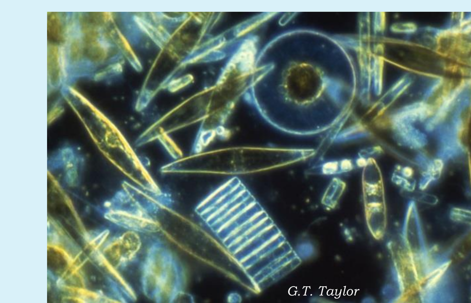
# A New Method to Measure Inter- and Intra-Specific Variability in Primary Productivity: Stable Isotope Probing and Single-Cell Resonance Raman Microspectrometry



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## Introduction

Understanding processes driving variability in phytoplankton productivity among cohabiting populations is fundamental to explaining the "paradox of the plankton" and to predicting overall ecosystem responses to episodic, seasonal and supra-annual environmental perturbations. In complex plankton communities, however, current oceanographic tools are rarely capable of addressing germane questions, such as:

- Are dominant taxa necessarily the fastest growing taxa of the moment?
- Does relaxed top-down control (predation & viral lysis) explain numerical dominance of specific taxa within natural communities?
- How does resource availability (light, N, P, Si, Fe, $B_{12}$) shape growth responses of specific taxa within natural communities?
- Is variability in single-cell growth rates necessarily greater between taxa than within individual taxa?
- How variable are intra-population single-cell growth rates among different phytoplankton taxa (e.g., rare vs common species)?

To address such questions, we developed the method described below to measure growth rates of individual photoautotrophic cells by combining Stable Isotope Probing (SIP) and Single-Cell Resonance Raman (SCRR) microspectrometry, fully described in *Taylor et al. (2017); doi: 10.3389/fmicb.2017.01449 and accessible here.*

## Approach

As proof-of-concept, we performed **SIP** experiments on isogenic cultures of the cyanobacterium, *Synechococcus* sp., grown with varying levels of $^{13}$C-bicarbonate.

- *A priori*, fractional $^{13}$C-labeling of cells ($f_{cell}$) is known to be a predictable function of $f_{media}$, isotopic fractionation ($\alpha$), ancestral fractional isotopic signature of the media ($f_0$), and generations completed ($n$) as illustrated in **Fig. 1**.
- Time course sampling demonstrated empirically that population growth rates were unaffected by fractional $^{13}$C-labeling of media ($f_{media}$) **(Fig. 2)**.
- For **SCRR** analysis, populations were subsampled every generation (♦ - **Fig. 2**), preserved with 2% formaldehyde, captured on GTTP membranes, and cells on membrane wedges were freeze-transferred onto mirror-finished stainless steel slides **(Fig. 3)**. {Note: FISH probes can be hybridized against cells on replicate membrane wedges for phylogenetic identification prior to freeze-transfer *(Huang et al. 2007; Environ. Microbiol. 9, 1878)*}.
- Target cells on dry slides were identified by epifluorescence or bright-field illumination **(Figs. 4A, 4B)** on confocal Raman microspectrometer stage **(Fig. 3D)** and locations recorded by mouse clicks. **SCRR** spectra were automatically acquired from all targets in a field (2 sec per cell) (e.g., **Fig. 4C**) using 514 nm laser excitation, then slide was advanced to next field. Three major resonance Raman peaks produced by carotenoids were analyzed for wavenumber (cm$^{-1}$) shifts that indicate degree of cellular isotopic labeling, $f_{cell}$ **(Fig. 4C)**.

Cell labeling described as: $$f_{cell} = \frac{\alpha f_{media}}{1+(\alpha-1)f_{media}} + \left(\frac{\alpha f_o}{1+(\alpha-1)f_o} - \frac{\alpha f_{media}}{1+(\alpha-1)f_{media}}\right)e^{-n\ln(2)}$$

