set to a 90° angle. This frequency of measuring, along with the very low initial cell counts (~10^5 cells/mL) present in the water column, resulted in the raw data containing noise.

Additionally, each raw optical density measurement was not necessarily directly comparable to another incubation due to inherent variability in the infrared and reference LEDs. To resolve this problem, a normalization method was used. For each incubation, the average of the first five values for optical density (over the first 25 seconds) was assigned the value of 1 as the basis for normalizing subsequent optical density measurements. The first 5 values were taken to account for the noise within each optical density reading. To better visualize the optical density trends within the incubation, a rolling average was taken with a 1-hour window (Figure 4).

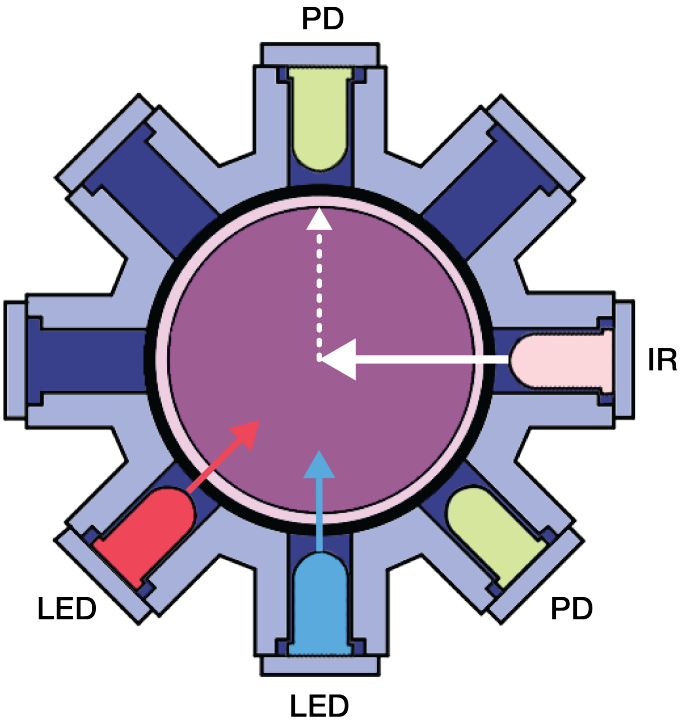


Figure 3. Diagram of infrared LED and reference photodiodes measuring optical density. The growth chamber schematic is shown in purple; not shown is the mixing bar at its base. The reference angle of 90° between the infrared (IR) photodiode and reference photodiode (PD) is shown with white arrows. The two LEDs (LED) used for the day-night cycle are shown in blue and red (figure adapted from pioreactor.com).

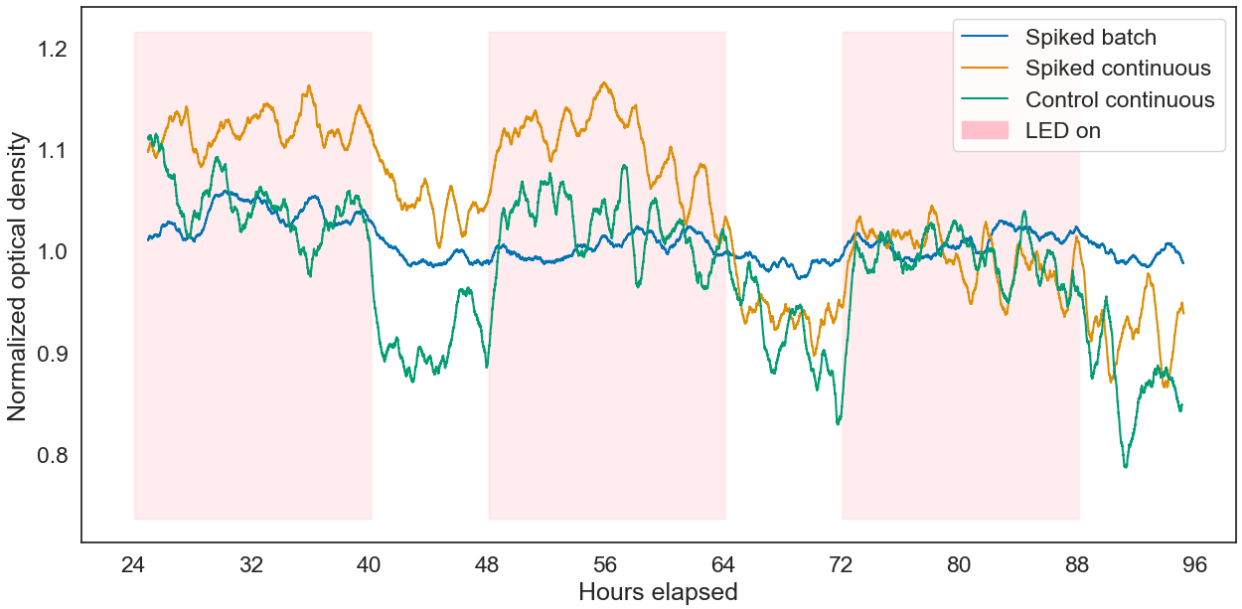
2.7 End point subsampling and flow cytometry

