

Macroalgal release of Dissolved organic carbon in coral reef and its interaction with the bacteria associated with the coral *Porites lutea*

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Abstract

Macroalgae supersede corals in the reefs worldwide, converting the coral dominant systems into algal dominant ones. Dissolved Organic Carbon (DOC) released by macroalgae play a prominent role in degrading the coral reefs by stimulating the bacterial growth and metabolism. However the long term remineralization of macroalgal DOC and their contribution to the carbon pool are least studied. In this study, we quantified the DOC released by five species of macroalgae that affected live corals through their physical contact and their subsequent remineralization for 100 days by coral mucus bacteria. Also, we analysed the changes in bacterial community structure after 30 days of exposure to the macroalgal DOC. All the macroalgae released a significant amount of DOC ranging from 2.2 ± 0.17 to $8.1 \pm 0.36 \mu\text{mol C g}^{-1} \text{ h}^{-1}$ (Mean \pm SD). After 100 days, between 9.2% and 30.9% of the macroalgal DOC remained recalcitrant to bacterial remineralization. There was no apparent change in the dominant bacterial groups exposed to the DOC released by the green macroalgae *Caulerpa racemosa* and *Halimeda* sp. In comparison, the Proteobacteria group decreased with a prominent increase in the Firmicutes, Planctomycetes and Bacteroidetes group in the samples exposed to DOC released by the brown macroalgae *Turbinaria ornata*, *Sargassum tenerrimum*, and *Padina gymnospora*. These inclusive data suggest that the DOC released by different species of macroalgae differed on their lability to microbial mineralization and highlight the comparable patterns in microbial responses to macroalgal exudates across different species.

Keywords: Corals, Macroalgae, Dissolved Organic Carbon, Remineralization, Bacterial community.

1 Introduction

Coral reefs are highly productive ecosystems, host a wide variety of flora and fauna, and offer numerous benefits to humankind in terms of fisheries, shoreline protection, and tourism (Cesar et al. 2003). Synergistic effects of climate and anthropogenic activities lead to unforeseen effects that rapidly degrade the coral reefs pushing them to the risk of extinction (Carpenter et al. 2008). Numerous studies have quantified the rate and extent of coral reef degradation but failed to focus on the underlying causes and the mechanisms of degradation (Hughes et al. 2010). In general, coral reefs respond to environmental perturbations by altering the community structure, often a shift from the coral dominant state to one dominated by macroalgae (Ledlie et al. 2007; Bruno et al. 2009; Fung et al. 2011). Though macroalgae are the natural components of a reef ecosystem, they are opportunistic and compete for space with other reef organisms, including corals. The macroalgae can take over the reef following a mass mortality of corals and prevent the recovery of a degraded reef by reducing the fecundity, and coral larval survival (Beatty et al. 2018; Birrell et al. 2008). The absence of corals in a reef affects the adjacent seagrass and mangrove ecosystems in a critical manner (Hoegh - Guldberg et al. 2007).

Gulf of Mannar Marine National Park (GoM), located on the Northern Indian Ocean, holds the ecological footprints of both climate and anthropogenic stressors. However, very few studies have captured the impacts of climate and anthropogenic stressors on the structure and function of these reef ecosystems (Pillai 1975, Arthur 2000, Kumaraguru et al. 2005). Recent studies shed light on the increasing dominance of macroalgae on the GoM reefs and their impacts on corals (Machendiranathan et al. 2016; Manikandan and Ravindran 2017). However, the effect of macroalgal dominance on the overall reef health is poorly addressed. In general, macroalgae are the important benthic producers contributing to the overall productivity of a reef ecosystem. Almost, 10-15% of the gross organic production by the reef benthic producers is released into the environment and act as the primary source of Dissolved Organic Carbon (DOC) in the reefs (Silveira et al. 2017).

DOC, a complex mixture of molecules produced by primary producers in the ocean, constitutes one of the largest bioactive carbon reservoirs in the ocean. Both particulate (POM) and dissolved organic matter (DOM) produced by photosynthetic organisms contribute to the temporary or permanent storage of carbon in the oceans through sedimentation and transport of water masses (Ducklow et al. 2001). Both POM and DOM undergo microbial mineralization in which the microbial population utilizes a part of organic matter, and the rest of the organic matter remains recalcitrant and resistant to the microbial mineralization for a longer duration (Carlson 2002). The DOC released by reef benthic producers stimulates the heterotrophic bacterial activity, and enable the bacterial communities to grow 50 times faster compared to the open ocean communities (Kirchman 2016;

Silveira et al. 2017). The interaction between microbes and organic matter influence various processes including the concentration and fate of atmospheric CO₂, accumulation of refractory carbon in the deep ocean, and carbon flux through food webs (Jiao et al. 2010).

DOC released by different benthic producers differs in their composition and the rate of DOC utilization. The recycling of DOC in a reef depends on the type of microbial communities associated with the corals, reef sediments and water column. For example, the DOC produced by certain benthic primary producers such as the macroalgae *Halimeda* and *Amansia* sp. comprises maximum labile fraction, and are readily utilized by the microbial communities. In contrast, DOC produced by other macroalgae such as *Turbinaria* sp. constitutes a significant fraction of complex molecules that remain resistant to bacterial action and contributes to the recalcitrant DOC (Haas et al. 2011). Quantitative data based on long-term experiments that expose what percentage of reef derived DOC is incorporated into the microbial food web and what percentage of DOC remains recalcitrant is limited (Tanaka et al. 2008).

The ever-growing dominance of macroalgae in reef ecosystems will substantially increase the amount of DOC released into the reef environment, with negative implications on the corals and overall reef health. Specifically, macro and turf algae induce biogeochemical changes through DOC release and enhance the heterotrophic microbial action that depletes oxygen and promote the expression of virulence factors in reef bacterioplankton (Roach et al. 2020; Roach et al. 2017; Cardenas et al. 2017). Studies so far characterized the effect of macroalgal DOC on the bacterial communities present in the reef water column, and there is a dearth of information on the effect of macroalgal DOC on coral mucus bacteria. Longer resident times of macroalgae in a reef and their direct contact with corals will continuously expose the coral-associated microbiome to the DOC released by the macroalgae. In this study, we quantified the amount of DOC released by two green and three brown macroalgal species that commonly grew over live corals in the GoM reef and studied the remineralization of the macroalgal DOC for 100 days by the bacterial community associated with the mucus of the coral *Porites lutea*. We also analyzed the changes in coral mucus bacterial community composition in response to carbon inputs from different species of macroalgae.

