Environmental Microbiome

RESEARCH Open Access

Development of a quantitative PMA-16S rRNA gene sequencing workflow for absolute abundance measurements of seawater microbial communities

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Abstract

Background Ecological risk assessments rarely consider the impacts of environmental stress on microbial communities. Incorporating microbial community responses into these evaluations requires establishing sensitivity thresholds based on the absolute abundance of viable taxa. While essential for describing microbial community dynamics, sequencing-based analyses are typically limited to relative proportions and fail to reveal the magnitude or directionality of abundance shifts. This study presents a workflow that combines propidium monoazide (PMA) treatment and microbial load estimates with 16S rRNA gene amplicon sequencing and quantitative microbiome profiling (QMP) to assess the absolute abundance of viable taxa in seawater microbiomes.

Results Using natural seawater, microbial load estimates from droplet digital PCR (ddPCR) and flow cytometry (FC) correlated strongly for total and intact cell counts, confirming the suitability of both methods for normalising 16S rRNA gene amplicon sequencing data. We demonstrated that PMA at concentrations of 2.5–15 µM effectively inhibited PCR amplification of DNA from membrane-compromised cells, reducing 16S RNA gene copies by 24–44% relative to untreated samples. Samples with known proportions of intact cells were generated by mixing heat-killed and natural seawater, enabling absolute abundance assessments by normalising 16S rRNA gene amplicon sequencing data to intact cell loads estimated via ddPCR and FC. This approach facilitated detailed comparisons of the effects of QMP versus relative microbiome profiling (RMP) on alpha and beta diversity metrics and on relative and absolute amplicon sequence variant (ASV) abundance profiles. Unlike RMP, QMP captured significant shifts in the microbial community composition across samples with decreasing proportions of intact cells. While RMP failed to detect abundance changes at ASV-level, QMP revealed consistent abundance declines.

Conclusion This workflow enhanced the accuracy in representing microbial community dynamics by addressing key limitations of RMP such as the inclusion of damaged cells or extracellular DNA and the misleading proportions

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of identified taxa. It is particularly suited for quantifying the magnitude and direction of changes in taxa abundance following stress exposure, making it directly applicable to microbial stress-response modelling.

Keywords Microbial ecotoxicology, Marine Microbiome, Response modelling, Cell viability, PMA, Absolute quantification, Flow cytometry, ddPCR

Background

Microbial ecotoxicology is an interdisciplinary field that integrates microbial ecology, biochemistry, and traditional (eco)toxicology, and aims to provide a holistic approach to understand microbial communities response dynamics under environmentally realistic conditions [1]. High-throughput sequencing technologies and associated bioinformatics have enabled the field to significantly advance and achieve detailed assessments of stressor effects at multiple biological levels [2–7]. However, current methods fail to meet the requirements for establishing regulatory guidelines for aquatic ecosystems, which demand quantitative derivation of environmentally relevant effect thresholds [8, 9]. To address this, cultivation-independent genomics tools should be integrated with traditional ecotoxicology modelling to establish quantitative microbial sensitivity thresholds for use in regulatory, management, and conservation frameworks [10–12].

Next-generation sequencing (NGS) techniques are widely applied to infer the relative composition of microbial communities by amplifying the 16S rRNA gene with universal primers and quantifying microbial taxa as proportions of the sample sequence library [13, 14]. While NGS amplification methods allow for culture-independent microbial community characterisation, they are restricted in differentiating viable or metabolically active cells from nonviable cells and extracellular DNA, leading to consistent overestimation of viable taxa fractions [15]. This limitation is particularly problematic for microbial ecotoxicology testing at the community level, where concentration-response modelling should solely focus on viable community members, analogous to survival assessments conducted with a single species [11].

Cell viability and activity assessments, compatible with subsequent NGS, range from RNA analyses and stable-isotope probing to DNA-binding dyes such as propidium monoazide (PMA) [15–18]. PMA is commonly used as an efficient and cost-effective method to discriminate intact cells based on membrane integrity by selectively binding to the DNA of membrane-compromised cells, thereby preventing PCR amplification [15, 16, 19, 20]. In combination with quantitative PCR (qPCR), 16S rRNA gene amplicon sequencing, and shotgun metagenomics, PMA has been applied to assess intact cell proportions in a variety of sample types, including soil [21], fish [22], faecal cells in seawater [23], and anoxic analogue environments such as permafrost, salt mines, acidic lakes, and sulfur springs [24]. However, factors including microbial community characteristics, dye concentration, light exposure, and sample conditions (e.g., turbidity and salt content) can influence PMA performance, highlighting the need for further optimisation, especially in low-biomass environments such as natural seawater [15, 25].

In addition to assessing cell viability, accurately quantifying the magnitude and direction of microbial responses requires converting relative abundances into absolute values. Since NGS data are inherently compositional, an increase in the relative abundance of one taxon inevitably leads to decreases in others, regardless of actual changes in population size [26–28]. To overcome this limitation, several approaches can be employed to determine absolute taxa abundance using defined "anchor" points, such as spike-in standards or microbial load estimates obtained through cell enumeration or molecular quantification techniques [27, 29–31]. For example, quantitative microbiome profiling (QMP), based on normalising sequencing data to absolute abundance values using microbial cell counts obtained via flow cytometry (FC), has shown utility across diverse sample types [27, 28, 32–34]. However, further validation is needed to apply this approach in stress-response modelling for microbial ecotoxicology testing.

This study presents a PMA-16S rRNA gene amplicon sequencing workflow, coupled with QMP, for application in microbial ecotoxicology using indigenous seawater microbiomes as model communities (Fig. 1). The objectives were to: (i) assess the effectiveness of cell-based anchoring measured by FC versus molecular-based anchoring measured by droplet digital PCR (ddPCR); (ii) determine optimal PMA concentration for excluding DNA from membrane-compromised cells and extracellular DNA; and (iii) evaluate how the abundance data transformation (relative vs. absolute) influences the interpretation of microbial community dynamics.

Methods

Seawater sampling

Seawater samples were collected from a nearshore coastal site (19°16′38.4"S, 147°03′32.1"E) located at the Australian Institute of Marine Science, Townsville, Queensland. Collection was performed using a submersible pump (750 GHP, Rule-Mate, Xylem Inc., USA) at a depth of 1 m and subsequently filtered with a canister filter equipped with a 4.5 µm absolute filter cartridge (Synopex, Parkway Process Solutions, Australia). For each sampling event, 40 L of seawater were collected into polyethylene carboy