At the beginning of the experiment, 4 mL of each 3 μm filtered seawater sample were transferred to 2 mL cryovials (2 mL per vial) for flow cytometry analysis. This process was repeated at the end of the experiment, when 4 mL of each Pioreactor growth chamber were pipetted into 2 mL cryovials (2 mL per vial). All cryovials were immediately stored at –80°C and transported back to land in a liquid nitrogen storage dewar. At the conclusion of the cruise, samples were stained with SYBR® Green I and counted on a flow cytometer (Guava easyCyte, Millipore Corporation, Billerica, MA). This process was conducted by the Morris Lab. Flow cytometry results provided estimated bacterial cell counts for each sample, as well as DNA content. Fluorescent SYBR® Green I binds to DNA, allowing for detection of all cells containing DNA within its range of measurement. DNA content is then calculated by estimating the relative ploidy number, and therefore DNA content in each cell (Darzynkiewicz et al., 2010).

3 Results

3.1 Optical density

After normalization and a rolling mean is applied to the optical density measurements (see section 2.6), three immediate trends are apparent. First, greater optical densities were observed while the LEDs were on (day-time) than when off (night-time). Second, the continuous incubations both had greater day-time optical density averages than the batch incubation. Finally, there was a noticeable decrease in day-time averages during the third day for both continuous incubations (Figure 4).



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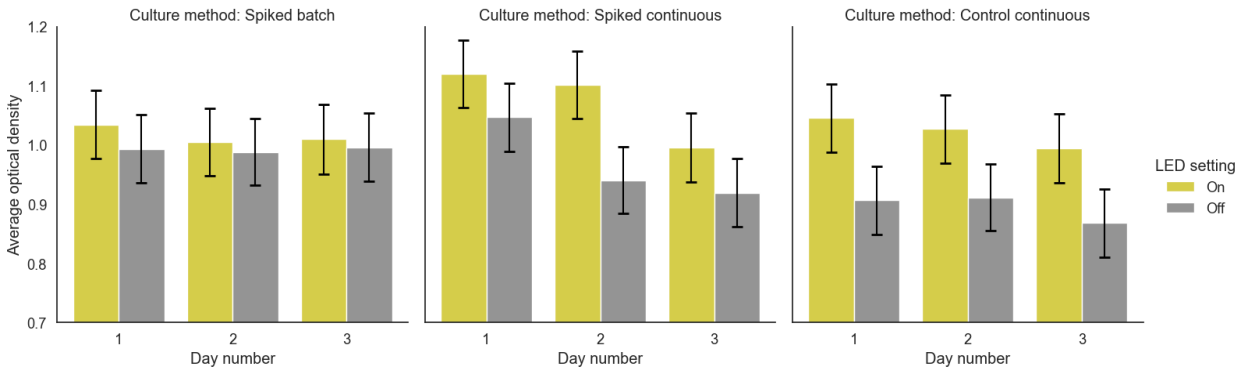
286 **Figure 4. Normalized 1-hour-window rolling average of optical density for all three**
287 **incubations.** The first 24 hours were omitted (see section 2.5). Pink highlighted sections
288 represent the simulated day-time phase. While this figure more clearly displays the trends within
289 the data, it is inherently reliant on the initial normalization.

290

291 Within both continuous incubations, a statistically relevant oscillation in optical density was
292 observed (p-value < 0.01 with independent t-test comparing the day and night phases for each
293 day). Although less visibly discernible, oscillation also occurred in the spiked batch system (p-
294 value < 0.01 with same test as above). The difference between the average normalized optical
295 density values for each incubation during the day and night is as follows: spiked batch, 0.022;
296 spiked continuous, 0.097; and control continuous, 0.115. The variability resolved by the optical
297 density for continuous incubations is 4–5 times greater than that of the batch incubation (Figure
298 5).



