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Carbon mass balance methodology to characterize the growth of pigmented marine bacteria under conditions of light cycling

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Abstract A carbon mass balance methodology employing minimal measurements was applied to heterotrophic and photoheterotrophic marine bacteria grown under constant dilution and exposed to 12-h intervals of light or darkness. Carbon mass balance calculations using measurements taken every 3 h closed to within 93–103% using dissolved organic carbon, biomass carbon and CO₂ production data only, indicating that background interference from dissolved inorganic carbon variations in the amended seawater medium was not significant. Neither strain was observed to sustain a net CO₂ fixation using paramagnetic measurement of oxygen uptake rates (OUR), indicating a need for more sensitive on-line measurement techniques for OUR. Photoheterotrophic growth demonstrated lower carbon-mole biomass yields (0.41 ± 0.026 vs. 0.64 ± 0.013 mol mol⁻¹) despite higher specific glucose uptake rates (0.025 vs. 0.02 mol mol⁻¹ h⁻¹), suggesting that bioreactor-based study of marine bacteria can present growth modes that are different from those encountered in the marine environment.

Keywords Carbon mass balance · Marine bacteria · Photoheterotrophy

Introduction

The contribution of marine bacteria to carbon and energy flows in the oceans is drawing increasing

attention [1]. Of particular interest is the contribution of photoheterotrophic bacteria conducting aerobic anoxygenic photosynthesis, which have recently been demonstrated to be ubiquitous in ocean surface waters, and may comprise as much as 11% of the marine bacterial community [2]. The aerobic anoxygenic phototrophs (AAPs) are strict aerobes, requiring oxygen for growth and photosynthetic electron transport [3]. These bacteria produce bacteriochlorophyll *a* (BChl *a*), as well as a wide variety of carotenoids (mainly zeaxanthin, bacteriorubixanthin and sulfated xanthophylls) needed to support photosynthetic electron transport. Photoheterotrophic AAPs can oxidize dissolved organic substrates in a heterotrophic mode, as well as derive energy from light via light harvesting pigments for increased metabolic efficiency. They display only low light-mediated carbon dioxide fixation, and contribute 2–5% of the total photolithoautotrophic electron transport fluxes in the upper-ocean [2]. In contrast, purely heterotrophic marine organisms are capable of growth on organic compounds, but are incapable of synthesizing carbon compounds from carbon dioxide or harvesting light for ATP production. Marine heterotrophs are found in a wide range of habitats, including sediments, biofilms, and open waters [4, 5].

The study of the regulation of energy metabolism and pigment production of photoheterotrophic marine bacteria in situ is challenging due to the complex nature of the marine food web. Hence, it would be useful to establish well-defined and controlled reactor-based culture techniques that permit investigation of photoheterotrophic organisms in relation to heterotrophs. Continuous culture is a cultivation technique suitable for this purpose as it permits environmental factors (e.g. nutrients, temperature, light cycling) to be varied at a constant growth rate, with carbon mass balancing often used to verify culture parameters. The carbon mass balance approach in continuous culture has been a tool frequently applied to freshwater cultivation of bacteria, yeast, mammalian, and insect cells [6]; its application as a tool to compare physiological

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response to external variables unique to the marine environment (e.g. light cycling) remains less explored despite the fact that chemostat culture has long been applied to the study of marine bacteria in isolation [7–13]. Hence, the primary goal of this work was to develop a simple carbon mass balance methodology that validates continuous culture as a means to quantify the physiological responses of marine bacteria, in particular to culture conditions that reflect their natural habitat such as light cycling. To do this, a marine heterotroph and a marine photoheterotroph were used as model systems. *Erythrobacter longus* NJ3Y, a strain recently isolated from waters near New Jersey (USA) [2], was used as the model photoheterotrophic organism while a pigmented marine strain (UHMJC1) was used as the model heterotroph. Using the methodology described herein, future studies will be able to address more specific issues of growth physiology and energy metabolism, such as the response of the photoheterotroph growth to varied cycles of light cycling i.e., dark, light and 12:12 light–dark cycling), with a focus on pigment production, carbon fixation, and energy metabolism.

Materials and methods

Media

Growth in batch and continuous cultivations used f/2 medium [14], modified by substitution of NH_4Cl for NaNO_3 and glucose as the organic carbon source. Specifically, the medium was composed of 0.2 μm filtered seawater amended with 10 mM NH_4Cl , 0.21 mM NaH_2PO_4 , 3.0 g l^{-1} glucose. To this medium, 2 ml (per liter) of a vitamin stock was added, and an additional 2 ml/l of a trace element solution. The vitamin solution was composed of cyanocobalamin (0.0005 g l^{-1}), thiamine HCl (0.1 g l^{-1}) and biotin (0.0005 g l^{-1}). The trace element solution was composed of Na EDTA (4.360 g l^{-1}), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (3.150 g l^{-1}), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.010 g l^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.022 g l^{-1}), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.010 g l^{-1}), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.180 g l^{-1}) and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.006 g l^{-1}). For growth of cultures on agar plates, yeast extract and peptone (0.1 g l^{-1}) replaced glucose and 1.5% agarose was added to make the f/2 media plates.

Strain selection and maintenance

The marine photoheterotroph *Erythrobacter longus* NJ3Y was isolated from waters near New Jersey and obtained as a generous gift (Paul G. Falkowski and Zbigniew S. Kolber, Rutgers University). The additional marine strain studied (UHMJC1) is a pigmented marine bacterium derived from a similar source. The 16S sequence (data not shown) of strain UHMJC1 was submitted to the ribosomal database project (Michigan

State University, Center for Microbial Ecology), and this isolate was tentatively identified (Similarity score = 0.986) as *Micrococcus* sp. and similar to those previously isolated in the Western Mediterranean Sea. The 16S sequence also showed homology to various *Arthrobacter* and *Citricoccus* sp. (Similarity score 0.870 and 0.961, respectively).

The purity of each cell line was verified in-house by dilution plating on f/2 media plates (0.1 g l^{-1} yeast extract, 0.5 g l^{-1} peptone). To guarantee that all experiments were performed with a genetically identical strain, master seed banks were prepared as follows. A pure colony was inoculated into 50 ml of f/2 medium supplemented with yeast extract/peptone (0.1 g l^{-1}) and grown to late exponential phase in shake-flask culture. Aliquots of this suspension (0.9 ml) were mixed with aliquots (0.6 ml) of a 50% glycerol-medium mixture in sterile Eppendorf tubes and the combined mixture (20% glycerol) was stored at -80°C .

Strain identification

The DNA was extracted from strain UHMJC1 using the Qiagen DNeasy kit as per the manufacturers' instructions (Qiagen, Inc. Chatsworth, CA, USA). Additional purification was performed using the DNeasy spin columns (Qiagen, Inc.). The 16S rDNA gene was amplified using platinum pfx DNA polymerase (Invitrogen Life Technologies), using the eubacterial-specific oligonucleotide primers 16S-27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 16S-1492R (5'-ACG GCT ACC TTG TTA CGA CTT-3') (IDT Integrated DNA Technologies Inc.). A 3-step cycling protocol that included denaturation at 94°C for 0.25 min, annealing at 55°C for 0.5 min, and extension at 68°C for 1.5 min was employed. PCR products were purified by electrophoresis in a 1% (w/v) agarose gel, with bands of approximately 950 base pairs excised and extracted using a gel extraction kit (QIAquick purification kit: Qiagen, Inc.). Direct sequencing of PCR product used dye terminator cycle sequencing kit as per the manufacturers' instructions (CEQ Beckman Coulter).