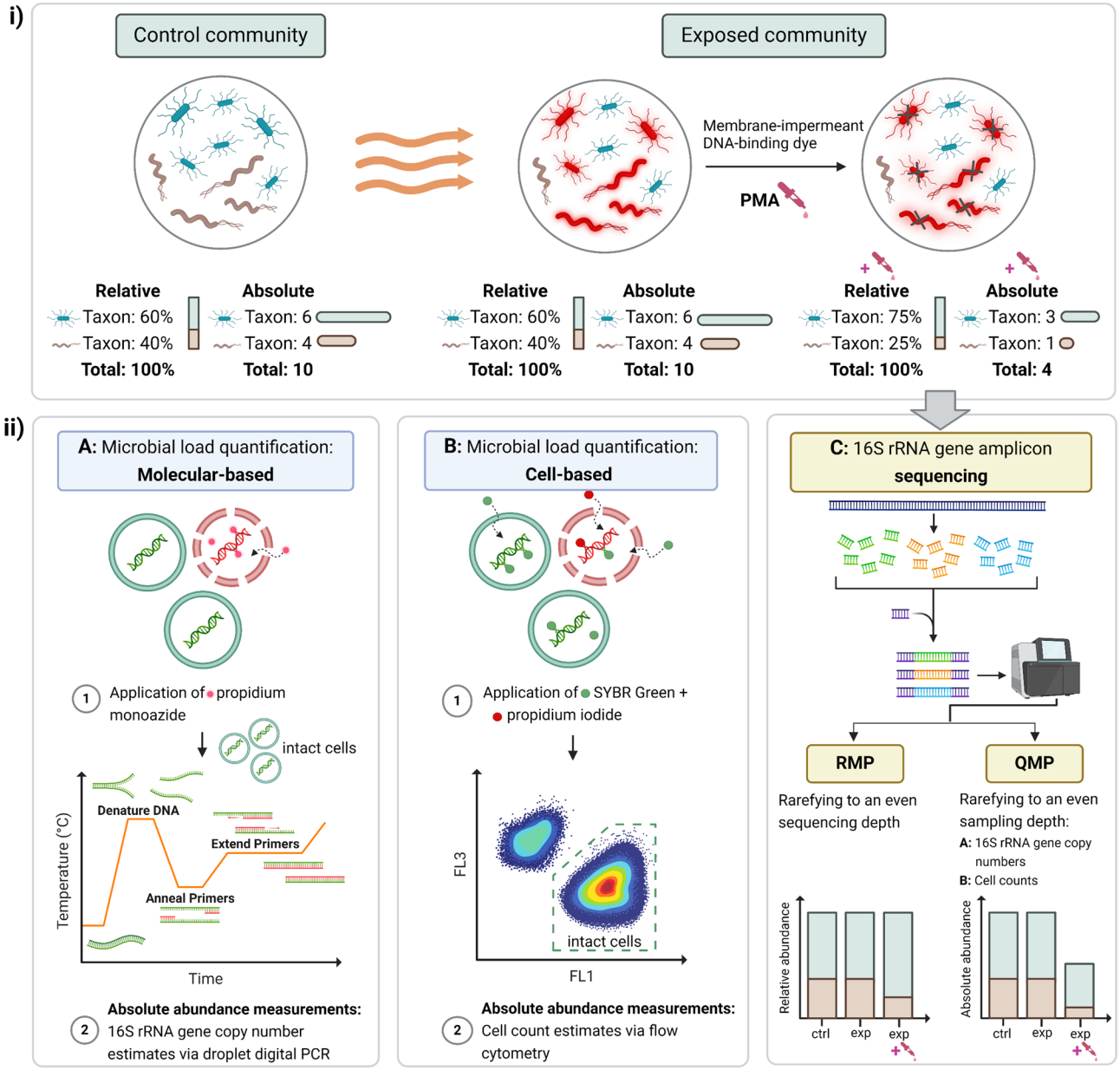

Fig. 1 Conceptual diagram illustrating the quantitative workflow for assessing changes in absolute abundances of seawater microbiomes. This approach combines propidium monoazide (PMA) treatment with 16S rRNA gene amplicon sequencing coupled with quantitative microbiome profiling (QMP). In the illustrated example, (**i**) the control community consists of two "viable" taxa in a ratio of 60:40% or 6:4 (relative or absolute). Following stress exposure, the relative and absolute ratios of the exposed community detected without PMA treatment remain unchanged; however, in the treated community, PMA binds to the DNA from cells compromised by the stress exposure. Microbial loads of intact cells can then be quantified with molecular techniques using droplet digital polymerase chain reaction (ddPCR). (**ii.A**) In the molecular-based workflow, PCR amplification of DNA from membrane-compromised cells is inhibited by PMA-treatment, allowing quantification of 16S rRNA gene copy number via ddPCR. (**ii.B**) In the cell-based workflow, Live/Dead staining (SYBR Green and propidium iodide) of independent sub-samples allows the quantification of intact cells by flow cytometry (FC). (**ii.C**) 16S rRNA gene amplicon sequencing data can be rarefied to an even sequencing depth resulting in relative microbiome profiling (RMP). In this example, RMP shows the same 60:40 ratio of taxa in both the control and stress-exposed communities. In contrast, sequencing data rarefied to an even sampling depth (= microbial sample load) obtained from the molecular- or cell-based quantification techniques results in QMP, which reveals a 50% decline in absolute abundance of both taxa in the stress-exposed community. This workflow outlines the necessary steps required to generate absolute abundance data suitable for application in stress-response modelling of viable microbial taxa in natural seawater microbiomes (created with BioRender.com)

containers. Sampling was conducted for each individual experiment ($n = 3$) over a period of one month, with samples collected on separate days according to experimental requirements. Seawater was directly transported by vehicle in sealed carboys from the collection site to the laboratory at AIMS (< 2 km) and processed immediately upon arrival.

Comparison of cell-based and molecular-based absolute quantification of seawater microbial communities

To evaluate the relationship between cell counts and absolute 16S rRNA gene copy numbers, varying proportions of natural seawater and artificial seawater (ASW, Aquaforest Reef Salt prepared at a salinity of 33 ppt, filtered to 0.22 µm) suspensions were measured with FC and ddPCR. Suspensions were prepared in triplicate with natural seawater proportions of 100, 80, 60, 40, 20, and 0% of a total 500 mL sample, with ASW making up the remaining proportion. For FC measurements, 0.5 mL sub-samples ($n = 18$) were taken from each treatment and stained with SYBR Green I (SG) to determine total microbial cell counts (see flow cytometry methods section for more details on the staining procedure). The remaining suspensions were filtered onto Sterivex filters (0.22 µm pore size, polyethersulfone membrane, Millipore Merck, Australia) using custom-built units for automatic filtration, snap frozen in liquid nitrogen and stored at −80 °C until DNA extraction and total 16S rRNA gene copy number quantification by ddPCR.

Optimisation of PMA treatment concentration for natural seawater samples

Multiple concentrations of PMA were tested to identify the optimum value for low biomass seawater samples. This step was necessary to ensure sufficient PMA was added to the samples to inhibit PCR amplification of DNA from membrane-compromised cells, while avoiding potential cytotoxic effects of PMA on membrane-intact cells [35]. To assess the efficiency of PMA in excluding membrane-compromised cells, another set of natural seawater samples was subjected to heat treatment at 85 °C for 5 min [36] using a water bath. Additional 0.5 mL sub-samples were taken from the natural and heat-treated ($n = 12$) seawater communities for counting total (SG stained) and intact microbial cells (Live/Dead staining; co-staining using SG and propidium iodide (PI), see flow cytometry methods section for more details on the staining procedure). Natural and heat-treated seawater communities ($n = 48$) were then filtered onto Sterivex filters and treated with PMA. Briefly, 2 mL aliquots of PMA working stocks (PMAxx Dye, 20 mM in H_2O, Biotium, Fremont, CA, USA) diluted in phosphate buffered saline (PBS, 33 ppt salinity, pH 8.0) at final concentrations of 0, 1.25, 2.5, 5, 15, 25, 50, and 100 µM were added to individual Sterivex filters. Samples were incubated in the dark for 10 min before exposure for 30 min to a 464 nm light LED Transilluminator (120 V, General Electric, USA) to achieve photo-induced PMA cross-linking to DNA from membrane-compromised cells and extracellular DNA. Sterivex filters were placed randomly on horizontal rollers rotating at 25 rpm to ensure homogeneous light exposure. Following light exposure, PMA solution was expelled using 5 mL syringes, and filters were snap frozen in liquid nitrogen and stored at −80°C until subsequent DNA extraction, ddPCR quantification, and 16S rRNA gene amplicon sequencing.

Selective detection of intact seawater microbiota

The performance of the PMA-ddPCR assay in selectively detecting intact cells in the presence of various proportions of damaged cells was tested. Defined ratios of natural and heat-treated seawater were mixed in triplicate to achieve a final natural seawater concentration of 100, 80, 60, 40, 20, 0% in a total 500 mL sample. Samples ($n = 18$) were then treated with a concentration of 2.5 µM PMA (chosen based on results from the PMA concentration-range-finding experiment) and processed as outlined in the previous section. PMA-untreated samples (0 µM PMA, $n = 3$) were included as controls for the 100% natural seawater treatment and processed in the same manner. To compare the performance of PMA-ddPCR quantification assay with the Live/Dead-FC quantification assay for microbial load anchoring in QMP, sub-samples ($n = 18$) were taken from each sample for Live/Dead staining and FC cell counting (see flow cytometry methods section for more details).

DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted from Sterivex filters using a phenol-chloroform extraction method, as outlined in the Supplementary Methods. Duplicate blank DNA extractions were included to identify reagent contamination. DNA quality and yield were quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Australia) and Qubit fluorometer (Thermo Fisher Scientific, Australia) using the 1X dsDNA High Sensitivity assay kit (Thermo Fisher Scientific, Australia). The samples were stored at −20 °C.

Amplicon library preparation and sequencing was performed at the Australian Centre for Ecogenomics in Brisbane, Queensland, where amplicon PCR was done on the V4 hypervariable regions of the 16S rRNA gene using the primer pair 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) [37] and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) [38]. Libraries were constructed following the Illumina 16S Metagenomic Sequencing Library Preparation Protocol (#15044223 B, see Supplementary Methods for more details) and sequenced using 2 × 300 bp paired-end

chemistry on the MiSeq platform. Appropriate positive and negative controls were included following the Australian Centre for Ecogenomics standard workflow.

Flow cytometry

Bacterial cell abundance in seawater samples was quantified using a BD Accuri C6 flow cytometer (BD Biosciences, USA), with 488-nm excitation from a blue solid-state laser. For total bacterial cell counts, seawater samples were 10-fold diluted in ASW and stained with the fluorescent dye SYBR Green I (SG: 10,000X concentrate in DMSO, Invitrogen, USA) at a final concentration of 1 × (1:10,000 dilution) [39]. For live/dead differentiation, samples were co-stained with SG and PI (Live/Dead staining, SGPI) at final concentrations of 1X SG and 15 µM PI. The PI concentration was selected based on a concentration-range finding experiment where PI concentrations of 2.5, 5, 10, 15, and 20 µM were tested. Concentrations less than 10 µM resulted in incomplete staining of damaged cells, while concentrations higher than 15 µM resulted in false PI-positive staining of intact cells (data not shown). Stained samples were incubated in the dark at room temperature for 15 min [39]. Forward scatter, side scatter, green (FL1, optical filter: 533/30) and red (FL3, optical filter: 670 LP) fluorescence were recorded with a set threshold of 1000 events (selected based on the background counts of blank samples = sterile ASW) on the FL1 channel. Measurements were performed at a medium flow rate of 35 µL min^{-1} by running a standardised 50 µL volume of sample. Duplicate blank samples using sterile ASW stained with SG were included to control sample background counts. Data was acquired on two-parameter density plots with an electronic gating to separate positive signals from instrument and sample background (Figure S1). Data was processed with the BD CSampler Plus software (BD Biosciences, USA).

