

Individual cell DNA synthesis within natural marine bacterial assemblages as detected by ‘click’ chemistry

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Supplement. Experimental procedures for testing EdU inhibition on bacterial protein production, as well as detailed descriptions of various tested techniques related to “click” chemistry and interpretation of the observed results. Figures and tables support the supplemental ‘Materials and methods’ and also results described in the main text.

Materials and methods

1. EdU inhibition test on bacterial protein production
2. Additional tests of click reaction application, membrane types, and permeabilization
3. Additional tests for cell counterstaining and slide preparation
4. Confounding effects between copper and DAPI counterstain
5. Apparent EdU labeling in controls

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Materials and methods:

EdU inhibition test on bacterial protein production

Seawater was collected from SIO Pier on October 21, 2009 (Table S1) and dispensed into duplicate polycarbonate bottles (Nalgene). The duplicate aliquots were amended with 0, 20 nM, 100 nM, or 1.0 μM EdU. Incubation bottles were maintained at 20°C. Subsamples (1.7 ml) were removed at 0, 1, 2, 4, and 7 h to determine protein production estimates via ³H-leucine

incorporation (Kirchman, et al. 1985, Simon & Azam, 1989) by the microcentrifugation protocol (Smith & Azam, 1992). Additional subsamples were preserved at the same timepoints by adding formaldehyde to 2% final concentration and cooling to 4°C. Aliquots (5 ml) from each preserved subsample were collected onto 0.2- μ m white polycarbonate filters (Millipore) for abundance estimates via DAPI staining (all timepoints) and for click reaction processing (7 h only).

Additional tests of click reaction application, membrane types, and permeabilization

In addition to the adopted ‘coverslip-inversion’ method described in the text, 2 other techniques for applying reaction cocktail were tested. One used a ‘pool’ approach in which ~100 μ l of reaction cocktail was added to the sample side of the filter piece on a glass slide. Surface tension kept the droplet pool in a dome so that the liquid did not diffuse laterally. A second technique used ‘membrane perfusion’ whereby 12 μ l of reaction cocktail was spotted onto a glass slide and the filter piece was placed atop the cocktail with the sample side up. This is similar to a method for applying the nucleic acid stain SYBRGreen I to enumerate virus particles (Noble & Fuhrman, 1998). The ‘coverslip-inversion’ technique was adopted because the coverslip limits exposure of the click reaction cocktail to air, which decreases the oxidation rate of Cu(I) to Cu(II), the former of which catalyzes the reaction and the latter of which does not. Furthermore, the coverslips provided sufficient coverage of the membrane filter surface. Additional tests of sterile hydrophobic coverslips (Hybrislip Hybridization Covers, 22 x 22mm, No. 247455) gave similar results as non-sterile coverslips.

Among 3 filter membrane types tested (see Main Text), Anodisc membranes were selected because fluorescent signals in cells were high for both DAPI and EdU-conjugated AlexaFluor-488. Labeled cells could be observed on white polycarbonate filters but the filters displayed higher background fluorescence relative to Anodiscs. Cells on black polycarbonate filters produced relatively dim DAPI and AlexaFluor-488 signals.

Polycarbonate and Anodisc filters treated with lysozyme to increase permeabilization of cells displayed high background fluorescence with variable and inconsistent signal from AlexaFluor-488 bound to EdU-labeled cells. In some regions of a single lysozyme-treated filter, labeled cells were abundant and bright against high background fluorescence, while in other regions labeled cells were few and dim against similarly high background fluorescence. Tests that combined pepsin with lysozyme did not reduce the background fluorescence. Conversely, cells treated with no permeabilization step became labeled and untreated filters displayed little background in both blue and green channels. We concluded that cell permeabilization was not necessary to detect EdU-labeled bacterial cells and that enzymatic treatments reduced detection sensitivity because they caused high fluorescence background.

Additional tests for cell counterstaining and slide preparation

Preliminary tests with the mountant VectaShield-DAPI (Vector Laboratories, H-1200) showed that DAPI fluorescence signals for bacteria and EdU-associated AlexaFluor-488 signals were both weak. By contrast, counterstaining via ‘membrane perfusion’ on a drop of DAPI solution followed by mounting in glycerol-based anti-fading solution provided intense cell DAPI fluorescence signal and intense AlexaFluor-488 signal, both with low background. Cell margins were well defined for both DAPI and AlexaFluor-488 signals. To test whether the membrane perfusion approach could be eliminated, we added DAPI directly to the EdU cocktail. Bacteria became stained with DAPI but there was high background fluorescence on Anodisc filters in both blue and green channels, which caused decreased signal-to-background ratios for EdU-labeled

cells. The cause of this may have been nonspecific binding of DAPI to the filter and/or nonspecific interactions of the AlexaFluor488-azide with adherent DAPI.