2 Materials and Methods

2.1 Sample collection

As a part of the continuous coral community monitoring program under the project "Dynamics of corals and macroalgal communities in Gulf of Mannar", a comprehensive survey was conducted around the Mandapam group of Islands in the Gulf of Mannar Marine National Park in 2019 (Fig 1)

to document the prevalence of macroalgae and their interaction with corals. The Mandapam group includes seven islands fringed with corals and hosts a rich diversity of flora and fauna. The corals are distributed in patches at the water depths between 0.5 - 5 m around the islands. Analysis of the photoquadrats (n=20; size = 1m²) taken at the sample collection location showed that macroalgae was the dominant benthic community (39±3.1%) followed by dead corals (20.5±2.5%) and live corals (19.3±6.5%). The monthly average insitu water temperature measured using the SBE56 temperature sensor (Seabird Electronics, USA) ranged between 27.5 °C (November) and 32.5 °C (May) in 2019. Further observations across the islands revealed the dominance of three brown algae species, including *Sargassum tenerrimum*, *Padina gymnospora*, *Turbinaria ornata*, and two green algae species, *Halimeda* sp, and *Caulerpa racemosa*, over the live coral colonies. Specimens of each of these macroalgae were collected from the reef flat of Pullivasal Island by skin diving, washed of loosely associated sediment particles and epifauna with a 0.2 µ filter-sterilized seawater and transported to the laboratory culture tank within 24 hours in polythene bags filled with seawater and stored in ice.

2.2 Experiment to determine the DOC release by macroalgae

The macroalgae samples collected in the field were acclimatized in an artificial reef mesocosm setup maintained using Artificial Seawater (ASW) for a week (Kram et al. 2016). Post acclimatization, each species of macroalgae was carefully observed for stress symptoms such as discoloration and shrinking of thallus and those specimens without any signs of stress were placed in an individual, acid-cleaned glass jar containing 1.2 liters of filtered ASW. Before introducing the macroalgae, a small volume of the seawater (20 ml) inside the glass jar was sampled using an acid-washed glass syringe and filtered through pre-combusted GF/F filter (Whatman, 0.7 µm) into an acid cleaned, pre-combusted DOC vials (450 °C for 5 h) and stored at -20 °C for determining the initial DOC concentration. The glass jars with macroalgae were sealed to prevent the exchange of water with surrounding medium and incubated inside the reef mesocosm setup for 12 hours during which the natural daylight was simulated using full-spectrum LED lights and the surrounding water temperature was maintained at 25 °C using aquarium heaters (Online resource 1). The temperature and Photosynthetically Active Radiation (PAR) inside the artificial reef mesocosm setup was measured using SBE 56 and ECOPAR sensors (Seabird Scientifics, USA) at 10 minutes interval and processed for determining the hourly average. After incubation, the macroalgae samples were removed from the glass jars using an acid-cleaned forceps, spinned in a salad spinner to remove excess water and dried in a laboratory oven at 40 °C for 48 h to determine the macroalgal biomass (dry weight) (Ross et al. 2017; Gupta et al. 2011). The remaining water inside the glass jar was sampled as described above to determine the final DOC. The net DOC production by each macroalgae species was measured by

calculating the difference between the initial and final DOC values. All the macroalgae incubation experiments were conducted in duplicates and the average amount of DOC released by each macroalgae species was reported as $\mu\text{mol C g}^{-1} \text{h}^{-1}$. Since the surface area of *C.racemosa* and *Halimeda* sp. could not be measured precisely, the DOC values were normalized for the macroalgae biomass to maintain uniformity in the representation of the data across all the macroalgae samples. The rest of the incubated water samples were filtered into acid-washed, autoclaved dark bottles using a sterile polyethersulfone (SUPOR, 0.2 μm , Pall Corporation) membrane pre-flushed with deionized water (Haas et al. 2010b) for microbial mineralization experiments.

2.3 DOC mineralization experiment

In situ observations on the field suggested that *Porites* sp. was the most vulnerable coral affected by all the macroalgal species selected for this study. Our field observations were in agreement with the studies of Thinesh et al. (2019) and Machendiranathan et al. (2016) which reported the increased vulnerability of massive coral forms such as *Porites* spp. to macroalgal contact. Therefore, the bacteria present in the mucus of the coral *P.lutea* was used as an inoculum for the mineralization experiment. The mucus sample was collected using a sterile syringe by sucking up the mucus sheets from the surface of a healthy *P.lutea* colony as described in Ravindran et al. (2013) and pooled together to ensure that each macroalgal exudates treatment receive the same starting pool of bacteria. Replicates of three ml of the mucus samples were filtered separately through sterile PVDF filters (0.2 μ) to retain the bacteria present in the mucus. Each experimental bottle was inoculated with bacteria by vigorously agitating the filter paper inside the water samples using acid cleaned forceps. After inoculation, the dark bottles were mixed well and 5 ml of the water was sampled from each dark bottle and fixed with paraformaldehyde (0.2% final concentration) for estimating the initial bacterial count. Also, 50 ml of the water was sampled in a sterile falcon tube and stored at -20 °C for characterizing the bacterial community composition before DOC mineralization. The dark bottles were incubated in a temperature-controlled room at 25 ± 2 °C and gently mixed before the removal of samples throughout the incubation period. The samples were removed as described above for DOC analysis and bacterial count on every alternate day for the first 30 days of the 100 days experiment. Assuming that most of the labile and semi-labile fraction of the DOC must be utilized by this time, 50 ml of the water was sampled on the 30th day from each experimental bottle and stored at -20 °C for characterizing the changes in bacterial community composition. Afterward, the samples were removed at weekly intervals from 31 to 100 days for the bacterial count and DOC analysis. The overall experimental procedure is summarized in Online resource 2.

The samples for bacterial counts were transferred to sterile corning tubes, fixed with paraformaldehyde (0.2% final concentration), and stored at 4 °C until analysis. DOC samples were filtered through 0.2 µm acrodisc syringe filters (SUPOR, 0.2 µm, Pall Corporation) and collected into an acid-cleaned 30 ml glass vials and immediately frozen upright at -20 °C until analysis. A Shimadzu Total organic carbon analyzer (TOC-L_{CPH}, Japan) was used to analyze DOC at 680 °C following the combustion catalytic oxidation method. Standardization was achieved using potassium hydrogen phthalate and a 5 point calibration curve was generated before each batch. CRM (Deep Sea standard; 41–44 µM for DOC; batch number 15-2015) procured from the University of Miami was run in each batch, and the accuracy of the analysis was ±1 µM. All the samples for DOC analysis and bacterial count were collected in duplicates. The total amount of DOC mineralized by bacteria for each macroalgal exudates was estimated by calculating the difference between the initial DOC value and the DOC value measured at the end of 100 days dark incubation and reported as the percentage of DOC utilized by the bacterial community. Decrease in the concentration of DOC due to bacterial degradation during the 100 days dark incubation was fitted with an exponential decay model and the degradation rates (k) were calculated using the first-order exponential decay formula with the refractory pool included in the equation (Banta et al. 2004).

$$\text{DOC}_{(t)} = \text{DOC}_{(0)} \times e^{-kt} + R$$

where $\text{DOC}_{(t)}$ is the DOC remaining at time t (days), $\text{DOC}_{(0)}$ is the DOC amount at day '0' ($\mu\text{mol L}^{-1}$), 'k' is the degradation rate (d^{-1}) and R = amount of DOC that remained resistant to bacterial action at the end of 100 days incubation experiment.