Droplet digital PCR

Total 16S rRNA gene copy numbers in seawater were quantified using ddPCR. PCR reactions of 22 µL were prepared in duplicate 96-well PCR plates with each well containing 11 µL QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories, USA), 0.3 µM of the primer pair 1406F (5′-GYACWCACCGCCCGT-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′) [40], 7.18 µL ultrapure water, and 2.5 µL extracted genomic DNA or ultrapure water/sterile ASW (negative template controls). Previously amplifiable DNA was used as a positive template control. Droplet generation was performed with the QX200 Automated Droplet Generator (Bio-Rad Laboratories, USA) that partitioned each sample into ~20,000 droplets in which nucleic acid molecules are randomly distributed. After droplet generation, the PCR plate was removed from the Droplet Generator and sealed with a PX1 PCR Plate Sealer (Bio-Rad Laboratories, USA). PCR amplification was run to end point in the C1000 Touch Thermal Cycler (Bio-Rad Laboratories, USA) with the following cycling steps: enzyme activation at 95 °C for 5 min, 40 cycles of paired denaturation at 95 °C for 30 s and annealing/extension across a temperature gradient from 51 to 61 °C for 1 min (temperature ramp rate: 1.5 °C s^{-1}), signal stabilisation at 4 °C for 5 min, followed by 5 min at 90 °C. Samples were held overnight at 4 °C until droplets were read. After thermal cycling, the sealed plate was transferred to the QX200 Droplet Reader (Bio-Rad Laboratories, USA) for subsequent droplet readings and data acquisition and analysis were performed using the QuantaSoft software (Bio-Rad Laboratories, USA, version 1.7.4). Concentrations of 16S rRNA gene copies per mL seawater were corrected for elution volume and losses during extraction before normalising to the filter volume as follows:

$$\begin{aligned} &Microbial\ load(16S\ rRNA\ gene\ copies\ mL^{-1}seawater) = \\ &\frac{ddPCR\ concentration * PCR\ dilution\ factor\ * DNA\ dilution\ * DNA\ elution\ volume}{DNA\ extraction\ volume} * \\ &\frac{1}{filter\ volume} \end{aligned} \quad (1)$$

Amplicon sequence data processing

Processing of demultiplexed sequencing data was performed with the QIIME 2 pipeline v2020.8 [41]. The plug-in demux (Boylen et al., 2019) was used to create an interactive plot to visualise the data and assess the quality of sequences. Amplicon primers were removed using the plug-in cutadapt [42]. Denoising of paired-end reads, chimera checking, trimming, and dereplication were performed with DADA2 [43], applying truncation lengths of 240 bases for the forward reads and 200 bases for the reverse reads (based on Q score > 30). Taxonomy was assigned to amplicon sequence variants (ASVs) using a naïve-Bayes taxonomy classifier [44], trained with the pre-formatted SILVA 138 release reference sequence and taxonomy files [45, 46] representing the 515F/806R region of the 16S SSU rRNA gene. The trained classifier was applied to the representative sequences to assign taxonomy using the classify-sklearn command [47] in the feature-classifier plugin based on 99% similarity. Additionally, ASVs arising from the blank DNA extractions were excluded with the decontam R package (v1.16.0) [48]. The sequence data generated in this study are available under NCBI BioProject ID PRJNA1176196 (accession numbers SRX2645979-SRX26459535).

Statistical analysis

All statistical analyses were performed using the R Statistical Software (v4.2.1) environment [49]. Data visualisation was performed with the ggplot2 package (v3.5.1) [51]. Regression analyses of 16S rRNA gene copy

numbers and cell counts were conducted using a linear model fitted with the lm() function from the stats package (v4.2.1) [49]. Pearson correlation coefficients were calculated, and p-values were determined with the stats package. Analysis of variance (ANOVA) was employed to test for significant differences between PMA concentrations, after confirming that assumptions of normality and homogeneity of variance were met. Post-hoc comparisons were conducted using Tukey's Honest Significant Difference (HSD) test with p-value adjustments for multiple comparisons (stats package).

For relative microbiome profiling (RMP), sequencing data were rarefied to the lowest sample sequencing depth using the phyloseq package (v1.40.0) [52]. Alpha diversity, measured by observed ASV richness and Shannon diversity index, were calculated (after rarefaction) using the phyloseq package. Statistical significance of differences in alpha diversity indices between groups was tested using a Kruskal-Wallis rank sum test (stats package). Pairwise differences were assessed using Dunn's test for multiple comparisons, with Benjamini-Hochberg correction for multiple testing using the FSA package (v0.9.5) [53]. Beta diversity was assessed based on Bray-Curtis dissimilarities at the ASV-level. Principal coordinate analysis (PCoA), limited to the first two dimensions, was used to visualise microbial community composition (phyloseq package). Permutational multivariate analysis of variance (PERMANOVA) and multivariate homogeneity of group dispersions (PERMDISP2) were performed using the vegan package (v2.6-6.1) [54], with 10,000 permutations. Venn diagrams were generated with the eulerr package (7.0.2) [55] to visualise unique and shared ASVs between PMA-treated and untreated communities. Differential abundance analysis was conducted at the ASV-level using the DESeq2 package (v1.36.0) [56].

For quantitative microbiome profiling (QMP), sample reads were rarefied to an even sampling depth based on the ratio between sequencing depth and gene copy numbers (ddPCR) or cell counts (FC) using the rarefy_even_sampling_depth function (seed = 711) [27]. This yielded absolute microbial taxa abundances per mL of seawater (cells or copies mL^{-1}). Scatterplots with smoothed lines (using the locally estimated scatterplot smoothing (LOESS) method) were created with ggplot2 to visualise relative and absolute ASV abundance changes.

Results

Comparison of total microbial loads in seawater estimated by FC and ddPCR

The predictive accuracy of the two methods (FC vs. ddPCR) was evaluated by analysing the relationship between the predicted and the observed average counts (relative to 100% natural seawater) (Figure S2). Regression analyses demonstrated significant positive linear relationships for both methods (FC: $F_{1,4}$ = 69960, $p < 0.001$, Figure S2A; ddPCR: $F_{1,4}$ = 486.5, $p < 0.001$, Figure S2B), with adjusted R^2 estimates close to 1: FC = 0.9999 and ddPCR = 0.9898 (Figure S2). The mean absolute error (MAE) and root mean square error (RMSE) for FC was MAE = 0.257 and RMSE = 0.346, while ddPCR showed higher MAE (3.25) and RMSE (4.64) values, indicating greater error magnitudes. Additionally, the coefficient of variation (CV) for repeated measures was 7.5-fold lower using FC (median CV = 1.9%) compared to ddPCR (median CV = 15%) (Fig. 2C).

The relationship between estimated counts by FC and ddPCR was further assessed using Pearson correlation and linear regression analysis (Fig. 2D). A strong positive correlation was observed, with a Pearson correlation coefficient of $r = 0.96$. The linear regression analysis further supported this finding, with the model explaining 93% of the variance in ddPCR counts ($F_{1,16}$ = 205.1, $p < 0.001$, adjusted $R^2 = 0.923$) (Fig. 2D). The slope of the regression line was 0.25 (95% CI: 0.209–0.282, $p < 0.001$), indicating that each additional cell measured by FC corresponds to an increase of approximately 0.25 gene copies detected by ddPCR (Fig. 2D).

Optimisation of PMA treatment for seawater samples

PMA treatment of the natural seawater community resulted in an average decline of 54–71% in DNA yields (Figure S3A) and an average 21–53% decrease in 16S rRNA gene copies (Fig. 3A), indicating a substantial reduction in detectable microbial loads after DNA from damaged cells was excluded from PCR amplification. The statistical significance of these observations was supported by ANOVA: DNA yields: $F_{7,16}$ = 9.819, $p < 0.001$, 16S rRNA gene copies: $F_{7,16}$ = 9.819, $p < 0.001$ (Table S2 & 2). Tukey's HSD post-hoc comparison further revealed that PMA concentrations of ≥ 2.5 µM (except for 15 µM) resulted in a significant reduction in DNA yields (Table S2) and 16S rRNA gene copy numbers (Table S3) compared to PMA-untreated samples. However, no significant differences in DNA yields were observed between all tested PMA concentrations (Table S2). The 16S rRNA gene copy numbers were not significantly different in samples treated with concentrations ≥ 2.5 µM, except for the comparison between the PMA concentrations of 15 µM and 25 µM (Table S3).

