Digging into DNA: Evaluating propidium monoazide as a tool to remove nonviable microbial DNA from deep-sea sediment cores

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ABSTRACT

Deep-sea sediments accumulate detrital material derived from downward particulate flux throughout the water column. An estimated 90% of DNA in deep-sea sediments is contained within dead cells or is extracellular DNA. Viability PCR using propidium monoazide (PMA) has been used to differentiate DNA from live cells and non-living material. We conducted five experiments to test the efficacy of using viability PCR (PMA-qPCR) in algal cultures and deep-sea sediments collected from the Monterey Bay submarine canyon. We tested two methods of DNA extraction and two general 16S bacterial primer sets for DNA quantification. Heat-killed treatments had significantly lower 16S gene copies (ANOVA, p-values << 0.0001) relative to a control treatment, showing that the 4 mM PMA concentration was sufficient to bind and inhibit PCR amplification. However, PMA treatment on live samples had no significant (ANOVA, p-values > 0.05), or barely significant (p-value ~ 0.05), change in gene copies relative to a control treatment, suggesting that there are little to no dead cells in the algal cultures and deep-sea sediments sampled. The DNA extraction method used for control and PMA treatments was insensitive to extracellular DNA due to pelleting. In comparison to standard methods of extraction without dilution and pelleting, there are significantly lower (ANOVA, p-values << 0.0001) 16S gene copies (54.4% lower on average) in the control and PMA treatments that were diluted for PMA photoactivation and pelleted for DNA extraction. This suggests a substantial amount of extracellular DNA despite no detectable dead cells present in the deep-sea sediments

sampled.

INTRODUCTION

Bacterial communities are essential drivers of biogeochemical processes in the marine environment (Azam and Malfatti, 2007). Advances in DNA sequencing technologies have granted independence from laborious cultivation-based methods and allowed for in-depth characterization of these often complex communities and their metabolic potential (Knight et al., 2018). However, current DNA sequencing methodology cannot distinguish between DNA from live versus membrane compromised cells or extracellular DNA (eDNA; Cangelosi and Meschke, 2014). These three groups have different contributions to metabolic processes and, thus, have different functional roles in the environment (Cangelosi and Meschke, 2014). Therefore, their distinction is important in order to properly characterize the living, or viable, community that is either actively growing and metabolizing or dormant.

Using sampling-based simulation models, Lennon et al. (2017) conclude that the bias of including nonviable DNA in community structure analyses are likely specific to ecosystem type. Sediments are particularly at risk of being confounded by nonviable DNA. Extracellular DNA can be released into the environment by both ruptured and living cells (reviewed in Torti et al., 2015), and is integral to lateral gene transfer and biofilm establishment (reviewed in Nielsen et al., 2007; Pietramellara et al., 2009). Effective preservation of eDNA within the sediment, due to complexation with organic and inorganic compounds, such as humic acids, leads to slow degradation rates and long residence times (Levy-Booth et al. 2007). Carini et al. (2016) found that this nonviable, or relic, DNA accounted for an average of 40% of all DNA from terrestrial soils and significantly inflated measurements of diversity. Marine sediments are further susceptible due to benthic-pelagic coupling facilitated by marine snow (Dell'Anno and

Danovaro, 2005). The estimated flux of eDNA to the sediments is 10 and 550 mg DNA m^{-3} $year^{-1}$ in open-ocean and continental margins, respectively (Dell'Anno et al., 2005; Dell'Anno et al., 1999). Recent studies have reported that more than 90% of DNA recovered from marine sediments is extracellular in origin (Dell'Anno and Danovaro, 2005; Lomstein et al., 2012) with a global estimate of 0.30–0.45 gigatons of eDNA in the uppermost 10 cm of deep-sea sediments (Dell'Anno and Danovaro, 2005). This estimate would make deep-sea sediment eDNA the largest reservoir of DNA in the world; at least three orders of magnitude higher relative to marine waters (Torti et al., 2015).

Despite our growing understanding on the importance of nonviable DNA, the scientific community has yet to establish a standard methodology for the differentiation of DNA from live cells and dead material. Several methods of viability analysis have been developed with notable tradeoffs, especially compatibility with flow cytometry for enumeration and sequencing for taxonomic information (reviewed in Cangelosi and Meschke, 2014; Emerson et al., 2017). There are three main types of viability tests: cultivation-, metabolism-, and membrane-based and methods. Cultivation detects living cells by population growth, but is restricted to cultivatable species. Metabolism-based methods use evidence of metabolic activity as evidence of cells being alive. These methods include stable isotope probing (SIP), molecular viability testing (MVT) which detects the synthesis of rRNA precursors, metatranscriptomics, metaproteomics, respiratory measurements such as changes in carbon dioxide, and cellular energy measurements such as ATP production. These methods can have false positives in part due to active extracellular enzymes (Al-Wahaibi et al., 2019; Baltar, 2018). The last set of viability analyses, membrane-based methods, use membrane integrity as a proxy for living cells. These methods include live/dead stains, DNase treatment, and viability PCR using ethidium monoazide (EMA)

or propidium monoazide (PMA). Propidium monoazide is a membrane impermeant, photoactivated dye that intercalates and covalently cross-links into double stranded DNA and inhibits PCR amplification (Nocker et al., 2007). This method is compatible with quantitative polymerase chain reaction (qPCR) and DNA sequencing. Here, we evaluated PMA-qPCR as a method of removing DNA from dead microbes and extracellular DNA in two algal cultures and three deep-sea sediment cores. We tested two methods of DNA extraction and two general bacterial primer sets for DNA quantification.