Cultivation

All experiments began with an individual Eppendorf tube removed from the master seed bank, thawed, and inoculated into 50 ml of f/2 medium (10 mM glucose). The inoculated culture was then shaken at 180 rpm and maintained at a temperature of 25°C . Incubation continued (4–6 days) until sufficient turbidity was achieved, at which time the suspension was transferred into the bioreactor.

All cultivations were conducted in 1.5-l spinner flasks (B. Braun, Germany), with dissolved oxygen (DO), pH and temperature monitored on-line. DO was maintained above 50% via inlet aeration (100–200 ml min^{-1}), pH

maintained at 8.0 by base addition (0.1 M NaOH) and temperature maintained at 25°C. Base addition was controlled using proportional integral feedback loops. Mixing was provided by agitation (200 rpm) of Rushton-style turbine comprised of four thin plastic blades of constant height (1") and diameter (2"). To avoid back growth, feed drippers in both inlet and exit lines were installed as close as possible to the reactor inlet and outlet. Light cycling was provided by wiring four 20-W incandescent tube-lights, placed on both left and right side of the reactor, to a self-regulating timer (Fig. 1). For the continuous culture experiments, the cultures were maintained for several days on a 12–12 light cycle before the measurement period began.

Continuous cultures were maintained at a dilution rate (D) of $\sim 0.01 \text{ h}^{-1}$ based upon preliminary batch tests, which indicated a maximum growth rate in the glucose-amended $f/2$ medium in the region of 0.02 h^{-1} for both strains (data not supplied). Sterile feed media was delivered through an inlet feed line using a peristaltic pump (Watson Marlow, USA). To maintain a constant reactor volume, spent medium was pumped from the reactor through an exit tube placed directly above the liquid surface at a height yielding a constant reactor volume (1.2 l). The pumping velocity of the outlet pump was set at 20% above the inlet pump to ensure the inlet rate never exceeded the exit rate. The inlet and outlet flow rates were quantified by placing both the inlet feed and spent medium reservoirs on top loading balances and measuring the weight loss or gain over defined periods of time (usually 18–24 h). Inlet air was sterilized through a 0.22- μm filter and controlled at a flow rate of 100–200 ml min^{-1} using mass flow controllers (MKS, USA).

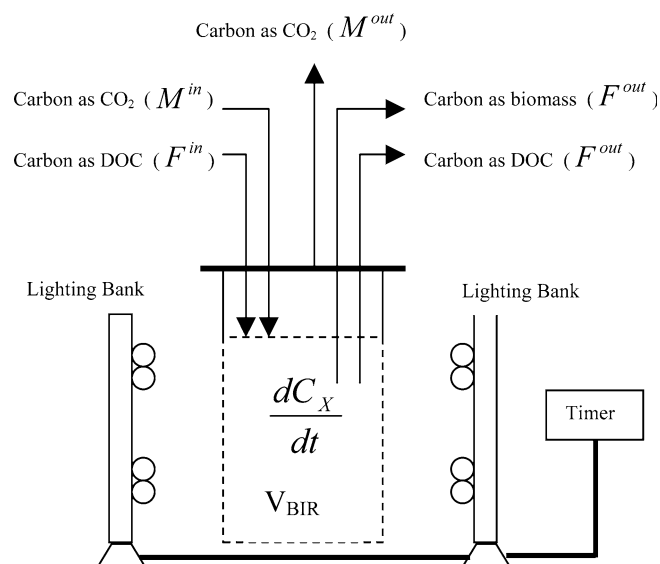


Fig. 1 Experimental setup for continuous cultivation of marine bacteria

Sampling

Two sampling frequencies were employed during continuous culture. The first, used during the approach to steady state, sampled twice per day. Steady state was accepted after either three reactor volumes of feed media had been pumped, or the optical density (OD) had remained constant over 3 days, whichever period was longer. Once steady state was achieved, an intensive sampling period was employed, during which time samples (4.5 ml) were taken every 3 h, resulting in a total of nine samples taken over each 24 h time period. This sampling frequency was calculated to permit the maximum number of samples to be taken without removing more than 10% of the total volume added over the sample period (3 h). This was done to minimize the effect of removing media from the reactor, an act that temporarily shifts the system from continuous to fed-batch cultivation. Samples were withdrawn sterile, using a standard sampling device attached to a filter syringe. The inlet tube was cleared with sterile air prior to and after all sample extractions.

From each sample, 0.5 ml was taken and diluted to 1 ml by the addition of an equal volume of filtered seawater, and its OD measured at 650 nm. The remaining sample (4 ml) was used for analysis of pigment and carbon–nitrogen (CN) concentrations. For pigment analysis, two samples (1 ml) were vacuum-filtered through 25 mm GF/F filters (previously dried for 18 h on aluminum trays at 80°C), and the filtrate frozen for later analysis of dissolved organic carbon (DOC) and glucose. For CN analysis, two samples (0.5 ml) were filtered through 25 mm GF/F filters (previously dried for 18 h on aluminum trays in an oven at 80°C) and their filtrate collected and frozen for later analysis. All filters were folded in half, wrapped in aluminum foil, labeled and stored at -80°C . To eliminate contaminants, the aluminum foil had previously been combusted at 450°C for 4 h.

Analytical

Primary biomass quantification was based on particulate organic carbon (POC) content from CN analysis. Additionally, dry cell weight (DCW) quantification of cultivation samples was conducted using OD readings. Calibrating the DCW to the OD was conducted with each experiment (data not shown). To eliminate the effect of salt residues from seawater on the DCW assay, three rinses of ammonium formate on the filter were applied. The ammonium formate maintains osmolarity (avoids cell lysis) but evaporates during drying. Cells were microcentrifuged at 6,000 rpm (or 2817 RCF), 0.9 ml of the supernatant was discarded, the cell pellets were resuspended with 0.9 ml of ammonium formate, and the mixture vortexed vigorously. Tests showed that three rinses were required until no decrease in dry weight was observed for the dried filters. This was

verified using controls on blank filters with and without seawater. Calibration curves were created by generating a dilution series (1:2) from shake-flask culture and measuring the OD of known aliquots (2 ml) before filtering them through pre-weighed 25 mm GF/F filters (previously dried for 18 h on aluminum trays at 80°C, cooled in a desiccator and then weighed). These filters were dried overnight at 80°C, cooled in a desiccator for at least 1 h and then weighed. The difference was divided by the volume (2 ml) to obtain the DCW per unit volume.

Culture glucose concentrations were determined by enzymatic assay as per manufacturers instructions (Boehringer Mannheim, Germany). Seawater interference was excluded from tests on 1.0 g l⁻¹ glucose standard in filtered seawater, which gave identical results to the 1.0 g l⁻¹ standard using distilled water.