In the heat-killed seawater community, which served as a control for the complete exclusion of membrane-compromised cells, PMA treatment significantly inhibited DNA extraction yields (Figure S3B), and amplification of 16S rRNA gene copies was almost eliminated across all PMA concentrations relative to the PMA-untreated samples (Fig. 3B).

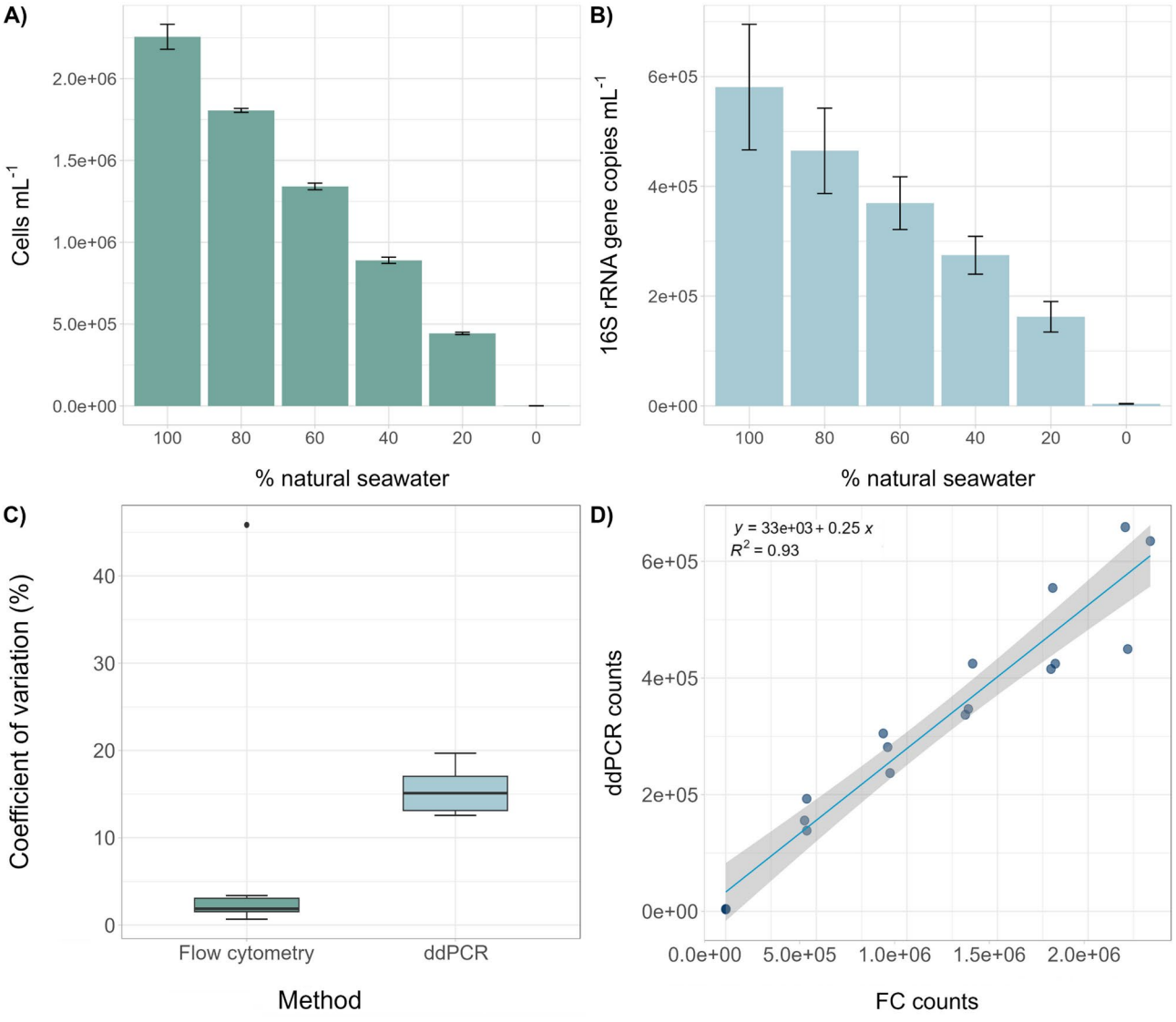

Fig. 2 Total microbial loads in seawater samples quantified by flow cytometry and droplet digital PCR (ddPCR). (**A**) Bar plot of average cells mL^{-1} ± SD ($n = 3$ replicates). (**B**) Bar plot of average 16S rRNA gene copies mL^{-1} ± SD ($n = 3$ replicates). (**C**) Boxplot of coefficient of variation (CV%, $n = 6$ per method), with whiskers = 10th and 90th percentiles, box = 25th and 75th percentiles, and horizontal line = median. (**D**) Linear regression analyses of cells mL^{-1} versus 16S rRNA gene copies mL^{-1}

To validate these PMA-ddPCR results, FC analysis was performed to quantify the proportion of intact cells after Live/Dead staining was applied to the same seawater samples. In the natural seawater community, FC measured 75% intact cells (SGPI, Fig. 3A), which was consistent with the proportional reductions in 16S rRNA gene copy numbers observed at lower PMA concentrations (1.25–15 µM). ANOVA ($F_{7,16} = 5.259$, $p < 0.01$) and Tukey's HSDpost-hoc comparison revealed no significant difference in the proportions of intact cells measured by ddPCR and FC, except for the 25 µM PMA concentration (Table S4). In the heat-killed seawater communities, FC indicated a 99% decline in the proportion of intact cells, which was consistent with the reduction in 16S rRNA gene copies across all PMA concentrations (Fig. 3B).

Effect of PMA on seawater microbiome composition

A total of 950,800 sequences were obtained from 24 seawater samples (minimum read depth: 32,041, maximum: 57,069, average: 39,617). After rarefaction to the minimum read depth (= 32,041 reads), 589 ASVs were recovered and assigned to 22 phyla.

PMA treatment affected the relative abundance of dominant taxa; however, the overall community composition was stable across different PMA concentrations and replicates, and no significant rank abundance shifts of the five most dominant taxa were observed