MATERIALS AND METHODS

Sample Collection

Sediment Cores

Three deep sea sediment cores within the Monterey Bay submarine canyon were collected for analysis (Fig. 1). Push core 52 was collected from 989 m at station "Extrovert Cliff" (36.775915° N, 122.08434° W) on 10 April 2019 aboard the RV Western Flyer cruise MMV19 using the ROV Doc Ricketts dive 1140. Push core 55 was collected from 385 m at a station 100 m from a whale fall (36.79018° N, 121.887262° W) on 11 April 2019 aboard the RV Western Flyer cruise MMV19 using the ROV Doc Ricketts dive 1141. Push core 1 was collected from 588 m at (36.712108° N, 121.995335° W) on 17 June 2019 aboard the RV Rachel Carson using the ROV Ventana dive 4200.

Algal Cultures

Two algal cultures were used for analysis. Samples from five non-axenic subcultures 201-205 (maintained for 133, 104, 72, 42, and 15 days, respectively) of *Bathycoccus prasinos* CCMP1898 were collected on 12 July 2019. Samples from one axenic subculture 350 (maintained for 5 days) of *Micromonas commoda* RCC299 were collected 18 July 2019.

Propidium Monoazide Treatment

Sediment Cores

Treatments are described pictorially in figure 2. Sediment cores were separated into four depth horizons: 0-3 cm, 3-6 cm, 6-9 cm, 12-15 cm. Six 0.1 mL samples were taken using a 1 mL syringe from each depth and added to 5 mL of pre-chilled 0.2 μm filter sterilized artificial seawater (ASW) in 15 mL clear polypropylene tubes. Replicates, in duplicate (PC 52 and 55) or triplicate (PC 1), were treated with 10 μL of 20 mM propidium monoazide (Biotium PMAxx) for a final concentration of 4 mM. The PMA was then photoactivated with 4 cycles of 30 s LED light exposure, 30 s LED light while vortexing, 30 s on ice in the dark. Sample tubes were taped together at the ends and affixed to a vortex adapter 30 cm from the LED light. Overhead laboratory lights were kept off for the duration of the PMA experiments. Another set of replicates, the "controls," were not treated with PMA but were exposed to the same 4 light cycles. The "standard" treatment replicates were not treated with PMA nor exposed to LED light cycles. For push core 1, three secondary control tests were included in triplicate from 3-6 cm: PMA with "1 light cycle," PMA with "10 light cycles," and a "heat killed" treatment which was boiled (5 min 95 °C) and freeze-thawed in liquid N_2 three times then treated with PMA and 4 light cycles. All samples were then stored at -80 °C until DNA extraction.

Algal Cultures

From each subculture, six 1.5 mL samples were transferred to 15 mL clear polypropylene tubes. Three were treated with 3 μL of 20 mM propidium monoazide (PMAxx) for an equivalent 4 mM final concentration. The PMA was photoactivated using the same method as the sediment cores. Three "control" samples were not treated with PMA but were exposed to the same 4 light cycles. For RCC299, two secondary control tests were included in triplicate: a "heat killed"

treatment which was boiled (5 min 95 °C) then treated with PMA and 4 light cycles, and a "heat killed control" treatment which was boiled (5 min 95 °C) and exposed to 4 light cycles but not treated with PMA. All samples were then stored at -80 °C until DNA extraction.

DNA Extraction

Sediment Cores

Several iterations of DNA extraction methods were tested using the DNeasy PowerSoil Pro Kit (Qiagen). Method 1: Samples were first thawed on ice, then vortexed and mixed vigorously to homogenize. 1 mL was removed with a pipette and added to the PowerSoil Pro bead tubes. From here, extraction was carried out according to the manufacturer's instructions beginning with 20 min of mechanical lysis by vortexing the bead tubes and concluding by eluting with 100 μL of solution C6. Method 2: Same as method 1 except bead tubes with sample were treated with 18 μL of 20 mg mL^{-1} proteinase K for a final concentration of 0.35 mg mL^{-1} and incubated at 55 °C while rotating for 2 h. In addition, two technical replicates were taken from each sample, lysed and washed separately, but applied to the same spin column. Samples were eluted with 30 μL of solution C6. Method 3: Same as method 2 except 3 mL of sample was first pelleted by centrifugation for 10 min at 10,000 x g. Supernatant was discarded and 800 μL of CD1 lysis buffer and glass beads from the PowerSoil Pro bead tubes were added to the pellet. Samples were then vortexed for 10 min for mechanical lysis. Samples were then treated with 45 μL of 20 mg mL^{-1} proteinase K for a final concentration of 1.06 mg mL^{-1} and incubated at 55 °C while rotating for 1 h. The manufacturer's instructions were then followed from step 3 using 600 μL for step 4 and 700 μL for step 6. Samples were eluted with 2 rounds of 20 μL solution C6 and waiting 5 min before centrifugation for a final elution volume of 40 μL. All extracted DNA was stored at -80 °C until quantification.