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300 **Figure 5. The average day and night optical densities for each incubation.** Error bars plot the
301 standard deviation of the mean for each day (n = 11520) and night (n = 5760) phase.

302

303 3.2 Cell counts

304 For all three incubations, the endpoint cell counts were greater than the initial seawater sample
305 (Figure 6). Additionally, all three incubations ended with different cell counts. The spiked batch
306 incubation ended with the highest cell densities, followed by the control continuous incubation,
307 and finally the spiked continuous incubation. Due to an error in the setup of the flow cytometer,
308 counts by cell size fractionation were not available for this experiment.

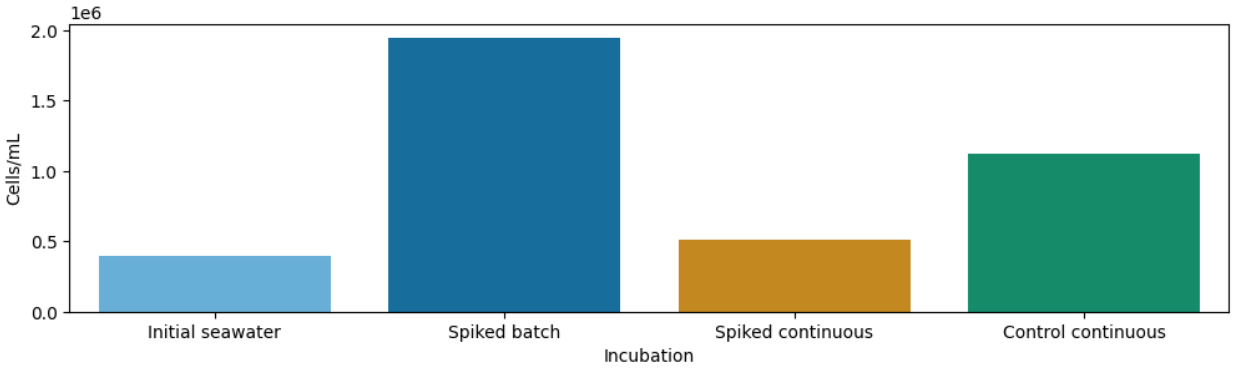

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Figure 6. Cell counts for initial and endpoint incubation subsamples. Plotted are the cell counts (cells/mL) by flow cytometry for the initial 3 µm filtered seawater, as well as the endpoint subsamples for each incubation method.

3.3 DNA content

All three endpoint subsamples contained a different DNA content, with the spiked continuous incubation having a greater DNA content than the control continuous incubation. The initial seawater DNA content was the lowest of the samples at a relative value of 9.07. The sample with the greatest DNA content was the spiked continuous incubation with a value of 39.27, while the control continuous incubation and spiked batch had similar values of 27.29 and 29.64, respectively (Figure 7).

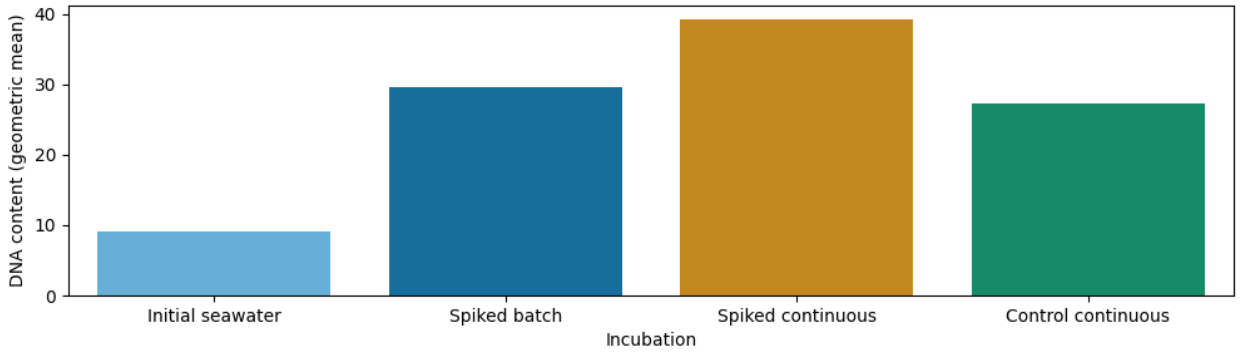

Figure 7. DNA content of incubation subsamples. Plotted are the DNA contents, measured as geometric mean by flow cytometry, for the initial 3 µm filtered seawater and the endpoint subsamples for each incubation method.