2.4 Bacterial abundance and community composition

The abundance of bacteria in each sample was estimated by flow cytometry. Prior to the analysis, the samples were thawed to room temperature, stained with freshly prepared SYBR Green I stain (Sigma-aldrich, Germany) at 1:10000 final concentration (Marie et al. 1999) and incubated in the dark for 15 minutes. After incubation, aliquots of 100 µl of the sample were loaded in 96 well plates and analyzed in BD Accuri C6 flow cytometer equipped with a 488 nm blue laser at a medium flow rate ($35 \mu\text{l minute}^{-1}$) with the sheath fluid provided by BD biosciences. Green fluorescence emission was collected in the FL1 channel ($533 \pm 30 \text{ nm}$) with a threshold value of 600 applied on the green fluorescence channel (FL1). Fluorescent beads (1µ size) (BD biosciences, USA) were used as an internal standard. The samples were agitated every 10 minutes to prevent the settling of cells. Between each sample run, one sterile distilled water sample was placed as blank to avoid carryover of the stain. The stained cells were differentiated based on the side scatter (SSC) and green fluorescence signal

(FL1) channels and the clustered cells were gated and quantified. The flow cytograms were analyzed using C-flow plus software and the bacterial abundance was expressed as cells ml⁻¹.

The bacterial community composition was analyzed using 16s rRNA gene amplicon sequencing. Due to financial limitations, this study involved sequencing only the six DNA samples without replicates comprising one coral mucus sample and five experimental water samples (30 days dark incubated) representing the zero-day bacterial community structure and structure after 30 days of DOC mineralization. DNA was isolated from the zero-day sample and 30 days dark incubated water samples using the reagents from Nucleospin kit (Clontech, Mountain view, CA) following the manufacturer's instructions. Briefly, the water samples were filtered using sterile 0.2µ cellulose nitrate membrane filters (Millipore) which were cut in to small stripes with sterile razor blades and transferred into sterile 2mL tubes. Lysis of the cells were achieved by incubating the samples in a proteinaseK/SDS solution. The lysate was mixed with chaotropic salts and ethanol to facilitate the binding of DNA to silica membrane in the Nucleospin column. Contaminations are removed by subsequent washing with wash buffers provided in the kit and the DNA was eluted using a slightly alkaline elution buffer. Library preparation and sequencing were done in Eurofins genomics India Pvt. Ltd using the Nextera XT Index Kit as per the 16S Metagenomic sequencing library preparation protocol (Illumina Inc). After quality and quantity check using nanodrop, the isolated DNA was used to amplify the V3 -V4 region of the 16S rRNA gene with the primers 16S rRNA F [5'-GCCTACGGGNGGCWGCAG-3'] and 16S rRNA R [5'-ACTACHVGGGTATCTAATCC-3'] under the following conditions: 95 °C for 3 min followed by 25 cycles of 95°C for 30 s; 55°C for 30 s; 72°C for 30 s after which the final elongation step at 72°C for 5 minutes. The amplicon libraries were purified using the AMPure XP beads and quantified using Qubit Fluorometer following the standard protocol (Illumina). This was followed by index PCR to attach the dual indices and Illumina sequencing adaptors, cleanup, library quantification, normalization and pooling outlined in the Illumina protocol. Thereafter the samples were loaded onto the MiSeq Illumina platform at an appropriate concentration of 10-20 pM for cluster generation and sequencing.

2.5 Sequence Analysis

The sequences were analyzed using QIIME2 (version 2-2019.10) and Phyloseq R Statistical software package (version 3.6.1). Overall, 959,334 demultiplexed sequences with an average length of 300bp were imported to QIIME2 in fastq format and the primers and adapters were removed using cut adapter command. After that, the sequences were quality filtered, de-replicated, chimera removed, and the paired-end reads were merged using the DADA2 algorithm (Callahan et al. 2016) with a p-trunc length of 260 and 270 bp. The representative sequences were taxonomically assigned using the

sklearn machine learning method (Pedregosa et al. 2011) with the Greengene reference database (99% clustering, all taxonomic level). The sequences were submitted to NCBI under the project PRJNA587491. The PICRUST2 software was used to infer the functional diversity of the bacteria with reference to carbon metabolism between the zero day sample and the samples exposed to macroalgal DOC (Douglas et al. 2020). The inferences on metabolic functions were made from KEGG annotated databases (Langille et al. 2013). The significance of the observed differences among the functional diversity of the samples exposed to green and brown macroalgae was analyzed using Tukey-Kramer parameter test (Tukey-Kramer 2013) in STAMP software package (Parks et al. 2014)

2.6 Data analysis

Interspecies variation in the net DOC release among the macroalgae was tested using a One-way Analysis of variance (ANOVA) followed by a Tukey Post hoc mean comparison. The bacterial community composition data comprising the number of sequences under each bacterial group were processed to obtain the relative percentage of each bacterial group in the samples incubated with DOC released by different macroalgae species. Linear regression analysis was performed to analyze the relationship between DOC concentration and bacterial abundance. All the analysis was performed using the statistical software SPSS V 22.

3 Results

3.1 DOC release by macroalgae

The concentration of DOC (mean \pm SD) in the filtered ASW before incubation with macroalgae was $63 \pm 2.61 \mu\text{mol l}^{-1}$. After 12 h incubation with different macroalgae species, the DOC concentration in the water samples increased up to 87.01 ± 0.72 to $439.3 \pm 16.9 \mu\text{mol l}^{-1}$ indicating the net DOC production by macroalgae and its subsequent release into the surrounding water. The amount of DOC released by macroalgae differed significantly between different species incubated under the same environmental conditions ($F_{4,5}=20.3$, $p=0.003$). The *P.gymnospora* released maximum DOC ($8.1 \pm 0.37 \mu\text{mol C g}^{-1} \text{h}^{-1}$) followed by *S.tenerrimum* ($6.6 \pm 1.68 \mu\text{mol C g}^{-1} \text{h}^{-1}$) and *T.ornata* ($4.8 \pm 0.25 \mu\text{mol C g}^{-1} \text{h}^{-1}$). The *Halimeda* sp. released DOC as low as $2.2 \pm 0.17 \mu\text{mol C g}^{-1} \text{h}^{-1}$ (Fig 2). A Tukey Post hoc test indicated that the green macroalgae *C.racemosa* and *Halimeda* sp. released DOC significantly lower than the brown macroalgae *S.tenerrimum* and *P.gymnospora* (p -value <0.05).