Algal Cultures

A modified DNeasy Mini Plant Kit (Qiagen) extraction method was used for algal culture extractions. Samples were first thawed on ice and vortexed to homogenize. The 1.5 mL sample was transferred to a 1.7 mL microcentrifuge tube and centrifuged at 10,000 x g for 10 min to pellet. The supernatant was removed and discarded with a 1 mL pipette. The pellet was resuspended with 400 μL solution AP1 and transferred to a 2 mL tube with autoclaved glass beads. For CCMP1898 only, the samples were then freeze-thawed three times in liquid N_2 and a 65 °C water bath. Then all samples were placed in a bead beater for 2 min for mechanical lysis. Samples were then treated with 45 μL of 20 mg mL^{-1} proteinase K for a final concentration of 2.25 mg mL^{-1} and incubated at 55 °C while rotating for 1 h. 4 μL of RNase A was added followed by another incubation at 65 °C for 10 min. Samples were vortexed 3 times during the incubation. Further extraction was carried out according to the manufacturer's instructions. Samples were eluted with 2 rounds of 25 μL TE (Tris-EDTA) buffer pre-heated to 65 °C and waiting 5 min before centrifugation for a final elution volume of 50 μL. All extracted DNA was stored at -80 °C until quantification.

Quantitative PCR Assay

Reagent Specifications

Two general bacterial 16S rRNA gene TaqMan real-time qPCR assays were used for analysis (Fig. 3). The first assay was adapted from Nadkarni et al. (2002) with the following sequence specifications. Forward primer 5'-TCCTACGGGAGGCAGCAGT-3'; reverse primer 5'-GGACTACCAGGGTATCTAATCCTGTT-3'; and TaqMan MGB probe (6-FAM)-5'-CGTATTACCGCGGCTGCTGGCAC-3'-(MGBNFQ) (Applied Biosystems). The second assay was adapted from Suzuki et al. (2000) with the following sequence specifications. Forward

primer 5'-CGGTGAATACGTTCYCGG-3'; reverse primer 5'-GGWTACCTTGTTACGACTT-3'; and TaqMan MGB probe (6-FAM)-5'-CCTGTACACACCGCCCGTC -3'-(MGBNFQ) (Applied Biosystems). Both primer sets were diluted to 9 μM with TE (Tris-EDTA) buffer at pH 7.5 for a final concentration of 0.9 μM in the assay and stored at -20 ℃. Both probes were diluted to 2.5 μM with TE buffer at pH 7.5 for a final concentration of 0.25 μM in the assay and stored covered at -20 ℃. TaqMan Universal PCR Master Mix was used at a final concentration of 1x in the assays.

Standards Specifications

Quantitative PCR assay standards were generated from plasmid amplification. A plated bacterial colony was added to 10 μL MilliQ water and heat shocked at 95 ℃ for 5 min. PCR was performed to amplify the 16S rDNA gene using 1 μL of the boiled colony and using either the Nadkarni et al. 2002 or Suzuki et al. 2000 primers as detailed above at a final concentration of 0.27 μM. The PCR reaction conditions for the Nadkarni et al. 2002 primers were 95 ℃ for 15 min, and 35 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min, followed by 72 ℃ for 7 min and 4 ℃ hold. The PCR reaction conditions for the Suzuki et al. 2000 primers were 95 ℃ for 15 min, and 35 cycles of 94 ℃ for 30 s, 56 ℃ for 30 s, and 72 ℃ for 1 min, followed by 72 ℃ for 7 min and 4 ℃ hold. Amplification was verified with gel electrophoresis by observing a band around the expected 466 bp range (Nadkarni et al. 2002) or 120 bp range (Suzuki et al. 2000). The amplified 16S gene was inserted into a plasmid using the TOPO TA kit. The reaction was carried out at room temperature for 30 min then stored at -20 ℃ overnight. The transformation was performed by adding 2 μL of the reaction to One Shot *E. coli*. The mixture was incubated on ice for 10 min, then heat shocked for 30 s at 42 ℃ in a water bath. 250 μL of S.O.C. medium at room temperature was added to the mixture and then incubated at 37 ℃ for 1

h continuously rotating. 200 μL of transformed plasmid was spread onto a LB plate treated with kanamycin and 40 μL X-Gal. *E. coli* carrying the plasmid were grown overnight at 37 °C. Plasmid insertion was verified from white colonies using PCR and gel electrophoresis. The remaining colony was suspended in 5 mL LB broth with 5 μL 50 mg L^{-1} kanamycin and incubated at 37 °C shaking 212 rpm overnight. 4 mL of the culture was centrifuged at 10,000 rpm for 3 min, and the supernatant discarded to concentrate the cells. The plasmid was then extracted using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions and eluted in 50 μL TE buffer pH 8. The plasmid was quantified using a Thermo NanoDrop One and diluted with TE buffer pH 8 to standard curve concentrations of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 plasmid copies per qPCR reaction well and stored at -20 °C.