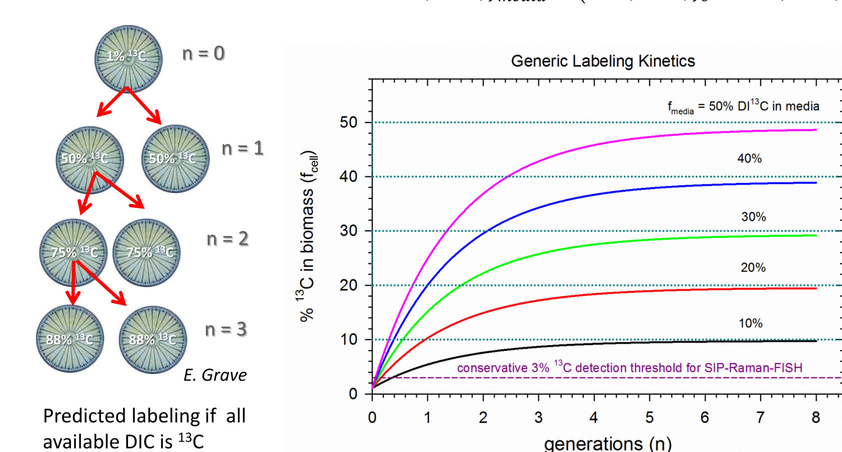



**Fig. 1.** Using growth kinetics to predict $f_{cell}$ through time. (fractionation factor, $\alpha$ = 0.976)

$f_{media}$ = $^{13}$C/($^{12}$C + $^{13}$C)

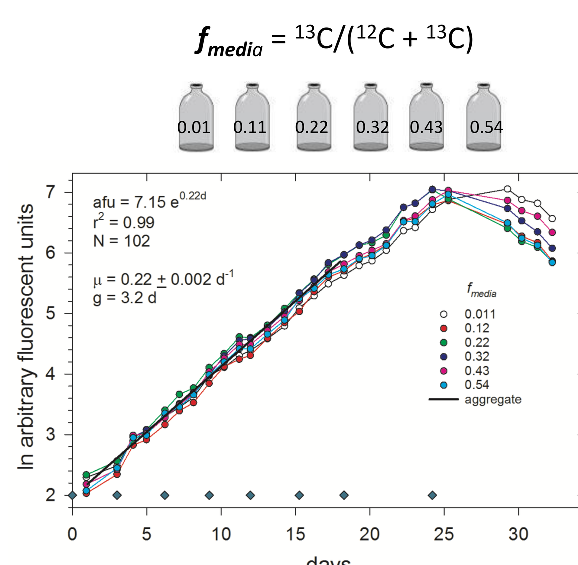



**Fig. 2.** Population growth curves of *Synechococcus* sp. cultured in varying proportions of $^{13}$C-bicarbonate ($f_{media}$) and measured by *in vivo* fluorescence. Total DIC was equivalent in all treatments. (♦ - SCRR samples withdrawn)

