Reports

Engineering an incubation environment that mimics in situ conditions for in vitro coastal microbiome studies

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ABSTRACT
Coastal environments are dynamic and can vary widely on short- or long-term scales depending on location and weather. Incubation equipment that reflects these changes through programmable gradient light and temperature cycles would permit more precise in vitro coastal microbiome studies. Here we present an open-source incubation environment that mimics in situ conditions for in vitro coastal microbiome studies using a modified shaking water bath that has fully customizable temperature and light gradients that can also mimic real-time field conditions. We compared coastal microbial community profiles incubated in situ and in our build mimicking field conditions over 48 h. Analyses of congruence indicated significant overlap (p > 0.2) between microbial communities incubated in situ and in vitro at each time point.

METHOD SUMMARY
We present schematics for modifying a shaking water bath that can be programmed to modulate photosynthetically active radiation from 0 to 160 μmol/m²/s as well as temperature cycling from 2 to 100 °C, which can be adjusted using live temperature data to mimic current field conditions, when data are available. This functionality permits microbiome studies where fluctuating light and temperature conditions are crucial for experimental conditions.

TWEETABLE ABSTRACT
Modification of a shaking water bath that can be programmed to have a fully customizable gradient light and temperature cycling from 2 to 100 °C, or that can scrape live data from and mimic current field conditions, when data are available.

KEYWORDS:
biological oceanography • coastal microbiomes • incubation environment • microbial ecology • shaking water bath

Coastal waters experience dynamic environmental fluctuations that occur on time scales ranging from seconds to seasons [1]. Coastal water temperatures can fluctuate by as much as 11 °C within a 24-h period [2–4] and also have a wide seasonal temperature variation [5–9]. Similarly, light levels vary significantly within days and across seasons. Only a minority portion of each daylight cycle has maximum photosynthetically active radiation (PAR) intensity, with sharp declines in intensity for the remainder of the daylight hours [10]. Across the year, daily light availability varies among latitudinal areas, as summer months observe up to double the daily light compared with winter months [11]. As coastal microbial communities are highly affected by the dynamic interactions with their environment [1,5], these environmental fluctuations can alter microbial community structure and function [5–9]. Changes in temperature and light availability alter taxonomic diversity and metabolic productivity rates [2,5,12]. For example, bacterial diversity within coastal environments has been found to be greater at night and in winter months [2]. Many coastal microorganisms are photoautotrophs or depend on photoautotrophs for their own metabolism [13]; therefore light availability is a crucial aspect to maintaining community functionality and diversity [14]. An average daily light cycle in coastal environments will also affect oxygen and nutrient availability within coastal waters, as rates of photosynthesis vary throughout each day and night [1]. In addition, increases in coastal temperature can lead to virulence in commensal bacteria [15] and changes to their growth rate [16]. Further, bacterial abundance and diversity significantly differ in varying coastal temperatures [17]. Temperature and light are therefore major contributing factors to coastal microbiome taxonomic and functional composition on short- and long-term scales [6,9,12,16,18,19], and they can be a larger contributor to coastal microbiome composition than geographic location [12].
Current studies that focus on the dynamics of coastal microbial communities often rely on in situ or laboratory-based experiments [2,9,13,20–22]. Field-based methods can be logistically challenging [23] and controlling experimental variables is difficult [9]. Closed-bottle systems are often used if experimental treatments need to be controlled or to protect the environment if external spike-ins are used to observe community effects [24]. If in situ incubations are not possible, in-lab experiments may be a more convenient alternative, but current in vitro equipment options are limited to isothermal conditions with a strict light/dark cycle that is not designed to emulate longitudinal, gradient light and temperature cycles [6,9,16,25–28]. To expand potential research capabilities, a combined laboratory–field model may prove to be beneficial in carrying out various coastal microbiome studies. Mesocosms provide a middle ground [29,30], but their set-up and maintenance can be prohibitive. Therefore, incubation environments that mimic real-time and/or more dynamic coastal environmental conditions could be an accessible option that would allow more precise in vitro, time-course microbial community studies.