4 Discussion

4.1 Method significance

The novel method utilized in this experiment has the potential to allow for a much greater understanding of microbial community dynamics across a wide range of locations. By utilizing the same seawater (prefiltered) from which the microbial community originated as the incubation media, a more accurate representation of in-situ conditions can be achieved. Additionally, the ability to gather real-time, high-resolution optical density measurements during an incubation, even with oligotrophic conditions and cell densities on the order of 10^5 mL^{-1}, is unprecedented. While these readings do not necessarily reflect all the changes the microbial community is undergoing (different microbes of different sizes scatter light differently), they allowed for a clear picture of overall community trends. Coupling the optical density measurements with additional instruments, such as a fluorometer, would be a natural next step for this type of experiment. The ability to control for light, temperature, stirring rate, and media dosing allows for a highly scalable system for future work across multiple fields of microbiology.

These continuous incubation methods represent a departure from traditional batch cultures and resulted in a different community response. While the approach was hindered by the incubation size of 15 mL, which restricted subsampling volumes, and the additional setup required to operate the system, it allowed for a far greater level of control over the variables that affected the incubation than can be achieved with other methods.

4.2 Comparing incubation methodologies

A batch incubation methodology yielded a different microbial growth response than a continuous incubation, both from an optical density and flow cytometry perspective. Continuous incubations had a very evident day-night oscillation in optical density, a feature that would be difficult to resolve without a live optical density reading. This result revealed a previously overlooked area

of incubation methodologies. Because of the continuously added media, the microbial community was able to respond to the day-night cycle differently than if no media had been supplied. Even if a well-controlled batch culture was analyzed consistently across multiple days with a day-night cycle, this response likely would have been underestimated if not undetected.

All incubations had greater endpoint cell counts than the initial seawater, implying that cell division had occurred during the incubation period. Each type of incubation ended with a different density of cells, likely due to the selection of different organisms with different growth rates. This is further supported by the differing DNA content across the three incubations, particularly between the spiked and control continuous incubations. The continuous systems had lower cell counts than the spiked batch. However, they were being diluted over the course of the experiment, while the spiked batch had no media added or removed.

4.3 Nutrient content changes the microbial community

For the two continuous incubations, the only difference was the addition of extra nitrate, silicate, and phosphate. While both incubations resulted in similar optical density curves, their flow cytometry cell counts and DNA content were quite different. The spiked continuous incubation ended with about 130% greater bacterial DNA content, but only about 50% of the cell density compared to the control continuous incubation. The end-point optical density of both incubations was very similar as well. DNA content can be a proxy for cell size (larger cells tend to have a higher ploidy, see Darzynkiewicz et al., 2010), which would explain the discrepancy between cell count and DNA content. Within the spiked continuous incubation, the dominant bacteria may have been larger in cell size but dividing less frequently than in the control continuous incubation. This interpretation would also align with my hypothesis that added nutrients would select for the picophytoplankton *Synechococcus*, which is larger than its common counterpart

Prochlorococcus (Morel et al., 1993). The other potential reason for the cell size, DNA content, and optical density discrepancies would be that while optical density captures the turbidity of everything inside the growth chamber, including live and dead cells, detritus, bubbles, etc., the flow cytometry measurements focused on bacteria, potentially missing some of the larger organisms (2–3 μm) that would have the greatest impact on optical density. In either case, the added nutrients affected the optical density, bacterial cell counts, and bacterial DNA content of the incubation, while still retaining the same general response to the day-night cycle as the control continuous incubation, which most closely resembled in-situ conditions.

5 Conclusion

This experiment revealed important differences between incubation methodologies, especially when attempting to replicate in-situ conditions. Traditional batch culturing (closed system) allows for the least number of controlled variables and the most distant set of conditions from the in-situ environment. Even a batch incubation of a microbial community is not a good analogue to determine in-situ responses to environmental changes, as the removal and replenishment of nutrients is a key factor in how microbes respond to their environment. While no experiment will perfectly replicate an in-situ environment, a continuous incubation does an effective job of simulating mixing. With a small, customizable culturing setup such as the Pioreactor system, field-based, continuous incubations with real-time optical density values can be achieved.

Optical density results display a prominent day-night cycle when the incubation has continuous media cycling, as opposed to a much lesser response in a batch incubation. Additionally, the differing final cell counts for each incubation method imply that different methods select for different microbes. Further studies are needed to determine how closely a continuous incubation experiment mimics in-situ selection.

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