3.2 DOC mineralization and bacterial abundance

The rate of mineralization of the DOC by the bacteria associated with the coral *P.lutea* differed between different macroalgae species and the DOC mineralization pattern over 100 days dark incubation was summarized in Figure 3. DOC released by *P.gymnospora* was rapidly mineralized

compared to the DOC released by other macroalgal species during the initial four days of the dark incubation. In total, 56.73% of the DOC produced by *P.gymnospora* was mineralized within 2 days with a corresponding increase in the bacterial population from 1.38 to 13.93x10⁶ cells ml⁻¹. Similarly, 41.6% of the DOC released by *T.ornata*; 30.18% of the DOC released by *Halimeda* sp.; 24.7% of the DOC released by *C.racemosa* and 31.4% of the DOC released by *S.tenerrimum* was mineralized within two days of dark incubation, with a corresponding increase in the bacterial abundance in the respective samples. Thereafter, the DOC concentration decreased gradually in all the samples and nearly 75% of the DOC released by *P.gymnospora* and *T.ornata* were mineralized at the end of 30 days. Whereas $\geq 60\%$ of the DOC released by *Halimeda* sp, *C. racemosa*, and *S. tenerrimum* were mineralized at the end of 30 days. The DOC degradation rate (*k*) for 100 days incubation was higher in the samples containing DOC released by *T.ornata* (0.023 d⁻¹) followed by *P.gymnospora* (0.020 d⁻¹) and *S.tenerrimum* (0.016 d⁻¹). The 'k' values were comparatively lower in the samples with DOC released by *C.racemosa* (*k* = 0.0117 d⁻¹) and *Halimeda* sp. (*k* = 0.0126 d⁻¹). After 100 days, the amount of DOC remained recalcitrant to bacterial action ranged between 32.2% (*C. racemosa*) and 9.4% (*T. ornata*) (Online resource 3). Turnover times were higher for the DOC released by the green macroalgae *C.racemosa* (85 d) and *Halimeda* sp. (79 d). Whereas the turnover times are calculated as 43, 62 and 50 d for the brown macroalgal species *T.ornata*, *S.tenerrimum*, and *P.gymnospora*, respectively. The abundance of bacteria in the experimental samples ranged between 0.87 and 1.85x10⁶ cells ml⁻¹ on the zero-day of dark incubation and it increased rapidly to a range of 3.68 to 5.72x10⁶ cells ml⁻¹ during the first four days of dark incubation (Fig 4) showing a linear relationship (Linear regression, R² = 0.71 (*S.tenerrimum*) - 0.94 (*T.ornata*)) with the decrease in DOC concentration. Thereafter, no significant increase in bacterial abundance was observed despite the gradual decrease in the DOC concentration.

3.3 Bacterial community composition

A total of 959,334 sequences from six samples were analyzed, and an average of 2485 Amplicon Sequence Variant (ASV) was obtained after quality filtering and chimera removal. The zero-day sample representing the bacterial community in coral mucus yielded 1548 ASVs. Whereas, the number of ASVs varied from 2214 (*C.racemosa*) to 9789 (*P.gymnospora*) in the samples exposed to macroalgal DOC. Analysis of the sequences revealed changes in the relative abundance of bacterial communities before and after exposure to the macroalgal DOC (Fig 5), which in turn are distinct between the DOC released by different species of macroalgae. Members of the class Gammaproteobacteria dominated the zero-day bacterial community (70.4% of all sequences) followed by Alphaproteobacteria (17.8% of all sequences) and Bacilli (4.4% of all sequences). At the family level, members of an unclassified family under Gammaproteobacteria (22.4%), Alteromonadaceae

(18.09%), Moraxellaceae (10.1%) and Pseudomonadaceae (8.2%) dominated the bacterial community and contributed to the overall dominance of Proteobacteria in the zero-day bacterial community (Online resource 4).

There was no major shift in the bacterial community composition before and after exposure to the DOC released by the green macroalgal species. Members of the class Gamma and Alphaproteobacteria were consistent with their dominance collectively accounting for 84.4% and 80.3% of all sequences, respectively. However, there was a prominent decrease in the members of the family Moraxellaceae (from 10.1% to 5.4 and 4.9%) and an unclassified gammaproteobacteria family (from 22.4% to 16.4 and 13.06%) in the samples exposed to *C.racemosa* and *Halimeda* sp. DOC respectively. A corresponding increase was noticed in the members of the family Alteromonadaceae, Pseudomonadaceae, and an Unclassified family within the class Chlamydia in the samples exposed to *C.racemosa* DOC. Likewise, members of an unclassified family under Alphaproteobacteria and Chlamydia, Rhodospirillaceae, and Flavobacteriaceae increased in their abundance in the samples exposed to the *Halimeda* sp. DOC (Online resource 4).

There was a major shift among the dominant bacterial groups in the samples exposed to the DOC released by the brown macroalgal species. The relative abundance of the class Gammaproteobacteria dropped markedly from 70.4% to 24.7%, 3.6% and 9.8% in the samples exposed to DOC released by *T.ornata*, *S.tenerrimum*, and *P.gymnospora* respectively. In the sample exposed to *T.ornata* DOC, members of the family Pirellulaceae, Flavobacteriaceae and Rhodospirillaceae increased with a significant decline in the members of the family Alteromonadaceae, *Pseudomonadaceae*, *Moraxellaceae* and an unclassified Gammaproteobacteria family (Online resource 5). Also, the Pirellulaceae family accounted for 32.06% of the total community and contributed to the increased abundance of the class Planctomycetia (37.8% of all sequences) within the Planctomycetes group in the sample exposed to the *S.tenerrimum* DOC. The relative abundance of the members of the class Clostridia and Bacteroidia increased from $\leq 0.1\%$ to 22.1% and 15.9%, respectively and Bacilli increased from 4.4% to 18.5% in the experimental sample exposed to the DOC released by *P.gymnospora*. Members of the family Lachnospiraceae, Ruminococcaceae, and unclassified family members within the order Bacteroidales of the class Bacteroidia dominated the bacterial community in the sample exposed to *P.gymnospora* DOC.

Comparison of the functional genes involved in carbon metabolism across the samples revealed that the proportional abundance of genes involved in carbon metabolism differed significantly between the zero-day sample and samples exposed to brown macroalgal DOC (Tukey Kramer, p value < 0.05). Whereas no such statistically significant difference was observed between the zero-day and green

macroalgal DOC incubated samples (Tukey Kramer, p value >0.05). Specifically, the metagenome dataset of zero-day sample representing the coral mucus bacterial community and green macroalgae DOC incubated samples were enriched with the Embden-Meyerhof-Parnas pathway (EMP) modules. Whereas the samples exposed to brown macroalgal DOC were enriched with less energy efficient Pentose Phosphate (PP) pathway and Glycogen synthesis pathway (Fig 6).

4 Discussion

Our study reports the long-term remineralization of DOC released by macroalgae and its influence on the bacterial community composition associated with the surface mucopolysaccharide layer of the coral *P.lutea*. Previous works focused on the short-term utilization of macroalgal DOC by the water column bacterial communities and documented the oxygen availability due to increased microbial action (Haas et al. 2011; Wild et al. 2010). Our results showed that different species of macroalgae released different amounts of DOC into the environment with differential effects on the coral-associated bacterial communities, which are considered the first line of defense for the corals (Shnit-Orland and Kushmaro 2009). The DOC released by different macroalgal species accelerated the bacterial growth to a different extent during the initial four days of dark incubation. After 30 days of exposure, there was a shift in the relative abundance of the dominant bacterial groups in the samples exposed to the DOC released by *T.ornata*, *S.tenerrimum*, and *P.gymnospora*. The changes in the bacterial community composition was characterized by an increase in a specific group of bacteria that were low in abundance before their exposure to the respective macroalgal DOC. However, the implications of such changes on coral health and the ability of corals to resist external disturbances are not known.