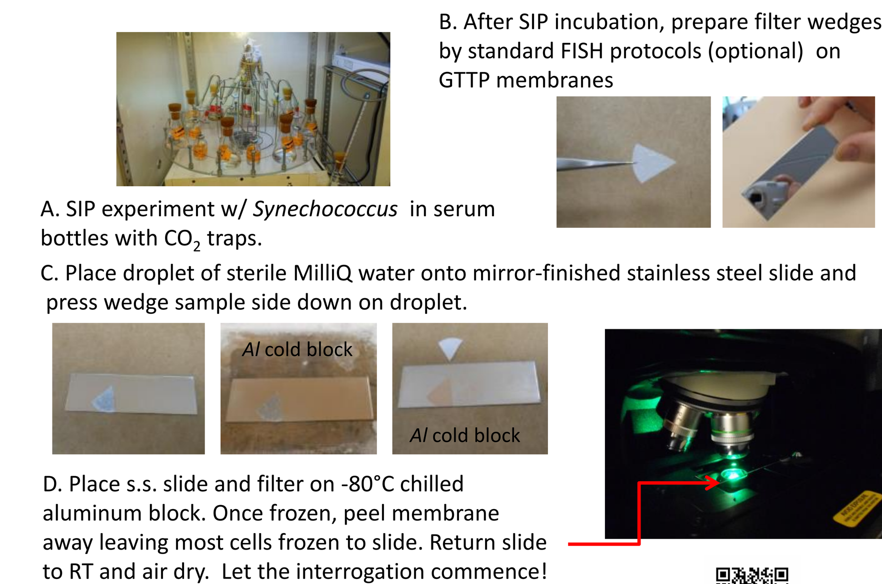



**Fig. 3.** Sample preparation for SIP-Raman-FISH

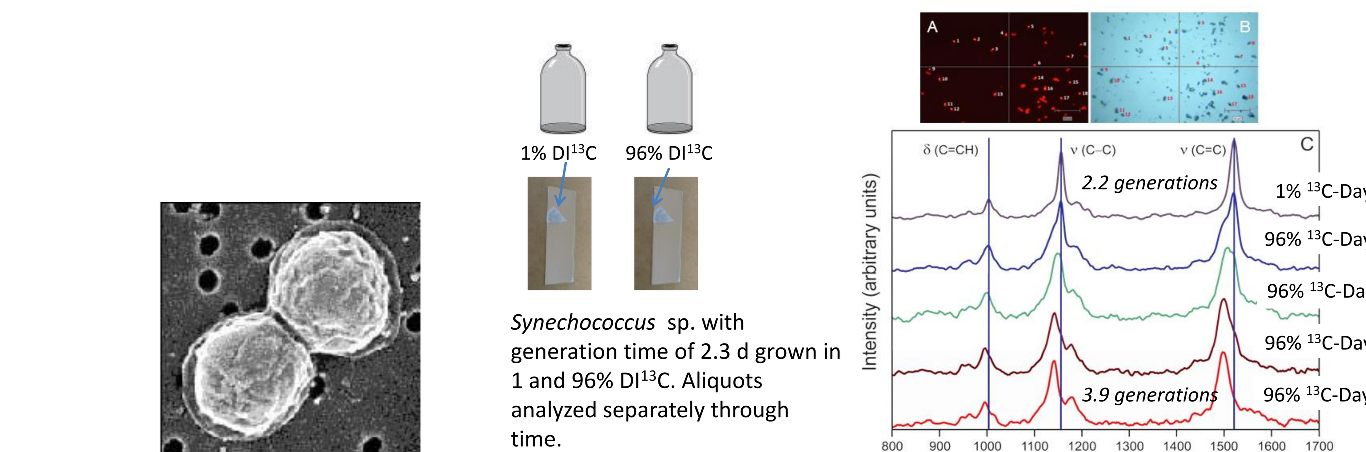



**Fig. 4.** Single-Cell Resonance Raman **(SCRR)** spectral detection of $^{13}$C acquisition through time. **SCRR** spectra of carotenoids were obtained before growth was detectable by *in vivo* fluorescence in dilute cultures.

## Results

**Fig. 5.** Examples of Raman shift peak position (cm$^{-1}$) response to cell acquisition of $^{13}$C in the $f_{media}$ = 0.32 treatment. {Each box and whisker includes 25 randomly selected cells. Circles, bars and boxes = 5th and 95th, 10th and 90th, and 25th-75th percentiles, respectively.}

Wavenumbers of all 3 diagnostic peaks red-shift as cells become isotopically heavier through time and approach minimum values as $f_{cell}$ approaches $f_{media}$ in 5-6 generations. Wavenumbers of all 3 peaks in negative controls (natural $^{13}$C abundances )are constant through time. (dotted lines represent theoretical predictions based on population growth rate).