PCR Conditions

The reaction conditions using the Nadkarni et al. 2002 primers were 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The reaction conditions using the Suzuki et al. 2000 primers were 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 56 °C for 1 min.

qPCR Efficiency Calculations

Inhibition was checked by spiking samples with 10^6 plasmid concentrations and calculating inhibition efficiency using 1-(Ct spiked – Ct of 10^6)/(Ct of 10^6)*100 with a 96% cutoff testing several dilutions. Primer-probe efficiency was calculated using (10^(1/slope) – 1)*100.

Statistical Analysis

All statistical analyses were performed using the R statistical software version 3.4.3. Data were natural logarithm, base ten logarithm, or square root transformed where necessary and

normality was assessed with the Shapiro–Wilk test. T-tests or analysis of variance (ANOVA) and Tukey post hoc tests were conducted to assess significance. P-values were adjusted for multiple comparisons. Statistical significance was based on the 0.05 cutoff for adjusted p-values.

RESULTS

DNA Extraction

Between the three methods evaluated for DNA extraction from sediment cores, methods 1 and 2 were ineffective while method 3 was effective at extracting DNA to a high enough yield for Qubit or qPCR quantification. In all cases, experimental and extraction blanks were below the detection limit of the Qubit and qPCR assay.

Quantitative PCR Assay

Between the two general bacterial 16S gene assays developed for qPCR, the primer/probe set based on Nadkarni et al. (2002) was ineffective with an amplified gene length of 466 bp, cycle threshold (CT) values between 24 and 44 cycles for the specified standards, and an average qPCR efficiency of 84.94%; while the primer/probe set based on Suzuki et al. (2000) was effective with an amplified gene length of 122 bp, CT values between 14 and 37 cycles for the specified standards, and an average qPCR efficiency of 98.21%. Standards 10^1 and 10^0 were not included in analyses since their CT values were close to the no template controls. Samples were diluted to DNA concentrations between 0.1 – 0.5 ng reaction^{-1} in order to reach inhibition efficiencies above 96%.

Experimental Treatments

Sediment Cores

PC 52: There was a significant difference in average 16S rRNA gene copies normalized to ng of DNA between the control and PMA treatment for the 0-3 cm depth horizon (Fig. 4-A;

ANOVA, p-value = 0.0466). There were significant differences between the standard and control and between the standard and PMA treatments (ANOVA, p-values << 0.0001) for the 0-3 cm depth horizon. All other depth horizons were not included in analysis.

PC 55: There were no significant differences in average 16S rRNA gene copies untransformed and normalized to ng of DNA between the control and PMA treatments for the 0-3 cm depth horizon (Fig. 4-B; ANOVA, p-value > 0.05). There were significant differences between the standard and control (ANOVA, p-value = 0.012) and between the standard and PMA treatments (ANOVA, p-value = 0.004) for the 0-3 cm depth horizon. All other depth horizons were not included in analysis.

PC 1: There were no significant differences in average 16S rRNA gene copies logarithm base 10 transformed and normalized to ng of DNA between the control, PMA, diluted standard, and ten light cycle treatments for the 3-6 cm depth horizon (Fig. 4-C; ANOVA, p-value > 0.05). There were significant differences between the heat-killed PMA treatment and the control, PMA, diluted standard, and ten cycle treatments (ANOVA, p-values < 0.0001) for the 3-6 cm depth horizon. All other depth horizons were not included in analysis.

Algal Cultures

CCMP 1898: Overall, there were no significant differences in average 16S rRNA gene copies natural logarithm transformed and normalized to ng of DNA between the control and PMA treatments for the 201-205 cultures (Fig. 5-A; ANOVA, p-values > 0.05). Separately, there was a significantly lower average for the PMA treatment for culture 202 (t = -6.759, df = 2, p-value = 0.0212). Only one biological replicate was analyzed for each treatment for each culture, so technical replicates were used for analysis.

RCC 299: There were no significant differences in average 16S rRNA gene copies square

root transformed and normalized to ng of DNA between the control and PMA treatments (Fig. 5-B; ANOVA, p-values > 0.05). There were significant differences between the heat-killed control treatment and the control, PMA, and heat-killed PMA treatments (ANOVA, p-values << 0.0001) and between the heat-killed PMA treatment and control and PMA treatments (ANOVA, p-values p-values << 0.0001).