Production and consumption of DOC constitute a vital process in the marine carbon cycle. In general, macroalgae release a significant amount of their photosynthetically fixed carbon as DOC into the external environment (Carlson and Carlson 1984; Wada et al. 2007; Reed et al. 2015), a part of which gets exported to the offshore waters (Watanabe et al. 2020). All the macroalgal species investigated in this study released a significant amount of DOC into the external environment. This is in agreement with previous studies that showed the release of photosynthetic products by macroalgae in the dissolved form, thereby providing carbon-rich compounds as a source of energy for the heterotrophic bacterial community associated with corals, water, and sediment surrounding the reefs (Nelson et al. 2013; Haas et al. 2010a). Majority of the previous studies normalized the amount of DOC released by macroalgae to their surface area (Haas et al. 2011; Villacorte et al. 2015; Brocke et al. 2015); thus the results of this study cannot be compared directly to the DOC release values presented

in the earlier studies. Very few studies normalized DOC values to the biomass of macroalgae (Wada and Hama 2013; Mueller et al. 2014).

The amount of DOC released by macroalgae differed significantly between different species, with the calcareous algae *Halimeda* sp. releasing the least and the thin brown algae *P.gymnospora* releasing maximum DOC into the water. This observation is in line with the experimental results of Mueller et al. (2014), in which *Halimeda* sp. released $1.1 \pm 0.3 \mu\text{mol C g}^{-1} \text{h}^{-1}$, the least among other macroalgae and benthic producers tested. Such variations in DOC release rate between different species of macroalgae are attributed to the difference in the rate of primary production and the surface area to volume ratios that facilitate the release of the metabolic products (Wada et al. 2007; Haas et al. 2011). Additionally, the calcification process in calcareous algae *Halimeda* sp. is an energy-demanding process requiring the photosynthates with minimal DOC release into the external environment. These variations between different species of macroalgae will have significant implications in the nutrient cycles and oxygen availability in the reef ecosystems (Wild et al. 2010) since different species of macroalgae exhibit marked seasonality in their occurrence and prevalence (Manikandan and Ravindran 2017).

Different compounds of DOC have been shown to enrich different bacterial groups and promote the growth of a dormant bacterial population, thereby imparting changes in the overall bacterial community composition (Jain et al. 2020; Nelson et al. 2013; Langenheder et al. 2005). After 30 days of dark incubation, the bacterial community exposed to DOC released by *C.racemosa* and *Halimeda* sp. retained a large proportion of Proteobacteria, Firmicutes, and an Unclassified bacteria that together contributed $\geq 85\%$ of the total sequences indicating minimal changes in the overall bacterial community composition. In contrast, the proportion of the bacterial groups Planctomycetes and Firmicutes increased significantly in the samples exposed to the DOC released by brown algae *T.ornata*, *S.tenerrimum*, and *P.gymnospora* with a subsequent decline in the abundance of Proteobacteria. A shift in the bacterial community structure in response to macroalgal DOC is often linked to the composition of DOC rather than the concentration, which in turn enhances the bacterial biomass (Landa et al. 2016; Li et al. 2012; Eiler et al. 2003).

Comparison of the functional potentials related to carbon metabolism showed that the genes affiliated with EMP pathway modules were abundant in the zero-day samples representing coral mucus bacteria. Whereas the proportional abundance of the genes associated with the PP pathway and glycogen synthesis pathway was enriched in the samples exposed to the brown macroalgal DOC. This is consistent with the results of Haas et al. (2016), which specified an increased abundance of less energy-efficient PP pathway genes in the algal-dominated reefs. The proportional abundance of the

genes associated with the Entner-Doudoroff (ED) pathway was low in the samples exposed to the DOC released by *P.gymnospora* and *T.ornata* compared to the zero-day sample indicating species-specific differences in regulating the functional diversity of coral-associated bacteria.

Our results showed that DOC released by *T.ornata* and *P.gymnospora* were mineralized rapidly by the coral mucus bacteria suggesting that the significant pool of the DOC released by these species might be in the labile form compared to the other macroalgal species. In line with this trend, $\geq 85\%$ of the DOC produced by both species were utilized within 100 days. However, between 30.9 and 9.1% of the macroalgal DOC remained resistant to bacterial action at the end of 100 days. Previous studies suggested that macroalgae release refractory compounds, both photosynthetically produced and stored in their thalli as DOC in to their environment (Wada and Hama 2013; Powers et al. 2019, Trevathan-Tackett et al. 2015). The temporal pattern of macroalgal DOC mineralization described in this study could differ from the natural environment since the macroalgal DOC released into the environment is primarily degraded by the water column bacteria (Jiao et al. 2010). However, the long-term persistence of macroalgae and their direct contact with corals will ensure the continuous exposure of coral mucus bacteria to macroalgal DOC with significant influence on their community structure and functions (Thurber et al. 2009). The DOC mineralization depends on the composition and the ratio of high and low molecular weight compounds present in it and the community composition of the bacteria involved in DOC remineralization (Pinhassi et al. 1999; Findlay et al. 2003; Nelson et al. 2013). Unfortunately, the composition and ratio of low and high molecular weight compounds of the DOC were not estimated in this study.

In the present study, the turnover times of DOC released by different species of macroalgae ranged between 15 and 31 d (k values = 0.031 - 0.0656 d⁻¹) during the first 30 days of incubation, shorter than the turnover times calculated for the other macroalgae such as *Ecklonia* kelp (24-172 d) (Wada et al. 2008) and *Sargassum horneri* (111-238 d) (Watanabe et al. 2020). With further incubation up to 100 days, the DOC turnover times extended between 43 and 85 days, longer than the turnover times of *Saccharina japonica* (Gao et al. 2021) and shorter than *Sargassum horneri* (Watanabe et al. 2020) for 150 d incubation. Collectively, these results indicate that the bioavailability of DOC differs between different species of macroalgae, and their recalcitrance varies over time being underestimated with short-term incubations (Trevathan-Tackett et al. 2020).

A stable microbial community confers the corals with resistance to thermal and acidification stress (Grottoli et al. 2018). However, this study shows that the DOC released by certain species of macroalgae can breakdown the stability of the microbial communities by inducing a shift among the dominant bacterial groups. Also, several studies showed an increase in microbial respiration when

exposed to DOC from macroalgae even in the absence of direct contact between them (Haas et al. 2011; Jorissen et al. 2016; Smith et al. 2006). Increased microbial respiration harms corals by reducing oxygen availability and creating hypoxic conditions that eventually lead to coral mortality and subsequently opens more space for the macroalgae (Smith et al. 2006). This forms the basis of the positive feedback loop mechanism, which states that macroalgae cause coral mortality by creating local hypoxia and mediating pathogens (Haas et al. 2016). However, the positive feedback loop mechanism cannot be generalized to all the reef environments where there is enough mixing of water that ensures the availability of oxygen. Moreover, field experiments also provide conclusive evidence that microbial respiration did not outweigh high oxygen production rates in the macroalgal-dominated reefs, thus enabling them to be net autotrophic (Haas et al. 2013). In such cases, attributing coral mortality to hypoxia created by microbial respiration need careful consideration.