Confounding effects between copper and DAPI counterstain

We observed that the click reaction caused diminished DAPI fluorescence intensity in cells and that pigmented picoplankton (e.g. *Synechococcus*) displayed diminished autofluorescence in the red channel. Parallel test incubations of reaction cocktail with or without copper demonstrated that it alone accounted for diminished fluorescence intensities. When Cu(II) is added to the reaction buffer, a portion of this is reduced to Cu(I) which is the reaction catalyst. The diminished DAPI and photopigment signals could have been caused by direct interactions between Cu(I) or Cu(II) with cellular macromolecules, though it is not clear. Notably, DAPI and AlexaFluor-488 signal intensities on membrane filters processed by click reaction decreased markedly within 2 d in storage at -20°C. However, for membranes stored dry at -20°C after the initial incubation and fixation steps and which then underwent click reaction processing, the cell signal intensities were bright (yet still faded as usual over ~2 h post-click reaction). In this regard, we observed EdU-labeled cells on membranes that had been stored dry for at least 18 months at -20°C. Thus, the method is amenable to field studies where time may be limited for sample processing.

Apparent EdU labeling in controls

A small but significant percentage of fluorescing cells were observed in unamended controls (Table 1). There are several possible sources. First, some heterotrophic bacteria might emit green autofluorescence. Indeed, *Synechococcus* and other picophytoplankton contain autofluorescent photosynthetic pigments detectable in the green channel, and we subtracted these signals from images during our quantification of labeled cells. However, on DAPI stained filters that did not undergo click reaction, we observed no green autofluorescing bacteria (though we did see green autofluorescent protists and amorphous particles). A second possibility is that wide fluorescence emission spectra from DAPI crossed-over into the green channel. However, this is inconsistent with our observation that nearly all DAPI-stained cells displayed no colocalized signal above background in the green channel for control treatments. A third possibility is that AlexaFluor-488 azide became bound non-specifically to cells without Cu(I) catalysis. A fourth possibility is that the AlexaFluor-azide conjugate underwent click reaction with naturally occurring alkynes in marine bacteria (see Main Text). Future studies should elucidate the mechanism(s) that contribute to background signaling. Such studies may be aided by the use of azides conjugated to other fluorophore colors, which may help differentiate potential interference effects from green and red autofluorescent pigments.

With regard to comparison with microradiography, we note that apparent labeling was higher in EdU control treatments (unamended controls) than for ³H-TdR control treatments (3% - 15% for EdU and 1% - 4% for ³H-TdR). It is not clear what caused the disparity between methods. It may be that the percentage of incorporation-positive cells is dependent on substrate concentration, incubation time, and for ³H-TdR, radiomicrograph exposure time. Previous microradiography studies generally report corrected values but do not report the percentage values for control treatments.

Table S1. Ancillary data for coastal seawater collection. Some values were not determined (nd).

Sample	Location	Depth (m)	Date	Local time	Irradiance ($\mu\text{E m}^{-2} \text{s}^{-1}$)	Chlorophyll ($\mu\text{g l}^{-1}$)	Salinity	Temp ($^{\circ}\text{C}$)
SP1	Scripps Pier	0.5	24. Aug 09	1130	nd	0.61	33.5	22.9
SP2	Scripps Pier	0.5	21. Oct 09	900	nd	0.63	33.5	19.1
SP3	Scripps Pier	0.5	06. Apr 10	800	nd	nd	nd	nd
SP4	Scripps Pier	0.5	13. Apr 10	815	nd	nd	33.4	nd
E1	Offshore (32.39° N, 117.29° W)	30	02. May 10	1840	nd	1.97 (± 0.04)	33.386	13.9
E2	Offshore (32.39° N, 117.29° W)	0.5	02. May 10	1900	4983	0.29 (± 0.001)	33.459	16.9

Table S2. EdU labeling for cultured growing cells (200 - 400 cells analyzed per strain). Cultures were amended with EdU for the duration of growth (9 h). EdU signals were determined at 5 h, and specific growth rates were determined from counts of DAPI-stained cells (experimental details in Main Text). In parallel incubations co-amended with both EdU (20 nM) and thymidine (1 μM), no cells were labeled in any of the 4 strains

Strain	% labeled cells	Labeled cell EdU signal intensity ($\times 10^2$ RFU)		Specific growth rate (h^{-1})
		Mean $\pm 95\%$ CI	Median	
<i>Vibrio corallilyticus</i>	99.2	4538 \pm 471	2786	nd
<i>Flexibacter</i> sp. SBD8	100	358 \pm 22	265	0.052
<i>Roseobacter</i> sp. BBAT1	100	145 \pm 13	128	0.112
Cytophaga strain SB19	not labeled	not labeled	not labeled	0.128