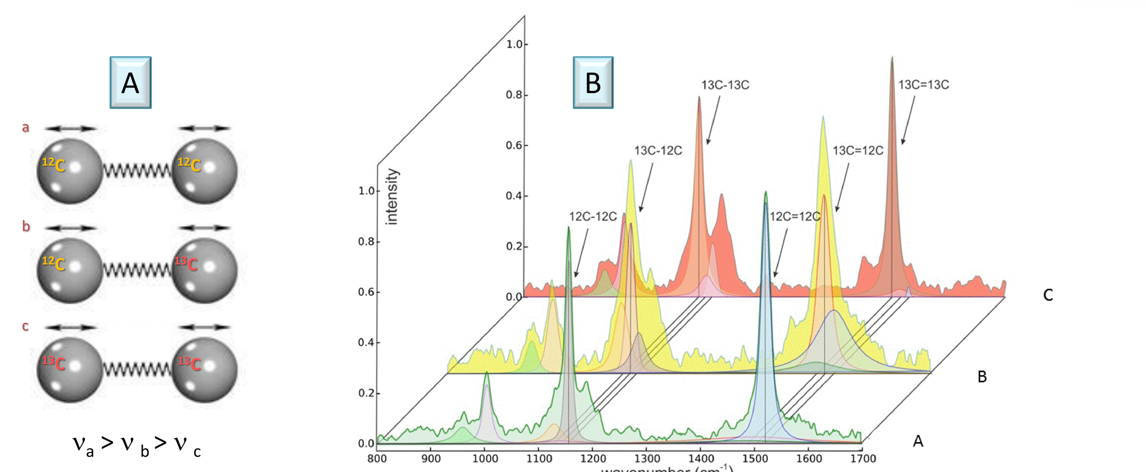
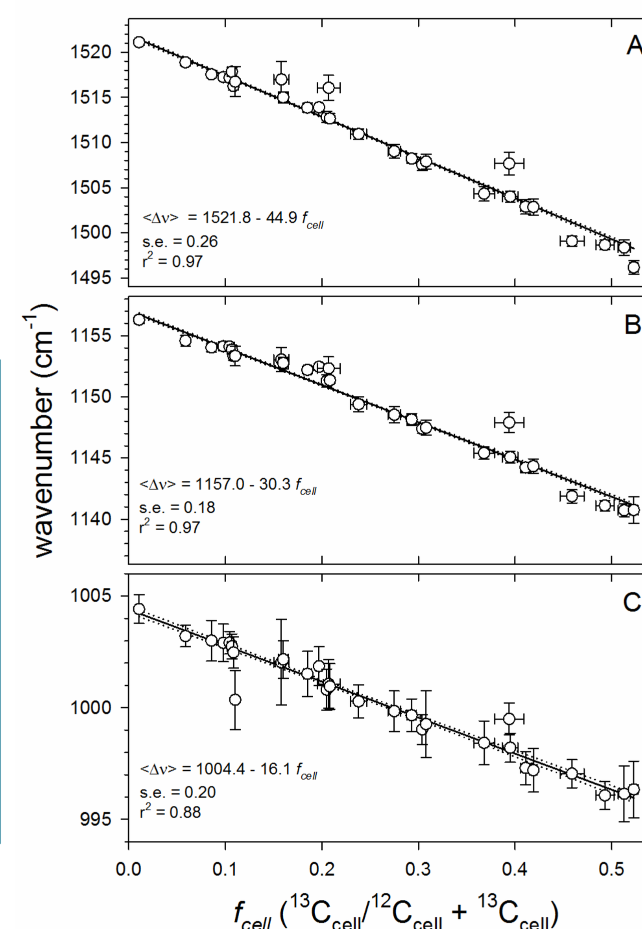



**Fig. 6. (A)** One-dimensional diatomic harmonic oscillator model illustrating how atomic masses of isotopologues dictate vibrational frequency. **(B) SCRR** spectra from *Synechococcus* sp. grown in 1 or 96% DI$^{13}$C analyzed through time.

Illustrates contributions of three isotopologues to triplet curve form as cells become $^{13}$C-enriched. A = natural $^{13}$C abundance, B: $f_{cell}$ = 0.50, C: $f_{cell}$ = 0.88.

**Fig. 7. SCRR** results from all SIP samples generated in time course experiment (♦ in **Fig. 2**). Each point is mean ± 1SD for 25 cells.




- Illustrates that **SCRR** peak positions, $\langle\Delta\tilde{\nu}_x\rangle$, for carotenoids vary predictably across a broad range in cellular $^{13}$C content ($f_{cell}$).
- Limit of detection (LOD) ≈ 3% changes in $f_{cell}$ with current method. Even at lowest $f_{media}$, $f_{cell}$ can be measured within 0.4 generations.

{LOD = 3SD/slope}

Compute single-cell growth rates ($\mu_{sc}$) from SIP-Raman experiment **(Fig. 7)**.