DISCUSSION

Deep-sea sediments accumulate detrital material leading to an expectation that DNA from dead cells or eDNA is present in the sediments. In our experiment using *Micromonas commoda* RCC299 cultures, heat-killed treatments had significantly lower 16S gene copies (ANOVA, p-value < 0.05) relative to a control treatment, showing that the 4 mM PMA concentration was sufficient to bind and inhibit PCR amplification. However, the PMA treatment had no significant (ANOVA, p-value > 0.05) change in gene copies relative to a control treatment in all experiments, except within PC 52 which was barely significant (p-value = 0.0466). It is likely that the algal cultures, particularly the relatively young subculture of *Micromonas commoda* RCC299 that was used, did not contain dead cells.

In the sediments, despite numerous studies that report significant eDNA and dead cell loads (Carini et al., 2016; Dell'Anno and Danovaro, 2005; Hellein et al., 2012; Lennon et al., 2017; Lomstein et al., 2012), our results suggest that there is no detectable dead biomass retaining DNA in algal cultures or deep-sea sediments. However, our extraction method, particularly the pelleting step, makes our evaluation insensitive to extracellular DNA that is not particle attached. If we compare our standard method of extraction from sediment, i.e. without suspension in artificial seawater, to our control treatment which is suspended and later pelleted, there is significantly more DNA and 16S gene copies (ANOVA, p-value < 0.05) recovered from

our standard method. This suggests that there is abundant extracellular DNA present in these sediments.

The detection of dead cells in our study may have been limited by the high biological variability across replicates. This variability could be minimized by mixing the sediments within the depth horizon before sample allocation to suppress small scale heterogeneity (as in Mincks et al., 2005). Furthermore, we were unable to recover eDNA in our control and PMA treatments because we needed to first pellet the samples before DNA extraction in order to get a reasonable DNA yield. We speculate that the salts in the artificial seawater interfered with the DNA extraction method. This could be resolved by using PBS which has a lower salt content and might allow for eDNA recovery using the PowerSoil kit. Another method of desalination might also allow for eDNA recovery. Nonviable DNA might be also lower than expected because of DNases (Al-Wahaibi et al., 2019).

Other studies have reported issues with PMA-qPCR (Elizaquivel et al., 2012; Li et al., 2017; Lovdal et al., 2011; Pan and Breidt, 2007; Varma et al., 2009) for samples with complex communities, high biomass, and a high ratio of dead to living material. For marine sediments, other techniques, such as treatment with DNase (Dell'Anno and Danovaro, 2005) or RNA-based sequencing (Li et al., 2017), might be more effective alternatives.

Establishing a standardized method of viability analysis that is compatible with DNA sequencing is imperative given the growing recognition of the importance of eDNA in structuring marine microbial communities, from supplying phosphorus (Dell'Anno and Danovaro, 2005), establishing biofilms (Pietramellara et al., 2009), laterally transferring genetic material (Nielsen et al., 2007), and documenting the past microbial community (Carini et al., 2016).