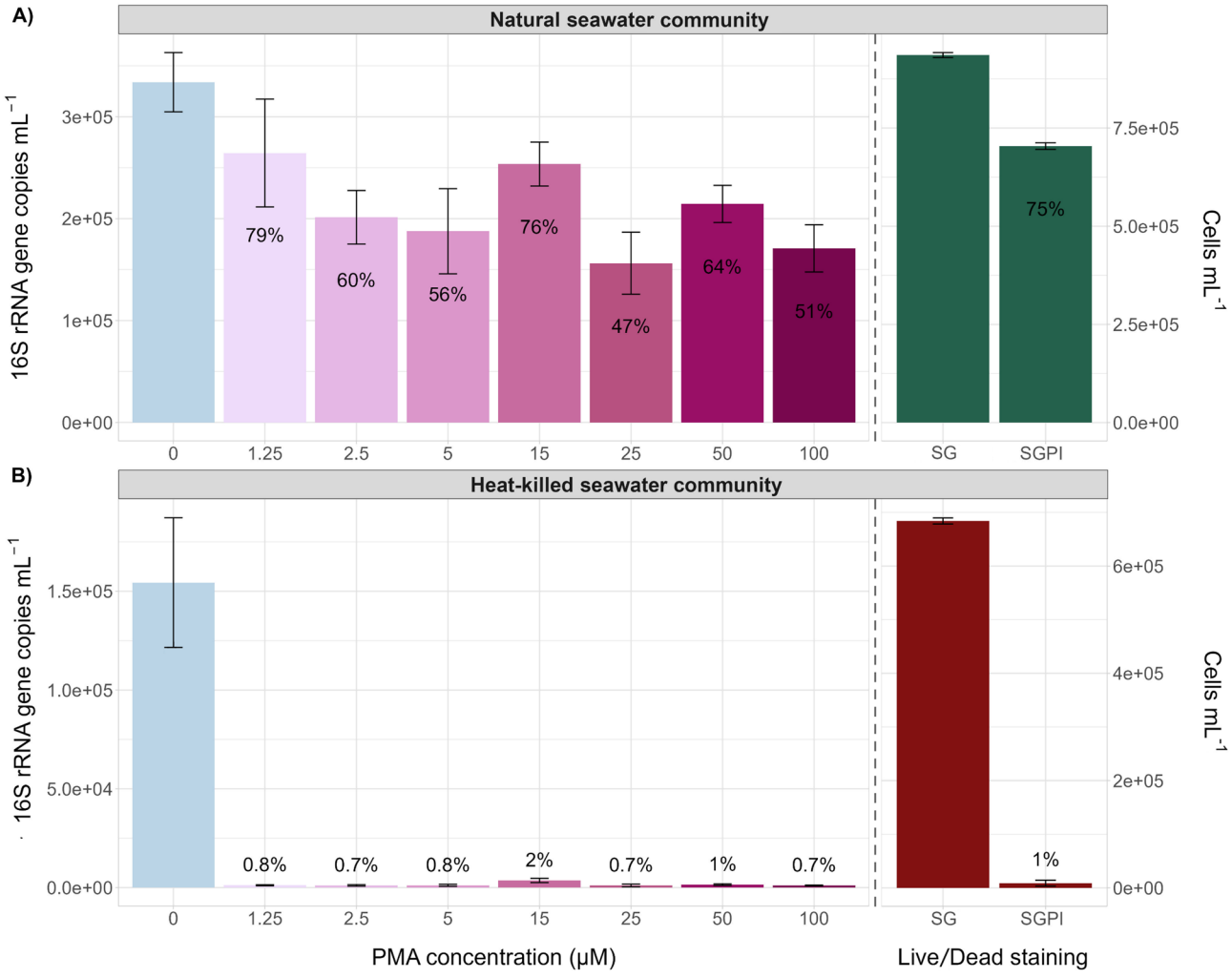

Fig. 3 Seawater microbial communities treated with a range of propidium monoazide (PMA) concentrations. (**A**) Bar plot showing average 16S rRNA gene copies mL^{-1} ± SD measured by droplet digital PCR (ddPCR) in natural seawater communities treated with varying PMA concentrations ($n = 3$ replicates). Green bars on the right represent average cells mL^{-1} ± SD measured with flow cytometry (FC) in natural seawater stained with SYBR Green I (SG) or SG and propidium iodide (Live/Dead staining; SGPI) ($n = 3$ replicates). (**B**) Bar plot displaying the average 16S rRNA gene copies mL^{-1} ± SD, quantified by ddPCR in heat-killed seawater communities treated with different PMA concentrations. Red bars on the right indicate average cells mL^{-1} ± SD measured by FC in heat-killed seawater stained with SG or SGPI. Percentages indicate the proportion of intact cells relative to PMA-untreated or SG-stained seawater communities

(Fig. 4A). For example, in the PMA-untreated samples, the top five dominant taxa were *Synechococcus* CC9902 (18 ± 4.2%; average relative abundance ± SD), SAR11 clade Ia (14 ± 0.13%), SAR86 clade (7.5 ± 3.8%), AEGEAN-169 marine group (6.0 ± 0.14%), and *Candidatus Actinomarina* (5.5 ± 0.19%) (Fig. 4A). Following PMA treatment, the average relative abundances of *Synechococcus* CC9902 and SAR11 clade Ia decreased to 9.4 ± 1.7% (range: 6.9–12%) and 6.9 ± 1.3% (range: 3.2–10%), respectively (Fig. 4A). In contrast, the average relative proportions of SAR86 clade, AEGEAN-169 marine group, and *Candidatus Actinomarina* increased to 9.5 ± 1.0% (range 8.2–12%), 9.3 ± 0.89% (range 6.9–11%), and 8.7 ± 1.3% (range 4.6–11%), respectively (Fig. 4A).

There were no statistically significant differences in the alpha diversity of seawater microbiomes between PMA-treated and untreated samples, as measured by the observed number of ASVs and Shannon diversity (Kruskal-Wallis rank sum test observed ASVs: $\chi^2 = 10.57$, df = 7, $p = 0.158$; Fig. 4B and Shannon diversity: $\chi^2 = 10.73$, df = 7, $p = 0.151$; Fig. 4C). However, microbial community structure (Bray-Curtis dissimilarities) at the ASV-level showed significant clustering according to PMA-treated and untreated samples, with the first two principal components explaining 85% of the observed variation (Fig. 4C). The statistical significance of sample groupings was verified with PERMANOVA on the distance matrix ($F_{7,16} = 7.143$, $R^2 = 0.758$, $p < 0.001$). PMA treatment explained 76% of the variation in community composition, with

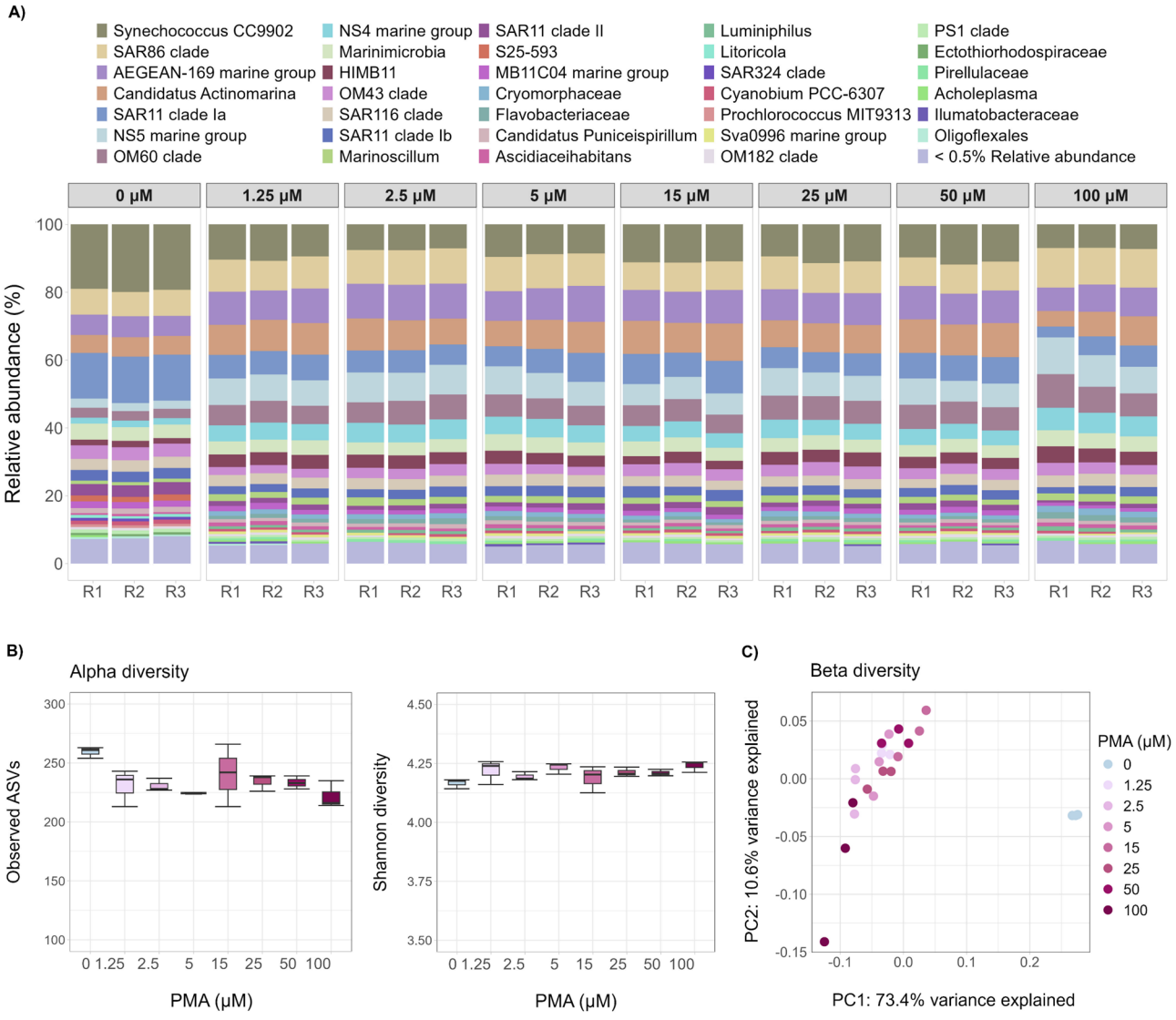

Fig. 4 Community profiling of seawater microbiomes treated with a series of propidium monoazide (PMA) concentrations. (**A**) Taxonomic bar plots generated at lowest taxonomic resolution displaying the relative abundance of microbial communities treated with PMA. (**B**) Boxplot of alpha diversity (observed number of amplicon sequence variants (ASVs) and Shannon diversity). The whiskers symbolize the 10th and 90th percentiles, the box denotes the 25th and 75th percentiles, and the horizontal line indicates the median ($n = 3$ replicates). (**C**) Principal coordinate analysis based on Bray-Curtis dissimilarities at ASV-level

24% of the variation attributed to residual factors. Additionally, there was no significant difference in sample group dispersions (PERMDISP, $F_{7,16} = 0.881$, $p = 0.543$), indicating that the variability within groups was homogeneously distributed.