$f_{cell}$ is derived from: $\Delta\tilde{\nu}_{C\text{-}C} = b_0 + b_1 f_{cell}$

*where* $b_0$ = 1157; $b_1$ = -30.3

Knowing $f_{cell}$ (Raman), $f_{media}$ (spiked), and $n = \frac{t}{g} = t\frac{\mu_{sc}}{\ln(2)}$

solve for $n$ (generations) $$n = \frac{1}{\ln(2)}\ln\left(\frac{f_{media} - f_o}{f_{media} - (1+(\alpha-1)f_{media})\frac{\langle\Delta\tilde{\nu}\rangle - b_o}{\alpha b_1}}\right)$$

Knowing $n$ and t, solve for $\mu_{sc}$ (specific growth rate, d$^{-1}$) $\mu_{sc} = 0.693\frac{n}{t}$

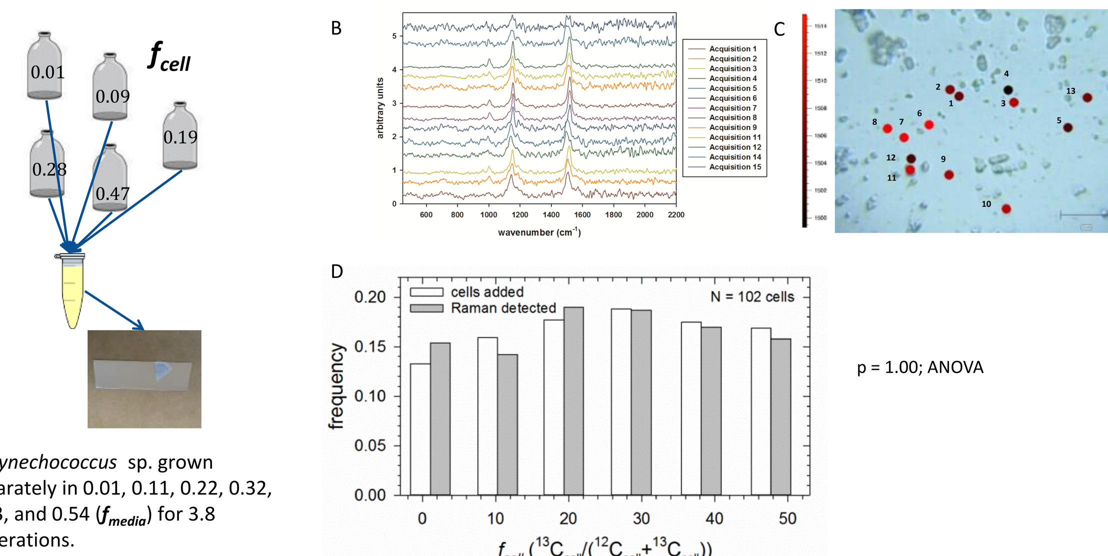



**Fig. 8. (A)** Constructed assemblage of *Synechococcus* sp. populations with distinct $f_{cell}$ labeling. **(B)** Stacked SCRR spectra from cells #1-13. **(C)** Bright field image of cells #1-13 targeted for Raman interrogation in a single field superimposed with $\langle\Delta\tilde{\nu}_{C-C}\rangle$ wavenumber color codes. **(D)** Frequency of occurrence of cells added from each $f_{cell}$ population (open bars) and those detected by SCRR (shaded bars).

- Frequency distributions of populations added and detected by **SCRR** are statistically indistinguishable (p = 1.00; ANOVA).