In conclusion, this study illustrated a significant variation in the amount of DOC released by different species of macroalgae in the reef environment. Between 9 and 32% of the DOC released by these species remained resistant to bacterial action for up to 100 days, which might contribute to the long-term carbon storage in recalcitrant form. Further, the DOC released by the brown macroalgal species was capable of reorganizing the community structure of bacteria associated with the mucus of the coral *P. lutea*. GoM hosts a rich diversity of macroalgae (106 species) (Narasimman and Murugaiyan 2016) whose seasonal dynamics are not characterized. These results suggest that the type of macroalgae and the amount of DOC they release into the environment will significantly influence the coral microbial community and vital reef functions such as carbon cycling involving the microbes. Further studies are warranted to analyze the chemical composition of DOC released by the macroalgae and the changes it will impart on the functional diversity of microbes and, thereby, the elemental cycling on reef ecosystems.

5 Declarations

Ethics approval and consent to participate: Not applicable

Consent for Publication: Not applicable

Availability of data and materials: Data pertaining to this work shall be made available by the corresponding author upon a reasonable request.

Competing interests: None declared

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Figure Legends

Fig. 1 Map showing the sample collection location at Gulf of Mannar Marine National Park, Southeast coast of India.

Fig. 2 Net Dissolved Organic Carbon (DOC) released by each species of macroalgae used in the experiment. Error bars indicate standard deviation. (Level of significance, ns- not significant; * - p value ≤ 0.05 ; ** - p value ≤ 0.01).

Fig. 3 Temporal pattern of DOC mineralization by the heterotrophic bacterial communities associated with the mucus of the coral *P.lutea* during 100 days dark incubation. Error bars represent the standard deviation between the duplicate samples.

Fig. 4 Time course of the abundance of the heterotrophic bacterial community associated with the coral *P.lutea* during 100 days dark incubation with DOC released by different species of macroalgae.

Fig. 5 Community composition of the 20 most abundant classes of bacteria present in the coral mucus ('zero-day' bacterial community structure) and 30 days dark incubated experimental water samples with DOC released by different species of macroalgae.

Fig. 6 The heatmap showing differences in the potential carbon metabolic pathways in the *P.lutea* mucus bacterial community on the zero-day and after 30 days exposure to macroalgal DOC. The abundance was represented between 0.2 to 0.7%.