The measurement of DOC was performed by the high-temperature catalytic oxidation method, as presented previously [15]. Elemental analysis of cellular carbon and nitrogen was carried out using a Perkin-Elmer 2400 Elemental Analyzer.

controllers, equal to the flow rates passed through the reactor.

Exit gas concentration of O₂ was recorded in percent L_{O₂}/L_{air} and converted to moles of O₂ per liter of air using the ideal gas law, as the exit line of the gas analyzer discharged to atmosphere. To relate this value to the measured oxygen in the bioreactor, one can multiply by the volumetric flow rate of the gas stream and divide by the volume of liquid in the bioreactor:

$$O_2^{\text{measured}} \left(\frac{\text{mmol}_{O_2}}{L_{\text{air}}} \right) = \frac{O_2^{\text{measured}} (L_{O_2}/L_{\text{air}})}{24.47 (L_{O_2}/\text{mol}_{O_2})} \times \frac{1,000 (\text{mmol}/\text{mol})}{V_{\text{BIR}} (L)} \times M \left(\frac{L}{h} \right) \quad (1)$$

where V_{BIR} is the volume of liquid in the bioreactor (Table 1). For CO₂ the equations are analogous. In the absence of O₂ losses due to secondary reactions in the liquid or gas phase, the volumetric OUR can be obtained by taking the difference between the gas phase O₂ measured in the inlet and exit gas streams:

$$\text{OUR} \left(\frac{\text{mmol}}{L \times h} \right) = \frac{(O_2^{\text{in}} (\text{mmol}/L) \times \dot{M}^{\text{in}} (L/h)) - (O_2^{\text{exit}} (\text{mmol}/L) \times \dot{M}^{\text{exit}} (L/h))}{V_{\text{BIR}} (L)} \quad (2)$$

Samples analyzed for pigments were retrieved from -80°C, thawed, extracted in acetone (containing an appropriate internal standard) with a tissue homogenizer, and then separated on a Varian 9012 HPLC system equipped with a ThermoSeparation UV2000 detector ($\lambda = 436$ nm for bacteriocarotenoids, and 360 nm for BChl *a*) and bacteriopheophytin *a*). Eluting pigments were identified on the basis of retention time, on-line diode array spectroscopy, co-injection analysis using authentic standards (if available), and LC-MS-MS analysis. Pigments were quantified using external standards as described previously [16, 17].

Gas phase analysis

Concentrations of oxygen and carbon dioxide in the gas stream exiting in the bioreactor were quantified by the passage through a paramagnetic/infrared gas analyzer (Siemens, Germany). Inlet gas was compressed house air passed through a pressure regulator that reduced the inlet air pressure to 20 kPa. The air was then delivered to the mass flow controller, which set the inlet mass flow rate as per required for the experiment (e.g., between 100 and 200 ml min⁻¹). The exit gas stream leaving the bioreactor was dehumidified by passage through 3 m of wound copper tubing packed in ice and subsequently through a 7.5 cm packed bed of Drierite. As the gas analyzer was sensitive to flow rates, the flow rate of the calibration gas was always set, using the mass flow

In those cases where the flow rates of the inlet and exit streams are equal, $M = M^{\text{out}} = M^{\text{in}}$ and the mass flow rate can be factored out of Eq. 2. The biomass-specific OUR is obtained by multiplying Eq. 2 by the molecular weight of O₂ and then dividing through by the biomass concentration in the bioreactor.

$$\text{OUR} \left(\frac{g_{O_2}}{g_{\text{dcw}} \times h} \right) = \frac{\text{OUR} (\text{mmol}/Lh) \times MW_{O_2} (g/\text{mol})}{1,000 \times \text{dcw} (g_{\text{dw}}/L)} \quad (3)$$

The CO₂ evolution rates are calculated analogously using gas phase measurement of CO₂ and assuming that no secondary reactions in the liquid or gas phase occur that act as sources or sinks of CO₂.

$$\text{CER} \left(\frac{g_{CO_2}}{g_{\text{dcw}} \times h} \right) = \frac{\text{OUR} (\text{mmol}/Lh) \times MW_{CO_2} (g/\text{mol})}{1,000 \times \text{dcw} (g_{\text{dw}}/L)} \quad (4)$$

The respiratory quotient (RQ) can be obtained by taking the ratio of the CO₂ evolution rate to the OUR. An alternative determination of RQ is possible via a generalized degree of reduction balance on glucose consumption, as adapted from [18]. First, the C-mole biomass yield is calculated from measured biomass carbon concentration and steady state glucose concentration during continuous steady state:

Table 1 Nomenclature of carbon mass balance terms

$d C_X/d t = C_X \times V_{BIR} \times \mu$ ($g h^{-1}$) is the rate of change of biomass within the bioreactor

$\dot{C}_X^{out} = C_X \times F^{out}$ ($g h^{-1}$) is the rate at which biomass carbon leaves through the liquid phase exit stream

$\dot{C}_{Glu}^{in} = C_{Glu}^{in} \times F^{in}$ ($g h^{-1}$) is the rate at which glucose carbon enters through the liquid phase feed stream

$\dot{C}_{Glu}^{out} = C_{Glu} \times F^{out}$ ($g h^{-1}$) is the rate at which glucose carbon leaves through the liquid phase exit stream

$\dot{C}_{Air}^{in} = C_{Air}^{in} \times M^{in}$ ($g h^{-1}$) is the rate at which rate CO₂: carbon enters through the feed gas stream

$\dot{C}_{Air}^{out} = C_{Air} \times M^{out}$ ($g h^{-1}$) is the rate at which rate CO₂ carbon leaves through the exit gas stream

$\dot{C}_{SW}^{in} = C_{SW}^{in} \times F^{in}$ ($g h^{-1}$) is the rate at which seawater carbon enters through the liquid phase feed stream

$\dot{C}_{SW}^{out} = C_{SW} \times F^{out}$ ($g h^{-1}$) is the rate at which seawater carbon exits through the liquid phase exit stream

$\dot{C}_{Acids}^{out} = C_{Acids} \times F^{out}$ ($g h^{-1}$) is the rate at which acid(s) carbon exits through the liquid phase exit stream

C_X ($g l^{-1}$) is the biomass carbon in the bioreactor volume

C_{Glu} ($g l^{-1}$) is the glucose carbon in the bioreactor volume

C_{Air}^{in} ($g l^{-1}$) is the CO₂ carbon in the inlet air

C_{Air} ($g l^{-1}$) is the CO₂ carbon in the gas phase of the reactor headspace

C_{SW}^{in} ($g l^{-1}$) is the seawater carbon in the inlet feed media

C_{SW} ($g l^{-1}$) is the seawater carbon in the bioreactor volume

C_{Acids} ($g l^{-1}$) is the acid(s) carbon present in the bioreactor volume

C_{Glu}^{in} ($g l^{-1}$) is the glucose carbon in the inlet feed media

F^{in} ($l h^{-1}$) is the inlet flow rate of feed media

F^{out} ($l h^{-1}$) is the outlet flow rate of liquid medium

M^{in} ($l h^{-1}$) is the flow rate of inlet gas entering the bioreactor (air)