To achieve this, we engineered a mock coastal environment using a standard water bath shaker modified with consumer hobbyist electronics and programming to create a custom thermally controlled gradient light-cycle incubation environment. This build contains commercial light-emitting diode (LED) grow lights which provide PAR that approximates daytime conditions from dawn until dusk. The device also contains heating/cooling temperature controls that can be fully preprogrammed on a schedule or mimic live coastal conditions when data are available. Further, the laboratory equipment used was modified without permanently altering individual components or voiding any warranties. To test how this build emulates real-environment experimental conditions, we performed a 48-h experiment comparing changes to coastal microbiomes at the Ellen Browning Scripps Memorial Pier in La Jolla, California, versus incubation in our build in the laboratory while mimicking live conditions at Scripps Pier in real time. This was followed by metagenomic sequencing across conditions and time points to observe changes to microbial community profiles. We observed similar profiles at each time point between conditions, indicating that this open-access build is a promising in vitro option for studies currently performed in coastal microbial ecology research.

Materials & methods

Coastal environment build

The full description and schematics of the build can be found at https://www.protocols.io/workspaces/mock-coastal-environment2. To summarize, a Fisher Scientific™ (MA, USA) Isotemp™ 27-L shaking water bath was used as the primary containment vessel (Figure 1A). Bungee cords or flask clamps were used to attach sample containers to the shaking rack to produce oscillating movement to mimic coastal currents. The original heating pad and thermometer attached to the bottom of the water bath were replaced with two 310-mm-square silicon adhesive-backed heating pads used for 3D printers by Richoose (Guangdong, CN, ASIN: B07FB1TZ25). This was the only modification to the original water bath and can be reversed to provide original functionality.

Cooling of the water bath was achieved using a LAUDA-Brinkmann (NJ, USA) WK 230 circulating chiller connected to a fabricated copper pipe system running below the shaking platform (Figure 1B) to facilitate heat exchange. Half-inch (1.27 cm) copper pipes and 90-degree elbows were soldered together in radiator-type loops to maximize the surface area of the water bath base while accounting for the movement of the shaking rack to avoid blocking the wheel tracks. The two ends of the pipe series were run vertically up the side of the water bath, ending in brass barb adapters for silicone tubing connected to the circulating chiller.

To facilitate the use of tall flasks within the water bath, the wall height of the water bath was extended by a frame fabricated from 3.625-inch (9.2 cm) galvanized steel metal studs. The frame adds vertical clearance to the water bath while providing space to mount internal lighting. Two Finnex (IL, USA) HLC aquarium lights (model#: HLC-24A) were mounted to this frame at 45-degree angles using zip ties to simulate gradient diurnal light cycles. These lights come preprogrammed with presets at eight time points with continuous light modulation between each, thereby simulating a natural 24-h lighting cycle that can be set to start in synchronization with the current time of day.

To evaluate the photosynthetic potential of the LED lighting system, one of the light panels was placed above a DLI-600 photosynthetic photon flux density meter (Apogee Instruments, UT, USA) [31] at a distance similar to that of the incubation environment. The LED lighting system was programmed to run the full gradient 24-h cycle, emulating the conditions of the build under test. Photosynthetic photon flux density values were tabulated and plotted to display the amount of PAR light dosed to the system over a whole day. The daily light integral (DLI) was calculated using the caTools (v.1.18.2) [32] ‘trapz’ function for trapezoidal approximation of definite integrals after recording a full 24-h light cycle. Known DLI readings of local areas were obtained using a publicly available DLI calculator (DLI Calculator, https://dlc.suntrackertech.com/) localized to the GPS coordinates of the sampling site (32° 52’ 10.6998” -117° 15’ 16.7112”). We then measured the emission spectra of sunlight at high noon and of the lighting system at six of the eight presets (12 am and 3 am presets are dark) using a ThorLabs CCS200 Spectrometer and ThorLabs OSA Software (https://www.thorlabs.com/software_pages/ViewSoftwarePage.cfm?Code=CCS), with intensity range set to 1 (the maximum value the sensor can read). The area under each spectral curve was then calculated to compare PAR spectra between noon sunlight and our system at the 12 pm preset (see code: https://github.com/BecketLab/Mock-Coastal-Environment/blob/main/SolarComparison.R)

The two 310-mm-square heating pads attached to the bottom of the water bath were connected to a four-channel DC 5V relay module allowing electronic control (Figure 1C). The circulating chiller was plugged into an integrated AC relay from Digital Loggers (CA, USA, product: IoT RELAY II) allowing it to be toggled with a 5-V DC signal. Both types of relays are connected to an Arduino (MA, USA) Mega 2560 Rev3 microcontroller to create a directed homeostatic system. A Gikfun (Jiang Su, CN) DS18B20 waterproof temperature probe
submerged in the water bath basin facilitated temperature input. The Arduino was programmed to operate within 0.5 °C of the set temperature to prolong the equipment life by minimizing excessive on/off cycling between heating and cooling.