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FIGURES

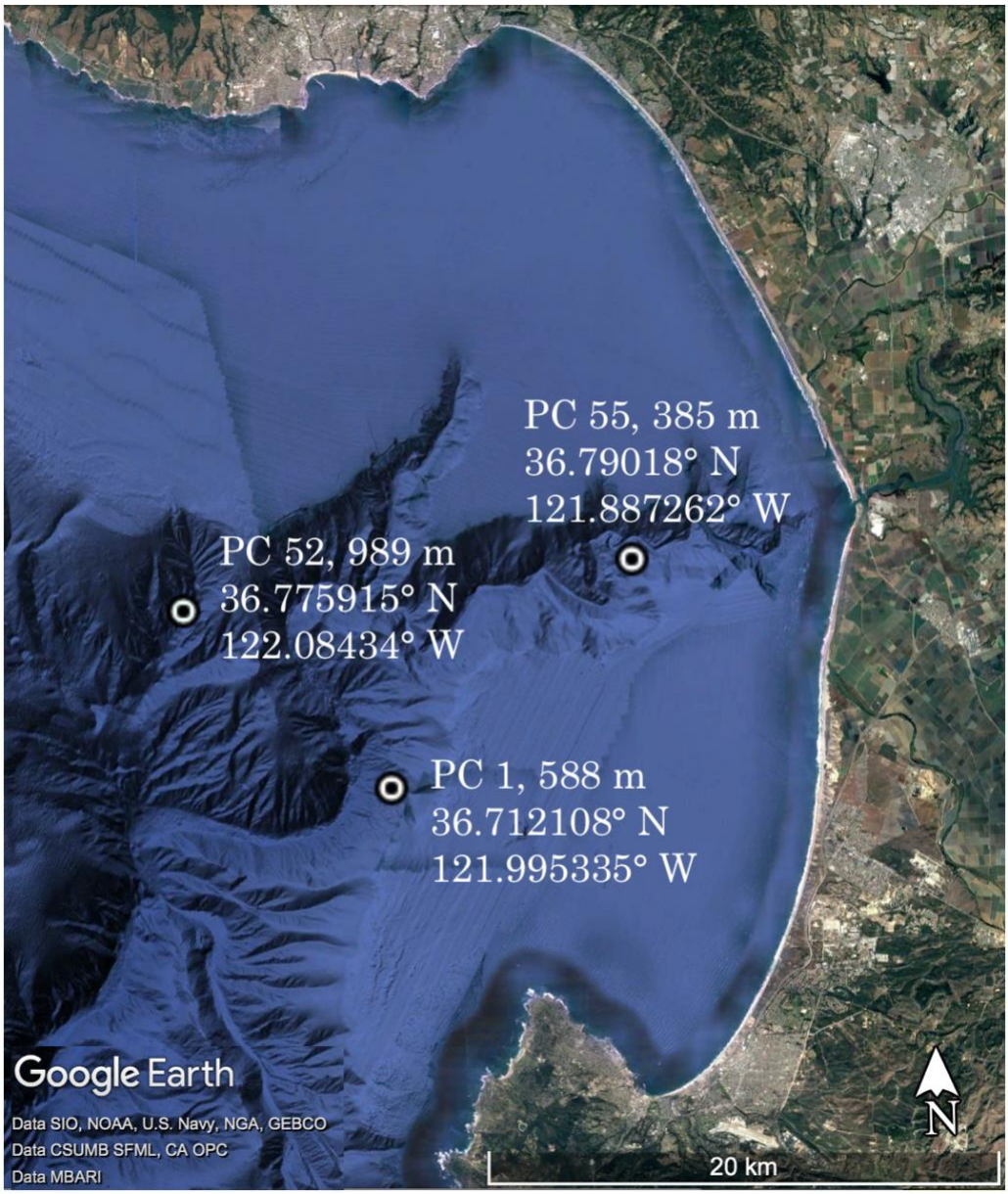

Figure 1. Push core collection locations in the Monterey Bay submarine canyon.

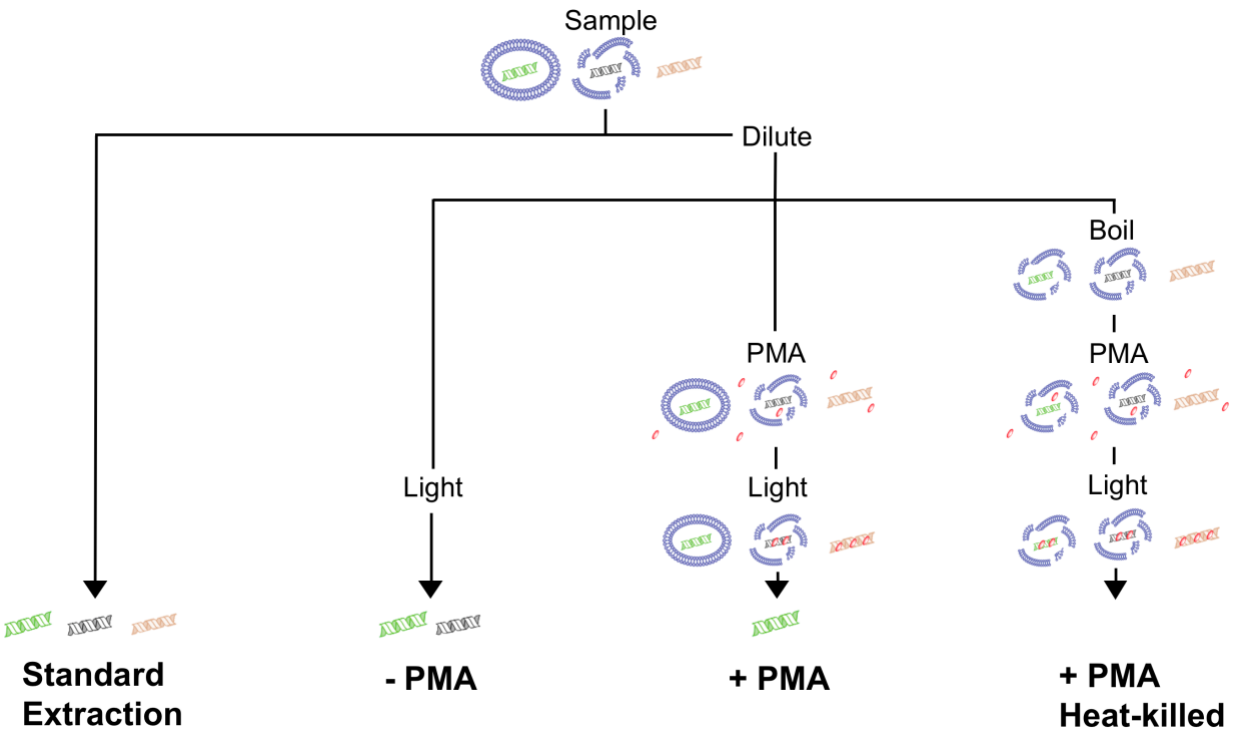

Figure 2. Schematic representation of sample processing for the PMA-qPCR viability assay. DNA yield from live (green), dead (grey), and extracellular DNA (orange) are shown at the terminal ends of the four treatments.