Figure 1

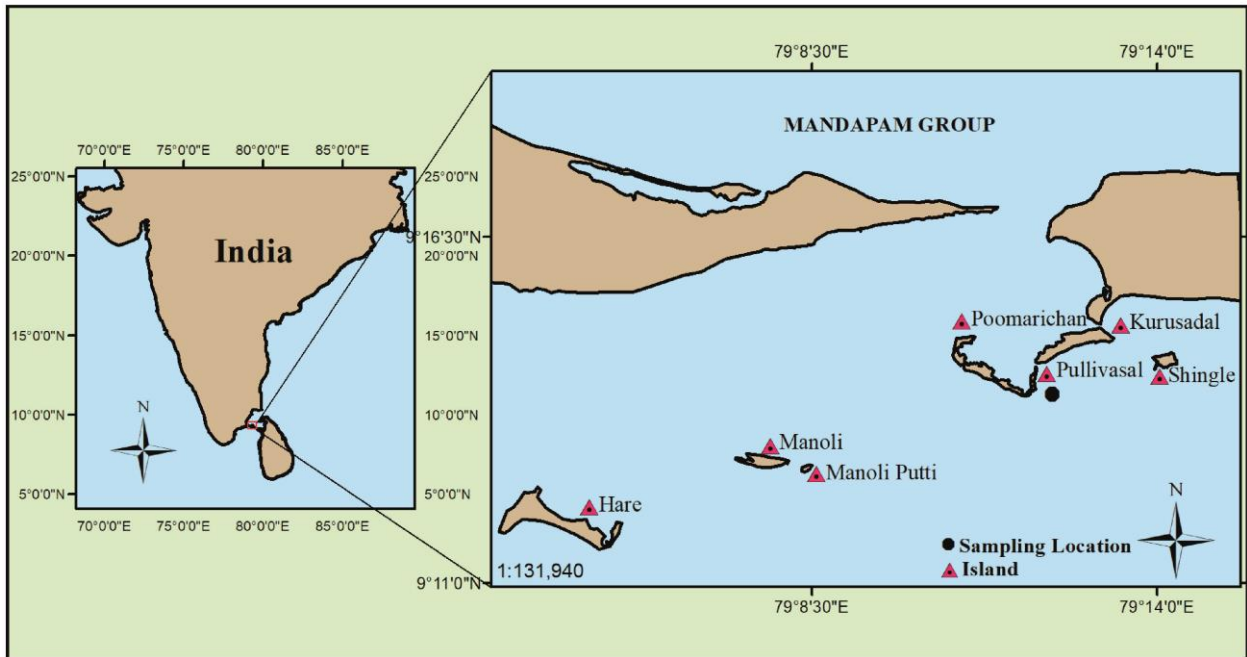


Figure 2

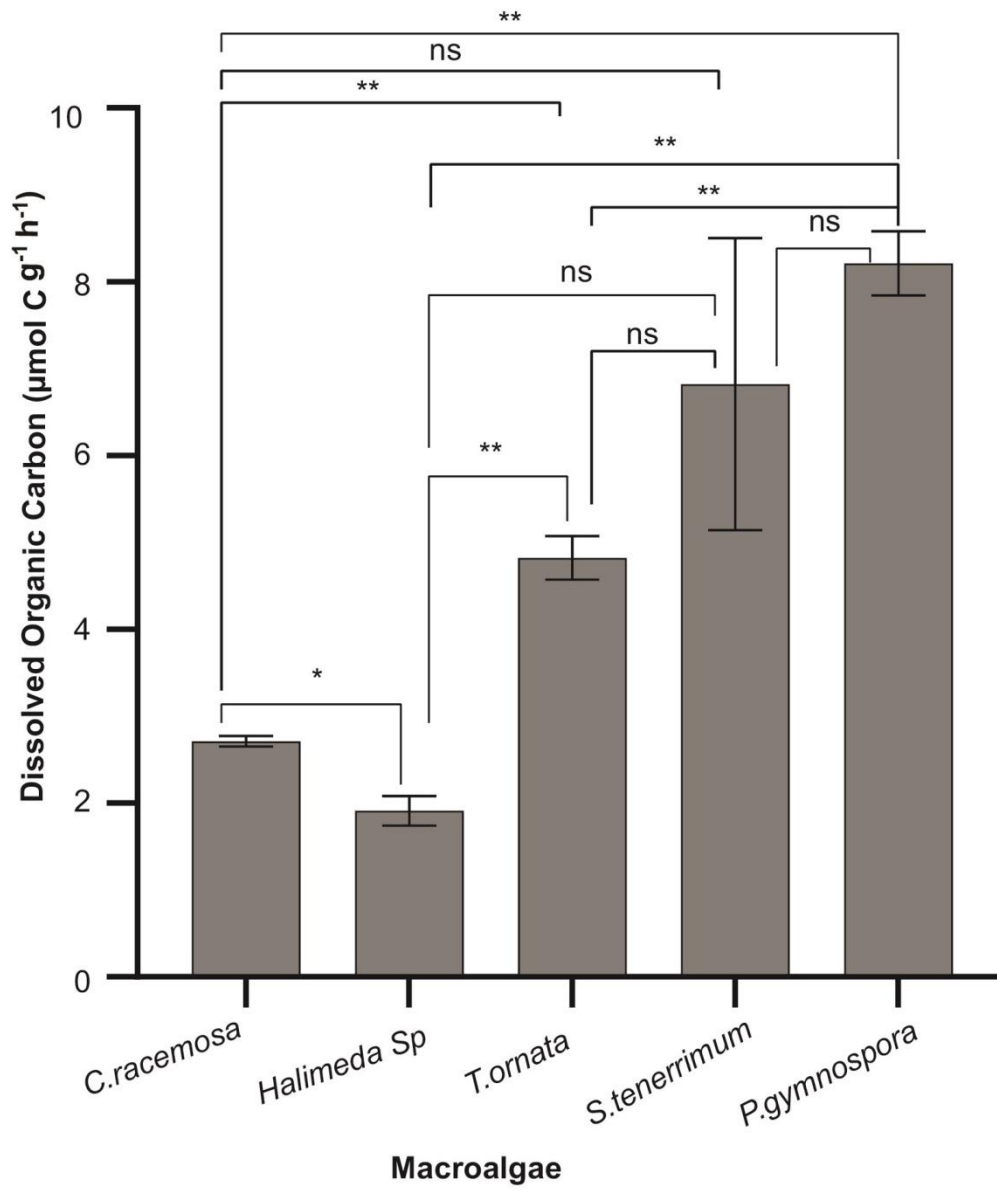


Figure 3

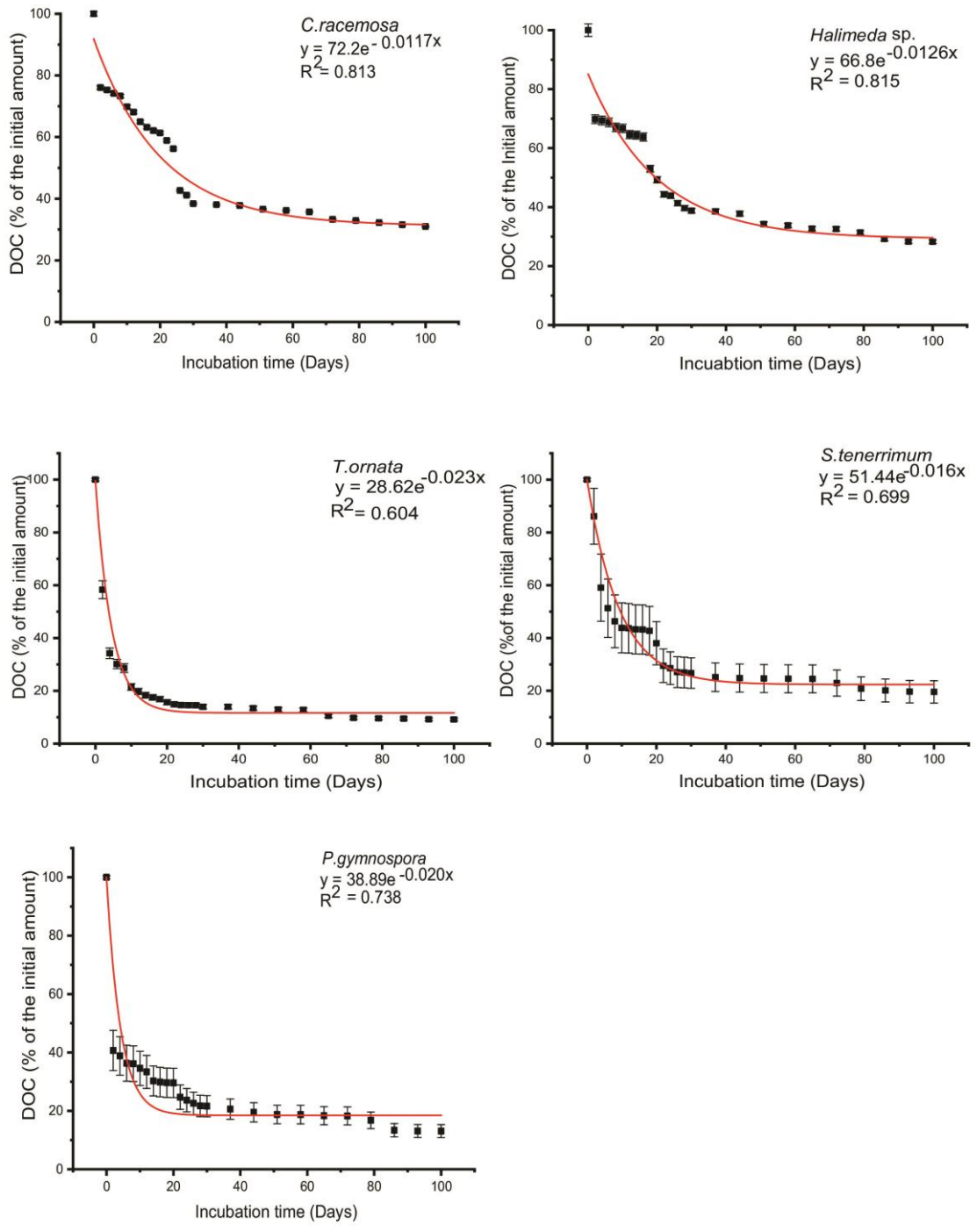