M^{out} ($l h^{-1}$) is the flow rate of exit gas leaving the bioreactor

V_{BIR} (l) is the liquid reaction volume of bioreactor culture

μ (h^{-1}) is the growth rate as defined by the steady state dilution rate

$$Y_{SX} \left(\frac{C_{mol_biomass}}{C_{mol_glucose}} \right) = \frac{C_x(g/L)}{12((g/C - mol))} \times \frac{30(g/(C - mol))}{Gl^{in} - Gl(g/L)} \quad (5)$$

where C_X is the biomass concentration in the bioreactor, Gl^{in} is the concentration of glucose in the inlet feed media and Gl is the glucose concentration in the bioreactor. From a carbon balance we find the C-molar yield of carbon dioxide to glucose as: $Y_{SC} = 1 - Y_{SX}$. The C-molar yield of O₂ on substrate is calculated via degree of reduction balance:

$$Y_{SO} \left(\frac{C_{mol_O_2}}{C_{mol_glucose}} \right) = \frac{1}{4} \times (4 - 4.20 \times Y_{SX}) \quad (6)$$

The respiratory quotient (RQ) is then calculated by taking the ratio Y_{SC}/Y_{SO} . All reported values for RQ in this work are calculated using this method.

Carbon mass balance

In our analysis the carbon balance is made around a control volume that surrounds the culture volume as follows (Table 1):

$$\frac{dC_X}{dt} = \dot{C}_{out}^{in} + \dot{C}_{Air}^{in} + \dot{C}_{SW}^{in} - \dot{C}_{Glu}^{out} - \dot{C}_{Air}^{out} - \dot{C}_{SW}^{out} - \dot{C}_{Acids}^{out} - \dot{C}_X^{out} \quad (7)$$

In continuous culture, and thus in the context of our analysis, the concentrations in a well-mixed reactor are assumed equal to those leaving the exit streams. The inlet flow rate of liquid volume, however, is not assumed to be equal to the exit liquid volume ($F^{in} \neq F^{out}$) as aqueous contents of the liquid volume can be absorbed by the air stream and thus reduce the exit liquid flow rate required to maintain exact volume. This factor can be significant at low dilution rates and high air inlet rates, as experienced with cultivation of slow growing marine bacteria, and thus the inlet and exit liquid flow rate should be measured to verify the validity of the assumption. Substituting the relationships from Table 1 into Eq. 7 yields the overall mass balance in terms of process variables relevant to the continuous cultures of marine bacteria:

$$0 = C_{Glu}^{in} \times F^{in} + C_{SW}^{in} \times F^{in} + C_{Air}^{in} \times M^{in} - C_{Glu} \times F^{out} - C_{SW} \times F^{out} - C_{Acids} \times F^{out} - C_{Air}^{out} \times M^{out} - C_X \times F^{out} \quad (8)$$

In the absence of a complete accounting of the acid products, a simplification can be made by grouping the terms. For example, the carbon flow of DOC entering the reactor through the feed inlet can be defined as:

$$C_{DOC}^{in} = C_{Glu}^{in} + C_{SW}^{in} \quad (9)$$

Similarly, the DOC leaving the reactor in the liquid phase exit stream can be defined as:

$$C_{DOC} = C_{Glu} + C_{SW} + C_{Acids} \quad (10)$$

Substituting Eqs. 9 and 10 into Eq. 8 permits the following simplification:

$$0 = C_{DOC}^{in} \times F^{in} + C_{Air}^{in} \times M^{in} - C_{DOC} \times F^{out} - C_{Air} \times M^{out} - C_X \times F^{out} \quad (11)$$

After rearranging terms as per Eqs. 9 and 10, Eq. 11 can be rearranged as:

$$C_{DOC}^{in} \times F^{in} + C_{Air}^{in} \times M^{in} = C_{DOC} \times F^{out} + C_{Air} \times M^{out} + C_X \times F^{out} \quad (12)$$

Equation 12 is in the form that will be used to verify the carbon mass balance in the current study.

Results and discussion

Verification of heterotrophic and photoheterotrophic growth

To verify the absence and presence of photoheterotrophic light-capture apparatus in the model systems, pigment profiles were measured for both strains.

When grown in continuous culture under the 12 h light, 12 h dark (12–12) light cycle, pigment analysis of the isolate UHMJC1 failed to show BChl *a* or zeaxanthin, although unidentified but assumed photoprotective pigments were present, and for this reason UHMJC1 was considered to be a strain incapable of BChl-based photoheterotrophy under the growth conditions employed in this study. Under identical culture conditions, *E. longus* NJ3Y was found to possess the expected complement of pigments (caloxanthin sulfate, bacteriopheophytin *a*, β -carotene, bacteriorubixanthinal), and possession of a full photoheterotrophic light capture apparatus: BChl *a* (780 ± 121 nmol DCW⁻¹) and zeaxanthin (298 ± 19 nmol DCW⁻¹). At a constant *D* of 0.1 h⁻¹, the relative pigment concentrations of *E. longus* NJ3Y did not significantly vary over 12–12 light cycling. Although a complete discussion of these pigments, their relative distribution, and relation to growth under varied light cycling is beyond the scope of this work, it will be presented in a subsequent publication.

Application of carbon mass balancing in marine media

Characterization work with recent marine organisms of interest has generally used filtered seawater amended with nutrients (i.e., semi-defined media), as such organisms are, in general, otherwise refractory to cultivation. Such media, especially when supplied in continuous culture at low *D* and over long durations, present unique challenges to the precise quantification of culture parameters, particularly as required for carbon flux mass balancing. Typical carbon flux profiles at single sampling time points are provided in Figs. 2 and 3 for the heterotrophic (UHMJC1) and photoheterotrophic (NJ3Y) strain, respectively.

For both strains mass balancing of input and output flows using total organic carbon, carbon dioxide production and biomass carbon were able to account for nearly 100% of the carbon flux (Figs. 2, 3), thus permitting reasonable confidence in measured culture parameters to define the metabolic response of the culture to select environmental pressures such as growth rate, feed nutrient concentration, and light cycling. Tables 2 and 3 present the measured process variables and carbon mass balance calculations at all time points for heterotrophic growth (UHMJC1), while Tables 4 and 5 present similar data for photoheterotrophic growth (NJ3Y). The successful closure of the carbon balance using a minimum of process measurement(s), validates the methodology used. Certain corrections to mass balancing process variables were required to accommodate the marine culture system, as will be detailed in the following sections.