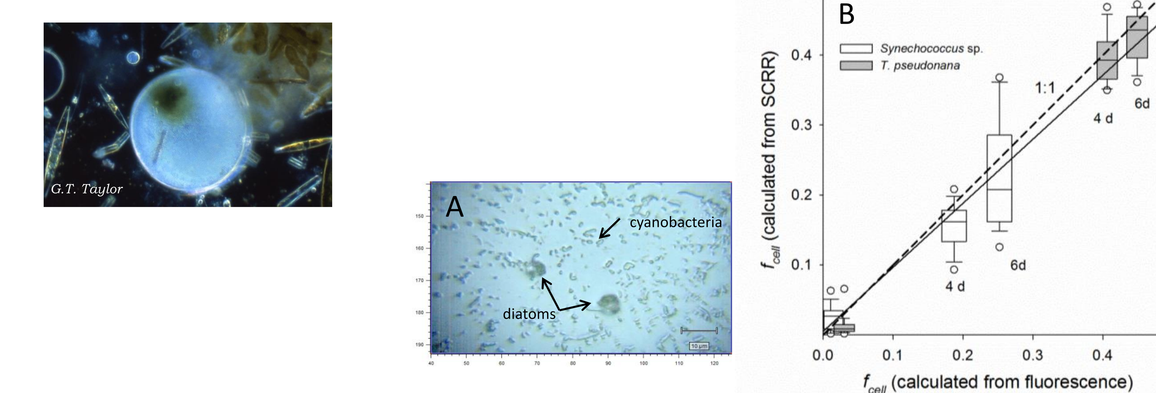



**Fig. 9. (A)** Bright-field image of interrogated cells and **(B)** comparison of measured and predicted $f_{cell}$ in constructed assemblages. Parallel cultures of a fast-growing diatom, *Thalassiosira pseudonana* ($\mu_{pop}$ = 0.48 d$^{-1}$) and a slow-growing cyanobacterium, *Synechococcus* sp. ($\mu_{pop}$ = 0.16 d$^{-1}$) in labeled F/2 media ($f_{media}$ = 0.48) were subsampled through exponential growth. Solid line represents linear regression of all observations (N = 253 cells) including natural $^{13}$C abundance controls ($f_{cell}$ ≈ 0.0107); $f_{cell}$(SCRR) = 0.005 + 0.92±0.02 $f_{cell}$ (fluor), $r^2$ = 0.92.

- Measured $f_{cell}$ values computed from SCRR peak positions $\langle\Delta\tilde{\nu}_{C\text{-}C}\rangle$ appear equivalent to predicted $f_{cell}$ values computed from $\alpha$, $f_{media}$, and $\mu_{pop}$ measured by *in vivo* fluorescence, within the uncertainty of all measurements.

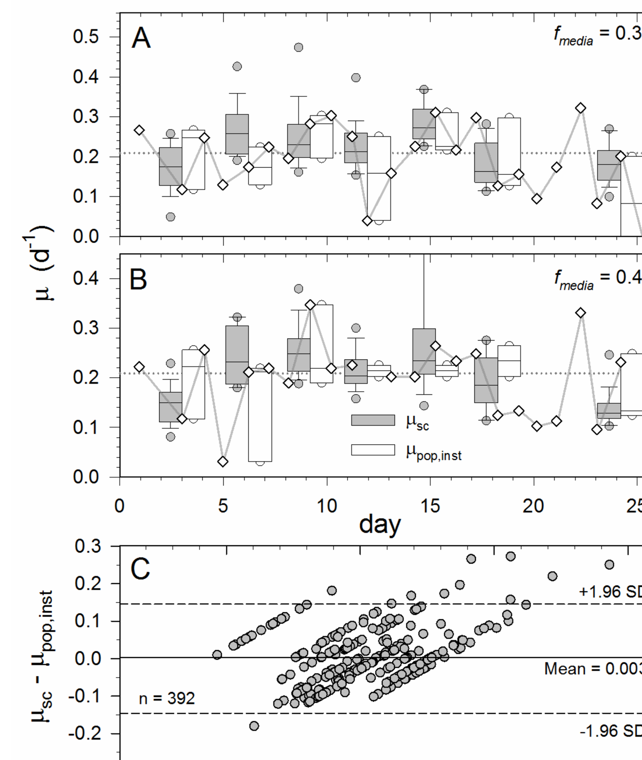


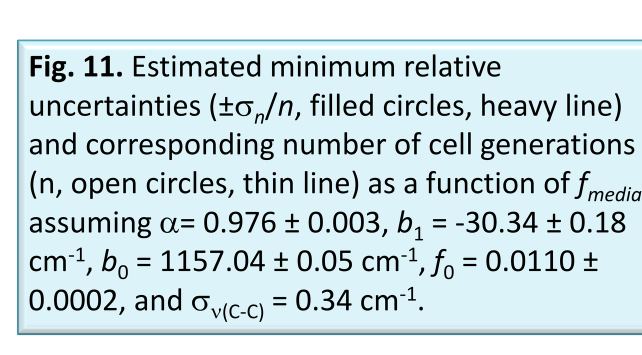

**Fig. 10.** Single-cell growth rates ($\mu_{sc}$) derived from SCRR $\langle\Delta\tilde{\nu}_{C\text{-}C}\rangle$ compared to daily population growth rates ($\mu_{pop,inst} - 0$) from *in vivo* fluorescence time courses for cultures incubated in $f_{media}$ = 0.32 **(A)** and 0.43 **(B)**.