PMA treatment at a concentration of 2.5 µM influenced the detection of specific ASVs, revealing 92 unique ASVs that were undetectable in PMA-untreated communities (Figure S4A). In contrast, PMA-untreated samples contained 115 unique ASVs not observed in PMA-treated communities. There were 215 shared ASVs between PMA-treated and untreated communities (~50% of ASVs) (Figure S4A). Differential abundance analysis identified 146 ASVs with significantly different abundances between PMA-treated and untreated samples (Figure S4B). Phyla such as Actinobacteriota and Bacteroidota exhibited pronounced negative log2 fold change values, indicating a significantly higher relative prevalence ($p < 0.05$) in PMA-treated samples (Figure S4B). Conversely, phyla including Cyanobacteria, Bdellovibrionota, and Verrucomicrobiota displayed significant positive log2 fold changes, suggesting a significantly higher relative abundance in PMA-untreated samples (Figure S4B). ASVs associated with Proteobacteria demonstrated a differential response to PMA within the same phylum (Figure S4B).

Assessing the performance of the PMA-ddPCR and Live/Dead-FC assays

The performance of PMA in detecting intact cells was evaluated using seawater sample mixtures that included defined ratios of intact and heat-killed cells. Absolute abundance estimates measured by the PMA-ddPCR assay were then compared with the Live/Dead-FC assay. For this experiment, a conservative PMA concentration of 2.5 µM was selected, as this was shown to effectively inhibit PCR amplification of membrane-compromised cells (see Results).

A strong positive correlation was observed between microbial loads determined by PMA-ddPCR and Live/Dead-FC methods, with a Pearson correlation coefficient of $r = 0.931$. Linear regression analysis further demonstrated a statistically significant positive relationship ($F_{1,16} = 104.1$, $p < 0.001$), with an adjusted $R^2 = 0.869$ (Figure S5). The slope of the regression line was 0.31 (95% CI: 0.245–0.374, $p < 0.001$), indicating that each additional intact cell detected by FC corresponded to an increase of approximately 0.31 copies measured by ddPCR (Figure S5). The predictive accuracy (relationship between predicted and relative averaged observed counts) of the two methods was further compared, revealing significant positive linear relationships for both ddPCR ($F_{1,4} = 137.3$, $p < 0.001$) (Figure S6A) and FC ($F_{1,4} = 13{,}550$, $p < 0.001$) (Figure S6B), with adjusted R^2 estimates close to 1: ddPCR = 0.9646 and FC = 0.9996 (Figure S6A & B). Additionally, the coefficient of variation (%CV) for repeated measures was 9.6-fold lower using FC (median CV = 2.6%) compared to ddPCR (median CV = 25%). These findings agreed with our previous results when the relationship between FC and ddPCR counts was investigated based on total microbial load quantifications only (no PMA or Live/Dead staining application).

Quantitative microbiome profiling influences microbiome analysis

A total of 698,727 sequences were obtained from 18 seawater samples (minimum read depth: 28,423, maximum: 49,554, average: 38,818). Rarefaction to an even sequencing depth of 28,423 reads yielded a total of 626 ASVs. The relative microbiome profiling (RMP) did not account for variations in microbial load across samples (Fig. 5A), and no significant relationship was observed between the average number of sequenced reads and absolute cell counts or gene copy numbers ($F_{1,4} = 3.66$, $p = 0.128$, adjusted $R^2 = 0.347$). Rarefaction to an even sampling depth resulted in the recovery of 449 ASVs when using ddPCR anchoring, compared to 417 ASVs retrieved from FC anchoring.

There were no significant differences in the observed number of ASVs independent of RMP or QMP normalisation (Kruskal-Wallis rank sum test: $p > 0.05$) (Table S5, Figure S7). Shannon diversity was insignificant for RMP (Kruskal-Wallis rank sum test: $p > 0.05$, Table S5, Table S7A) but indicated significant differences for QMP (Kruskal-Wallis rank sum test: $p < 0.05$, Table S5, Figure S7B & C). However, post hoc comparison demonstrated that only group comparison 100% versus 0% intact cells was significant for both anchoring methods (Table S5). When assessing Bray-Curtis community dissimilarities at the ASV-level, RMP sample groupings were only significant for the 0% intact cell group relative to other groups (PERMANOVA $p < 0.001$, Fig. 5C left plot, Table S6). After QMP, the community structure showed significant clustering according to the % of intact cells (PERMANOVA $p < 0.001$, Fig. 5C middle and right plot, Table S7). Correlation analysis further revealed significant associations of 16S rRNA gene copies and cell counts with Bray-Curtis dissimilarities (Pearson correlation coefficient: $r_{FC} = -0.975$, $r_{ddPCR} = -0.954$), indicating that reduced microbial abundances, identified by QMP, was a key driver of dissimilarity within the community.

RMP abundance curves remained generally stable across samples, only decreasing or increasing in the virtual absence of intact cells (e.g., 0% intact cells, Fig. 6A). In contrast, QMP revealed predictable quantitative declines in ASV abundances as the percentage of heat-killed cells increased (Fig. 6B & C). QMP anchoring to cells mL^{-1} estimated via FC (Fig. 6C) yielded more reliable abundance relationships, with substantially narrower confidence intervals compared to QMP anchoring to 16S rRNA gene copy numbers (Fig. 6B).

Discussion

Quantifying changes in microbial taxa in response to environmental disturbances requires accurate estimation of the absolute abundances of viable community members [27, 28]. However, DNA sequencing-based microbiome studies overwhelmingly rely on relative abundance data, which generally include DNA from dead cells and extracellular sources, potentially leading to erroneous interpretations [15, 57]. In this study, we presented a workflow for quantifying absolute abundance of microbial taxa in seawater microbiomes. PMA effectively inhibited PCR amplification of DNA from membrane-compromised cells in natural seawater microbial communities. Anchoring 16S rRNA gene amplicon sequencing data to microbial loads of membrane-intact cells allowed the transformation of relative (RMP) to absolute (QMP) abundance data. The developed PMA-16S sequencing workflow, combined with QMP, overcomes several compositional biases inherent in RMP analyses, allowing for more accurate and reliable assessments of microbial