Dissolved inorganic carbon

The f/2 seawater-based media employed in the current study contained approximately 25 mg l⁻¹ dissolved inorganic carbon (DIC), which functioned as the pri-

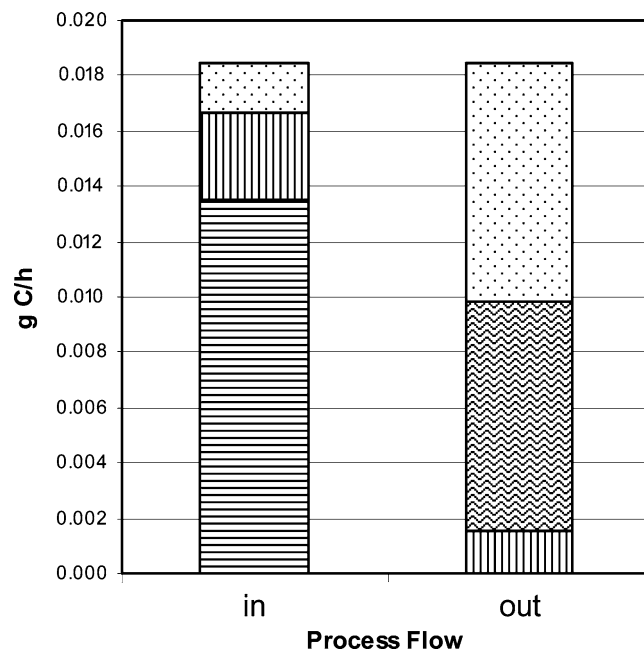


Fig. 2 Input and output carbon mass balance for heterotrophic marine strain UHMJC1, $t=3$ h, dotted area, CO₂ carbon; vertical stripes, non-glucose DOC; horizontal stripes, glucose DOC; wavy stripes, biomass carbon

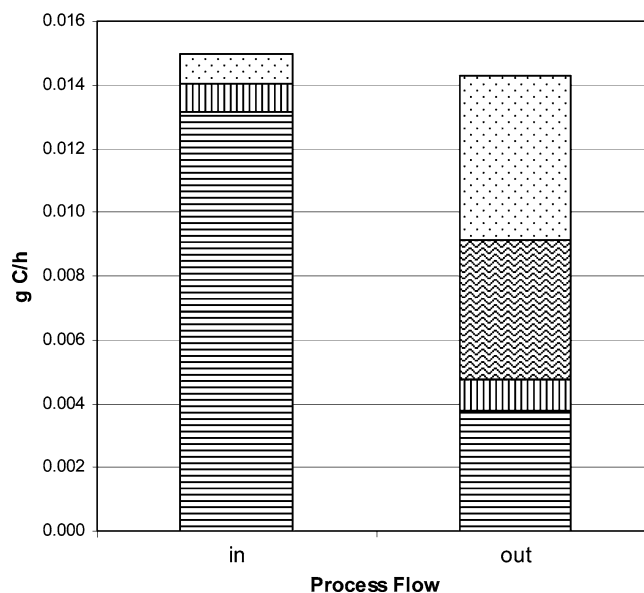


Fig. 3 Input and output carbon mass balance for photoheterotrophic marine strain NJ3Y, $t=3$ h, dotted area, CO₂ carbon; vertical stripes, non-glucose DOC; horizontal stripes, glucose DOC; wavy stripes, biomass carbon

mary seawater buffer system as depicted by the standard equations.

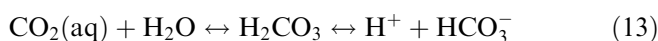


Table 2 Measured variables from 12 h light to 12 h dark cycle applied to continuous culture of the marine heterotroph UHMJCI

Time (h)	D (h^{-1})	Glucose (g l^{-1})	DOC (g l^{-1})	X (g l^{-1})	C_X (g l^{-1})	N_X (g l^{-1})	C/N (g g^{-1})	$Y_{X/S}$ (C-mol/C-mol)	SPGUR (h^{-1})	RQ (mol mol^{-1})
0.0	0.010	0.0049	0.098	1.41	0.70	0.10	7.0	0.62	0.021	1.09
3.0	0.010	0.0049	0.134	1.41	0.70	0.10	7.3	0.62	0.020	1.09
6.0	0.010	0.0082	0.091	1.46	0.72	0.10	7.4	0.64	0.019	1.10
9.0	0.010	0.0115	0.079	1.46	0.71	0.10	7.0	0.64	0.019	1.10
12.0	0.011	0.0049	0.092	1.41	0.73	0.10	7.0	0.65	0.021	1.10
15.0	0.010	0.0049	0.074	1.47	0.72	0.11	6.7	0.64	0.019	1.10
18.0	0.010	0.0066	0.062	1.46	0.72	0.10	7.0	0.64	0.019	1.10
21.0	0.010	0.0049	0.059	1.45	0.73	0.11	6.7	0.65	0.019	1.10
24.0	0.010	0.0033	0.062	1.47	0.74	0.11	7.1	0.66	0.019	1.11

Lights turned off at 7 h and turned on at 19 h. Reactor volume 1.2 l; mass flow rate of inlet air, 200 ml min^{-1} ; inlet feed total DOC, 1.39 g l^{-1} ; inlet feed glucose 2.82 g l^{-1} . Time zero reflects the initiation of the light cycle. Biomass yields ($Y_{X/S}$) are quoted in

C-mol carbon biomass per C-mole glucose, specific glucose uptake rates (SPGUR) are quoted in C-mol glucose per C-mol biomass per hour

Table 3 Carbon mass balance for 12 h light–12 h dark cycle applied to continuous culture of the marine heterotroph UHMJCI

Time (h)	F^{in} (l h^{-1})	F^{out} (l h^{-1})	CO_2 (%)	C_{Air} (g l^{-1})	C_X (g l^{-1})	C_{DOC} (g l^{-1})	Carbon in (g h^{-1})	Carbon out (g h^{-1})	Closure (%)
0	0.0123	0.0121	0.149	0.0007234	0.7004	0.0982	0.0189	0.0183	96.8
3	0.012	0.0118	0.148	0.0007258	0.6992	0.1336	0.0185	0.0185	100.0
6	0.012	0.0118	0.158	0.0007748	0.7191	0.0905	0.0185	0.0188	101.7
9	0.012	0.0118	0.156	0.0007650	0.7134	0.0791	0.0185	0.0185	99.9
12	0.0127	0.0125	0.154	0.0007552	0.7278	0.0925	0.0194	0.0192	99.0
15	0.012	0.0118	0.154	0.0007552	0.7176	0.0742	0.0185	0.0183	99.3
18	0.012	0.0118	0.158	0.0007748	0.7179	0.0616	0.0185	0.0184	99.7
21	0.012	0.0118	0.166	0.0008141	0.7344	0.0595	0.0185	0.0191	103.2
24	0.012	0.0118	0.153	0.0007503	0.744617388	0.061706844	0.0185	0.0184	99.9
Average							0.0186	0.0186	99.9
SD							0.0003	0.0003	1.77

Lights turned off at 7 h and turned on at 19 h. Reactor volume 1.2 l; mass flow rate of inlet air, 200 ml min^{-1} ; DOC in feed inlet, 1.39 g l^{-1} ; inlet feed glucose 2.82 g l^{-1} . The exit liquid phase flow rate has been corrected for water loss due to gas stripping

Table 4 Measured values from 12 h light–12 h dark cycle applied to continuous culture of the marine photoheterotroph *E. longus* NJ3Y