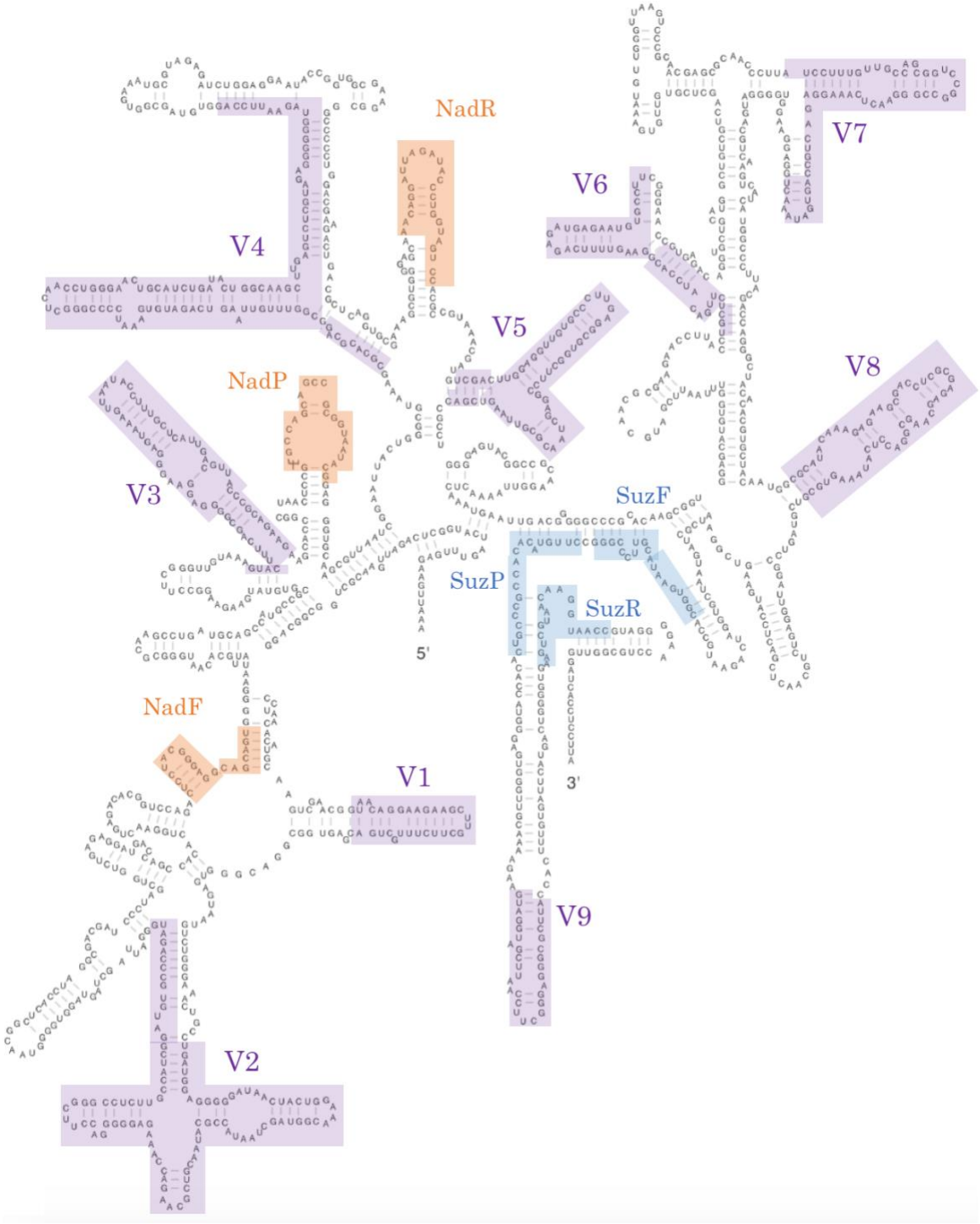

Figure 3. Primer (F/R) and probe (P) locations on the 16S rRNA gene. Orange and blue locations based on Nadkarni et al. (2002) and Suzuki et al. (2000), respectively. Purple locations correspond to hypervariable regions. Secondary structure of the 16S rRNA of *Escherichia coli* based on Yarza et al. (2014).