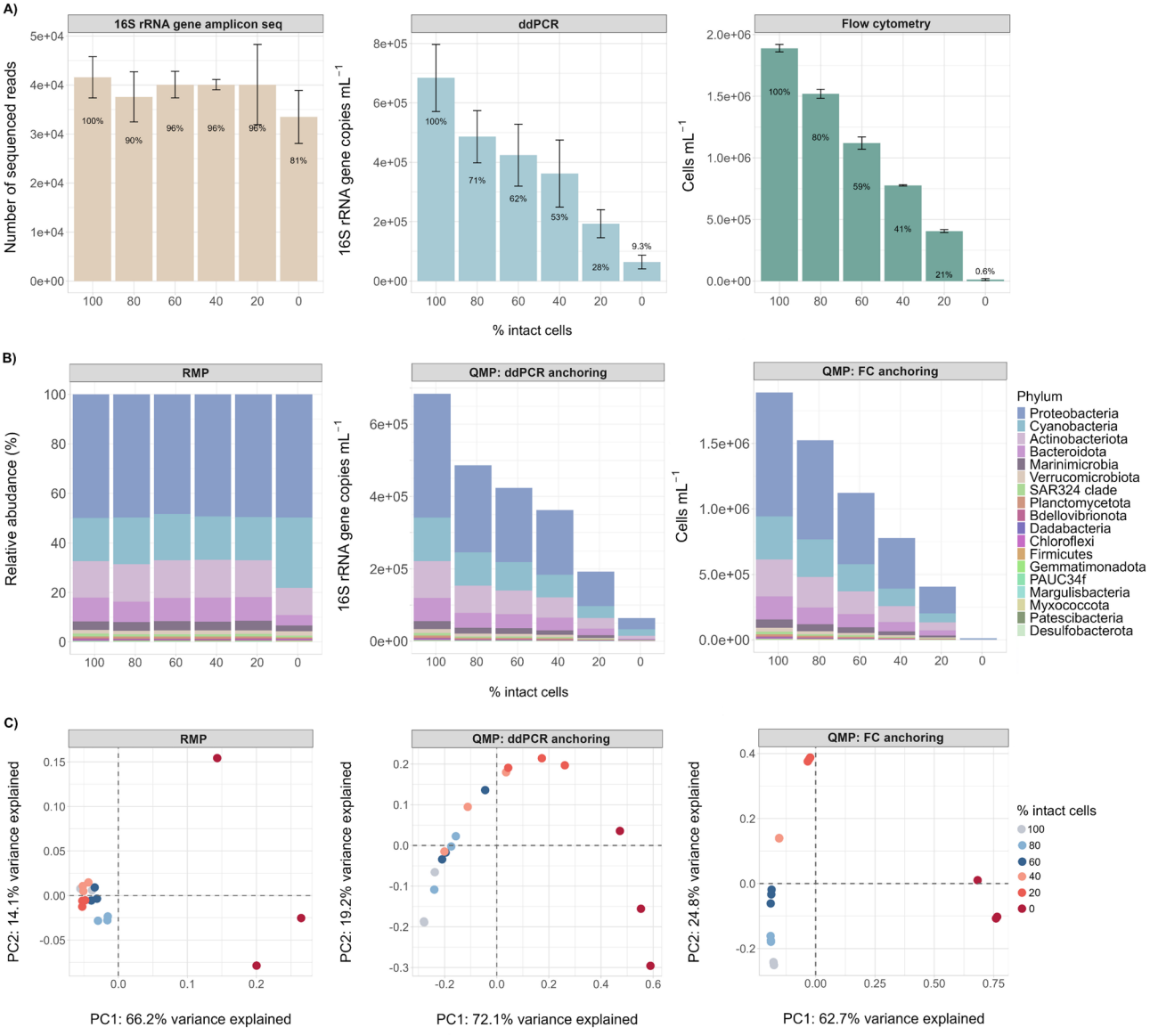

Fig. 5 Influence of quantitative microbiome profiling on microbiome analysis: Illustrated using a series of defined ratios of heat-killed and intact cells. (**A**) Bar plots showing the average number of 16S rRNA gene amplicon sequencing reads ± SD (left), average 16S rRNA gene copies mL^{-1} ± SD (middle), and average cells mL^{-1} ± SD (right). (**B**) Taxonomic bar plots generated at the phylum-level, displaying relative microbiome profiling (RMP, left) and quantitative microbiome profiling (QMP) anchored to either 16S rRNA gene copies (middle) or cell counts (right). (**C**) Principal coordinate analysis based on Bray-Curtis dissimilarities at ASV-level after rarefying 16S rRNA gene amplicon sequencing data to an even sequencing depth (RMP, left) or even sampling depth anchored to 16S rRNA gene copies mL^{-1} (QMP: ddPCR anchoring, middle) or cells mL^{-1} (QMP: FC anchoring, right)

abundance changes in response to environmental stressors.

PMA effectively reduces the contribution of compromised cells in seawater samples

Natural microbial assemblages may display varying metabolic activity and states of viability. However, DNA-based molecular technologies alone cannot distinguish between intact and compromised cells, as the persistence of nucleic acids after cell death limits accurate estimations of the intact microbial population [16, 20]. PMA has been used with various environmental water sample types, often in conjunction with species-specific primers to selectively detect intact faecal indicator bacteria such as *Escherichia coli*, *Enterococcus*, and *Bacteroides* in wastewater and seawater [23, 36, 58–61]. Yet, PMA has not previously been applied to seawater samples to assess the responses of entire microbial communities to stress conditions. In this context, PMA may facilitate the establishment of quantitative stress-response relationships by enabling accurate measurement of changes in the abundance of intact microbial taxa.

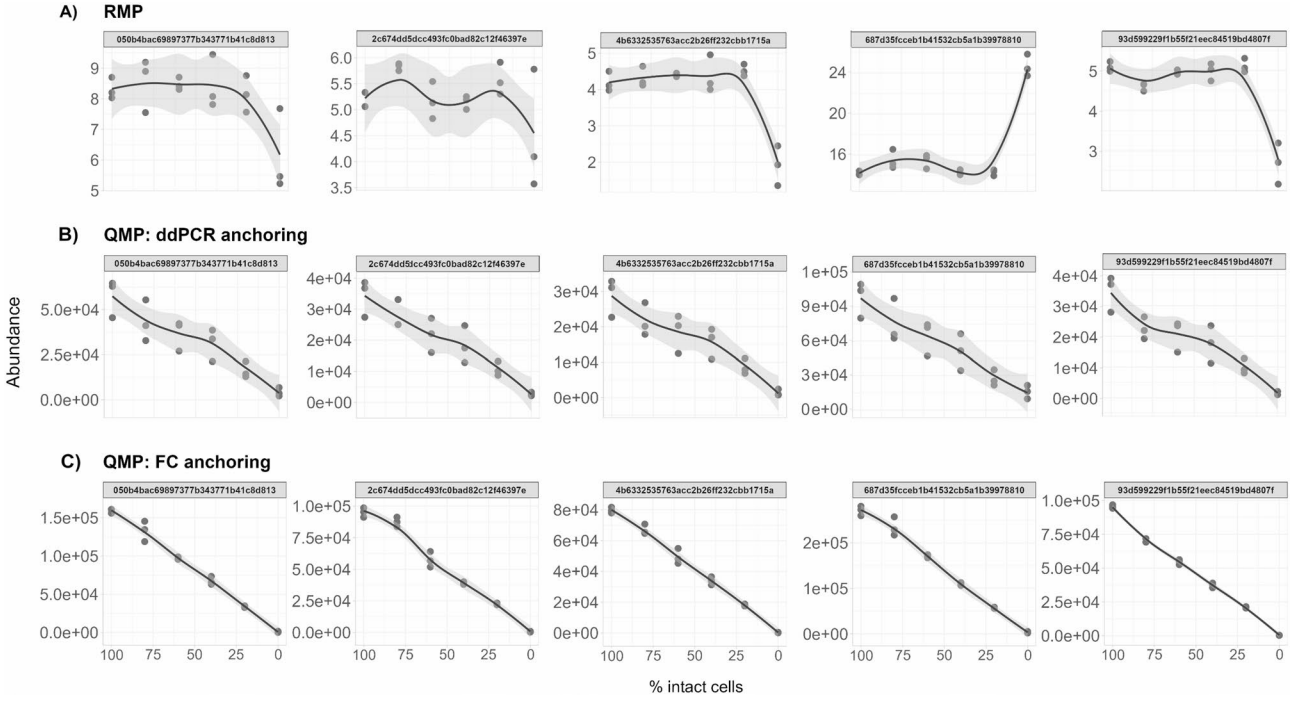

Fig. 6 Comparative analysis of relative microbiome profiling (RMP) and quantitative microbiome profiling (QMP). Differences in relative (%) and absolute (cells or gene copies mL^{-1}) abundance changes of five representative ASVs after normalisation of 16S rRNA gene amplicon sequencing data. (**A**) RMP: Rarefaction to an even sequencing depth resulting in percent relative abundance. (**B**) QMP: Rarefaction to an even sampling depth anchored to 16S rRNA gene copies mL^{-1}, estimated via droplet digital PCR (ddPCR). (**C**) QMP: Rarefaction to an even sampling depth anchored to cells mL^{-1}, quantified by flow cytometry (FC)

In this study, a range of PMA concentrations significantly reduced 16S rRNA gene copy numbers in natural seawater microbiomes to 47–79% of those in untreated communities (Fig. 3A). FC analysis using Live/Dead staining revealed that 75% of the same seawater community was composed of intact cells, consistent with the reduction in 16S rRNA gene copy numbers at PMA concentrations ≤ 15 µM (Fig. 3A). These results demonstrate that about one-third of the microbial community consisted of damaged cells, which should be excluded from studies that require precise measurements of absolute declines. While differences in gene copy number estimates across the tested PMA concentrations were only minor, higher concentrations may increase the permeability of intact cell membranes or induce cytotoxic effects, potentially causing false-negative results [35, 62]. Based on these findings, we concluded that PMA concentrations between 2.5 and 15 µM are optimal for minimising the contribution of membrane-compromised cells in seawater samples.

Low PMA concentrations effectively inhibited DNA amplification from membrane-compromised cells in seawater; however, PMA performance can be influenced by treatment conditions, including dye concentration, light exposure, incubation duration, and biological, chemical, and physical factors such as biomass, community composition, sample turbidity, pH, and salt content [15, 25, 61]. For example, higher concentrations (25–100 µM) may be required for more complex or high-biomass samples, such as estuarine benthic mud, marine sediments [19], or tissue-rich samples from corals [63] and sponges [64], where PMA can be lost to surfaces, particles, or biological material [23, 35, 65, 66]. PMA also tends to perform better with less diverse microbial communities [25], a characteristic of the seawater microbial communities in this study. Chemical and physical conditions of marine samples, such as high salinity concentrations and elevated total suspended solids (TSS) typical of inshore waters, can negatively impact PMA efficiency [23, 61]. For instance, Bae and Wuertz [23] reported that complete PMA suppression of DNA from heat-killed cells was only achievable in samples with TSS concentrations ≤ 100 mg L^{-1}. High salt concentrations may create osmotic pressure, dehydrating the cell membrane and altering its permeability, which could prevent PMA from accessing target DNA, even in membrane-compromised cells [67]. However, we did not encounter negative interactions between these factors and PMA efficiency in our samples, with PCR amplification of DNA from heat-killed cells completely inhibited at all tested PMA concentrations (Fig. 3B). This may have been facilitated by the initial filtration step, which reduced the chemical

and physical complexity of the sample by removing particles and biota larger than 4.5 µm in size. Seawater from areas with significant anthropogenic influence, however, is often characterised by high planktonic biomass and elevated TSS concentrations. Therefore, sample-specific factors should be carefully considered, and PMA concentrations adjusted accordingly [35].