Time (h)	D (h^{-1})	Gl (g l^{-1})	DOC (g l^{-1})	X (g l^{-1})	C (g l^{-1})	N_X (g l^{-1})	C/N (g g^{-1})	$Y_{S/X}$ (C-mol/C-mol)	SPGUR (h^{-1})	RQ (mol/mol)
0.0	0.0087	0.88	0.45	0.842	0.42	0.050	8.5	0.46	0.019	1.04
3.0	0.0087	0.92	0.46	0.851	0.43	0.050	8.5	0.48	0.018	1.05
6.0	0.0087	0.95	0.53	0.769	0.38	0.048	7.9	0.43	0.020	1.04
11.2	0.0087	0.96	0.51	0.768	0.38	0.049	7.9	0.44	0.020	1.04
14.0	0.0087	1.05	0.52	0.732	0.37	0.046	7.9	0.43	0.020	1.04
17.3	0.0087	0.99	0.54	0.743	0.37	0.045	8.3	0.43	0.020	1.04
20.5	0.0087	0.97	0.56	0.681	0.34	0.039	8.7	0.39	0.022	1.03
24.0	0.0087	0.99	0.53	0.707	0.35	0.043	8.2	0.41	0.021	1.04

Lights turned off at 7 h and turned on at 19 h. Reactor volume 1.2 l; mass flow rate of inlet air, 100 ml l^{-1} ; DOC in feed inlet, 1.349 g l^{-1} ; inlet feed glucose 3.16 g l^{-1} . Time zero reflects the in-

itiation of the light cycle. Biomass yields ($Y_{X/S}$) are quoted in C-mol carbon biomass per C-mole glucose, specific glucose uptake rates (SPGUR) are quoted in C-mol glucose per C-mol biomass per hour

At typical seawater pH levels (~ 8.2), the major species of DIC present is HCO_3^- . However, dynamic change in system conditions, such as net production or consumption of CO_2 , can cause a net increase or decrease of DIC within the reactor control volume. Although this change could be accounted for in mass balancing via off-line DIC assay of the culture liquid, detection of transients using off-lines measurement is difficult. Theoretically, it is possible to minimize the effect of variations in DIC in continuous culture through careful maintenance

of pH control (to avoid DIC fluctuations) and steady state dilution rates. Feedback control of pH using standard probes, however, is problematic due to salt interference on the absolute measurement and requires use of specialized probes such as Ross electrodes. Closure of the carbon mass balances, however, adds another independent check as it is based on input/output parameters only (Figs. 2, 3).

The ability to execute carbon mass balancing without DIC quantification of the bulk reactor liquid increases

Table 5 Carbon mass balance for 12 h light–12 h dark cycle applied to continuous culture of the marine photoheterotroph *E.longus* NJ3Y

Time [h]	F^{in} (l h ⁻¹)	F^{out} (l h ⁻¹)	CO ₂ (%)	C_{Air} (g l ⁻¹)	C_X (g l ⁻¹)	C_{DOC} (g l ⁻¹)	Carbon in (g h ⁻¹)	Carbon out (g h ⁻¹)	Closure (%)
0.0	0.01042	0.01033	0.1860	0.000903	0.4214	0.4610	0.0150	0.0145	96.8
3.0	0.01042	0.01033	0.1760	0.000863	0.4257	0.4590	0.0150	0.0143	95.4
6.0	0.01042	0.01033	0.1710	0.000839	0.3845	0.5320	0.0150	0.0144	96.6
11.2	0.01042	0.01033	0.1690	0.000829	0.3839	0.5100	0.0150	0.0142	94.7
14.0	0.01042	0.01033	0.1640	0.000804	0.3660	0.5230	0.0150	0.0140	93.4
17.3	0.01042	0.01033	0.1600	0.000785	0.3713	0.5350	0.0150	0.0140	93.8
20.5	0.01042	0.01033	0.1570	0.000770	0.3404	0.5620	0.0150	0.0139	92.9
24.0	0.01042	0.01033	0.1630	0.000799	0.3533	0.5340	0.0150	0.0139	93.1
Average							0.0150	0.0141	94.6
Std dev.							0.0000	0.0002	1.57

Lights turned off at 7 h and turned on at 19 h. Reactor volume 1.2 l; mass flow rate of inlet air, 100 ml l⁻¹; DOC in feed inlet, 1.349 g l⁻¹; inlet feed glucose 3.16 g l⁻¹. The exit liquid phase flow rate has been corrected for water loss due to gas stripping

the applicability of this methodology to continuous cultivation of marine bacteria. However, the DIC fed to the reactor through the inlet feed will tend to dynamically oppose rapid changes in the rate of measured culture carbon dioxide evolution rate, which can interfere with the rapid detection of changes in respiration by photoheterotrophic bacteria in response to light transitions. In the current study, no significant light-mediated decrease in respiration was observed for the NJ3Y, as has been observed in previous studies [2], which may have been due to this dampening effect.

Water crossover between liquid and gas flows

The precision of carbon mass balancing requires precise quantification of inlet and outlet flows of gas and liquid. Although precise calibration of aeration mass flow controllers and feed pumps define inlet parameters, effects of humidification of the gas stream and consequent loss of reactor liquid on exit flows should be considered when low dilution rates are employed, especially in the presence of relatively high aeration rates. Such consideration is particularly relevant for marine cultures in which loss of reactor H₂O will alter the salinity, thus potentially impacting cell viability, culture responses to environmental conditions, and carbonate and buffer balances. These changes were considered for both heterotrophic (UHMJC1) and photoheterotrophic (NJ3Y) continuous culture mass balance calculations, as presented below using continuous cultivation of UHMJC1 as the working example.

When aerated, the potential loss of liquid to the air stream needs to be addressed. The continuous culture of the heterotroph UHMJC1 was aerated at 200 ml min⁻¹ (compressed air) and the inlet flow rate of feed media set at 12 ml h⁻¹ ($D \sim 0.01$ h⁻¹). At 200 ml min⁻¹, a totally dry air stream can extract a maximum of 6.2 ml min⁻¹ water gas (saturation partial pressure of water 3.1% at 25°C, 1 bar), assuming ideal transfer. Applying the ideal gas law (1-mol water gas occupies 24.5 l at 25°C, 1 bar), and including the molecular density of water (1-mol liquid water occupies 18 ml), an upper limit of

0.27 ml h⁻¹ of liquid water that can be removed from the reactor via the gas stream can be calculated. In actuality, the removal rate should be lower as the inlet air is partially saturated. To experimentally verify water transfer effects on the exit liquid flow rate, several trials were run on separate days in which house air was passed through sterile water for 24 h and the volume loss determined. The actual loss of moisture from the reactor to the air stream was experimentally measured to be 0.18 ml h⁻¹. This corresponded to an inlet humidity of 31% in the compressed air stream. To account for this rate of moisture loss, we estimate that the outlet liquid phase flow rate (at 12 ml h⁻¹ inlet) should be 98.5% of the liquid inlet flow rate for mass balance calculations. Altering the mass balance to account for this term, lowered the closure just at 1%. While this does not represent a large correction, it may be significant when fully dry air is used or when considered in combination with other corrections, and when carbon closures as near to 100% as possible are desired. As will be discussed below, in those cases where the oxygen uptake is quite low, other techniques to maintain aeration can be used that both calculate the OUR, control the DO levels, eliminate the need to physically aerate the culture with a moving gas stream.