The Arduino was also programmed to accept target temperatures over a serial connection by USB cable, which allows for both isothermal and dynamic operation. A Raspberry Pi 4 model b connected to the internet and programmed to scrape temperature data (https://erddap.scoos.org/erddap/tabledap/; see code https://github.com/BecketLab/Mock-Coastal-Environment/blob/main/Temp%20Scripts/ScrippsTemp.py) from a buoy off Scripps Pier provided temperature measurements sent to the Arduino to mimic temperature fluctuations in real time (Figure 1D).

Experimental design
More than 10 L of coastal water were captured in a sterile 20-L polycarbonate carboy from the side of Scripps Pier in La Jolla, using a rope to raise/lower and an anchor tied to the carboy neck to facilitate subsurface water capture. The captured water was then split across five 2-L polycarbonate cylindrical bottles. Three bottles were placed in a mesh bag with an anchor and hung over the side of the pier at a minimum of approximately 2 m below the surface at low tide. The remaining two bottles were immediately placed in a portable incubator set at 19 °C (reflecting current water temperature) while being transported to the laboratory, then placed horizontally.
in the incubation environment build. The build was preprogrammed to mimic the live temperature at Scripps Pier, with a 24-h gradient in lighting conditions, and shaking set at 30 rpm. Bottles were incubated for 48 h, and 50 mL of water was filter-captured on 0.22-μm 25-mm mixed cellulose ester (MCE) filters at 0 h (from the original 10 L captured), and at 24 and 48 h from each 2-L bottle. Filters underwent microbial DNA extraction using the ZymoBiomics DNA Microprep Kit (Zymo Research, CA, USA), and Illumina shotgun sequencing libraries were generated using the Swift Biosciences (MI, USA) 2S Turbo DNA Library Kit (both kits following manufacturer’s protocol – see Murguia et al. [34] for detailed protocol). Barcoded samples were pooled and underwent 2 × 150-bp sequencing on an Illumina (CA, USA) iSeq 100 at CSU San Marcos for low-pass sequencing. Paired-end reads were trimmed using Trimmomatic (v.0.38) [35] using Illumina TruSeq adapter sequences for adapter removal. Reads were then quality-checked using FastQC (v.0.11.9) [36] to confirm the removal of all adapter sequences and assembled using MEGAHIT (v.1.2.9) [37]. Assemblies then underwent taxonomic classification using Kraken2 (v.2.0.8) [38] using the Genome Taxonomy Database (release 202) [39] as the reference database. Supplementary Table 1 shows sequence read and classification statistics. Kraken2 outputs were converted and combined into a single biom table using Kraken-biom (v.1.0.1) [40]. We performed a centered log ratio transformation of the biom table to normalize the resulting compositional abundance dataset [41] assisted by zComposition (v.1.4.0-1) [42] and Phyloseq (v.1.38.0) [43] in R (v.4.2.1). We then calculated Aitchison distance on the centered log ratio-transformed species level data and generated Non-metric Multidimensional Scaling plots with R packages vegan (v.2.6-2) [44], ggrepel (v.3.3.6) [45] and ggforce (v.0.3.3). Statistical analysis of congruence between time periods and within incubation sites was produced using both analysis of similarities and permutational multivariate analysis of variance with the analysis of similarities and adonis2 functions in the vegan package. Complete code for these data processing and analysis steps can be found at: https://github.com/BecketLab/Mock-Coastal-Environment.

Results & discussion
To create a coastal incubation environment, we modified an isothermal shaking water bath to create a custom thermally controlled gradient light-cycle in vitro environment that can be preprogrammed on a schedule or mimic live coastal conditions (Figure 1). Because of how quickly coastal water temperatures can fluctuate, we opted for a water bath setup (instead of an air chamber) to facilitate more rapid and accurate transfer of thermal energy. The build was further integrated with a heating and cooling unit connected via relays (Figure 1C), which allowed for preprogrammed or live-mimicking fluctuating temperature conditions between 2 and 100 °C of the reported temperature in order to reduce the number of times the heating pads and chiller had to be cycled on/off; however, this feature can be modified to replicate temperatures more precisely.