Shaded boxes represent 25 single-cell growth rates ($\mu_{sc}$) and open boxes represent daily population growth rates ($\mu_{pop,inst}$) at $t_{x-1}$, $t_x$ and $t_{x+1}$. Horizontal broken line is mean $\mu_{pop}$ over exponential growth phase (0-18 d).

Bland-Altman plot **(C)** compares results obtained from two independent measurements of growth. (broken horizontal lines =95% C.I.).

- Only 17 of 392 observations fell outside the 95% C.I.
- On average, $\mu_{sc}$ returned a 0.3% higher result than $\mu_{pop,inst}$.
- Variations in $\mu_{sc}$ and $\mu_{pop,inst}$ are statistically indistinguishable.

**Fig. 11.** Estimated minimum relative uncertainties (±$\sigma_n$/n, filled circles, heavy line) and corresponding number of cell generations (n, open circles, thin line) as a function of $f_{media}$ assuming $\alpha$= 0.976 ± 0.003, $b_1$ = -30.34 ± 0.18 cm$^{-1}$, $b_0$ = 1157.04 ± 0.05 cm$^{-1}$, $f_0$ = 0.0110 ± 0.0002, and $\sigma_{\nu(C\text{-}C)}$ = 0.34 cm$^{-1}$.




As a practical compromise between measurement performance, costs, and incubation artifacts such as varying photoperiodicities and pH, we advocate 24 h incubations and $f_{media}$ values between 0.3 and 0.5, where the minimum theoretical relative uncertainty (CV) for the optimum $n$ value (1.5) is between 0.11 and 0.066.

## Summary

- SIP-Raman-FISH sensitively detects stable isotopic assimilation in specific microbial cells and is one of the few tools to directly link function with phylogeny.
- Analysis of wavenumber shifts $\langle\Delta\tilde{\nu}_{C\text{-}C}\rangle$ in Raman scattered photons enables quantitative determination of degree of biomass labeling ($f_{cell}$), a virtual single-cell mass spectrometer.
- Single-cell specific growth rates ($\mu_{sc}$) computed from $f_{cell}$ match $\mu_{pop}$ determined fluorometrically from entire populations.
- However, **SCRR** enables examination of intra- and inter-specific variability in growth, microbial processing of carbon, and other biogeochemically important elements.
- Total propagated analytical error (CV) is typically less than **2%**.
- Sample preparation requirements are relaxed, i.e., live, dried, preserved, frozen, and probe-hybridized cells can all be interrogated under ordinary lab conditions.
- SIP-Raman revealed that single-cell growth rates within a given isogenic *Synechococcus* sp. population could vary by **~27%** (CV) around the mean at any particular time point.
- Similar cell-to-cell variability has been reported for cultures of *Chlorella* and *Chlamydomonas* within mineral oil-encapsulated droplets of media in a microfluidic devices (CV = 27-35%) *(Dewan et al., 2012; Biotechnol. Bioeng. 109, 2987; Damodaran et al., 2014; PLoS ONE 10:e0118987)* and in chemostat cultures of bacteria (CV = 19-51%) using SIP-nano-SIMS *(Kopf et al., 2015; Environ. Microbiol. 17, 2542)*.
- The few lab studies available all demonstrate that even within isogenic populations subjected to uniform environmental conditions, a range of growth phenotypes emerge. Therefore, the range of growth phenotypes and variability of their responses to heterogeneous seascapes in nature can scarcely be assessed with existing information.

## What's Next?

We are keenly interested in forging new collaborations with groups studying or planning to study: (i) parsing of productivity among cohabiting phytoplankton populations, (ii) bottom-up and top-down factors controlling productivity, (iii) plankton responses to heterogeneous microenvironments, (iv) application of agent-based models to planktonic systems, (v) diazotrophy, (vi) C and N flow among planktonic functional groups.

Intrigued? Contact Gordon (gordon.taylor@stonybrook.edu)

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