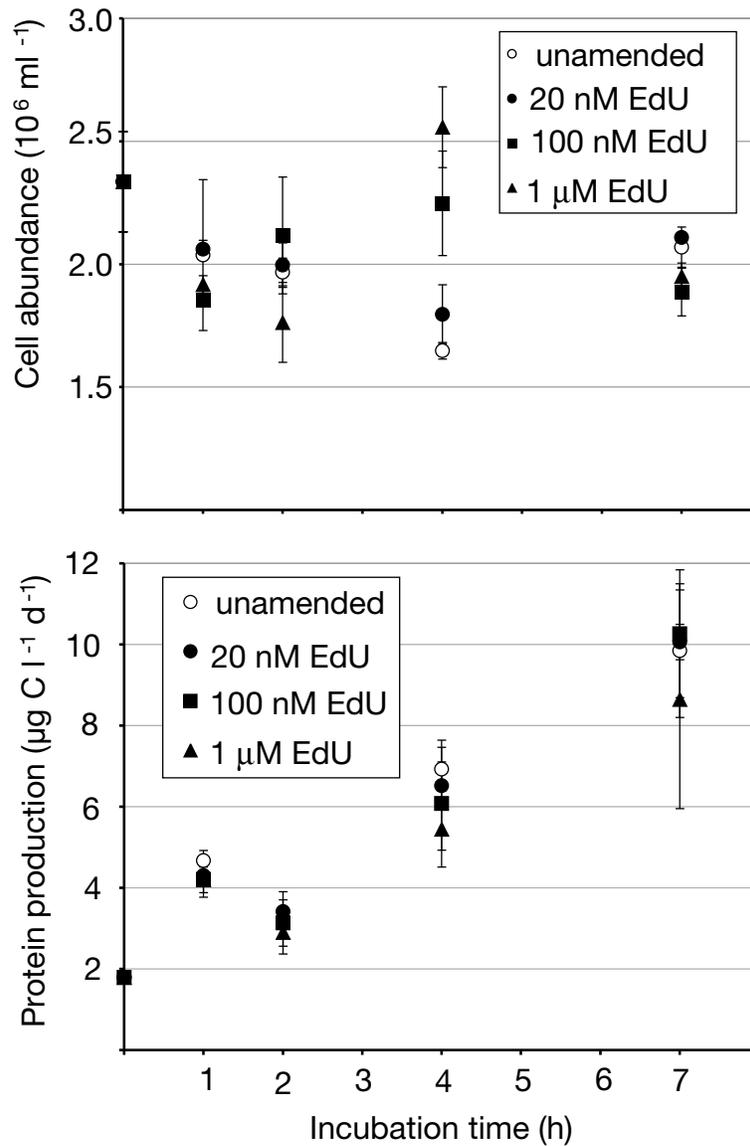


Fig. S1. Time course showing bacterial cell abundance (top) and protein production (bottom) in coastal seawater sample SP-2 at 4 concentrations of ethynyldeoxyuridine (EdU). Error bars show \pm SE of replicate membrane filters.

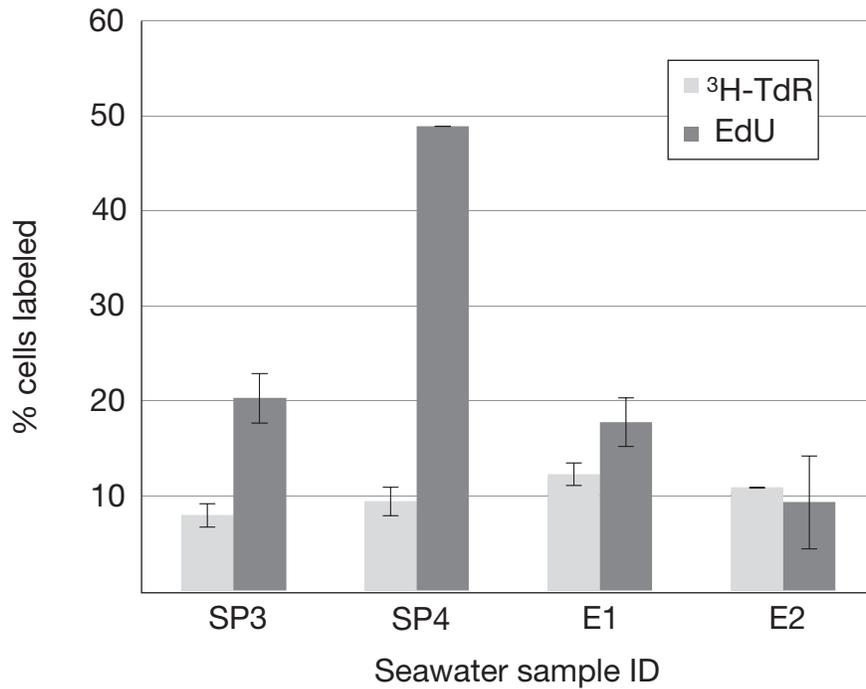


Fig. S2. Comparison of ³H-TdR microradiography and EdU methods for cell labeling on 4 seawater sampling dates. Mean labeling for duplicate membranes is shown (\pm min/max) as determined from \sim 20 microscopic fields per membrane. Method-specific controls were subtracted, i.e. mean percentage values for unamended treatments in each method for each sample.

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