Figure 4

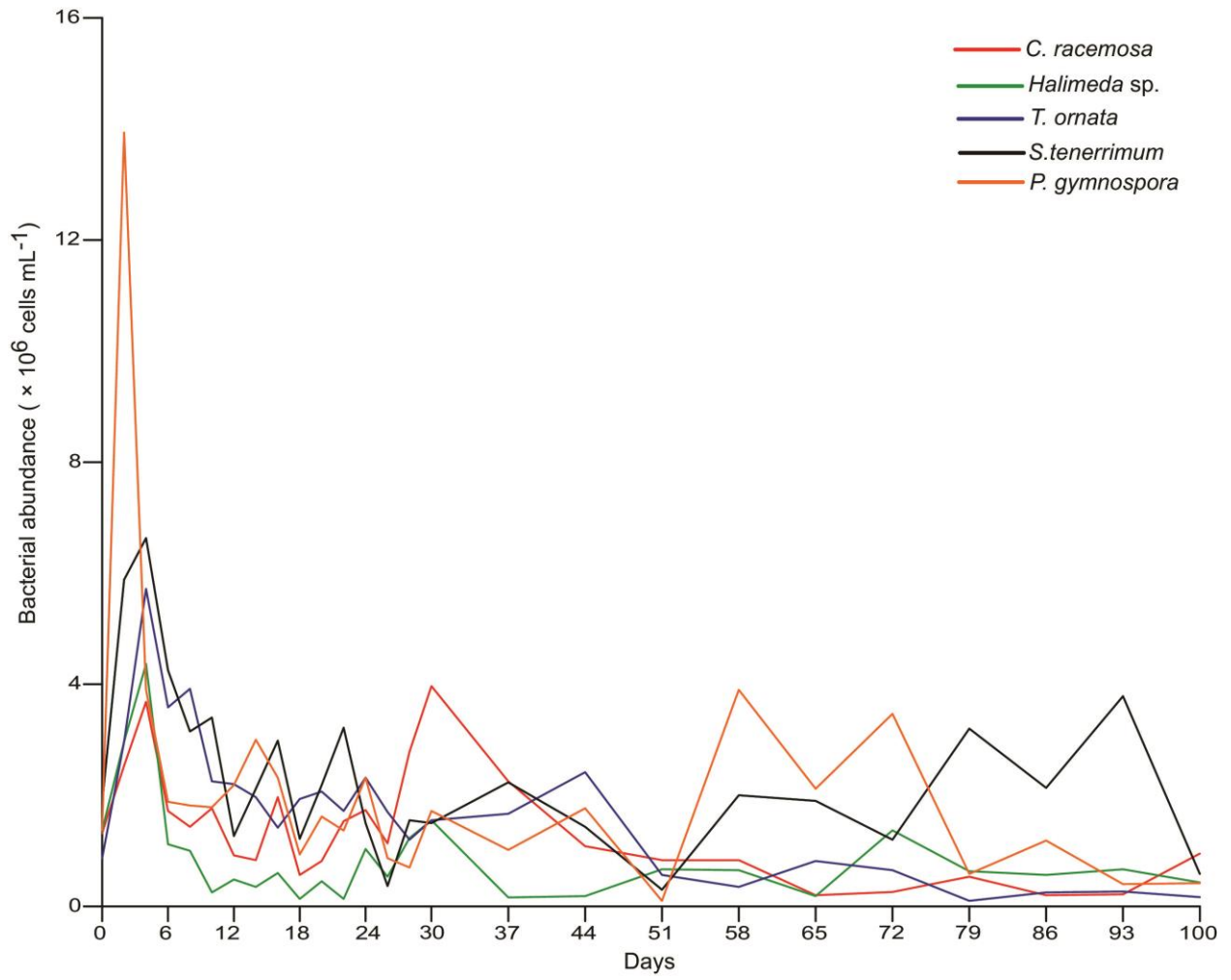


Figure 5

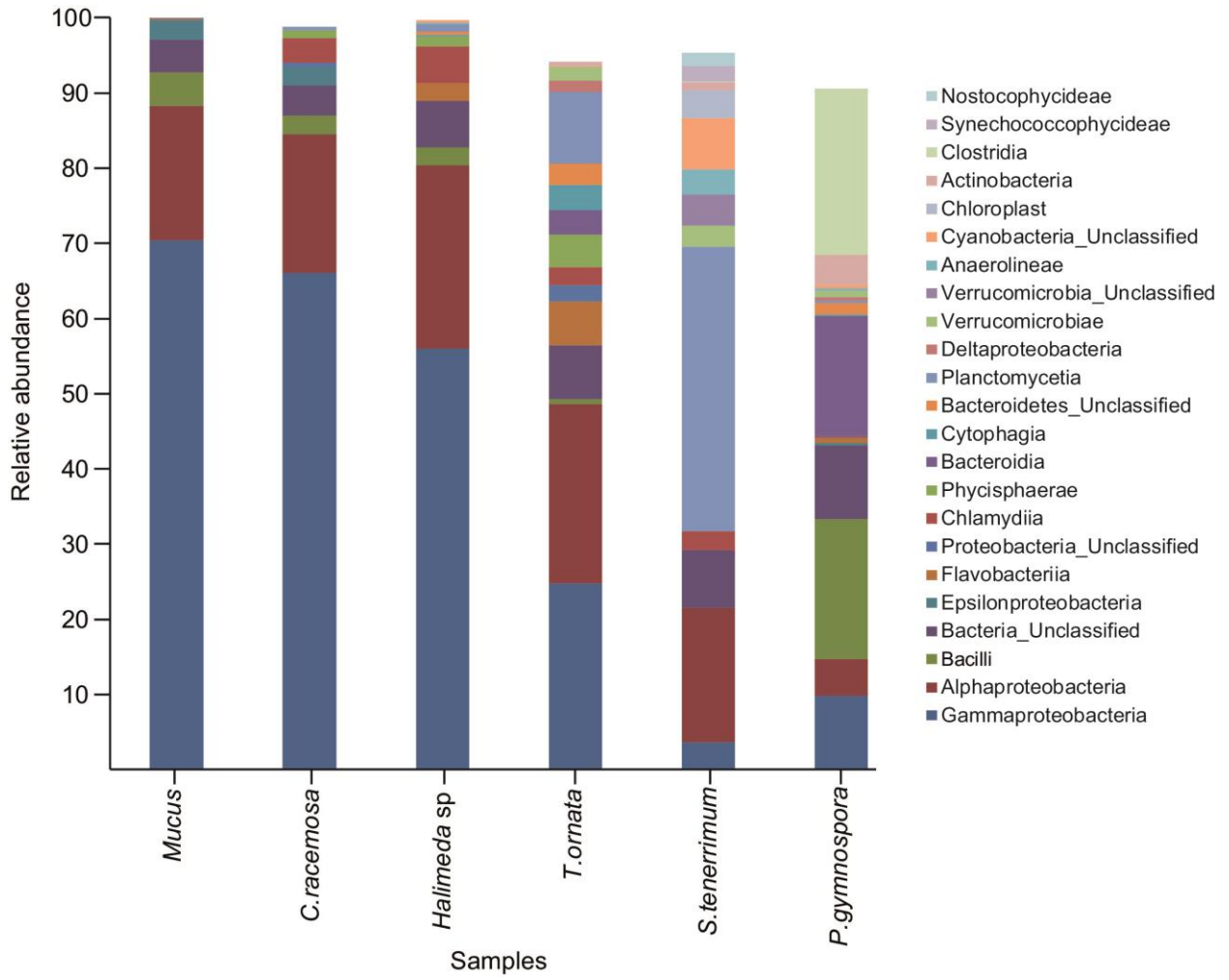


Figure 6