Loss of culture water to the vapour phase will also increase the concentration of salts in the media with time. Although this effect is mitigated in continuous culture by media exchange, it can be exacerbated at low dilution rates as required for cultivation of certain marine bacteria. Assuming an inlet flow of 12 ml h⁻¹, an outlet flow of 11.8 ml h⁻¹ (i.e., 98.5% reduction as previously derived, representing maximum water loss via vapor), an inlet media salinity of 35 g l⁻¹ (total salts), and a D of a 0.01 h⁻¹, the steady state salt concentration will be increased from 35.0 to 35.6 g l⁻¹. This represents a relatively low increase in culture media salt gain and the relatively constant growth yield for both strains over 24 h growth periods reassured minimal concern for this variable. Reactor DOC concentration will also be concentrated via the same mechanism, however the effect of this change is compensated by use of the corrected exit flow rate.

Addition or removal of water vapor to the gas stream passing through the bioreactor can alter both the partial pressures of oxygen/carbon dioxide and exit gas flow rate fed to the gas analyzer; their effect should be considered, particularly for growth of marine bacterial strains with low growth rates and therefore low gas uptake and evolution rates. This is especially true when using paramagnetic measurement of oxygen, as the inlet air possesses a relatively high percentage of oxygen and changes in this value due to oxygen uptake by marine bacteria can be near or below instrument sensitivity. As all gas measurements were undertaken on filter dried gas streams (required for analysis using paramagnetic oxygen and infrared carbon dioxide detection), the inlet aeration rate was corrected to represent the dry air input only. For an inlet air rate of 200 ml min^{-1} , this corresponded to 198 ml min^{-1} dry air, with 2.1 ml min^{-1} being water vapor. In the context of the carbon balance this correction lowered the percent closure by 0.5%.

Although corrections to the carbon balance as a result of liquid/gas crossover were not large, they assume constant humidity. In the current case where the experiments are run in a location where the average humidity can alter dramatically over the course of days, changes to liquid/gas crossover can be quite significant. For example, in Hawaii the humidity can vary from 30% to 80%, and depending upon the quality of the house air compression system, the percentage of inlet air that is water can prove quite significant. To counter these and liquid/gas crossover effects altogether, one can filter-dry the incoming air (prior to entry to the inlet mass flow controller) as well as the outgoing air (prior to gas analyzer entry), although this technique would increase evaporative reactor liquid loss and require an additional inlet gas humidifier just prior to the reactor (although after the inlet mass flow controller). With such a gas/liquid system, no corrections to calibrated inlet and outlet flows would be necessary. An additional consideration for the accuracy of typical exit gas analyzers, are errors in calibration due to changes in ambient laboratory pressure (due to air-conditioning cycling, etc.). Such errors may be minimized by frequent recalibration of the gas analyzers during data collection, or periodic gas sampling preceded by calibration at each step. Otherwise, corrections for changes in barometric pressure should be included.

Heterotrophic and photoheterotrophic growth under light cycling in continuous culture

In general, culture parameters for both strains UHMJC1 and NJ3Y remained constant throughout the entire 12–12 h light–dark cycle (Tables 2 and 4). Steady state was confirmed, as change in OD (650 nm) was less than 5% over the 24-hour cycle for each cultivation. This result was expected for the UHMJC1, as pigmentation for this strain was attributed to photo-protection only. The lack of lighting transition response was observed for NJ3Y,

which was demonstrated to possess a light harvesting pigment apparatus. Despite the fact that light cycling has previously been linked to dynamic reduction in respiration after the commencement of lighting [2], no corresponding dynamic change in RQ for NJ3Y was observed in the current study. This suggests that a sampling interval of once per 3 h is not suitable for detecting dynamic respiratory response to lighting transitions within a single 24-hour cycle period. Part of the failure of the continuous culture system to detect rapid changes can be attributed to DOC load in the amended seawater medium, coupled with inability for direct OUR measurement, as will be discussed in detail below.

Direct on-line quantification of the OUR was not possible via exit gas measurement of oxygen in continuous cultivation of either strain due to insufficient respiration induced deviation from the baseline aeration oxygen concentration (20.95% in air). This occurred even at low aeration rates (100 ml min^{-1} for NJ3Y cultivation), and is attributed to respiration rates below our limits of detection. The problem was far less pronounced for carbon dioxide measurement, as the baseline content of carbon dioxide in air was low (0.031%) and the sensor was more able to detect small deviations. Solutions include aerating the culture at low rates with a feed gas of low oxygen concentration (e.g. 5% oxygen in nitrogen) and measuring respiration directly using winkler titration of DO, or indirectly using one of a variety of redox dyes like CTC, INTA. An alternative method would be to precisely control the oxygen transfer rate (OTR) via membrane oxygen transfer in order to determine OUR. This technique, which tracks (through the opening and closing of a solenoid valve) the amount of high-purity oxygen required to maintain a steady state DO setpoint, was successfully applied to mammalian cell culture [20]. The third solution, used in the present study, is to accurately calculate the OUR and RQ from glucose consumption via degree of reduction balance from [18]. However, results of the present study suggest that this method may require a rate of sampling that is more frequent than reasonable, and is hence not suitable for online monitoring directed toward detection of transients. Hence, it is recommended that future work on slow growing photoheterotrophic strains employ the membrane oxygenation techniques to accurately measure OURs.

The C/N values (g/g) for NJ3Y (8.2 ± 0.3) were significantly higher than those previously reported in batch culture for this strain (3.7–4.1, [2]), suggesting nitrogen or phosphate limitation. Additionally, maximum growth rates for NJ3Y in the current study (glucose-amended f/2 medium) were approximately a factor of five lower than those reported for NJ3Y in yeast extract plus peptone-amended f/2 medium [2]. These results are supported by preliminary batch culture experiments, which yielded maximum growth rates in our glucose-amended f/2 medium in the region of 0.02 h^{-1} for both strains (data not supplied). This finding suggests that ammonium supports lower growth rates than peptone.

While the purpose of this work was to develop a protocol to study these organisms in continuous culture using defined media, with a particular emphasis towards supporting studies concerning the effect of light cycling on pigment production, media optimization (including consideration of alternative nitrogen sources in defined media) experiments might allow greater maximum growth rates than those observed in the current study, and hence greater continuous culture dilution rates.

Some discussion regarding the effect of light cycling on biomass concentration is warranted. Assuming a 5% conversion of electrical energy into light energy from the four 20-W incandescent lights, and that only 50% of the available light shines on the bioreactor, 1.6 W of light energy should be available for absorption by the bacteria. If we further assume that only 10% of this absorbed light energy is converted (stored) as chemical energy in the form of biomass, we have 160 mW (or 0.576 kJ h^{-1}) in this set up going towards biomass production. If the enthalpy of combustion is taken to be 500 kJ per C-mol biomass, one obtains an estimated value of 1.15×10^{-3} C-mol biomass produced per hour. If we further assume a C-mol biomass of roughly 30 gdw per C-mol, we have about 34.5 mg dry weight produced per hour, or $28.74 \text{ mg h}^{-1} \text{ l}^{-1}$. For heterotrophic growth, we achieved about one-half this value ($14.1 \text{ mg h}^{-1} \text{ l}^{-1}$) and for photoheterotrophic growth, about one third ($7.4 \text{ mg h}^{-1} \text{ l}^{-1}$). These numbers suggest that sufficient energy (as light) was available for biomass growth and that we should have observed increased biomass in the photoheterotroph during the light cycle.