We sought to compare the intensity and spectrum of our lighting system with that of natural sunlight. To examine the photosynthetic potential of the LED lighting system, photosynthetic photon flux density values were obtained over a 24-h period and plotted (Figure 2A) to display the amount of PAR light dosed to the system over a whole day, resulting in a DLI of 8.02 mol/m²/day (4.01 per light fixture). For comparison, known DLI readings of the sampling location over the span of 1 year are plotted in Figure 2B. Further characterization of various commercial grow lights would be useful to achieve parity with local measurements of actual sunlight DLI. We then compared the emission spectra between our system and natural sunlight by obtaining the combined LED emission spectra at six of the system’s eight preset time points (12 am and 3 am were dark) across a 24-h cycle. The lighting system covers 54.25% of the PAR spectrum of sunlight (Figure 2C), at a ten-times lower intensity per light strip than sunlight at the Scripps Pier at the same time of year (Figure 2A & B). The intensity of each LED type (red, green, blue, white) can also be individually adjusted beyond presets, but the cumulative intensity of the light rig is still four-times lower than that of the lowest annual sunlight intensity at the sampling site. Future iterations of this device will focus on matching the adjustable intensity and spectrum of natural sunlight; however, in situ samples are usually below the ocean surface and therefore lighting intensity and spectra are affected by occlusions of the water, bringing the parity of actual PAR dosages closer to the build described here. Additionally, examining the impact said disparity may have on the microbial communities would be beneficial for future investigations.

To test how this build mimics conditions for microbiome experiments, we incubated coastal water in situ off the Scripps Pier and in our build mimicking live pier conditions over 48 h (see Figure 3A for setup and workflow). Non-metric Multidimensional Scaling plots from shotgun metagenomic sequencing of microbiome samples taken at 0, 24 and 48 h demonstrated that the samples formed distinct clusters based on time points yet did not cluster between the in situ and in vitro samples (Figure 3B). Analyses of congruence revealed that clustering within each time point between conditions (in situ vs in vitro) was not statistically different (p = 0.2 and 0.1 for conditions at 24 and 48 h; respectively; 99 permutations; Table 1). However, there was a significant difference in the communities between time points sampled (0, 24 and 48 h; p = 0.01; Table 1). Collectively, these data indicated that during the tested time period, while the microbial community did change with time, the communities within our build were statistically similar between in situ and in vitro conditions tested, and these similarities were consistent across each time point.

In addition to the convenience of performing experiments in the lab, an added flexibility to the described build is that studies can be performed with samples that are aerated (i.e., using flasks with air-permeable plugs) or connected to a chemostat, which may not be feasible in field studies. However, closed-system/bottled field studies are often performed in oceanographic research [24,46], particu-
Figure 2. Characterization of grow lights and daily light integrals of sample site. (A) Photosynthetic photon flux density measurement of a single Finnex light fixture across the entire 24-h diel cycle. (B) Daily light integrals at Scripps Pier across a calendar year. (C) Sunlight emission spectra overlaid with the combined light-emitting diode emission spectra of six of the lighting system's eight preset time points (12 am and 3 am were dark) across a 24-h cycle.

Table 1. Analyses of congruence of centered log ratio-transformed taxonomic abundance profiles across experimental conditions and time.

| Conditions analyzed        | ANOSIM | PERMANOVA |
|---------------------------|--------|-----------|
| All time points           | 0.01   | 0.03      |
| 24 h vs 48 h              | 0.01   | 0.0077    |
| 24 h (in situ vs in vitro)| 0.2    | 0.21      |
| 48 h (in situ vs in vitro)| 1      | 1         |

ANOSIM: Analysis of similarities; PERMANOVA: Permutational multivariate analysis of variance.
In vitro incubation of sea water in coastal mock environment

Day 0 filter capture

Filter capture 50 mL samples through 0.22 μm MCE filters days 0–2 (every 24 h)

Illumina 2 × 150 bp sequencing and data analysis

Incubation of sea water from pier

10 L seawater captured and split into five 2 L bottles

In situ: samples incubated hanging from the Scripps Pier; Initial: microbiomes captured at time zero before splitting seawater into bottles.

NMDS: Non-metric Multidimensional Scaling.