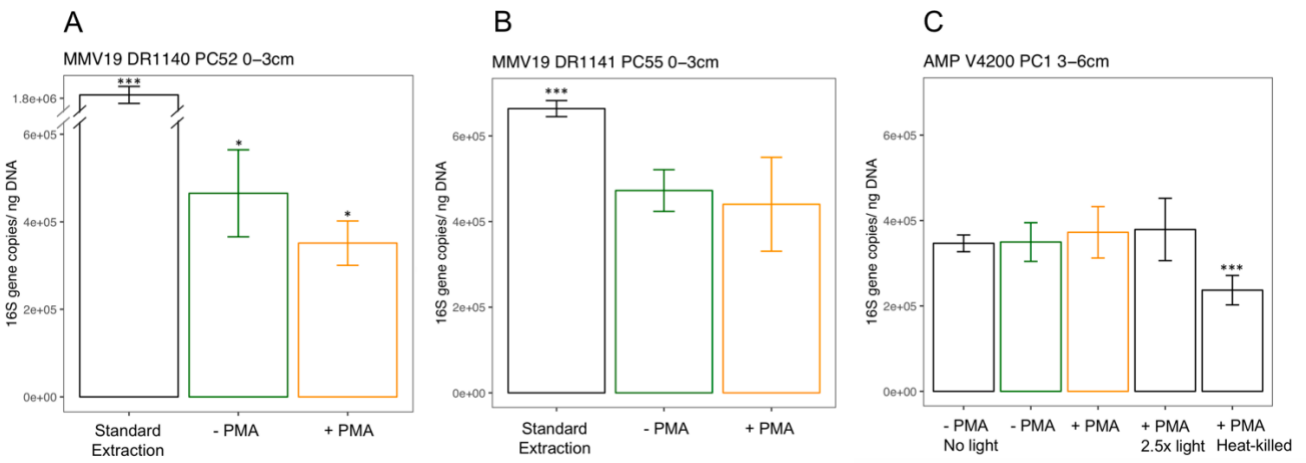

Figure 4. Average 16S gene copies normalized to ng of DNA for sediment push cores 52 (A), 55 (B), and 1 (C). Control treatments are highlighted with green bars and PMA treatments with orange. Error bars represent one standard deviation from the mean. For push core 52 and 55, the standard extraction treatment had one replicate and the two other treatments had two replicates, each with three technical replicates. For push core 1, each treatment had three biological replicates with three technical replicates. Asterisks denote treatments that are significantly different from all the other treatments (ANOVA, * p-value < 0.05, *** p-value < 0.0001).

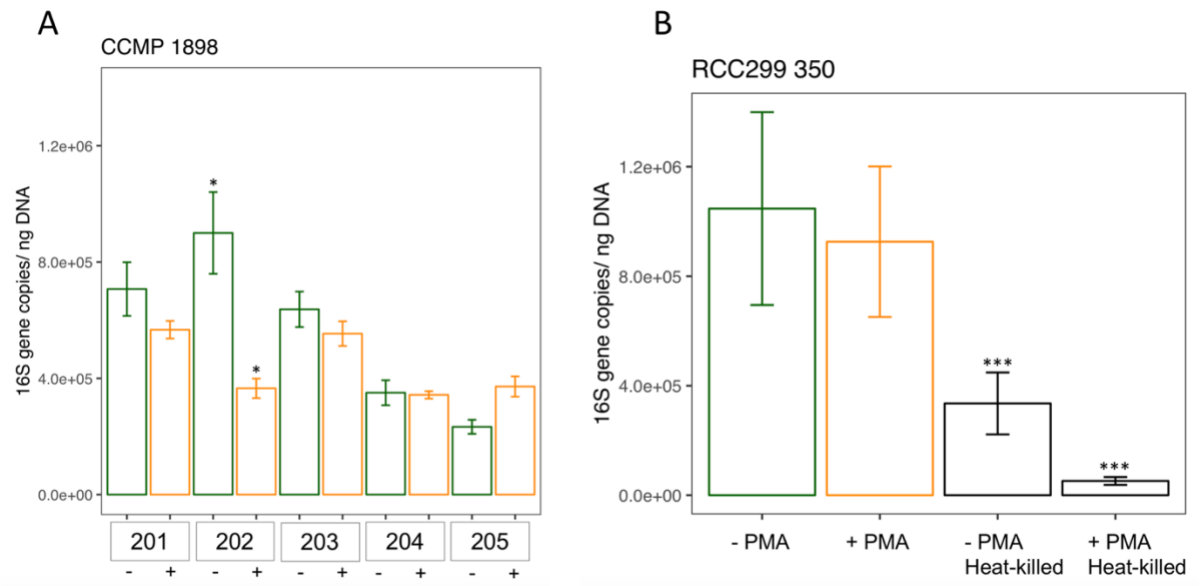

Figure 5. Average gene copies normalized to ng of DNA for algal cultures CCMP 1898 (A) and RCC 299 (B). Control treatments are highlighted with green bars and PMA treatments with orange. Error bars represent one standard deviation from the mean. Each treatment had three biological replicates with three technical replicates, except for CCMP 1898 where only one biological replicate was analyzed from each treatment. Asterisks denote treatments that are significantly different from all the other treatments (ANOVA, * p-value < 0.05, *** p-value < 0.0001).