The data for photoheterotrophic growth (Table 4) show a weak trend of increased biomass during the light phase followed by a concomitant decline during the dark phase. However, the data also show that the increase in biomass is both relatively low and somewhat lagging the light cycle. Pigment data compared against similar data taken for cultures grown in full light and full darkness (data not shown), clearly show the expected production of light-assisted pigments during light cycling. While a detailed discussion of this phenomenon is beyond the scope of this presentation, it does confirm that our photoheterotroph culture is responding to light, in terms of pigment production. The lack of an expected response in terms of biomass productivity is more likely due to poor light penetration into the center of the bioreactor. This would significantly lower the estimated efficiency (10%) and suggests that one would need to wait a longer time than 12 hours to measure a significant increase in biomass density in chemostat culture. It is suggested that all future chemostat work applied to the study of photoheterotrophs be performed in air-lift bioreactors that are designed to maximize the percentage of culture that is located near the surface of the outer wall, and therefore in the path of light penetration. This will require a relatively large height to diameter ratio.

Average carbon-mole biomass yields were lower for the photoheterotroph ($0.41 \pm 0.026 \text{ mol mol}^{-1}$) than for the heterotroph ($0.64 \pm 0.013 \text{ mol mol}^{-1}$). The average

specific glucose uptake rates were slightly lower for the heterotroph ($0.02 \text{ mol mol}^{-1} \text{ h}^{-1}$) as compared to the photoheterotroph ($0.025 \text{ mol mol}^{-1} \text{ h}^{-1}$) (Tables 2, 4). These findings were unexpected, as the provision of light in an environmentally appropriate cycle (i.e. 12 h light, 12 h dark) was expected to benefit the photoheterotrophic strain, which possesses a photosensitive pigment apparatus suitable for harvesting light for ATP production. As stated above, part of this result can be attributed to inefficient light penetration. The specific glucose uptake rates (per unit C-mol) were also higher for the heterotroph strain.

It is suggested that the culture system selected (i.e., glucose-amended seawater applied at a constant feed rate in continuous culture) may have biased the system toward competitive advantage for heterotrophy, by providing an excessive level of reduced carbon/energy source. Given that the natural ocean environment from which these organisms were isolated contains a paucity of reduced DOC, it is entirely likely that marine heterotrophs possess a greater affinity for reduced carbon uptake (e.g., lower K_m values in the Monod kinetic description of growth) relative to their photoheterotrophic counterparts. One example of this would be our reported carbon to biomass yields of 0.41 and $0.64 \text{ mol mol}^{-1}$. These yields, which reflect growth on glucose, are quite high to those normally reported for open ocean carbon to biomass yields ($\sim 0.1 \text{ mol mol}^{-1}$) which are reflected for growth on highly oxidized and poor energy carbon sources. This effect highlights a disadvantage of applying conventional pure culture systems to the study of marine microbial physiology; that artificial stresses of bioreactor-based culture can yield measurements of metabolic performance that are ecologically irrelevant. For example, focusing on the specific glucose rate could suggest that the heterotroph is more competitive when in reality several studies have suggested the distinct advantage of mixotrophy within the context of open ocean water [21, 22]. While isolated continuous culture may be a useful technique to isolate and study a specific pathway (i.e., the fermentation production industry), it may not yield a relatively realistic comparison between marine heterotrophs and photoheterotrophs.

As per metabolic calculation of the respiratory quotient, carbon dioxide fixation was not detected for NJ3Y (average RQ not significantly less than 1.0), indicating that if the light harvested via photopigments was used for this purpose, it was a transient phenomenon that could not be detected by the methodology applied in this study. For the current study, which used carbon-rich glucose-amended media, carbon dioxide fixation could be considered unnecessary and may have been down-regulated by NJ3Y in response to culture conditions.

Summary and conclusions

Certain marine bacteria present unique challenges to parameter quantification in continuous culture, pri-

marily due to slow growth rates and the effects of amended seawater media. Slow maximum growth rates require low dilution rates (e.g. 0.01–0.02 h⁻¹) and hence, such cultures exhibit low levels of measurable metabolic parameters. In the current study this made conventional OUR measurement (via exit gas oxygen) unreliable, and an alternative method (generalized degree of reduction balance) was required to calculate respiration rate, and hence RQ. However, this technique was shown to be unsuited for detecting rapid dynamic changes in respiration or RQ (e.g., in response to light cycling) if they exist. Specialized gas exit analysis systems intended for low metabolic activity application, such as described in [20], would be useful to directly characterize dynamic respiration changes. Additionally, measurement of DI¹⁴C uptake may be employed to accurately measure carbon fixation independent of respiration data.

The methodology used for carbon mass balancing was not disrupted by the effect of variations of DIC concentration in the amended seawater medium used. This was due to the provision of steady state continuous culture with good pH control. Thus, for marine continuous culture systems where exit gas analysis of CO₂ is not available, this work has shown that carbon dioxide release levels could be computed via subtraction from DOC, substrate and biomass data. However, background seawater DIC load does appear to dampen the dynamic effects on CO₂ release, which presents an additional disadvantage to detecting rapid culture responses to events such as lighting transitions.

Overall, the continuous culture system described above, coupled with the carbon mass balancing methodology, proved useful in characterizing metabolic profiles of marine bacterial cultures undergoing light cycling changes, and was able to characterize and contrast the metabolism of a marine heterotrophic and a marine photoheterotrophic bacteria grown in continuous cultures under 12–12 light cycling. However, paramagnetic analysis of gas phase oxygen as well as degree of reduction balancing on discrete samples, were unable to detect rapid transients in respiration metabolism, and carbon dioxide fixation (as measured through a reduction in the RQ value) was not detected even though pigment analysis verified the presence of light-capturing apparatus. Part of this failure was attributed to DOC load in the amended seawater medium, which may have masked the transient behavior. Certain physical limitations such as inefficient light penetration also contributed to an inability to well characterize the response of biomass productivity to light.

A final comment addresses the accuracy of carbon balancing, which depends upon the resolution of the measurement and the productivity of the system monitored. To increase the latter, one is confronted with the need to deviate from the natural habitat of the cultures being studied. While this is not preferred it makes mass balancing more difficult. Improving nutrition and light supply to increase productivity will permit better measurements and the confidence of better data, but these

conditions do not duplicate environmental conditions or predict what will happen in situ. As bioreactor-based studies of marine bacteria increase with the discovery of new organisms, it is worthwhile noting that most regions of the oceans represent relatively extreme environments. The surface of the oceans are exposed to cycles of intense light, the open ocean water columns are nutrient-poor environments, and the ocean floors are without light, under increased pressure, and in the case of hydrothermal vents exposed to severe gradients of temperature. These microorganisms will not respond to bioreactor-based studies in manners similar to most commonly studied terrestrial organisms and will require careful strategies and new tools to study their physiology in a manner that is both accurate and reflective of their natural environment.

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