Figure 3. Comparison testing of microbial communities between in situ and in vitro conditions. (A) Schematic of experimental approach to compare microbial community profiles in water incubated in situ or in our build. (B) Species-level NMDS plot from centered log ratio-transformed taxonomic profiles of each sample/time point, with groups highlighted in colored ellipses based on day sampled and on incubation condition. In vitro: samples incubated in our incubation environment; In situ: samples incubated hanging from the Scripps Pier; Initial: microbiomes captured at time zero before splitting seawater into bottles.

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larly when spiking water samples with materials like antibiotics, prebiotics or microplastics [24,47] to observe effects on the microbial community. Many in vitro coastal microbiome studies attempt to mimic coastal environments by replicating and maintaining the initial in situ water temperature, without accounting for the changes in temperature throughout the day [6,26,27], which can vary by as much as 11 °C in some locations [2–4]. Additionally, current in vitro systems vary significantly in light intensity, spectral distribution, photoperiod and method of illumination, with PAR values ranging from ~10 to 40% that of natural sunlight [16,25–27]. However, incorporating this diel variability is important. For example, variability in solar radiation on hourly timescales can influence microbial community composition and function [48,49]. Furthermore, marine bacterial production and community composition have been shown to be affected by differences in in situ and static, in vitro conditions [50].

**Conclusion**

We have developed an open-access build as an affordable (∼US$600, minus water bath [US$2203] and chiller [donated and discontinued, but can be obtained for US$200–1000]) modification to common lab equipment that can be utilized by researchers to permit precise in-lab microbiome experiments in a controlled and accessible environment. This build can be preprogrammed with constant or fluctuating temperature and lighting conditions, or it can mimic live conditions when data are available. This build includes a water bath instead of air chambers for more direct thermal transfer from fluctuating temperatures. Upon comparing coastal water microbial community profiles between samples incubated in the field or in our build, the relative compositions were congruent across both conditions and changed similarly over time. Further experiments over extended time frames and comparing conventional laboratory testing methods are needed to further explore the efficacy of the programmed conditions, but these data serve as an initial proof of principle for an open-access set of modifications that can be added to common laboratory equipment to bolster complex long-term microbial ecology studies.

**Future perspective**

The build presented herein will permit microbiome studies where custom fluctuating light and temperature conditions are crucial experimental conditions. Complete hardware and software information is presented so that any research group can replicate this build.

**Executive summary**

**Background**

- Coastal environments are dynamic and can vary widely on short- or long-term scales depending on location and weather.
- Incubation equipment that reflects these changes through programmable gradient light and temperature cycles would permit more precise in vitro coastal microbiome studies.

**Methods**

- We constructed an incubation environment that mimics in situ light and temperature conditions for in vitro coastal microbiome research.
- We used a modified shaking water bath that has fully customizable temperature and light gradients that can also mimic real-time field conditions.

**Results & discussion**

- We compared coastal microbial community profiles incubated in situ and in our build mimicking field conditions over 48 h.
- Analyses of congruence indicated significant overlap (p > 0.2) between microbial communities incubated in situ and in vitro at each time point.

**Conclusion**

- This open-access build is an affordable modification to common lab equipment that can be utilized by researchers to permit precise in-lab microbiome experiments in a controlled and accessible environment.

**Supplementary data**

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2022-0080

**Author contributions**

B Hunter: designed and built the incubation environment; performed sequencing, informatics and statistical analyses; and drafted the protocols, scripts and manuscript. J Flury: drafted the manuscript, procured all the references and assisted in experiments conducted. S Cocobi: performed the LED light experiment and contributed associated text in the manuscript; contributed to manuscript development. M Cope-Arguello: designed and built the incubation environment hardware and contributed to manuscript development. J Helms and K Garcia: performed field experiments and contributed to manuscript development. G Dominguez: guided initial design of the build and contributed to manuscript development. D Taniguchi: guided and provided expertise for experiment planning and design, provided field access and materials for experiments performed and guided manuscript development. E Becket was the principal investigator on the project, oversaw all experiments and guided manuscript development.
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Data sharing statement
Raw sequencing reads can be accessed under the SRA accession number PRJNA860963. Details of the build hardware and electronic diagrams can be found at https://www.protocols.io/workspaces/mock-coastal-environment2. All scripts relating to the build and analyses described herein can be found at https://github.com/BecketLab/Mock-Coastal-Environment.

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