Uniform exclusion of DNA from compromised cells across diverse taxa by PMA

Compositional analysis of PMA-treated seawater samples revealed consistent taxonomic profiles across all tested concentrations, with a high degree of similarity observed between replicates (Fig. 4A). There were no statistically significant differences in the observed number of ASVs between PMA-treated and untreated samples and, although Shannon diversity increased with PMA-treatment, this change was not statistically significant (Fig. 4B). However, notable differences in microbial community dissimilarities were observed between PMA-treated and untreated samples (Fig. 4C). This outcome was expected, as PMA-treated samples mainly represent intact cells, while the untreated samples include DNA from both membrane-compromised cells and extracellular sources, influencing relative abundance profiles and compositional similarities. ASVs that were differentially abundant between PMA-treated and untreated samples were not associated with any specific taxonomic group (Figure S4B), indicating that compromised cells, excluded by PMA, represented multiple taxa. Furthermore, 21% of unique ASVs were found exclusively in PMA-untreated samples, suggesting these ASVs originated from DNA of membrane-compromised cells or extracellular DNA (Figure S4A). In contrast, 27% of ASVs were detected only in the PMA-treated samples (Figure S4A) but these ASVs were predominantly low abundance ASVs. Similar findings have been previously reported and are likely linked to PCR amplification efficiency, where sequences from highly abundant taxa are preferentially amplified in PMA-untreated samples. Following PMA treatment, DNA from damaged cells is inaccessible, allowing the amplification of DNA from low-abundance taxa that were previously overshadowed [19, 68, 69]. These results suggest that PMA treatment does not affect the overall number of ASVs but selectively removes the contribution of DNA from damaged cells, enhancing the detection of low-abundance taxa [63, 70, 71]. Some studies have also reported the presence of PMA-resistant microbes, including some Gram-positive and Gram-negative bacteria, with differences in cell membranes influencing the effectiveness of PMA treatment [20, 35, 72]. Although not specifically assessed in this study, we found that *Actinobacteriota* showed significantly higher relative prevalence in PMA-treated samples. These taxa typically possess a Gram-positive cell envelope, consisting of a plasma membrane and a thick peptidoglycan layer [73], which may reduce the effectiveness of PMA in penetrating their membranes. However, PCR amplification of DNA from the heat-killed community was effectively eliminated at all PMA concentrations compared to the untreated samples, suggesting that these effects were likely minor, and that PMA treatment interacted similarly across microbial taxa, with no clear taxon-specific biases.

While PMA treatment effectively excludes membrane-compromised cells from analysis, it does not provide precise insights into the active fraction of the microbial community. Viable cells can be metabolically active or inactive, with respect to processes such as substrate uptake, respiration, or biogeochemical cycles [17]. When assessing microbial stress responses, the effectiveness of PMA treatment likely depends on the mode of action of the stressors, as some may impact cells without compromising their membranes. Therefore, membrane integrity alone does not confirm metabolic activity, and additional viability assessment techniques, such as multi-omic approaches, could provide a more comprehensive understanding of microbial viability. For example, combining PMA-seq with metatranscriptomic or metaproteomic profiles can overcome some limitations that are not captured by PMA-16S rRNA gene sequencing alone [15, 25]. Despite these limitations, PMA treatment of seawater samples remains valuable when differences in intact/damaged cell ratios are quantitatively important, particularly for assessing microbial responses to increasing stressor levels.

Converting relative to absolute abundance in seawater microbiomes with ddPCR and FC-anchoring

QMP offers a valuable approach for determining absolute microbial abundances from NGS data, addressing key limitations of compositional data analysis and improving microbial community assessments. Total 16S rRNA gene copy numbers estimated in seawater by ddPCR were highly correlated with total cell loads measured using FC (Fig. 2A & B), indicating that either method could be used to normalise NGS data. We also assessed the ability of each method to detect predicted proportions of intact cells in mixtures of heat-killed and natural seawater communities. As with total microbial load estimates, FC and ddPCR were strongly correlated in quantifying intact cells, reinforcing the suitability of both methods for QMP in microbial ecotoxicology. However, ddPCR exhibited greater variability between replicates, higher uncertainty, and lower predictive accuracy compared to FC (Fig. 2), suggesting that FC may provide more precise estimates of total microbial loads in seawater samples. This level of precision is particularly important for establishing reliable relationships between taxon abundance and stressor

levels in microbial ecotoxicology. Additionally, FC was less technically demanding than ddPCR and has already been effectively applied in QMP across different microbiome sample types, including agricultural soil [74], faecal material [27, 75], river water [33], and chicken gut [34].

It has been recommended that RMP should be normalised to the sequenced microbial load, rather than the microbial load in the original sample, as FC may not accurately reflect the microbial load being sequenced [76]. While ddPCR uses the same DNA extraction inputs as NGS, method-specific protocols can also impact amplification consistency between ddPCR and NGS. Another challenge in applying ddPCR for absolute quantification is the variability in 16S rRNA gene copy numbers among different taxa [77]. Despite the availability of databases cataloguing variation in ribosomal RNA operons (rrn) for bacteria and archaea, many ASVs remain unclassified [78], and the limited number of rrn entries from sequenced genomes likely does not capture the full range of rrn copy number variability using universal 16S rRNA primers[78].

In the context of microbial ecotoxicology testing, these specific limitations and biases of FC and ddPCR may be less critical. The primary focus is on assessing proportional changes (declines or increases) of specific taxa relative to their absolute abundance, rather than comparing absolute abundances across taxa. As such, method-specific variations in sample volumes, input material, or gene copy numbers are less important than maximising consistency in exposure, sampling, quantification, and sequencing across treatments. Although FC and ddPCR showed a high correlation in measuring absolute microbial loads in seawater, the advantages of FC make it a more practical and reliable choice for routine microbial load quantification, particularly in scenarios requiring high sample throughput and assessments across multiple stressor levels.

QMP allows for quantitative assessment of microbial abundance changes

Substantial discrepancies were observed between QMP and RMP in assessing microbial abundances within natural seawater communities. QMP accurately identified absolute microbial abundance trends in samples with predefined ratios of intact and heat-killed cells. In contrast, RMP was unable to capture microbial abundance changes, resulting in markedly different distributions of taxa abundances between the approaches, as previously reported [27, 75]. While rarefying sequencing outputs to an equal number of reads per sample is common practice in microbiome research [79, 80], variations in sequencing depths often stems from technical artifacts during DNA library pooling [81, 82]. This was confirmed in the current study, where sample sequencing depths did not correspond to trends in absolute microbial loads. Furthermore, RMP underestimated variations in the microbial community structure, as samples with different proportions of intact cells clustered closely together, distinguishing only from the 100% heat-killed community (Fig. 5C left plot). By contrast, microbial abundance emerged as a significant driver of community dissimilarity after absolute abundance normalisation (Fig. 5C middle and right plot). Additionally, RMP was unable to identify declines in ASV abundance as the proportion of intact cells declined from 100 to 0% in the mixed community (Fig. 6A), while QMP accurately captured the proportional declines in absolute ASV abundance (Fig. 6B & C).

Conclusion

The PMA-16S rRNA gene amplicon sequencing workflow, coupled with QMP, enabled accurate quantification of absolute microbial abundances in natural seawater microbiomes. Treating seawater samples with low concentrations of PMA effectively reduced the contribution of DNA from compromised cells and extracellular DNA, as validated by parallel FC Live/Dead staining. This step is crucial for describing effects of environmental stress on microbial communities, ensuring only intact cells contribute to quantitative abundance estimates. Stressors like contaminants or heat events are likely to compromise cell integrity, and this workflow enhances the reliability of interpreting stress effects at the community level. It is particularly well-suited for quantifying the magnitude and direction of ASV abundance changes following stress, making it directly applicable to stress-response modelling for individual taxa. By identifying microbial stress thresholds based on absolute abundance changes, this workflow can facilitate the incorporation of microbial sensitivities into regulatory guidelines aimed at protecting ecosystem health.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-025-00741-2.

Supplementary Material 1

Acknowledgements

We thank Sara Bell for sharing her expertise and protocol for DNA extraction from Sterivex filters. We also thank Frederik Hammes, Jean-Baptiste Raina, and Jason Doyle for their insights into optimising the flow cytometry and ddPCR protocols.

Author contributions

MCT, GW, IV, NSW, APN and HML conceived and designed the study; MCT and GW developed and validated the experimental methods. MCT and KD collected and processed samples; MCT analysed the data; MCT and KD interpreted the data; MCT wrote the first draft; all authors revised the manuscript and approve of the submitted version.

Funding
This work was supported by the Australian Research Council under the project DP200100790: Quantifying the impacts of environmental stress on marine microorganisms, the Australian Institute of Marine Science, the Marine Strategic Funding from the Australian Centre for Genomics, University of Queensland, the Australian Government Research Training Program Fee Offset and Scholarship program from the University of Queensland (Australia).

Data availability
The sequence data generated and analysed in this study are available under NCBI BioProject ID PRJNA1176196 (accession numbers SRX2645979-SRX26459535). QIIME2 and R code can be found on MarieCThomas/Quantitative-PMA-16S-rRNA-gene-sequencing-workflow- (github.com).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 14 November 2024 / Accepted: 13 June 2025
Published online: 01 July 2025

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